

## Characterization of the MTL complex and AtVPS13M1: two actors involved in phosphate starvation-induced membrane lipid remodeling in Arabidopsis thaliana

Sébastien Leterme

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#### THÈSE

Pour obtenir le grade de



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Caractérisation du complexe MTL et de AtVPS13M1 : deux acteurs impliqués dans le remodelage des lipides membranaires induit par la carence en phosphate chez Arabidopsis thaliana

Characterization of the MTL complex and AtVPS13M1: two actors involved in phosphate starvation-induced membrane lipid remodeling in Arabidopsis thaliana

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## **Table of contents**

Ge	nera	l introduction1
1	End	losymbiotic organelles in plants: origin, functions and biogenesis
2	Mei	nbrane contact sites6
3	Nor	-vesicular glycerolipid transport in plant cells8
4 in	Nor tramit	-vesicular lipid transport at endoplasmic reticulum – mitochondria and ochondrial contact sites
	4.1	Functions of endoplasmic reticulum – mitochondria contact sites79
	4.2	Glycerolipid synthesis and trafficking to and in plant mitochondria79
	4.3 lipid tr	Proteins involved in ER-mitochondria and intramitochondrial contact site tethering and ansport
	4.3.	1 Tethering at ER-mitochondria contact sites
	4.3.	2 Tethering at intramitochondrial contact sites
	4.3.	Phosphatidylserine transport to the mitochondrial inner and outer membranes
	4.3.	Phosphatidic acid transport to the mitochondrial inner and outer membranes
	4.3.	5 EMCS lipid transport proteins with general or unknown glycerolipid specificity91
	4.3.	5 Concluding remarks
5	Pho	sphate starvation-induced membrane lipid remodeling in Arabidopsis thaliana93

## 

1 <b>B</b> i	ological material and culture conditions	
1.1	Arabidopsis thaliana	
1.	1.1 Arabidopsis thaliana plants	
1.	1.2 Arabidopsis thaliana calli	
1.2	Nicotiana benthamiana plants	
1.3	Escherichia coli	
1.4	Agrobacterium tumefaciens	
1.5	Saccharomyces cerevisiae	
2 M	olecular biology	
2.1	Arabidopsis thaliana genomic DNA extraction from callus	
2.2	Extraction of total RNAs from Arabidopsis thaliana calli	
2.3	Generation of cDNA from Arabidopsis thaliana calli	
2.4	Molecular cloning	

	2.4.	1 Plasmids	107
	2.4.	.2 Polymerase chain reaction	112
	2.4.	.3 DNA fragment purification on agarose gel	113
	2.4.	.4 DNA fragment assembly by the Gibson method	113
	2.4.	.5 Gateway recombinational cloning	113
	2.4.	.6 <i>Escherichia coli</i> transformation and selection of transformants	113
	2.4.	7 Plasmid DNA preparation from <i>E. coli</i>	114
	2.4.	.8 Sanger sequencing of plasmids and DNA fragments	114
	2.4.	9 Cryopreservation of <i>Escherichia coli</i> and <i>Agrobacterium tumefaciens</i> strains	114
	2.5	Agrobacterium tumefaciens transformation	114
	2.6	Transient transgene expression in Nicotiana benthamiana by agroinfiltration	115
	2.7	Saccharomyces cerevisiae transformation	115
	2.8	Arabidopsis thaliana stable line generation by floral dip	116
3	Pla	nt phenotype analysis	116
	3.1	Cold stress	116
	3.2	Arabidopsis thaliana pollen collection and pollen tube germination	117
4	Sub	ocellular fractionation	117
	4.1	Mitochondria purification on a discontinuous Percoll gradient	117
5	4.1 Met	Mitochondria purification on a discontinuous Percoll gradient	117 <b>119</b>
5	4.1 Met 5.1	Mitochondria purification on a discontinuous Percoll gradient thods for protein and protein complex purification, analysis and quantification Total protein extraction from <i>Arabidopsis thaliana</i> calli	117 <b>119</b> 119
5	<ul><li>4.1</li><li>Met</li><li>5.1</li><li>5.2</li></ul>	Mitochondria purification on a discontinuous Percoll gradient thods for protein and protein complex purification, analysis and quantification Total protein extraction from <i>Arabidopsis thaliana</i> calli Protein quantity determination by a colorimetric protein assay with Coomassie blue	117 <b>119</b> 119 120
5	<ul> <li>4.1</li> <li>Met</li> <li>5.1</li> <li>5.2</li> <li>5.3</li> </ul>	Mitochondria purification on a discontinuous Percoll gradient thods for protein and protein complex purification, analysis and quantification Total protein extraction from <i>Arabidopsis thaliana</i> calli Protein quantity determination by a colorimetric protein assay with Coomassie blue Protein sample concentration through a centrifugable molecular cutoff filter	117 119 120 120
5	<ul> <li>4.1</li> <li>Met</li> <li>5.1</li> <li>5.2</li> <li>5.3</li> <li>5.4</li> </ul>	Mitochondria purification on a discontinuous Percoll gradient thods for protein and protein complex purification, analysis and quantification Total protein extraction from <i>Arabidopsis thaliana</i> calli Protein quantity determination by a colorimetric protein assay with Coomassie blue Protein sample concentration through a centrifugable molecular cutoff filter Protein expression systems	117 119 120 120 120 120
5	4.1 Met 5.1 5.2 5.3 5.4 5.4.	Mitochondria purification on a discontinuous Percoll gradient         thods for protein and protein complex purification, analysis and quantification.         Total protein extraction from Arabidopsis thaliana calli         Protein quantity determination by a colorimetric protein assay with Coomassie blue         Protein sample concentration through a centrifugable molecular cutoff filter         Protein expression systems         .1         Protein expression in the yeast Saccharomyces cerevisiae	117 119 120 120 120 120 120
5	4.1 Met 5.1 5.2 5.3 5.4 5.4 5.4.	Mitochondria purification on a discontinuous Percoll gradient         thods for protein and protein complex purification, analysis and quantification.         Total protein extraction from Arabidopsis thaliana calli         Protein quantity determination by a colorimetric protein assay with Coomassie blue         Protein sample concentration through a centrifugable molecular cutoff filter         Protein expression systems         .1       Protein expression in the yeast Saccharomyces cerevisiae         .2       Cell-free protein expression	117 119 120 120 120 120 120 121
5	4.1 Met 5.1 5.2 5.3 5.4 5.4. 5.4. 5.5	<ul> <li>Mitochondria purification on a discontinuous Percoll gradient</li></ul>	117 119 120 120 120 120 120 121
5	4.1 Met 5.1 5.2 5.3 5.4 5.4. 5.4. 5.5 5.6	<ul> <li>Mitochondria purification on a discontinuous Percoll gradient</li></ul>	117 119 120 120 120 120 120 121 121 121
5	4.1 Met 5.1 5.2 5.3 5.4 5.4. 5.4. 5.5 5.6 5.6 5.6.	<ul> <li>Mitochondria purification on a discontinuous Percoll gradient</li></ul>	117 117 119 120 120 120 120 121 121 122 122
5	4.1 Met 5.1 5.2 5.3 5.4 5.4 5.4 5.5 5.6 5.6 5.6 5.6.	<ul> <li>Mitochondria purification on a discontinuous Percoll gradient</li></ul>	117 117 119 120 120 120 120 121 121 122 122 122
5	4.1 Met 5.1 5.2 5.3 5.4 5.4 5.4 5.5 5.6 5.6 5.6. 5.6. 5.6.	<ul> <li>Mitochondria purification on a discontinuous Percoll gradient</li> <li>thods for protein and protein complex purification, analysis and quantification .</li> <li>Total protein extraction from <i>Arabidopsis thaliana</i> calli</li> <li>Protein quantity determination by a colorimetric protein assay with Coomassie blue</li> <li>Protein sample concentration through a centrifugable molecular cutoff filter</li> <li>Protein expression systems</li> <li>.1 Protein expression in the yeast <i>Saccharomyces cerevisiae</i></li> <li>.2 Cell-free protein expression</li> <li>Protein purification by nickel affinity chromatography</li> <li>.1 Extraction of mitochondrial membrane complexes</li> <li>.2 Size exclusion chromatography</li> <li>.3 Sucrose density gradient ultracentrifugation</li> </ul>	117 117 119 120 120 120 120 121 121 122 122 123 124
5	4.1 Met 5.1 5.2 5.3 5.4 5.4 5.4 5.5 5.6 5.6 5.6. 5.6. 5.6. 5.6.	<ul> <li>Mitochondria purification on a discontinuous Percoll gradient</li></ul>	117 117 119 120 120 120 120 121 121 121 122 122 123 124 124
5	4.1 Met 5.1 5.2 5.3 5.4 5.4 5.4 5.5 5.6 5.6 5.6. 5.6. 5.6. 5.6. 5.6. 5.6.	<ul> <li>Mitochondria purification on a discontinuous Percoll gradient</li></ul>	117 117 119 120 120 120 120 120 121 121 121 122 123 124 124 n125
5	4.1 Met 5.1 5.2 5.3 5.4 5.4 5.4 5.5 5.6 5.6 5.6. 5.6. 5.6. 5.6. 5.6. 5.6. 5.6. 5.6.	<ul> <li>Mitochondria purification on a discontinuous Percoll gradient</li></ul>	117 117 119 120 120 120 120 120 121 121 121 122 123 124 n125 126
5	4.1 Met 5.1 5.2 5.3 5.4 5.4 5.4 5.5 5.6 5.6 5.6. 5.6. 5.6. 5.6. 5.6. 5.6. 5.6. 5.6. 5.7	<ul> <li>Mitochondria purification on a discontinuous Percoll gradient</li> <li>thods for protein and protein complex purification, analysis and quantification</li> <li>Total protein extraction from <i>Arabidopsis thaliana</i> calli</li> <li>Protein quantity determination by a colorimetric protein assay with Coomassie blue</li> <li>Protein sample concentration through a centrifugable molecular cutoff filter</li> <li>Protein expression systems</li> <li>Protein expression in the yeast <i>Saccharomyces cerevisiae</i></li> <li>Cell-free protein expression</li> <li>Protein purification by nickel affinity chromatography</li> <li>MTL complex purification and analysis</li> <li>1 Extraction of mitochondrial membrane complexes</li> <li>2 Size exclusion chromatography</li> <li>3 Sucrose density gradient ultracentrifugation</li> <li>4 Anion exchange chromatography</li> <li>5 Clear native polyacrylamide gel electrophoresis for MTL complex visualizatio</li> <li>6 Cryo-electron microscopy</li> </ul>	117 117 119 120 120 120 120 120 120 121 121 122 122 124 n125 126 126
5	4.1 Met 5.1 5.2 5.3 5.4 5.4 5.4 5.5 5.6 5.6 5.6. 5.6. 5.6. 5.6. 5.6. 5.6. 5.6. 5.6. 5.6. 5.6. 5.8	<ul> <li>Mitochondria purification on a discontinuous Percoll gradient</li></ul>	117 117 119 120 120 120 120 121 121 121 122 122 123 124 n125 126 126 126

6	Lip	oid an	alysis	
	6.1	Gly	cerolipid extraction	
	6.1	.1	Glycerolipid extraction from biological samples by the Folch method	
	6.2	Met	hanolysis of total lipid extracts	129
	6.3	Fatt	y acid and lipid quantification	129
	6.3. dete	.1 ection	Quantification of fatty acid methyl esters by gas chromatography coupled 129	to ion flame
	6.3. to t	.2 riple o	Quantification of glycerolipids by high performance liquid chromatogra quadrupole mass spectrometry (HPLC-MS/MS)	aphy coupled 130
7	Ima	aging	techniques	
	7.1	Las	er scanning confocal microscopy	131

## Part 1 Characterization of the mitochondrial transmembrane

## 

1	The	discovery of the mitochondrial transmembrane lipoprotein (MTL) complex	.135
2	Res	ults	138
	2.1	Rationale	138
	2.2	MTL complex stability in solution	139
	2.3	MTL complex purification by size exclusion chromatography	
	2.4	Redefining the apparent size and hydrodynamic radius of the MTL complex	
	2.5	Sucrose gradient ultracentrifugation combined with size exclusion chromatography	
	2.6	Anion exchange chromatography combined with size exclusion chromatography	.146
	2.7	Proteomic analysis and cryo-electron microscopy	.150
3	Disc	cussion and perspectives	
	3.1	Size of the MTL complex	
	3.2	The MTL complex is divided into different sub-populations	.155
	3.3	Lipids might hold the MTL complex components together	.157
	3.4	Conclusions and perspectives	157

## Part 2 Identification and characterization of AtVPS13M1, a VPS13

homol	og in Arabidopsis thaliana	
1 VI	PS13 proteins – hydrophobic tunnels for bulk lipid transport betwee	n membranes161
1.1	Initial discovery and historical overview	

	1.2 conser	VPS ved t	513s are huge proteins localized at membranes and membrane contact sites and hroughout eukaryotic evolution
	1.3	VPS	13 proteins are lipid transport proteins164
	1.3.	1	Insight on VPS13 lipid transport function from physiological data164
	1.3.2	2	Insight on VPS13 lipid transport function from <i>in vitro</i> and <i>in vivo</i> biochemical data 167
	1.3.	3	Insight on VPS13 lipid transport function from structural data167
	1.3.4	4	Emerging model for VPS13 bulk lipid transport168
	1.4	Org	anization and structure of VPS13 proteins169
	1.4. prot	l eins	Structure of the hydrophobic tunnel and general domain organization of VPS13 169
	1.4.2	2	VPS13 proteins belong to the repeating $\beta$ -groove lipid transport protein family172
	1.5 localiz	VPS ation	513 hydrophobic tunnels are decorated by functional domains that determine their
	1.5.	1	The N-terminal domain anchors VPS13 proteins to the endoplasmic reticulum176
	1.5.2	2	The VPS13 FFAT motifs interact with VAPs in the endoplasmic reticulum176
	1.5.	3	The VAB domain interacts with multiple proteins at organelle surfaces177
	1.5.4	4	The ATG2_C domain forms a bundle of amphipathic helices179
	1.5.: bind	5 ling r	The VPS13 pleckstrin homology-like domains have multiple lipid- and protein- nodes
	1.5.	6	The APT1 region presents affinity for phosphoinositides
	1.5.	7	Non-canonical functional domains
	1.5.	8	Reading the complexity of VPS13 localization determinants
	1.6	VPS	13 lipid transport mechanism and directionality184
	1.7	VPS	13 proteins in plants
2	Res	ults .	
	2.1 possib	Co-i le int	immunoprecipitation using Tom40-HA as bait identifies At4g17140/AtVPS13M1 as a eractant
	2.2	Lon 188	g read sequencing reveals AtVPS13M1 real splice variant composition and abundance
	2.3	AtV	PS13M1 subcellular localization193
	2.3.	1	Different regions of AtVPS13M1 protein localize to the endoplasmic reticulum194
	2.3.2	2	Anti-AtVPS13M1 polyclonal antibody production198
	2.4 BiFC	Ana 199	lysis of AtVPS13M1 interactions with other proteins of interest using rBiFC and classic
	2.5	Inve	estigation of AtVPS13M1 in vitro activity
	2.5.	1	AtVPS13M1 binds glycerolipids in vitro
	2.6	Iden	tification of four AtVPS13M1 T-DNA insertion knock out mutants

2.7	AtVP	S13M1 partic	ipates in Ar	rabidopsis lipid homeo	stasis		207
2.8	AtVP	S13M1 muta	nt phenotyp	e analysis			211
2.8	8.1 A	tVPS13M1	olants grow	th exhibit no growth pl	nenotype		211
2.8	8.2 0	old stress					211
2.8	8.3 F	ollen tube ge	rmination				212
3 Di	scussion	and perspec	tives				214
3.1	AtVP	S13M1 gene	holds an un	expected diversity of s	plice variants		214
3.2	AtVP	S13M1 subce	llular locali	ization remains elusive			215
3.3 home	AtVP: eostasis <i>i</i>	513M1 bind <i>n vivo</i>	s multiple	phosphoglycerolipids	<i>in vitro</i> and	participates	in lipid 218
3.4	atvpsl	3m1 mutant	plants phen	otype analysis			223
3.5	Concl	uding remark	s and future	e prospects			224
Gener	al con	clusion a	and pers	spectives			227
Refere	ences	•••••					233

Scientific publications and communications		
1	Scientific publications	
2	Scientific communications	

## Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
aa	Amino acid
AAC1/2/3	ADP/ATP carrier 1/2/3
AAPT1/2	Aminoalcohol phosphotransferase
ABC	ATP binding cassette
ACP	Acyl carrier protein
ADK1	Adenylate kinase 1
ADP	Adenosine diphosphate
AEC	Anion exchange chromatography
AI	Artificial intelligence
ANT	Adenine nucleotide translocase
APS	Ammonium persulfate
APT1	Aberrant pollen transmission 1
Arf1	ADP-ribosylation factor
AsmA	Assembly suppressor mutation A
At	Arabidopsis thaliana
ATG2/9	Autophagy related 2/9
ATG2_C	Autophagy related 2 C-terminus homology domain
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BAP31	B cell receptor-associated protein 31
BiFC	Bimolecular fluorescence complementation
BLTP	Bridge-like lipid transport protein
<b>BN-PAGE</b>	Blue native polyacrylamide gel electrophoresis
BSA	Bovine serum albumin
CaMV 35S	Cauliflower Mosaic Virus 35S promoter
CCT1/2	Phospho-choline cytidyltransferase
cDNA	Coding deoxyribonucleic acid
CDP	Cytidine diphosphate
CDP-Cho	Cytidine diphosphate choline
CDP-DAG	Cytidine diphosphate diacylglycerol
CDP-DAGS	Cytidine diphosphate diacylglycerol synthase
CDP-Etn	Cytidine diphosphate ethanolamine
Chorein_N	Chorein N-terminus homology domain
CL	Cardiolipin
CLS	Cardiolipin synthase
CN-PAGE	Clear native polyacrylamide gel electrophoresis
CoA	Coenzyme A
Col0	Colombia-0 Arabidopsis thaliana ecotype
COXII	Cytochrome c oxidase subunit 2
CPN10	10 kDa chaperonin
CPY	Carboxypeptidase Y
Cryo-ET	Cryo-electron tomography
Csf1	Cold sensitive for fermentation

Ct	Chaetomium thermophilum
CTAB	Cetyltrimethylammonium bromide
CTP	Cytidine triphosphate
Da	Dalton
DAG	Diacylglycerol
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
Dd	Dictyostelium discoideum
DDM	n-dodecyl-β-D-maltoside
DEPC	diethyl pyrocarbonate
DGD1/2	Digalactosyl diacyglycerol synthase 1/2
DGDG	Digalactosyl diacylglycerol
DGPS	Diphosphatidylglycerol synthase
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonucleic acid nuclease
dNTP	Deoxyribonucleotide triphosphate
DPG	Diphosphatidylglycerol
DSP	Dithiobis(succinimidyl propionate)
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
DUF	Domain of unknown function
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EM	Electron microscopy
EMC	ER membrane complex
EMCS	Endoplasmic reticulum-mitochondria contact site
ER	Endoplasmic reticulum
ERMES	Endoplasmic reticulum mitochondria encounter structure
ERMIONE	ER-mitochondria organizing network
ESCRT	Endosomal sorting complexes required for transport
E-Syt	Extended synaptotagmin
FA	Fatty acid
FAME	Fatty acid methyl esters
FAS	Fatty acid synthase
FFA	Free fatty acid
FFAT	Two phenylalanines in an acidic tract
FIS1	Mitochondrial fission 1
Fmp27	Found in mitochondrial proteome 27
FPLC	Fast protein liquid chromatography
FRET	Fluorescence resonance energy transfer
Fzo1	Fuzzy Onions homolog 1
G3P	Glycerol-3-phosphate

GC-FID	Gas chromatography coupled to ion flame detection
gDNA	Genomic deoxyribonucleic acid
GDP	Glycerophosphodiester
GDPD	Glycerophosphodiester phosphodiesterase
Gem1	GTPase EF-hand protein of mitochondria 1
GFP	Green fluorescent protein
GPAT	Glycerol-3-phosphate acyltransferase
GPI	Glycosylphosphatidylinositol
GRP75	75-kDa glucose-regulated protein
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
h	hour
HDX-MS	Hydrogen-deuterium exchange mass spectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIC	Hydrophobic interaction chromatography
Hob1/2	Hobbit 1/2
HOPS	Homotypic fusion and protein sorting complex
HPLC-MS/MS	High performance liquid chromatography coupled to triple quadrupole mass
	spectrometry
HRP	Horseradish peroxydase
Hs	Homo sapiens
iBAQ	Intensity-based absolute quantification
IE	Chloroplast inner envelope membrane
IM	Mitochondrial inner membrane
IP3R	Inositol 1,4,5-trisphosphate receptors
kb	Kilobase
KD	Knock down
kDa	Kilodalton
KIP	Kinky pollen
КО	Knock out
LAH	Lipid acyl hydrolase
Lam4/6	Lipid transfer protein anchored at membrane contact sites 4/6
LB	Lysogeny broth
LD	Lipid droplet
LECA	Last eukaryotic common ancestor
LPAAT	Lysophosphatidic acid acyltransferase
LPS	Lipopolysaccharide
LPT	Lipopolysaccharide transporter
LPTD1	Lipopolysaccharide transport-like protein 1
LTP	Lipid transport protein
MALLS	Multi-angle laser light scattering
Mcp1	Mdm10 complementing protein 1
MCS	Membrane contact site
Mdm	Mitochondrial distribution and morphology
MELL1	Mitochondria-ER localized LEA-related LysM domain protein 1
MES	2-(N-morpholino)ethanesulfonic acid

METALIC	Mass tagging-enabled tracking of lipids In cells
MFN1/2	Mitofusin 1/2
MGD1/2/3	Monogalactosyl diacyglycerol synthase 1/2/3
MGDG	Monogalactosyl diacylglycerol
Mgm1	Mitochondrial genome maintenance 1
MIB	Intermembrane space bridging complex
MIC60	60 kDa subunit of the mitochondrial contact site and cristae organizing system
MICOS	Mitochondrial contact site and cristae organizing system
MIGA	Mitoguardin
min	minute
MIRO1/2/3	Mitochondrial Rho GTPase 1/2/3
Mm	Mus musculus
Mmm1	Maintenance of mitochondrial morphology 1
Mmr1	Mitochondrial Myo2p receptor-related 1
MOPS	3-(N-morpholino)propanesulfonic acid
MOSPD2	Motile sperm domain containing 2
mRNA	Messenger ribonucleic acid
MS	Murashige & Skoog
MSP	Major sperm protein
mtCK	Mitochondrial creatine kinase
MTL	Mitochondrial transmembrane lipoprotein
Mtx	Metaxin
Nb	Nicotiana benthamiana
NBD	Nitrobenzoxadiazole
NDPK	Nucleoside diphosphate kinase
NMR	Nuclear magnetic resonance
NPC	Non-specific phospholipase C
nsLTP	Non-specific lipid transport protein
NTA	Nitriloacetic acid
NVJ	Nuclear vacuole junction
OD	Optical density
OE	Chloroplast outer envelope membrane
OM	Mitochondrial outer membrane
OMP24	24 kDa outer membrane protein
ORP	Oxysterol-binding protein (OSBP) related proteins
OSBP	Oxysterol-binding protein
Osh	Oxysterol-binding protein homolog
OXPHOS	Oxidative phosphorylation
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PAP	Phosphatidic acid phosphatase
P-Cho	Phospho-choline
PCR	Polymerase chain reaction
PDB	Protein databank
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), Zonula occludens-1 protein (zo-1)

PDZD8	PDZ domain-containing protein 8
PE	Phosphatidylethanolamine
PEAMT	Phospho-ethanolamine methyltransferase
PECT1	Phospho-ethanolamine cytidyltransferase
PEG	Polyethylene glycol
PEP9	Peptidase 9
PERK	Protein kinase RNA-like ER kinase
P-Etn	Phospho-ethanolamine
PG	Phosphatidylglycerol
PGM	Pollen germination medium
PGP	Phosphatidylglycerol phosphate
PGP1	Phosphatidylglycerol phosphate synthase 1
PGPP1	Phosphatidylglycerol phosphate phosphatase 1
PH	Pleckstrin homology domain
Pi	Phosphate
PI	Phosphatidylinositol
PI(4,5)P <sub>2</sub>	Phosphatidylinositol 4,5 diphopshate
PIP	Phosphatidylinositol phosphate
PI-PLC	Phosphatidylinositol phospholipase C
PLC	Phospholipase C
PLD	Phospholipase D
PM	Plasma membrane
PMD2	Peroxisomal and mitochondrial division factor 2
PMSF	Phenylmethylsulfonyl fluoride
PRELI	Protein of relevant evolutionary and lymphoid interest domain
PRELID	PRELI domain-containing protein
PS	Phosphatidylserine
PSD1/2/3	Phosphatidylserine decarboxylase 1/2/3
PVP25	Polyvinylpyrrolidone 25
PxP	Proline-x-proline
QC	Quality control
RBG	Repeating β-groove
rBiFC	Ratiometric bimolecular fluorescence complementation
RFP	Red fluorescent protein
RISP	Rieske iron-sulfur protein
RNA	Ribonucleic acid
RNAse	Ribonucleic acid nuclease
SAM	Sorting and assembly machinery
SATAY	Saturated transposon analysis in yeast
Sc	Saccharomyces cerevisiae
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
sec	second
Sec14	Secretory 14
SHR	SHORT ROOT
SHR-BD	SHORT ROOT-binding

SMP	Synaptotagmin-like mitochondrial-lipid binding protein
sn	Stereospecific numbering
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
SOI1	Suppressor of the rapid onset of impotence 1
Spo71	Sporulation 71
SQDG	Sulfoquinovosyl diacylglycerol
ssDNA	Simple strand deoxyribonucleic acid
StARD	Steroidogenic acute regulatory protein
StART	Steroidogenic acute regulatory protein-related transfer
SYNJ2BP	Synaptojanin-2-binding protein
TAG	Triacylglycerol
TAIR	The Arabidopsis Information Resource
TamB	Translocation and assembly module B
Taq	Thermus aquaticus
TBS	Tris buffer saline
T-DNA	Transfer deoxyribonucleic acid
TEMED	Tetramethylethylenediamine
TGD	Trigalactosyl diacylglycerol complex
TIC	Translocon of the inner chloroplast membrane
TIM	Translocase of the inner mitochondrial membrane
TM	Transmembrane
TMEM41B	Transmembrane protein 41B
TOC	Translocon of the outer chloroplast membrane
TOM	Translocase of the outer mitochondrial membrane
TRB1	TraB family protein 1
TRIAP	TP53-regulated inhibitor of apoptosis 1
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
TSG101	Tumor susceptibility gene 101
Tt	Tetrahymena thermophila
U	Enzyme unit
UBA	Ubiquitin associated domain
Ugo1	Ugo (Japanese for fusion) 1
Ups	Unprocessed
UV	Ultraviolet
V	Volt
VAB	VPS13 adaptor binding domain
VAD1	Vascular associated death1
VAMP	Vesicle-associated membrane protein
VAPA/B/27	Vesicle-associated membrane protein-associated protein A/B/27
vCLAMP	Vacuole and mitochondria patch
VDAC	Voltage dependent anion channel
VMP1	Vacuole membrane protein 1
vpl	Vacuolar protein localization
VPS13/39/62	Vacuolar protein sorting 13/39/62
vpt	Vacuolar protein targeting

VPT2	Vacuolar protein targeting 2
WT	Wild type
WWE	Tryptophan-Tryptophan-Glutamate motif-containing domain
XK	X-linked Kx blood group antigen
YFP	Yellow fluorescent protein
YPD	Yeast extract peptone dextrose
Ypt7/35	Yeast protein two 7/35

**General introduction** 

# 1 Endosymbiotic organelles in plants: origin, functions and biogenesis

Eukaryotic cells are divided into multiple compartments delineated by one or multiple membranes. Compartmentalization is essential to divide the cell in a multitude of physico-chemical environments allowing the separation of chemical reactions and biological processes demanding specific conditions, which would otherwise be incompatible. While separating a bigger space in smaller entities is convenient to precisely regulate a set of processes occurring in a confined space, compartmentalization comes with a price: the exchange of molecules between organelles is more complex and requires the establishment of specific transport routes.

The evolutionary path leading to the development of a complex intracellular structure will probably remain forever mysterious as no experiment can reproduce in the present what happened in the past. However, observing contemporary organisms reveals numerous clues on events that might have happened. The acquisition of mitochondria and plastids by two independent endosymbiotic events has been extensively studied and can be considered an established fact (Maréchal, 2018; Martin et al., 2015). How the endosymbionts could be integrated in such a complex membrane system as the eukaryotic cell is a question that has not yet reached a consensus as multiple convincing models have been presented (Martin et al., 2015). The inside-out scenario is a particularly interesting one because it simultaneously explains the incorporation of endosymbionts and the appearance of the endomembrane system (Baum & Baum, 2014) (Figure 1). In this scenario, the ancestor of eukaryotic cells, which was devoid of nuclear membrane, engaged into mutualistic associations with the endosymbiont ancestor (Figure 1A). To expand the mutualistic contacts, the proto-eukaryote projected membrane protrusions that progressively engulfed the proto-endosymbiont (Figure 1B). The process continued until the proto-endosymbiont was completely surrounded by the host membrane leaving just a narrow cleft for it to communicate with the outside (Figure 1C). The fusion of the protrusions then completely sealed off the endosymbionts creating a new plasma membrane (Figure 1D). The clefts between the protrusions likely became the endoplasmic reticulum (ER), which is continuous with the ancient plasma membrane that has become the nuclear envelope and gave rise to the whole endomembrane system. The eukaryotic cell can therefore be divided into two types of compartments of distinct origins: the endomembrane system and the endosymbiotic organelles, mitochondria and plastids.

Despite their extrinsic origin, plastids and mitochondria have evolved numerous indispensable functions for eukaryotic life, which likely constituted the key for their successful incorporation. Plastids are most known for their capacity to perform photosynthesis. The photosynthetic membranes harvest light energy to convert it to chemical energy that is used to fix atmospheric carbon into sugar molecules, thus laying the foundation for photoautotrophic life. But they also accomplish numerous other important cellular functions including synthesis of vitamins and pigments or starch storage (Rolland et al., 2018). Plastids



**Figure 1** Inside-out scenario for the origin of the endomembrane system and endosymbiotic organelles (Baum & Baum, 2014). Figure inspired by Baum & Baum, 2014 and Kornmann, 2020. (A) An ancestor of eukaryotic cells engages into mutualistic interactions with the ancestor of an endosymbiont (proto-endosymbiont). (B) The eukaryote ancestor develops protrusions to expand and tighten the mutualistic interactions with the proto-endosymbiont. (C) The protrusions have developed all around the proto-endosymbiont so as to totally wrap around it. A narrow cleft (conduit) remains between the protrusions allowing the proto-endosymbiont to interact with the environment. (D) The protrusions have fused leading to the final incorporation of the endosymbionts. The cleft has become the endoplasmic reticulum which is continuous with the nuclear envelope and gives rise to the endomembrane system.

are also of the utmost importance for membrane biogenesis in plant cells since fatty acids are synthetized in the stroma and since they are the only site of galactolipid synthesis (Li-Beisson et al., 2013). Mitochondria are the central compartment of cell bioenergetics. The mitochondrial respiratory chains use chemical energy stored in diverse biomolecules including sugars and amino acids to create a proton motive force that can be converted to usable energy in the form of ATP. Mitochondria also play major roles in calcium signaling, apoptosis and redox balance (Spinelli & Haigis, 2018). Additionally, mitochondria are important metabolite synthesis hubs including nucleotides, amino acids, vitamins and some lipids (Spinelli & Haigis, 2018).

Given their extrinsic origin, neither mitochondria nor plastids are connected to the endomembrane system. However, vesicles budding from mitochondria have been observed and seem to play a physiological role (McLelland & Fon, 2018) and glycosylated proteins seem to transit from the Golgi complex to plastids through vesicles (Baslam et al., 2016). Despite the existence of vesicular trafficking

implying mitochondria and plastids, this process likely accounts for a very low fraction of the transport required for their biogenesis and maintenance. Additionally, mitochondria and plastids produce important metabolites implied in numerous cellular processes that have to be exported to the cytosol. Furthermore, both mitochondria and plastids are delineated by two membranes, likely derived from their bacterial ancestors. Hence, cells developed ingenious systems to allow the exchange of sugars, lipids, proteins and other metabolites with mitochondria and plastids.

The biogenesis and maintenance of organelles necessitates a tight regulation of membrane dynamics and a tremendous input of biomolecules. Plastids and mitochondria are semi-autonomous organelles. While organelles belonging to the endomembrane system can be regenerated from the ER when damaged or lost during cell division, endosymbiotic organelles can only be derived from the same compartment type by division. Thus, mitochondria and plastid division processes are carefully and tightly regulated. Both plastids and mitochondria have retained the capacity to divide autonomously using their own division machinery regulated by the physiological state of the cell (Yoshida, 2018). Both endosymbiotic organelles also possess a residual genome able to replicate on its own and that still encodes several active proteins. Most of the plastidial and mitochondrial proteins, however, are encoded by genes that have been transferred to the nuclear genome in the course of evolution. Thus, mitochondria and plastid biogenesis requires a huge import of nuclear-encoded proteins through dedicated protein import systems. Import of proteins in mitochondria and plastids are well-studied and well-understood processes. Proteins targeted to mitochondria or plastids carry specific sequences allowing their recognition by the respective import machineries (Michaud & Duchêne, 2012). Proteins targeted to mitochondria transit through the TOM, TIM and SAM machineries to attain the mitochondrial matrix or to be inserted in the OM or IM (Murcha et al., 2014). Similarly, proteins targeted to plastids are transported by the TOC and TIC machinery (Richardson & Schnell, 2020).

Lipids are hydrophobic or amphiphilic molecules mostly insoluble in water. In contrast to other biological molecules, lipids are defined by their physical properties rather than by their structure. In fact, the molecules considered as lipids present a huge structural diversity. Mitochondrial and plastidial membrane compositions are widely dominated by glycerolipids, even though they also contain trace amounts of sterols. Therefore, this study focuses on the biosynthesis and transport of glycerolipids. The lipid composition of a biological membrane is of utmost importance to define its identity (Casares et al., 2019), integrity and biophysical properties (Ibarguren et al., 2014; Maulucci et al., 2016). Thus, the membrane lipid homeostasis of the different cellular compartments is highly regulated by intricately linked processes including lipid biosynthesis, lipid modifications and turnover, lipid degradation and lipid transport. The lipid synthesis activity is particularly unevenly distributed among organelles. In plant cells, the chloroplast is the predominant site of fatty acid synthesis. While the ER is the main site of phosphoglycerolipid synthesis, the chloroplast is the only organelle sustaining galactoglycerolipid synthesis (see **section 3**). Mitochondria, on the contrary, have only retained a very limited lipid synthesis

capacity (see section 4.2). Similarly, lipid degradation by  $\beta$ -oxidation and their turnover are confined to specific organelles or membrane domains. These highly disproportionate lipid synthesis and processing capacities between organelles highlight the absolute necessity for lipid transport between the cell compartments in membrane homeostasis. In contrast to the already extensively studied protein targeting mechanisms, the transport of lipids to and from plastids and mitochondria is much less characterized. Two main types of mechanism can mediate lipid transport. Vesicular transport implies the budding of a membrane domain from a donor organelle, its transport through the cytoplasm and its fusion with an acceptor membrane. While this mechanism takes place extensively between endomembranous organelles, it is almost absent in endosymbiotic organelles and is inconsistent with the tremendous amounts of lipids that have to be transported to and from plastids and mitochondria. Non-vesicular lipid transport implies the direct transport of a lipid molecule from one membrane to another through the cytosol. Given the hydrophobic nature of lipids, non-vesicular lipid transport is facilitated by lipid transport proteins (LTPs). To reduce the distance of transport and allow the direct channeling of lipid molecules from one compartment to another, non-vesicular lipid transport takes place at sites at which both the donor and acceptor membranes are in close apposition, named membrane contact sites (MCSs). The concepts of membrane contact site and non-vesicular lipid transport will be addressed in the two following sections.

#### 2 Membrane contact sites

The eukaryotic cell is divided into multiple organelles each presenting a particular biochemical environment allowing the compartmentalization and hence the regulation of specific biological processes. This compartmentalization has long held the view that each cell compartment performs its own biological function disconnected from the other organelles and that the metabolites and other signaling molecules travelled long distances to create interactions between the cell compartments (Scorrano et al., 2019). Even though the first descriptions of membrane contact sites date back to the 1950s (Bernhard & Rouiller, 1956; Copeland & Dalton, 1959), it is not until the 1990s that MCSs have been described as cellular entities in their own right carrying out specific functions (Rizzuto et al., 1998; Vance, 1990). Since their discovery, the definition of membrane contact sites was rather unclear and mostly based on the distance between the membranes of adjacent organelles measured on micrographs. However, organelles could simply appear in close proximity by chance, especially in plant cells where the vacuole takes up all the available space and compresses the other organelles at the cell periphery.

In 2019, Scorrano et al. gave a clear definition of MCSs in which the presence of tethering and functional effectors surpass the mere distance of membrane apposition (Scorrano et al., 2019). First, two apposed membranes form a contact site if they are maintained in close proximity (usually between 10 nm and 30 nm but sometimes up to 80 nm) by tethering forces. MCS tethers are usually proteins or protein complexes that use protein-protein or protein-lipid interactions to generate the required tethering force.

Defining a MCS tether is a challenging task as the overall tethering force may result from the combined interactions of multiple proteins (Eisenberg-Bord et al., 2016). Furthermore, most MCS functional effectors may as well contribute to tethering. Second, membranes forming a functional MCS are not fusing. This excludes the proximity of membranes during vesicular trafficking events. Importantly, MCSs must carry out a specific function. This condition allows to consider MCSs as distinguishable cellular entities with crucial physiological/biochemical roles. All organelles are able to engage into contacts with others to exchange metabolites, to allow the synthesis of components necessitating a particular enzymatic or biochemical environment or for signal transduction. It is important to keep in mind that many MCS effectors may have different functions when located at contact sites than when located elsewhere. Finally, MCSs harbor a defined proteome and/or lipidome. Even though the composition of a MCS may vary over time or depending on the cell physiological condition, specific tethers, effectors and membrane composition characterize a MCS, likely to create a biochemically unique environment in which particular processes can be carried out without disturbing the rest of the cellular environment.

Since their discovery, an increasing number of cellular functions have been assigned to membrane contact sites. Briefly, functions of MCSs can be divided in four main categories (Prinz et al., 2020): metabolic channeling, cell signaling, regulation of membrane dynamics and lipid transport. Metabolic channeling at MCSs allows to directly pass on metabolites between enzymes or enzyme complexes located in different cell compartment, hence increasing the efficiency of a metabolic route and minimizing off-pathway reactions. The proximity of two or more organelles also facilitates the transmission of various signals or can be used as a platform to assemble specific protein and/or lipid environments in response to extra- or intracellular stimuli. For example, Ca<sup>2+</sup> signaling takes place at ER-mitochondria and ER-plasma membrane contact sites to efficiently transduce stress signals. MCSs also contribute to organelle membrane dynamics for example by marking sites of organelle fission and fusion, by influencing the position of organelles in the cell or by regulating autophagy. Finally, MCSs are sites at which non-vesicular lipid transport takes place and is regulated.

The general functions of MCSs are thought to be conserved in plants (Pérez-Sancho et al., 2016). While in yeast and mammals the field of MCS research is starting to get a complete picture of the existing organelle contacts (Gatta & Levine, 2017; Prinz et al., 2020; Wenzel et al., 2022), plant MCSs have been much less investigated. Many concepts and plant MCS effectors have been inferred from other eukaryotes by homology (Pérez-Sancho et al., 2016). However, plant cells harbor some specific organelles or cell structures, like chloroplasts and plasmodesmata, increasing the complexity and the possibility of MCSs formation and functions. Plasmodesmata are tight openings between neighboring cells allowing cytoplasmic continuity and communication. Plasmodesmata also allow the passage of ER tubules that form ER-plasma membrane contact sites directly in the plasmodesmal conduit (Tilsner et al., 2016). These plasmodesmal ER-plasma membrane contact sites play crucial roles in the regulation of cell-to-cell communication (Tilsner et al., 2016). Chloroplasts form a plant-specific compartment that is known to interact with almost all the other cell compartments and fulfill a vast array of functions ranging from lipid neosynthesis to nucleus signaling or positioning in response to light (Mueller-Schuessele & Michaud, 2018). Interestingly, plastids are known to form stroma-filled protrusions called stromules (stroma-filled tubules) that are able to form contact sites with other organelles, including mitochondria (Kwok & Hanson, 2004; Schattat et al., 2011). Another important aspect of plant MCSs is their intimate link to stress response (Michaud & Jouhet, 2019; Mueller-Schuessele & Michaud, 2018; Pérez-Sancho et al., 2016). Upon biotic stress, high light or oxidative stress, chloroplast stromules contact the nucleus, presumably to favor stress signal transduction. Light stress further induces the tight association between chloroplasts, peroxisomes and mitochondria presumably to promote photorespiration (H. Gao et al., 2016; Oikawa et al., 2015). Mitochondria have also been observed to directly associate to chloroplasts upon phosphate starvation (see section 5), presumably to promote digalactosyl diacylglycerol (DGDG) non-vesicular transport to mitochondria (Jouhet et al., 2004). Like in yeast and mammals, plant MCSs are sites of non-vesicular lipid transport (Leterme & Michaud, 2022). Beside ER-mitochondria contact sites, ER-chloroplast contact sites form a highly active lipid transport platform indispensable for plant lipid biosynthesis (see section 3).

## 3 Non-vesicular glycerolipid transport in plant cells

This section is composed of the fourth chapter of Advances in Botanical Research, volume 101 (Leterme & Michaud, 2022). This book chapter starts with an overview on the diversity and nomenclature of glycerolipids as well as an introduction to their neosynthesis in plant cells. After a concise introduction on membrane contact sites, non-vesicular lipid transport and the general features of lipid transport proteins, the focus is laid on the thorough description of LTPs present in plants by analogy with what is known in yeast and mammals. For each type of LTP, the general protein organization and available physiological data in plants are described. For each LTP family, a table gathering the known or predicted representatives in plants as well as their general protein domain organization is given.

We classified lipid transport proteins according to their lipid transport domain and/or lipid transport mechanism (**Figure 2**). The first part describes LTPs with known lipid transport domains. The second part focuses on enzymes of the lipid metabolism and flippases that are known to be important for lipid trafficking while the last part describes lipid transport protein complexes.

A section of this book chapter is dedicated to VPS13-like proteins. At the time of writing (spring 2021), knowledge on plant VPS13 proteins as well as on other VPS13-like proteins was scarce. VPS13 proteins and other repeating  $\beta$ -groove proteins are thoroughly discussed in **part 2** of this manuscript.



**Figure 2** Schematic representation of the Leterme & Michaud, 2022 ABR book chapter organization. Protein structures are represented on the right as an example for the different lipid transport protein classes if available.

## Non-vesicular glycerolipids transport in plant cells

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## Contents

1.	I. Introduction	
	1.1 Glycerolipid synthesis in plant cells	122
	1.2 Non-vesicular glycerolipid transport in plant cells	124
2.	Lipid transport mediated by LTPs	128
	2.1 The SMP family	129
	<b>2.2</b> ORP	143
	2.3 VPS13-like protein family/Chorein_N domain-containing proteins	147
	2.4 The Sec14-superfamily	150
	<b>2.5</b> nsLTPs	156
3.	Lipid transport mediated by other proteins	158
	3.1 NDPK-D	158
	3.2 Enzymes involved in lipid metabolism	161
	3.3 Enzymes flipping lipids between membrane leaflets	163
4.	Lipid transport mediated by protein complexes	164
	4.1 The TGD complex	164
	4.2 The MTL complex	167
5.	Conclusion and perspectives	168
Fu	Fundings	
Re	ferences	170

#### Abstract

In Eukaryotic cells, organelles are delineated by one or several membranes that are composed of a specific assembly of lipids and proteins ensuring the identity of each cell compartment and regulating their biogenesis and function. Lipids regulate many aspects of cell biology including vesicular trafficking, protein targeting, programmed cell death or signaling. They also play key roles in response to stress, which is particularly important for organisms such as plants that constantly cope with a multitude of environmental variations. Membranes are mainly composed of glycerolipids, sterols and sphingolipids. Lipid precursors are primarily synthesized in plastids and in the endoplasmic reticulum. Then, they have to be specifically directed to other cell compartments where they can be modified and transported to other organelles to regulate membrane

biogenesis and function. Thus, lipid transfer is a key process involved in the maintenance of membrane homeostasis, in particular in stress conditions. Lipid exchanges between organelles can rely on vesicular or non-vesicular transport. Whereas vesicular transport concerns mainly organelles connected to the endomembrane system, such as the Golgi, vacuoles or the plasma membrane, non-vesicular lipid trafficking concerns virtually all organelles. This lipid transfer mechanism is thought to occur at membrane contact sites, corresponding to specific cell structures formed by the close apposition of two membranes at a distance  $\leq$ 30 nm. In this chapter, we will summarize our current knowledge about the mechanisms involved in non-vesicular lipid transport in plant cells, with a particular focus on glycerolipids, the main components of organelle membranes.

## 1. Introduction

## 1.1 Glycerolipid synthesis in plant cells

Glycerolipids are composed of a glycerol backbone esterified with two fatty acids (FAs) in position sn-1 and sn-2 (Fig. 1A). FAs are defined by the length of their carbon chain and the number of double bonds (or unsaturations). In Arabidopsis thaliana, glycerolipid acyl-chains are mainly composed of 16 or 18 carbons (C16, C18) with up to three unsaturations (C16:0 to C16:3 and C18:0 to C18:3) (Li-Beisson et al., 2013). The nature of the polar head associated to the position sn-3 will define the class of the glycerolipid molecule. Three main classes are found in plants: (1) the phospholipids, containing a phosphate (Pi) moiety, (2) the galactoglycerolipids, containing one or two galactose residues for the monogalactosyldiacylglycerol (MGDG) or the digalactosyldiacylglycerol (DGDG), respectively, and (3) the sulfolipid sulfoquinovosyldiacylglycerol (SQDG), containing a sulfoquinovosyl group in its polar head (Fig. 1B). Galactoglycerolipids and SQDG are the main constituents of plastid membranes, where they play a major structural and functional role in photosynthesis, whereas phospholipids are the main glycerolipids found in extra-plastidial membranes. Enzymes involved in the synthesis of galactoglycerolipids, SQDG and of plastidial phosphatidylglycerol (PG) are localized in plastid envelopes (Boudière et al., 2014; Li-Beisson et al., 2013). Enzymes involved in phospholipids synthesis are mainly located in the endoplasmic reticulum (ER) and some are also found in mitochondria or in the endomembrane system (Li-Beisson et al., 2013; Michaud, Prinz, & Jouhet, 2017). In plants, FA biosynthesis occurs in the plastid stroma and gives rise to C16:0-acyl carrier protein (ACP) and C18:1-ACP (Li, Kabbage, Liu, & Dickman, 2016; Li, Xu,



**Fig. 1** Glycerolipid families found in plant cells. (A) Glycerolipids are composed of a glycerol backbone esterified with two fatty acids (FA) in *sn-1* and *sn-2* positions. In *Arabidopsis thaliana*, the main FAs found in glycerolipids are 16 (C16) or 18 (C18) carbons-long and have up to three unsaturations (C16:0 to C16:3 and C18:0 to C18:3). In *A. thaliana*, the length of the carbon chain in *sn-2* position defines the origin of the glycerolipid backbone: a C16 is a signature of a backbone synthesized by the plastid by the Prokaryotic pathway while a C18 is a signature of a backbone synthesized in the endoplasmic reticulum by the Eukaryotic pathway. (B) Three main glycerolipid families are found in plants depending on the nature of the polar head located in *sn-3* position of the glycerol backbone: (1) the phospholipids, all presenting a phosphate group in their polar head, (2) the galactoglycerolipids, having a polar head containing one or two galactose residues, and (3) the sulfoglycerolipid, with a sugar-based polar head containing a negatively charged sulfate group.

Li-Beisson, & Philippar, 2016). These acyl-chains can then be directly used to feed the synthesis of plastidial lipids by the so-called Prokaryotic pathway or they can be exported in the cytosol and integrated into glycerolipids in the ER by the so-called Eukaryotic pathway (LaBrant, Barnes, & Roston, 2018; Li-Beisson et al., 2013). These two pathways lead to the biogenesis of phosphatidic acid (PA) and diacylglycerol (DAG), two precursor molecules that will be used to synthesize the whole set of plant glycerolipids (Fig. 1). Importantly, after their synthesis, glycerolipid molecules can be subjected to a wide variety of modifications, such as acyl-chain remodeling or polar head modifications, combined to their transport to different cell compartments or translocation from one membrane leaflet to another. The vast variety of pathways involved in these processes highlights the complexity of maintaining membrane lipid homeostasis.

## 1.2 Non-vesicular glycerolipid transport in plant cells 1.2.1 Where does non-vesicular glycerolipid transport occur in plant cells?

Lipid transport can be mediated by vesicular or non-vesicular pathways. The vesicular pathway involves the transport of lipids, as well as proteins and soluble molecules, in vesicles that will emerge from a donor organelle, be transported through the cytosol and finally fuse with the acceptor organelle to deliver their content. This trafficking route concerns organelles connected to the endomembrane system, such as the ER, the vacuole, the Golgi or the plasma membrane (PM) (Cevher-Keskin, 2020). In plants, vesicular transport also occurs in plastid stroma where this process is involved in the synthesis of thylakoids (Lindquist & Aronsson, 2018). Non-vesicular lipid transport corresponds to the direct transfer of lipid molecules from one membrane to another. This process is involved in the biogenesis of virtually all organelles, but those not connected to the vesicular transport pathway, like mitochondria or plastids, are completely dependent on this lipid transport route to ensure their biogenesis and function (Bayer, Sparkes, Vanneste, & Rosado, 2017; Hurlock, Roston, Wang, & Benning, 2014; LaBrant et al., 2018; Michaud et al., 2017). Non-vesicular lipid transport is thought to occur at membrane contact sites (MCSs), corresponding to particular cell structures formed by the close apposition of two membranes at a distance  $\leq 30 \,\mathrm{nm}$  (Michaud & Jouhet, 2019; Petit, Immel, Lins, & Bayer, 2019; Scorrano et al., 2019). The short distance between the two membranes at MCSs is supposed to decrease the soluble interface that amphiphilic lipid molecules have to cross from the donor to the acceptor membranes. Three types of proteins can be found at MCSs: (1) the tethers, which are proteins able to bridge and maintain the two membranes in close apposition, (2) the effectors, corresponding to proteins providing functionality to the MCSs, and (3) proteins that share both functions (Bohnert & Schuldiner, 2018; Prinz, Toulmay, & Balla, 2020; Scorrano et al., 2019). Beside lipid transport, MCSs play essential roles in many cellular processes including ion exchanges, programmed cell death, organelle division, and appear now as cellular hubs for the regulation of organelle biogenesis and function and cell signaling (Michaud & Jouhet, 2019; Prinz et al., 2020; Scorrano et al., 2019). Contact sites between many organelles have been observed in plants, but we are just starting to understand their role and importance. Plant MCSs are highly dynamic and regulated during development and in response to many biotic and abiotic stresses (Liu & Li, 2019; Mathur, 2020; Michaud & Jouhet, 2019). In this chapter, we will mainly focus on their role in plant lipid transport. For recent reviews about plant MCSs, see Liu and Li (2019), Michaud and Jouhet (2019), Mueller-Schuessele and Michaud (2018), Perez-Sancho et al. (2015), and Petit et al. (2019).

In plants, lipid transport could virtually occur between all organelles. However, the currently available information about the transported molecules and the mechanisms involved are scarce. By analogy to yeast and animal systems, it can be suspected that huge lipid transport between the ER and mitochondria and the ER and the PM occurs, but direct proofs of such transport and the nature of the transported lipids are still missing (Michaud et al., 2017; Perez-Sancho et al., 2016; Petrungaro & Kornmann, 2019; Saheki & De Camilli, 2017a). The most investigated non-vesicular lipid trafficking route in plants corresponds to the ER to chloroplast transport of lipid precursors to synthesize plastid lipids. As described above, plastids have the ability to synthesize *de novo* lipid precursors by the Prokaryotic pathway and to import precursors synthesized in the ER from the Eukaryotic pathway. Due to the specificity of the plastidial and ER sn-2acyltransferases, glycerolipids arising from both pathways have an acyl-chain signature at the sn-2 position corresponding to a C16 or a C18 for the glycerolipids arising from the Prokaryotic or the Eukaryotic pathways, respectively (Fig. 1A) (Hurlock et al., 2014; LaBrant et al., 2018). In A. thaliana, around 50% of plastidial lipids come from the Eukaryotic pathway whereas in other plants, like grasses, they almost all arise from this pathway (Browse, Warwick, Somerville, & Slack, 1986; Mongrand, Bessoule, Cabantous, & Cassagne, 1998). Thus, an extensive transfer of lipids from the ER to plastids is required to sustain chloroplast biogenesis. The identity of the transported precursor molecule is still under debate, but it is now widely accepted that different precursors, such as phosphatidylcholine (PC), lyso-PC (i.e., a PC molecule with one acyl-chain), PA or DAG, could be imported into plastids by several co-existing pathways.

The particularity of plant organisms is their ability to adapt to a wide variety of stresses including pathogen attacks, nutrient starvation, extreme

temperatures, high light exposure or drought. The modification of plant lipid composition is a widespread phenomenon involved in cell adaptation to stress (Liu et al., 2019; Liu & Li, 2019; Michaud & Jouhet, 2019; Moellering & Benning, 2011). The modification of the plant lipidome can play a role in signal transduction by the generation of lipid signaling molecules such as PA, lyso-lipids, free FAs or phosphatidylinositide phosphates (PIPs) (Hou, Ufer, & Bartels, 2016). It can also play a direct role in the adaptation of plant cells to environmental changes, for instance by modifying membrane fluidity during cold or heat stress or by triggering energy storage by the accumulation of triacylglycerols (TAGs) into lipid droplets (LDs) in response to nitrogen starvation (Liu et al., 2019; Meï et al., 2015; Upchurch, 2008). The best-studied lipid remodeling process occurs in response to phosphate (Pi) starvation, a frequent nutrient stress encountered by plants in nature. Phospholipids retain up to one third of the intracellular Pi content and constitute an important Pi reserve (Poirier, Thoma, Somerville, & Schiefelbein, 1991). During Pi starvation, plants partially degrade their phospholipids to remobilize intracellular Pi (Plaxton & Tran, 2011; Poirier et al., 1991). To maintain membrane integrity, the degraded phospholipids are replaced by DGDG (Hartel, Dormann, & Benning, 2001; Jouhet, Maréchal, Bligny, Joyard, & Block, 2003; Tjellström, Andersson, Larsson, & Sandelius, 2008). Since DGDG is exclusively synthetized in plastids, a massive DGDG export from this organelle to extra-plastidial membranes like vacuole, mitochondria and PM takes place (Andersson, Larsson, Tjellström, Liljenberg, & Sandelius, 2005; Jouhet et al., 2004). The transfer of DGDG from plastids to mitochondria is thought to occur by non-vesicular routes at MCSs between the two organelles as their number significantly increases in response to Pi starvation (Jouhet et al., 2004). However, the mechanisms involved in DGDG transport from plastids to vacuoles and PM (i.e., vesicular and/or non-vesicular) are still unknown.

#### 1.2.2 How does non-vesicular glycerolipid transport occur in plant cells?

The non-vesicular transport of lipids can be divided in three steps: (1) the desorption of the lipid from the donor membrane, (2) its transport through the cytosol and (3) its insertion into the acceptor membrane. The limiting step is the desorption of the lipid from the donor membrane because of the high-energy barrier linked to the extrusion of amphipathic molecules from their hydrophobic environment (Somerharju, 2015). Different mechanisms



**Fig. 2** Mechanisms involved in non-vesicular glycerolipid transport at membrane contact sites. (A) Spontaneous transfer of glycerolipids between membranes. (B–E) Lipid transport mediated by lipid transfer proteins (LTPs). LTPs have a soluble domain with a hydrophobic cavity accommodating lipid molecule(s). They can be soluble (B), membrane bound (C) or anchored at two membranes (D), to shuttle lipids between membranes, or they can adopt a bridge-like structure forming a hydrophobic tunnel for lipid channeling between membranes (E). (F) Lipid transport can be mediated by multi-subunits complexes crossing two or several membranes. (G) Some membrane protein can promote non-vesicular lipid transport by favoring lipid extrusion or insertion from the donor or the acceptor membranes, respectively. The different proteins and complexes using each mechanisms that will be discussed in this chapter are indicated.

have been proposed to mediate non-vesicular lipid transport in cells. Spontaneous transport of glycerolipids can occur (Fig. 2A), but this process is likely irrelevant in the context of cell division as the half time of spontaneous PC transport in vitro ranges from several hours to days (Somerharju, 2015). Obviously, many factors can influence the rate of spontaneous transport of glycerolipids, including the composition of the donor membrane and the hydrophobicity of the transported molecules (length and unsaturation level of the acyl-chains). For instance, the *in vitro* spontaneous transfer rate of lyso-lipids significantly decreases compared to glycerolipids, ranging from seconds to minutes (Somerharju, 2015). It has also been proposed that MCSs might form microdomains with a specific lipid and protein composition favoring non-vesicular lipid transport (Gaigg, Simbeni, Hrastnik, Paltauf, & Daum, 1995; Pichler et al., 2001; Somerharju, 2015), but this remains to be determined. In vivo, many proteins, such as lipid transfer proteins (LTPs) (Fig. 2B–E), as well as multi-subunit complexes that can cross at least two membranes (Fig. 2F), are involved in non-vesicular lipid transport. In addition, some membrane proteins can act in concert with LTPs to promote membrane lipid desorption or insertion (Fig. 2G). In this chapter, these mechanisms will be discussed in plants. As our current knowledge is scarce, we will rely on the lipid transfer processes described in other organisms to draw a more global picture of the putative mechanisms involved in non-vesicular glycerolipid transport in plant cells.

## 2. Lipid transport mediated by LTPs

LTPs can be divided into different families depending on their structure and mechanism of lipid transport, but they all share common features: (1) they possess a soluble domain with a hydrophobic cavity accommodating one or several lipid molecules, favoring the transport of lipids through soluble compartments, and (2) they are able to significantly increase the rate of glycerolipid transfer between membranes (Reinisch & Prinz, 2021; Somerharju, 2015; Wong, Gatta, & Levine, 2019). Some LTPs have also been proposed to directly facilitate membrane lipid desorption or to associate to membrane proteins favoring lipid extrusion (Lipp, Ikhlef, Milanini, & Drin, 2020; Reinisch & Prinz, 2021; Wong et al., 2019). Two main mechanisms have been proposed for LTPs: (1) the shuttle mechanism, where the lipid-binding domain of LTPs is able to diffuse through the soluble compartment to shuttle lipids from one membrane to another (Fig. 2B–D) and (2) the bridge-like mechanism in which LTPs are able to form hydrophobic tunnels contacting simultaneously two membranes between which lipids will be transported (Fig. 2E) (Reinisch & Prinz, 2021; Wong et al., 2019). LTPs can be soluble and transiently associated to membranes *via* protein-lipid or protein-protein interactions (Fig. 2B) or anchored to membranes by transmembrane (TM) domains (Fig. 2C). Indeed, as further described in this chapter, the lipid-binding domain of LTPs is often associated to additional domains, the most frequent being the pleckstrin-homology (PH) domain, which specifically binds to PIPs, and the C2 domains that can bind a wider range of membrane lipids. These additional domains allow LTPs targeting to particular organelles or MCSs (Reinisch & Prinz, 2021; Wong et al., 2019). Furthermore, some LTPs are able to simultaneously bind two membranes and are able to act as lipid transporters and membrane tethers at MCSs (Fig. 2D). In plants, a few LTPs have been characterized until now but they are involved in a wide variety of cellular processes beside lipid transport including lipid signaling, plant resistance to pathogens, adaptation to many abiotic stresses or regulation of gene expression (Table 1).
Roles	Characterized LTP proteins in plants	
Lipid transfer between membranes	Syt1, Syt3, At1g73200, ORP3A, AtSfh1, AtPITP11, Ssh2, NbSec14	
Membrane tethering	Syt1, Syt 3, Syt5, Syt7, At1g73200	
Signaling	AtSfh1, Ssh1, AtPIPT7, MtN5	
Vesicular trafficking	Syt2, AtPATL1, AtPATL2, AtPITP7	
Plant development	PiORP3, AtSfh1, GhLTPG1	
Abiotic stress response	Syt1, Syt3, AtPATL1, Ssh1, AsE246, MtN5	
Biotic stress response	Syt1, Syt5, Syt7, VAD1, BAGP1, ERD2, ORPs, AtPATL3, AtPATL6, NbSec14	
Regulation of gene expression	Syt7, GL2, HD-StART, GmOSBP	

**Table 1** Roles of the different lipid transfer proteins (LTPs) characterized until now in plants.

A detailed description of the role of these LTPs can be found in this chapter.

# 2.1 The SMP family

The SMP (synaptotagmin-like mitochondrial-lipid binding protein) domain is a lipid binding/transport domain belonging to the TULIPs (tubular lipid binding proteins) superfamily of LTPs (Kopec, Alva, & Lupas, 2010; Wong & Levine, 2017). SMP domain-containing proteins are evolutionarily conserved among Eukaryotes (Craxton, 2004; Lee & Hong, 2006). This family has been mostly studied in yeast and mammals and shown to be located at various MCSs: (1) at ER-PM MCSs, for the Extended synaptotagmins (E-Syts), also called Tricalbins in yeast (Perez-Sancho et al., 2015; Saheki & De Camilli, 2017b), (2) at ER-mitochondria MCSs, for some ERMES (ER-mitochondria encounter structure) complex subunits in yeast and the PDZD8 protein in mammals (Hirabayashi et al., 2017; Kornmann et al., 2009), (3) at ER-Golgi MCSs for Nvj2 in yeast (Liu, Choudhary, Toulmay, & Prinz, 2017) and (4) at the ER-endosomes MCSs for PDZD8 (Elbaz-Alon et al., 2020; Shirane et al., 2020). The SMP proteins are mostly predicted to be anchored to membranes by one TM segment linked to the SMP domain by a flexible linker (Lee & Hong, 2006; Saheki & De Camilli, 2017b). Other domains can also be associated, as exemplified by C2 domains located in *E*-Syts allowing the binding of the protein to a second membrane enriched in PIPs (see below). Cristal structures of the SMP

domains show that they fold into a  $\beta$ -barrel structure oligomerizing to form hydrophobic tunnels that can accommodate phospholipid molecules (Jeong, Park, Jun, & Lee, 2017; Saheki & De Camilli, 2017b; Schauder et al., 2014). SMP domains are able to form homodimers or heteromultimers (AhYoung et al., 2015; Schauder et al., 2014). Analyses of lipids bound to purified proteins as well as in vitro transfer assays support the role of SMP proteins in phospholipid transport between membranes at MCSs, independently of the polar head, suggesting that SMP proteins are non-specific glycerolipid transporters (AhYoung et al., 2015; Jeong et al., 2017; Saheki et al., 2016). The flexible linkers located between the TM and the SMP domain of these proteins allow the movement of the lipid binding domain in the cytosol to shuttle lipids from one membrane to another in an ATP-independent manner (Fig. 2D) (Jeong et al., 2017; Schauder et al., 2014). By searching in the literature and in domain databases, 13 proteins containing a SMP domain can be identified in A. thaliana (Table 2) (Ishikawa et al., 2020; Perez-Sancho et al., 2016). Seven show a domain organization similar to *E*-Syts and two to Nvj2 proteins.

#### 2.1.1 E-Syts

E-Syts contain a TM domain anchoring the protein in the ER, and one or several C2 domains located in the cytosol (Perez-Sancho et al., 2016; Saheki & De Camilli, 2017b). C2 domains are able to bind PIPs-enriched PM in a Ca<sup>2+</sup>-dependent manner, allowing the localization of E-Syts at ER-PM MCSs (Fig. 2D). At this junction, E-Syts play a dual role as tethers and lipid transporters (Jeyasimman & Saheki, 2020; Saheki et al., 2016). Whereas yeast and human genomes contain three E-Syt genes (Reinisch & De Camilli, 2016), this family is expanded in plants, with seven members found in A. thaliana genome (Table 2) (Ishikawa et al., 2020). Among this family, Syt1, Syt3, Syt5 and Syt7 (also called AtCLB1) are enriched at ER-PM MCSs and are ubiquitously expressed at a high level (de Silva, Laska, Brown, Sederoff, & Khodakovskaya, 2011; Ishikawa et al., 2020; Lee et al., 2020). Intriguingly, Syt7, which binds ceramides, was also observed in the nuclear envelope of root tip cells. Syt7 associates to AtTASH1 promoter to negatively regulate the expression of this gene involved in the synthesis of triterpenoids required for root development (de Silva et al., 2011). The regulation of the localization of this membrane protein to either ER-PM MCSs or the nucleus remains to be investigated. Syt2 is highly expressed in pollen and localized in Golgi and PM (Wang et al., 2015; Zhang et al., 2011). Syt2 is involved in non-conventional secretion pathways and plays a role in pollen development and pollen tube

<b>Table 2</b> Gen <b>AGI</b>	ies of the synaptot <b>Name</b>	agmin-like mit <b>Sub-family</b>	ochondrial-lipid binding protein (SM Localization	IP) family present in the A. <i>thali</i> Domain organization	<i>ana</i> genome. <b>References</b>
At2g20990	SYT1/SYTA	E-Syt	ER/ER-PM MCSs	TM-SMP-C2-C2	Perez-Sancho et al. (2015)
At1g20080	SYT2/SYTB		Golgi/PM	TM-SMP-C2-C2	Wang et al. (2015) and Zhang et al. (2011)
At5g04220	SYT3/SYTC		ER-PM MCSs	TM-SMP-C2-C2	Ruiz-Lopez et al. (2020)
At5g11100	SYT4/SYTD		ER (prediction)	TM-SMP-C2-C2	
At1g05500	SYT5/SYTE		ER/ER-PM MCSs	TM-SMP-C2-C2	Ishikawa, Fukao, and Shimada (2020) and Lee et al. (2020)
At3g18370	SYT6/SYTF		ER (prediction)	TM-SMP-C2-C2-CC	
At3g61050	Syt7/AtCLB1		ER/ER-PM MCSs/nucleus	TM-SMP-C2-CC	Ishikawa et al. (2020)
At3g60950			Mitochondria (prediction)	SMP-C2-DNaseI-like	
At3g61030			Mitochondria (prediction)	SMP-C2-DNaseI-like	
At1g73200			ER-Golgi MCSs (yeast complementation)	TM-PH-SMP	Liu et al. (2017)
At1g17820			ER-Golgi MCSs (by homology to At1g73200)	TM-PH-SMP	
At1g50260	NTMC2T5.1		Plastids (prediction)	TM-SMP-TM-TM-C2- TM	
At3g19830	NTMC2T5.2		Plastids (prediction)	TM-SMP-TM-TM-C2- TM	
Protein localiza Aryamanesh, & TM: transmem	tion were determined Millar, 2017; Schwac brane domain.	experimentally or ke et al., 2003). T	the consensus localizations from SUBA and he domain organization found for each prote	ARAMEMNON websites (prediction ein is also indicated. CC: coiled-coil d	1) are indicated (Hooper, Castleden, Tanz, omain, PH: pleckstrin homology domain,

elongation (Wang et al., 2015; Zhang et al., 2011). These results show that, in contrast to yeast and mammals, plant *E*-Syts have a broader subcellular localization and could be involved in the regulation of a wider range of cellular processes besides ER-PM tethering and lipid transport, including gene expression regulation.

The most characterized E-Syt in plants is Syt1 in A. thaliana. Syt1 is anchored in the ER by its N-terminal TM segment and is able to bind negatively charged lipids in the PM via the two C2 domains protruding in the cytosol (Fig. 3A) (Lee et al., 2020; Perez-Sancho et al., 2015; Ruiz-Lopez et al., 2020; Schapire et al., 2008; Yamazaki, Kawamura, Minami, & Uemura, 2008; Yamazaki, Takata, Uemura, & Kawamura, 2010). Whether the PM binding is  $Ca^{2+}$  dependent or not is still a matter of debate (Perez-Sancho et al., 2015; Ruiz-Lopez et al., 2020; Schapire et al., 2008). The localization of Syt1 to ER-PM MCSs also requires VST (VAP-related suppressor of TMM) proteins, which are peripheral PM proteins implicated in stomatal development (Ho, Paciorek, Abrash, & Bergmann, 2016). Syt1 is involved in many aspects of plant development and biotic and abiotic stress response. Indeed, ER-PM proximity is highly regulated during plant development and in response to different stresses exerting huge constraints on the PM, such as high salinity, osmotic stress, mechanical stress or freezing (Ho et al., 2016; Lee et al., 2019, 2020; McFarlane et al., 2017; Perez-Sancho et al., 2015). In such situations, increasing of ER-PM MCSs is thought to stabilize the PM and to favor its recovery after damages. In accordance with the key role of Syt1 in these processes: (1) Syt1 is overexpressed during cold acclimation or mechanical stress, (2) syt1 mutants are sensitive to freezing, high NaCl concentration, drought, osmotic or mechanical stress and (3) a decrease of PM integrity is observed in syt1 mutants (Perez-Sancho et al., 2015; Ruiz-Lopez et al., 2020; Schapire et al., 2008; Yamazaki et al., 2008). Syt1 is also located at ER-PM MCSs close to plasmodesmata (PD) entries. PDs are channels crossing the plant cell wall, ensuring a continuity of PM, ER and cytosol between plant cells and allowing cell-to-cell communication (Petit et al., 2019). In PDs, ER diameter is reduced and ER-PM MCSs mediated by Syt1, as well as by Syt5 and 7, are suspected to play a role in ER constriction to enter and cross PDs (Ishikawa et al., 2020). In contrast to what is observed in the case of abiotic stress, syt1 mutants exhibit a higher resistance to fungi and viral infection, notably by reducing pathogen entry into cells or limiting cell-tocell transmission of viral particles through PDs (Ishikawa et al., 2020; Kim et al., 2016; Levy, Zheng, & Lazarowitz, 2015). Syt1 is able to interact with



**Fig. 3** Proteins involved in non-vesicular glycerolipid transport at different membrane contact sites (MCSs) in plant cells. (A) Transport of diacylglycerol (DAG) by Syt1/3 at endoplasmic reticulum (ER)-plasma membrane (PM) MCSs. Syt1/3 are anchored to the ER by a transmembrane domain and to the PM by the binding of the C2 domains *(Continued)* 

three other E-Syts, Syt3, Syt5 and Syt7, suggesting it could also form heteromultimeric complexes at ER-PM MCSs, maybe to promote lipid transfer as in mammals (Ishikawa et al., 2020; Lee et al., 2020; Ruiz-Lopez et al., 2020). Recently, it has been shown that Syt3 has a redundant function with Syt1. Both proteins are involved in ER-PM MCSs formation and are more abundant in these sites during cold stress (Ruiz-Lopez et al., 2020). Interestingly, lipidomic analyses of the double syt1/3 mutant under cold stress show no changes in the whole leaf lipidome but a specific accumulation of DAG and PC in PM fraction, suggesting that DAG clearance from PM is impaired in the double mutant (Ruiz-Lopez et al., 2020). DAG accumulation in PM could be responsible for the cold hypersensitivity of the syt1/3 mutant by altering PM integrity. In addition, Syt1 co-purifies with DAG (Ruiz-Lopez et al., 2020), further supporting a role of Syt1, and likely Syt3, in DAG transport at ER-PM MCSs during cold stress (Fig. 3A). This first study confirms that, like in yeast and metazoans, E-Syts are involved in both ER-PM tethering and lipid transport, opening important perspectives about the role of these proteins in PM biogenesis and response to stress. Interestingly, five mRNA splice variants were detected for Syt3

to phosphatidylinositides (PIPs). DAG transport is mediated by the Fig. 3—Cont'd synaptotagmin-like mitochondrial-lipid binding protein (SMP) domain. (B) Putative transport of sitosterol by ORP3A at ER-golgi MCS. ORP3A interacts in the ER with the PVA12 VAP protein. (C) (i) Putative transport of cardiolipin at mitochondrial (M) outer (OM)-inner (IM) membrane MCSs mediated by NDPK-3. NDPK-3 interacts in the IM with the ANT translocator. (ii) Digalactosyldiacylglycerol (DGDG) and phosphatidylethanolamine transport at OM-IM MCSs mediated by the mitochondrial transmembrane lipoprotein (MTL) complex during phosphate (Pi) starvation. In this complex, AtMic60 interacts with Tom40 and plays a key role in the lipid transport process. Because of the presence of chloroplast (C) proteins in the MTL complex, it could also be located at chloroplasts-mitochondria MCSs to mediate DGDG import during Pi starvation. (D) Lipid transport at ER-chloroplasts MCSs and outer (OE)-inner (IE) envelopes MCSs. (i) Putative lipid transport mediated by phosphatidylcholine (PC) remodeling at ER-chloroplasts MCSs. BnCLIP1 lipase could form lyso-PC from PC that could be acylated in the OE by lyso-PC acyltransferases (LPCATs) using a local pool of acyl-CoA generated by the long-chain acyl-CoA synthetases LACS4 and LACS9. (ii) The DGDG synthase DGD1 might play a role in OE-IE lipid transport thanks to its N-terminal extension. (iii) The ALA10 flippase could promote PC transport from the ER to the chloroplasts by promoting enrichment of 18:2-PC in the cytosolic leaflet of the ER. (iv) Import of lipids precursors in chloroplasts IE by the TGD complex and the LptD1 protein. TDG1, 2 and 3 form an ABC transporter in the IE whereas TGD5 bridges the IE and OE via its interaction with TGD1/2/3 and TGD4. LptD1 is a homolog of TGD4 and might be involved in galactoglycerolipid synthesis during Pi starvation.

(Ruiz-Lopez et al., 2020), increasing the putative number of Syts present in plant cells and the functions they can perform.

# 2.1.2 Other SMP-domain containing proteins

The localization and role of the six other A. thaliana SMP domain-containing proteins are currently unknown. At1g73200 and At1g17820 have a similar domain organization than yeast Nvj2 with a TM domain separated of the SMP domain by a PH domain (Table 2). Nvj2 is involved in the formation of ER-Golgi contacts and ceramide transport to Golgi to alleviate the ER cytotoxicity linked to ceramide accumulation (Liu et al., 2017). At1g73200 is able to restore ceramide transport and ER–Golgi tethering in the yeast  $\Delta nvj2$ mutant, suggesting that At1g73200 and At1g17820 might be Nvj2 orthologs (Liu et al., 2017). The At1g50260 and At3g19830 proteins present an unusual domain organization with four predicted TM domains flanking a SMP and a C2 domain (Table 2). This organization seems to be found only in plants (Lee & Hong, 2006). The At3g60950 and At3g61030 proteins are the only A. thaliana SMP proteins that do not contain a TM domain (Table 2). The SMP domain is associated to a C2 and an endonuclease/exonuclease/phosphatase super family domain that was not found associated to SMP domain in other organisms. No putative orthologs of the ERMES complex subunits or PDZD8, involved in ER-mitochondria and ER-endosomes lipid transport in yeast and mammals, respectively, were found in A. thaliana genome.

# 2.1.3 The StARkin super-family

The StARkin (relatives (kin) of steroidogenic acute regulatory protein (StAR)) is a large superfamily of LTPs that gather families sharing a common lipid binding/transfer domain folding despite a low sequence conservation (Hanada, 2018; Wong & Levine, 2016). The StARkin lipid binding/transfer domain is composed of a curved  $\beta$ -sheet containing seven strands and of  $\alpha$ -helices that form a hydrophobic cavity accommodating amphiphilic molecules (Wong & Levine, 2016). StARkin super family includes the StARTs (StAR related transfers), the LAMs (lipid transfer at membrane contact sites), the PITPs (phosphoinositol (PI) transfer proteins), the Ups/PRELI and the Bet-V1 protein family. Bet-V1 (also called PR10) proteins are plant-specific. They are able to bind a wide variety of amphipathic molecules and are involved in the synthesis of many compounds such as flavonoids, alkaloids, sporopollenin or polyphenolics, for instance in response to pathogen infection. As their main substrates are not glycerolipids, this family will not be further described in this chapter (for a recent review about Bet\_V1

proteins, see Morris, Caldo, Liang, & Facchini, 2021). PITPs with a StARkin domain play a role in the transport of PI, PC, PA or sphingolipids in different cell compartments and are involved in many cellular processes such as PIPs signaling, vesicular trafficking or sphingolipid synthesis. This family is mainly present in metazoans but is not conserved either in plants or in yeasts and thus will not be further discussed in this chapter (for recent reviews, see Grabon, Bankaitis, & McDermott, 2019; Selitrennik & Lev, 2016).

#### 2.1.4 The LAM family

LAM proteins are lipid transporters located at MCSs and conserved throughout the Eukaryotic lineage. All the LAM proteins studied until now are sterol transporters (Khafif, Cottret, Balague, & Raffaele, 2014; Wong & Levine, 2016). These proteins have a conserved organization with (1) a GRAM (glucosyltransferases, Rab-like GTPase activators and myotubularins) domain corresponding to a PH-like domain involved in protein/protein or protein/ lipid interactions, (2) one or two VASt (VAD1 analog of StAR-related lipid transfer) domains, involved in lipid binding and transport and (3) a C-terminal TM domain (Hanada, 2018; Wong & Levine, 2016). The three human LAMs, as well as the yeast Lam1-4 proteins are located at ER-PM MCSs, whereas Lam5 and Lam6 are detected at multiple MCSs in yeast (Elbaz-Alon et al., 2015; Gatta et al., 2015; Murley et al., 2015; Sandhu et al., 2018). They are anchored in the ER by their TM domain but the domains and mechanisms involved in their association with a second organelle remain elusive. Lam6 associates to mitochondria and vacuole through interactions with the protein import receptors Tom70/71 and Vac8, respectively (Murley et al., 2015). In vitro, the VASt domains of Lam2/4 is able to bind phosphatidylserine (PS)-containing liposomes. The transport efficiency of the VASt domain of GRAMD1 proteins is enhanced by the addition of anionic lipids (PS or PIPs) in the acceptor liposome (Horenkamp, Valverde, Nunnari, & Reinisch, 2018; Tong, Manik, & Im, 2018), suggesting that the anchoring to a second membrane could also be achieved by interactions of LAMs with lipids. Structural analyses of the VASt domain of several LAM proteins show it is monomeric and folds into a hydrophobic pocket accommodating one sterol molecule (Gatta, Sauerwein, Zhuravleva, Levine, & Matthews, 2018; Horenkamp et al., 2018). In vitro, the VASt domain of most of the LAMs is able to bind and transport sterols between membranes in an ATP-independent manner (Gatta et al., 2015, 2018; Horenkamp et al., 2018; Murley et al., 2015). This transport activity was also

confirmed *in vivo*, where LAM proteins are able to shuttle sterols at ER-PM or ER-vacuole junctions (Gatta et al., 2015; Murley et al., 2017; Sandhu et al., 2018). In yeast, Lam2/4 and Lam6 trigger the formation of sterol-enriched microdomains in the PM and in the vacuole, respectively, playing a role in signaling during stress response (Murley et al., 2015, 2017).

*A. thaliana* genome contains four putative LAM proteins (Khafif et al., 2014; Wong & Levine, 2016). Besides VAD1, plant LAMs have an unusual domain organization compared to yeast and mammals with the presence of additional C2 domains and two TM domains located in the middle of the protein for At1g03370 and At5g50170 (Table 3). The C2 domains might be involved in protein binding to membranes enriched in negatively-charged lipids, such as the PM. Few data are available concerning plant LAMs. VAD1-RFP fusion does not show a punctate pattern and seems to be localized in different cell compartments, including, PM, Golgi, tonoplast, ER, cytosol or nucleus when overexpressed (Khafif et al., 2017). This localization pattern might be linked to the RFP fused in C-terminal, close to the TM domain, which might alter membrane targeting of VAD1. VAD1 is a negative regulator of the programmed cell death hypersensitive response (HR) occurring in response to pathogen invasion to avoid their propagation

AGI	Name	Localization	Domain organization	References
At1g02120	VAD1	PM, ER, golgi, cytosol, tonoplast, nucleus	GRAM-VASt- TM-CC	Khafif, Balague, Huard-Chauveau, and Roby (2017)
At1g03370		Cytosol (prediction)	C2-VASt- TM-TM-C2- GRAM-VaST	
At3g59660	BAGP1	PM (prediction)	TM-C2- GRAM-VASt	
At5g50170		Cytosol (prediction)	C2-VASt- TM-TM-C2- GRAM-VaST	

**Table 3** Genes of the lipid transfer at membrane contact sites (LAM) family present inthe A. thaliana genome.

Protein localization were determined experimentally or the consensus localizations from SUBA and ARAMEMNON websites (prediction) are indicated (Hooper et al., 2017; Schwacke et al., 2003). The domain organization found for each protein is also indicated. CC: coiled-coil domain, GRAM: glucosyltransferases, Rab-like GTPase activators and myotubularins domain, TM: transmembrane domain, VASt: VAD1 analog of StAR-related lipid transfer domain.

in plant tissues (Khafif et al., 2017; Lorrain et al., 2004). Because of its role in cell death regulation, VAD1 is involved in pathogen resistance and the *vad1* mutant shows an enhanced resistance to pathogen attack linked to constitutive cell death activation (Khafif et al., 2017; Lorrain et al., 2004). Interestingly, the role of VAD1 in HR, as well as its nuclear localization, are dependent on the VASt, but not on the GRAM domain, suggesting that VAD1 localization and function might be regulated by lipid binding to the VASt domain (Khafif et al., 2017). The second LAM protein studied in A. thaliana is BAGP1, which also plays a role in biotic stress response by promoting basal immunity against the pathogenic fungus Botrytis cinerea. In response to pathogen infection, BAGP1 interacts with the protease APCB1 to promote the cleavage of BAG6 (Bcl2-associated athanogene), an autophagy activator inducing basal immunity against fungi (Li, Kabbage, et al., 2016; Li, Xu, et al., 2016). However, the localization of BAGP1, how it regulates BAG6 cleavage and whether lipids are involved in the regulation of its function are still unknown. Overall, LAM proteins seem to play important roles in biotic stress response in plants, but despite their homology with yeast and mammalian LAMs, their localization and their ability to bind and/or transport lipids remain to be determined.

### 2.1.5 The Ups/PRELI family

Proteins of the Ups/PRELI family are soluble proteins located in the intermembrane space (IMS) of mitochondria acting in heterodimers with the molecular chaperone Mdm35/TRIAP1 to shuttle lipids from the outer (OM) to the inner (IM) membrane. Three Ups/PRELI and one Mdm35/ TRIAP1 proteins exist in yeast (Ups1-3, Mdm35) and mammals (PRELID1, PRELID3a, PRELID3b, TRIAP1). The Ups1/PRELID1 and Ups2/PRELID3b proteins regulate mitochondrial CL and PE metabolism, respectively, by promoting the transport of lipid substrates, PA and PS, respectively, from the OM to the IM where cardiolipin (CL) and phosphatidylethanolamine (PE) synthesizing enzymes are located (Aaltonen et al., 2016; Connerth et al., 2012; Miyata, Watanabe, Tamura, Endo, & Kuge, 2016; Osman et al., 2009). Mdm35/TRIAP1 acts as a chaperone facilitating import and stability of Ups/PRELI proteins in the IMS (Potting et al., 2013; Potting, Wilmes, Engmann, Osman, & Langer, 2010). Mdm35/TRIAP1 dissociates from Ups/PRELI upon binding to membrane enriched in anionic lipids, such as CL, allowing lipid releasing and protein recycling (Aaltonen et al., 2016; Connerth et al., 2012; Watanabe, Tamura, Kawano, & Endo, 2015). The Ups/PRELI proteins fold is characteristic of the StARkin domain superfamily with a sevenstranded  $\beta$ -sheet decorated with three flexible helices forming a hydrophobic cavity (Miliara et al., 2019; Watanabe et al., 2015). Interestingly, in plants, only one Ups/PRELI (At5g13070) and one Mdm35/TRIAP1 (At4g33100) homologs are found (Wideman & Muñoz-Gómez, 2016). At5g13070 protein is uncharacterized in A. thaliana but is predicted to be located in mitochondria IMS (Hooper et al., 2017; Schwacke et al., 2003). Sequence analysis suggests that At5g13070 is closer to the Ups2 than the Ups1 family, and might transport PS because of the absence of a conserved lysine residue (K58), present in Ups1 family, which impaired PS transport (Miliara et al., 2019; Wideman & Muñoz-Gómez, 2016). In vitro, yeast Ups2 can also transport PA, but in vivo, no decrease of CL is observed in  $\Delta ups2$  showing that Ups2 does not play an essential role in PA transport (Miyata et al., 2016; Osman et al., 2009). Thus, the plant Ups/PRELI protein might be involved in the transport of both PS and PA and/or alternative pathways might exist to transport PA for CL synthesis in the IM.

#### 2.1.6 The StART family

StART domain-containing proteins are conserved throughout evolution (Ponting & Aravind, 1999). StART family is expanded in higher plants with 35 and 29 members in A. thaliana and in rice, respectively, whereas 15 members are found in humans and only one in yeast (Clark, 2020; Schrick, Nguyen, Karlowski, & Mayer, 2004). StART proteins are divided into subfamilies according to their domain organization and lipid binding specificity (Clark, 2020; Schrick et al., 2004). They are able to bind different ligands such as sterols, ceramides or phospholipids (Clark, 2020). As only members of the StARD2 subfamily have been shown to transport phospholipids (Kang, Wei, & Cohen, 2010), we will focus on this subfamily (for reviews about StART proteins, see Clark, 2012, 2020). In humans, there are three phospholipid-binding StART proteins (StARD2, StARD7 and StARD10) also called PC-TPs (PC transporter proteins), which are small proteins (around 200-300 aa) only composed of a StART domain (Clark, 2020; Schrick et al., 2004). StARD2 binds and transports PC between liposomes in vitro (Wirtz, 1991). It folds into a nine-stranded curved  $\beta$ -sheet with four helices forming a binding pocket accommodating one PC molecule (Roderick et al., 2002). This protein is localized in the cytosol and the nucleus and has been shown to regulate lipid droplet (LD) size in rat liver as well as the interplay between glucose and lipid metabolism (Clark, 2020;

Kang et al., 2010; Kanno et al., 2007; Nicholls, Hornick, & Cohen, 2017). In the nucleus, StARD2 also interacts with the Homeodomain (HD) transcription factor Pax3, involved in tissues and organs development (Buckingham & Relaix, 2007), and stimulates its activity (Kang et al., 2010; Kanno et al., 2007). Thus, it seems that StARD2 is more likely a PC-sensor regulating different cellular pathways than a PC transporter between membranes. In contrast, StARD7 is involved in PC transport from the mitochondrial OM to the IM in mammals (Horibata et al., 2016; Saita et al., 2018). Stard7 KO or KD cell lines present a variety of mitochondrial defects including a reduced respiration rate and ATP level, mtDNA damages and defects in mitochondrial cristae structure, highlighting the importance of this PC-transporter for mitochondria biogenesis and functions (Flores-Martín, Reyna, Ridano, Panzetta-Dutari, & Genti-Raimondi, 2016; Horibata et al., 2016; Saita et al., 2018; Yang et al., 2017). Two isoforms, StARD7-I, with a 75aa N-terminal mitochondrial targeting sequence, and StARD7-II, co-exist in cells and are able to bind PC and ceramides, but only PC is transported between liposomes (Bockelmann et al., 2018; Horibata & Sugimoto, 2010; Saita et al., 2018). StARD10 is able to bind and transport PC and PE in vitro and its transport activity is inhibited by phosphorylation, showing that lipid transfer and membrane binding can be regulated by post-translational modifications (Olayioye et al., 2005, 2007). In lung alveolar type II epithelial cells, StARD10 interacts with the ER lyso-PC acyltransferase 1 (LPCAT1) and promotes lipid transport on lamellar bodies (LB) where lipids are stored until secretion in the lung surfactant (Goss, Hunt, & Postle, 2013; Lin, Ikegami, Moon, Naren, & Shannon, 2015).

Six homologs of the PC-PT family can be found in *A. thaliana* (Table 4). They are longer than their yeast and human counterparts (around 400aa) and predicted to possess one or several TM domains (Schrick et al., 2004). TM domain-containing StART proteins are also found in rice and chickpea (Satheesh et al., 2016; Schrick et al., 2004). They are expressed at different stages of plant development and expression of some of them are induced or repressed in response to biotic or abiotic stress (Satheesh et al., 2016). Information about the function, localization and lipid binding properties of these proteins is scarce. 3D homology modeling of the At3g13062 shows that the StART domain overlaps with the crystal structure of StARD2 despite a low primary sequence similarity (Schrick et al., 2004). None of them is predicted to be targeted in mitochondria, questioning the existence of a StARD7 homolog in plants. Concerning the other StART domain-containing proteins, five members in *A. thaliana* have in C-terminal a

AGI	Name	Localization	Domain organization	References
At1g05230		Nucleus (prediction)	HD-ZLZ-StART	
At1g17920		Nucleus (prediction)		
At1g34650		Nucleus (prediction)		
At1g73360	HDG11	Nucleus		Khosla et al. (2014)
At1g79840	GL2	Nucleus	_	Schrick et al. (2014)
At2g32370		Nucleus (prediction)	_	
At3g03260		Nucleus (prediction)		
At3g61150		Nucleus (prediction)		
At4g00730	ANL2, AHDP	Nucleus (prediction)		
At4g04890	PDF2	Nucleus (prediction)	_	
At4g17710		Nucleus (prediction)		
At4g21750	ATML1	Nucleus (prediction)		
At4g25530	FWA	Nucleus (prediction)		
At5g17320		Nucleus (prediction)		
At5g46880		Nucleus (prediction)	_	
At5g52170		Nucleus (prediction)		

**Table 4** Genes of the StAR related transfers (StART) family present in the A. thaliana genome.

Continued

AGI	Name	Localization	Domain organization	References
At1g30490	PHV-ATHB9	Nucleus (prediction)	HD-ZIP-StART	
At1g52170		Nucleus (prediction)		
At2g34710	PHB, ATHB-14	Nucleus		Lee et al. (2006)
At4g32880	ATHB-8	Nucleus		Lee et al. (2006)
At5g60690	REV, IFL1	Nucleus		Lee et al. (2006)
At2g28320		Cyto/nucleus (prediction)	PH-StART-DUF1336	
At3g54800		Cyto/Mito/ soluble (prediction)		
At4g19040	EDR2	ER, PM, endosomes		Vorwerk et al. (2007)
At5g45560		Mito (prediction)		
At5g35180		Cyto/plaste/ nucleus (prediction)	StART-DUF1336	
At1g55960	Putatives	PM (prediction)	TM-StART	
At1g64720	PC-PT	Vacuole (prediction)	TM-StART	
At3g13062		PM (prediction)	TM-StART	
At3g23080		PM (prediction)	TM-StART-TM	
At4g14500		PM (prediction)	TM-StART-TM	
At5g54170		PM (prediction)	TM-StART-TM	
At4g26920		PM (prediction)	TM-StART	
At5g07260		Cyto/Mito (prediction)	StART	
At5g49800		Nucleus/ Chloroplast (prediction)	StART	

**Table 4** Genes of the StAR related transfers (StART) family present in the *A. thaliana* genome.—cont'd

Protein localization were determined experimentally or the consensus localizations from SUBA and ARAMEMNON websites (prediction) are indicated (Hooper et al., 2017; Schwacke et al., 2003). T- he domain organization found for each protein is also indicated. HD: homeodomain, PH: pleckstrin homology domain, TM: transmembrane domain, ZIP: leucine zipper, ZLZ: leucine zipper-loop-zipper.

domain of unknown function (DUF1336), which is plant-specific (Table 4) (Schrick et al., 2004). One member, EDR2, is a negative regulator of cell death during pathogen attack and binds to PI-4-monophosphate (PI(4)P) via its PH domain (Vorwerk et al., 2007), but the role of PI(4)P binding or of the StART domain has not been further investigated. It has to be noted that plants possess a unique subfamily of transcription factors composed of a StART domain associated to an homeodomain (HD) (21 members in A. thaliana, Table 4) (Schrick et al., 2004). In accordance with their putative role in the regulation of gene expression, they are predicted to be located in the nucleus and this localization was confirmed for some of them (Khosla et al., 2014; Lee et al., 2006; Schrick et al., 2014). Mutant analyses highlighted the importance of these transcription factors in plant development (Schrick et al., 2014). However, the role of the StART domains in the regulation of plant development, as well as their ligands, are still poorly understood. The StART domain of Glabra 2 (GL2), is required for the function of this transcription factor but not for its nuclear localization (Schrick et al., 2014). Interestingly, the StART domain of the mouse cholesterol transporter StAR can functionally replace GL2 StART domain and a mutation affecting StAR cholesterol binding site prevents functional complementation, indicating that sterol binding to GL2 is required for its transcriptional activator function (Schrick et al., 2014). In the same study, the authors analyzed the metabolites associated to the StART domain of three A. thaliana proteins (GL2, PDF2 and At3g13062) expressed and purified from yeast cells. Most of the detected metabolites are lipids. GL2 and PDF2 are associated to similar metabolites including lyso-PC, PC, DAG or sphingolipids whereas the putative PC-PT At3g13062 was associated to DAG and TAG (Schrick et al., 2014). However, because the study was performed in yeast, which contains only one sort of sterol (ergosterol) and missed some plant-specific lipids such as galactoglycerolipids, the lipidbinding ability of these A. thaliana StART domains might be misevaluated. Because of the association of a StARkin with a HD domain, regulation of gene expression by these plant-specific transcription factors might involve lipid binding.

#### 2.2 ORP

ORPs (Oxysterol-binding protein (OSBP) related proteins), also called Osh (Oxysterol-binding protein homolog), are a widespread family of LTPs that regulate sterol and phospholipid homeostasis (Lipp et al., 2020; Moser von

Filseck & Drin, 2016; Pietrangelo & Ridgway, 2018; Santos, Girik, & Nunes-Hasler, 2020). Twelve and seven homologs are found in human and yeast genomes, respectively, and most of them are located at MCSs and are able to bind and transport sterols and PIPs between different organelles (Moser von Filseck & Drin, 2016; Pietrangelo & Ridgway, 2018; Schulz & Prinz, 2007). Some of them, Osh6/7 in yeast and their homologs in human ORP5/8, are also able to bind and transport PS and will be further described below (Lipp et al., 2020; Santos et al., 2020). ORP proteins all contain an ORD (oxysterol-binding related domain), which folds into a hydrophobic  $\beta$ -barrel accommodating one lipid molecule with a flexible N-terminal lid that regulates the opening/closing of the tunnel entry (Dong, Wang, Ye, & Zhang, 2020; Im, Raychaudhuri, Prinz, & Hurley, 2005; Maeda et al., 2013). The ORD domain can be associated to other domains regulating membrane binding and protein sub-cellular localization, such as a PH domain, a FFAT (Phenylalanine-Phenylalanine Acidic Track) motif targeting proteins to ER, or a TM domain (Pietrangelo & Ridgway, 2018; Schulz & Prinz, 2007). Interestingly, some of them (ORP5/8 and OSBP in human and Osh4/6/7 in yeast) use an original counter-exchange mechanism to transport lipids from one membrane to another against their concentration gradient (Chung et al., 2015; de Saint-Jean et al., 2011; Lipp et al., 2020; Moser von Filseck et al., 2015; Moser von Filseck & Drin, 2016). Osh6/7 and ORP5/8 are located at ER-PM MCSs where they play a role in PS transport to PM, PS being enriched in the cytosolic leaflet. Those proteins are able to bind PS or PI(4)P, which are abundant in the PM, by their ORD domain in a mutually exclusive manner (Maeda et al., 2013; Moser von Filseck et al., 2015). In vivo, Osh6/7 and ORP5/8 extract PS from its site of synthesis, the ER, to shuttle and release it into the PM where PI(4)P is extracted and shuttled back to the ER (Maeda et al., 2013; Moser von Filseck et al., 2015). The hydrolysis of PI(4)P by the ER phosphatase Sac1 is suspected to provide sufficient energy for PS transport and to maintain low PI(4)P content in the ER compared to PM, favoring directional PS transport (Lipp et al., 2020; Moser von Filseck & Drin, 2016). Interestingly, ORP5/8 are also located at ER-mitochondria and ER-LD MCSs. ORP5 interacts with LD via an amphipathic helix in the ORP domain and has been shown to regulate LD PS and PI(4)P content and LD size (Du et al., 2020). At ER-mitochondria MCSs, ORP5 and ORP8 interact with each other and with PTPIP51, an OM protein involved in mitochondria-ER tethering via its interaction with the ER protein VAP-B (Galmes et al., 2016; Stoica et al., 2014). ORP5/8 are involved in PS transport at ER-mitochondria to

fulfill PE synthesis in the IM by PSD1 (Galmes et al., 2016; Rochin et al., 2020). Interestingly, most of the ORP5 proteins located at ERmitochondria MCSs are close to cristae junctions (CJ), which are OM-IM MCSs maintained by the IM MICOS complex (Mukherjee, Ghosh, & Meinecke, 2021; Rochin et al., 2020), suggesting that ORP5/8 might link PS transport from ER to the mitochondrial IM. As PIPs are barely detected in mitochondria, the transport of PS might be directed by a concentration gradient of PS rather than by a counter-exchange mechanism. In addition to its role in PS transport to mitochondria and PM, ORP5 is also involved in cholesterol transfer from low density lipoproteins (LDL) to late endosomes/ lysosomes in Niemann Pick C (NPC) fibroblasts (Du et al., 2011) and regulates Ca<sup>2+</sup> accumulation in the mitochondria and in the PM microdomains called caveolae (Pulli et al., 2018; Rochin et al., 2020). This example shows the versatility of functions and localizations associated to ORP proteins.

A. thaliana and rice genomes encode 12 and six ORP domain-containing proteins, respectively (Skirpan et al., 2006; Umate, 2011; Ye, Ji, Guo, & Jiang, 2020). A. thaliana proteins cluster in four groups and five proteins contain a PH domain and a FFAT motif that might be involved in their localization to ER and endomembrane compartments whereas the other members only have an ORD domain (Table 5) (Ye et al., 2020). Interestingly, when phylogeny analyses are performed with yeast and mammalian proteins, no A. thaliana proteins group with the PS transfer proteins ORP5/8 and Osh6/7 (Skirpan et al., 2006; Ye et al., 2020), suggesting that ORP proteins might not transport PS in plants or that this transport is mediated by a more distantly related family of ORPs. All ORPs are expressed in different tissues and developmental stages, ORP3 group being the most highly expressed (Skirpan et al., 2006; Umate, 2011). Most of the ORPs genes are induced by biotic and/or abiotic stress as well as in response to toxic compounds such as metals (Umate, 2011). In accordance with this, GmOSBP is highly induced during high salt stress and senescence and its PH domain targets GFP protein to the nucleus in onion epidermal cells, suggesting the protein might be involved in gene expression regulation in soybean (Li et al., 2008). In petunia, PiORP1 is highly expressed in pollen tubes where it interacts with the receptor-like kinase PRK1, a protein essential for pollen tube development, leading to PiORP1 phosphorylation (Skirpan et al., 2006). PiORP1-GFP localizes in the cytosol and forms PH domain-dependent punctates in the PM, suggesting a localization at PM MCSs. However, the lipid binding specificity of PiORP1 and its role in pollen tube development, maybe to provide lipids to PM for elongation, is

AGI	Name	Localization	Domain organization	References
At2g31020	ORP1A	Cyto/Chloro (prediction)	PH-ORD	
At2g31030	ORP1B	Cyto (prediction)	ORD	
At4g08180	ORP1C	Nucleus/Chloro (prediction)	PH-ORD	
At1g13170	ORP1D	Nucleus/Chloro (prediction)	PH-ORD	
At4g22540	ORP2A	Nucleus (prediction)	PH-ORD	
At4g12460	ORP2B	Nucleus (prediction)	PH <b>-</b> ORD	
At5g02100	ORP3A	ER	ORD	Saravanan et al. (2009)
At3g09300	ORP3B	Cyto (prediction)	ORD	
At5g59420	ORP3C	Cyto (prediction)	ORD	
At4g25860	ORP4A	Cyto (prediction)	ORD	
At4g25850	ORP4B	Cyto (prediction)	ORD	
At5g57240	ORP4C	Cyto (prediction)	ORD	

 Table 5 Genes of the OSBP related protein (ORP) family present in the A. thaliana genome.

Protein localization were determined experimentally or the consensus localizations from SUBA and ARAMEMNON websites (prediction) are indicated (Hooper et al., 2017; Schwacke et al., 2003). The domain organization found for each protein is also indicated. ORD: oxysterol-binding related domain, PH: pleckstrin homology domain.

still unknown. In *A. thaliana*, ORP3A is localized in the ER *via* a WFDE motif required for its interaction with the ER VAP protein PVA12 (Saravanan et al., 2009). When this interaction is disrupted, ORP3A localizes to the Golgi compartment. *In vitro*, ORP3A ORD domain binds sitosterols but PVA12 interaction as well as the ER localization of ORP3A are independent of its ability to bind lipids (Saravanan et al., 2009). All these results suggest that ORP3A could shuttle sitosterol from ER to Golgi (Fig. 3B). Plants ORP proteins could also be involved in the replication of some viruses that require specific lipids or membrane compartments for replication (Barajas et al., 2014; Nagy, 2015; Rodriguez-Peña, Mounadi, & Garcia-Ruiz, 2021). For instance, the bushy stunt virus (TBSV), a plant (+)RNA virus belonging to the Tombusvirus family, induces the formation of specific compartments arising from peroxisome

membrane invagination, called spherules. Within spherules, the assembly of the virus replicase complex (VRC) and the replication process occur, protected from degradation (Nagy, Barajas, & Pogany, 2012; Rodriguez-Peña et al., 2021). The TBSV protein p33 is involved in the recruitment of the VRC to peroxisome membrane. Using a yeast model system to investigate the mechanism of TBSV replication, it has been shown that sterols are redistributed from the PM to the virus replication site and that sterols are essential for efficient replication of the virus (Barajas et al., 2014). Interestingly, p33 interacts with six A. thaliana ORPs (ORP3A-C and ORP4A-C) as well as with five VAP proteins and ORP3A and PVA12 have been shown to co-localize with p33 close to VRCs in Nicotiana benthamiana (Barajas et al., 2014). In both yeast and plant systems, the presence of a replicative TBSV virus induces ER-peroxisomes MCSs formation close to spherules, p33 being located in both peroxisomes and ER membranes (Barajas et al., 2014). This suggests that p33 induces peroxisome-ER MCSs formation and recruits ORP proteins to promote sterol transfer from ER to peroxisomes and to stimulate TBSV replication and spherule formation.

## 2.3 VPS13-like protein family/Chorein\_N domain-containing proteins

VPS13-like proteins are highly conserved from yeast to human. This protein family is composed of two main protein types: the huge (>3000 aa) vacuolar protein sorting 13 (VPS13) and the autophagy-related 2 (ATG2) proteins. Among all the functional domains found in VPS13-like proteins, the N-terminal Chorein\_N domain and the adjacent VPS13\_N domain, both involved in lipid binding, are the best conserved (De et al., 2017; Pfisterer et al., 2014). In VPS13 proteins, these domains are usually followed by a long region containing repeated coiled regions and the characteristic VPS13 adaptor binding (VAB) domain. Their C-terminus is typically composed of an APT1-homology domain, an ATG-homology domain (ATG\_C) and finally a C-terminal PH domain. Most of these domains are involved in the subcellular targeting of VPS13 proteins (Dziurdzik & Conibear, 2021). VPS13-like proteins associate to membranes and MCSs and are implicated in a large variety of cellular processes like sporulation and trans-Golgi network homotypic fusion in yeast (De et al., 2017; Park & Neiman, 2012; Rabitsch et al., 2001) or regulation of autophagy, cytoskeletal architecture and cell survival in humans (Föller et al., 2012; Muñoz-Braceras, Calvo, & Escalante, 2015). The Saccharomyces cerevisiae genome

contains only one VPS13 protein, which localizes to nuclear-vacuolar junctions, mitochondria-endosome and vacuole-mitochondria MCSs depending on the cell physiological condition (Bean et al., 2018). Four homologs have been identified in human which also localize to different MCSs (for recent review, see Dziurdzik & Conibear, 2021). Yeast and human genomes encode one and two ATG2 protein, respectively, that are localized to ERautophagosome MCSs (Graef, Friedman, Graham, Babu, & Nunnari, 2013; Suzuki, Akioka, Kondo-Kakuta, Yamamoto, & Ohsumi, 2013) and are part of the autophagosome forming machinery (Nakatogawa, 2020). Both VPS13 and ATG2 proteins play a major role in lipid transport. In vitro, the N-terminal parts of Chaetomium thermophilum VPS13 and human ATG2A are able to bind at least 10 glycerolipids and to transport them between liposomes, strongly suggesting they are non-specific glycerolipid transporters (Kumar et al., 2018; Valverde et al., 2019). Structural analyses show that the N-terminal extremity containing the Chorein\_N domain forms a scooplike hydrophobic cavity that could presumably accommodate glycerolipids and that is extended by a long hydrophobic groove with a rod-like  $\beta$ -strand structure (Kumar et al., 2018; Li, Lees, Lusk, & Reinisch, 2020; Osawa et al., 2019; Valverde et al., 2019). Due to their localization at MCSs, their lipid-friendly elongated groove structure and their size exceeding 20nm (De et al., 2017), it has been broadly hypothesized that VPS13 proteins form bridges between membranes at MCSs to mediate non-specific bulk glycerolipid transport by a bridge-like mechanism (Fig. 2E) (Dziurdzik & Conibear, 2021; Kumar et al., 2018; Li et al., 2020; Reinisch & Prinz, 2021).

Nothing is known about VPS13-like plant homologs. In A. thaliana, five Chorein\_N domain-containing proteins have been identified, three of which have already been previously identified (Table 6) (Michaud et al., 2017; Velayos-Baeza, Vettori, Copley, Dobson-Stone, & Monaco, 2004). Three unknown proteins (At4g17140, At1g48090 and At5g24740) harbor the typical VPS13 protein conserved domains, especially the Chorein\_N domain and the VPS13\_N domain present at their N-terminal extremity. Given the high degree of homology found with other VPS13 proteins, we propose to rename At4g17140, At1g48090 and At5g24740, respectively, AtVPS13A, AtVPS13B and AtVPS13C. Currently, nothing is known about AtVPS13A-C cellular localization. Predictions suggest a localization in the Golgi for all three proteins. Surprisingly, AtVPS13A and AtVPS13B have an additional VPS62-homology domain of unknown function in the middle of the protein. AtVPS13A has an additional predicted PH domain in the repeating coiled region and AtVPS13B harbors a C2 domain just before the VAB domain while AtVPS13C lacks the C-terminal PH domain

AGI	Name	Localization	Domain organization
At4g17140	AtVPS13A	Golgi (prediction)	Chorein–VPS13_N–RCR–PH– RCR–VPS62–VAB–APT1– ATG_C–PH
At1g48090	AtVPS13B	Golgi (prediction)	Chorein–VPS13_N–RCR–VPS62– C2–VAB–APT1–ATG_C–PH
At5g24740	AtVPS13C, SHBY	Golgi (prediction)	Chorein–VPS13_N–RCR–RCR– VAB–APT1–ATG_C
At3g19190	ATG2/ PEUP1	Nucleus/Golgi (prediction)	Chorein–VPS13_N–ATG2_CAD– ATG_C
At3g20720		Mitochondria/ Cytosol (prediction)	Chorein–VPS13_N

Table 6Genes of the VPS13-like/Chorein\_N family present in the A. thaliana genome.AGINameLocalizationDomain organization

Protein localization corresponds to the consensus localizations from SUBA and ARAMEMNON websites (prediction) (Hooper et al., 2017; Schwacke et al., 2003). The domain organization found for each protein is also indicated. APT1: Aberrant pollen transmission 1, PH: pleckstrin homology domain, RCR: Repeated coiled region, VAB: Vps13 adaptor binding.

(Table 6). The conservation of the domains involved in VPS13 protein targeting in yeast and mammals suggest that plant VPS13 proteins are also associated to multiple cell compatments, including MCSs. In addition, the presence of slight differences in domain organization and the presence of additional domains, like C2 or VPS62-like domains, preclude a higher complexity in the pattern of VPS13 protein localization and their regulation in plant cells. Interestingly, AtVPS13A/At4g17140 was identified in the mitochondrial OM proteome of A. thaliana (Duncan et al., 2011). Even though the GFP fusions of the 200 N-terminal and C-terminal amino acid residues failed to give a consistent localization, this study gives a first hint for the potential mitochondrial localization of AtVPS13A. AtVPS13A was also found in the A. thaliana mitochondrial complexome and migrated at a molecular weight of 650kDa on BN-PAGE (Senkler et al., 2017). A FFAT motif localized in the N-terminal region of AtVPS13A was also identified, suggesting an association with the ER membrane (Slee & Levine, 2019). In light of these preliminary results, AtVPS13A-C seem to be good candidates as novel VPS13-family LTPs in plants and require further investigation. At3g19190 corresponds to a plant ATG2, also known as PEUP1. Its domain organization and its cellular functions are similar to yeast and mammalian ATG2 proteins (Chen, Dong, & Wang, 2021; Ding, Zhang, & Otegui, 2018).

#### 2.4 The Sec14-superfamily

The Sec14 domain is widely conserved in Eukaryotes and the Sec14superfamily is expanded in higher Eukaryotes where more than 20 members can be found in mammals and plants whereas yeast contains only six Sec14 proteins (Bankaitis, Mousley, & Schaaf, 2010). In lower Eukaryotes, proteins of this family usually contain only one Sec14 domain whereas in higher Eukaryotes, the Sec14 domain is mainly associated to other domains including the GOLD (Golgi dynamics) domain, the PRELI domain or the plant-specific nodulin domain (Bankaitis et al., 2010). Because of their high number and huge diversity in domain organization, the Sec14 proteins are involved in a wide range of cellular processes including membrane trafficking, lipid metabolism and signaling pathways by regulating PIPs levels and the activity of different GTPases (Bankaitis et al., 2010). Sec14 domains fold in an approximatively 280 as globular structure with two lobes, formed by an assembly of  $\alpha$ -helices and one  $\beta$ -sheet (for a review, see Bankaitis et al., 2010). The lipid-binding pocket is composed of a  $\beta$ -sheet defining the pocket floor and of  $\alpha$ -helices forming the wall, the number of  $\beta$ -strands and the orientation and number of  $\alpha$ -helices varying among the Sec14 proteins. A helical flexible gate regulates the entry of the cavity with an "open" conformation in absence of lipids and a "closed" conformation when a lipid molecule is inserted in the binding pocket (Bankaitis et al., 2010). Sec14 proteins are able to bind and transport PI, as well as a second ligand that can be diverse hydrophobic compounds like PC, PS, PE, sterols, squalene or  $\alpha$ -tocopherol (Li et al., 2000; Schaaf et al., 2008; Tripathi et al., 2019). The role and mechanisms of action of Sec14 PIPTs have been mostly studied in yeast where they are involved in PIPs metabolism and signaling, vesicular trafficking, lipid droplet biogenesis or PS synthesis (Lipp et al., 2020; Ren et al., 2014; Schaaf et al., 2008). It appears that Sec14 proteins are likely involved in regulatory processes rather than in interorganellar bulk lipid transport. The most studied protein is yeast Sec14, that regulates PIPs metabolism and vesicular trafficking at the trans-Golgi network (TGN) (for reviews, see Bankaitis et al., 2010; Grabon, Khan, & Bankaitis, 2015; Lipp et al., 2020). Sec14 binds and transports PC and PI in an ATP-independent manner and regulates PI(4)P level in the Golgi by transporting PI in this organelle and promoting its phosphorylation by the PI(4) kinase Pik1 (Bankaitis, Aitken, Cleves, & Dowhan, 1990; Lipp et al., 2020). The delivery of PI to the Golgi is coupled to the exchange with one PC molecule (Schaaf et al., 2008). From structural analyses of PC- or PI-bound Sec14 and Sfh1, the closest Sec14 homolog in yeast, it was possible to establish PCand PI-binding barcodes, corresponding to aa residues establishing specific interactions with PI or PC polar head (Lipp et al., 2020; Schaaf et al., 2008). The main role of Sec14 is not to transport PI per se, but to render PI accessible to Pik1 for phosphorylation which otherwise happens with very low efficiency when PI is buried in a lipid bilayer (Lipp et al., 2020; Schaaf et al., 2008). This mechanism is called the "presentation model" in the literature (Kf de Campos & Schaaf, 2017; Lipp et al., 2020). It has to be noticed that efficient PI presentation to Pik1 requires PC binding, explaining why PC transfer activity is required for regulation of PIPs signaling by Sec14 and why PC metabolism is coupled to this signaling pathway (Grabon et al., 2015; Kf de Campos & Schaaf, 2017; Schaaf et al., 2008). A. thaliana and rice genomes encode for 32 and 27 Sec14 domain-containing proteins, respectively (Table 7) (Huang, Ghosh, & Bankaitis, 2016). They are classified into three different families depending on domain organization and sequence similarities.

#### 2.4.1 The Sec14-nodulin family

Fourteen proteins (AtSfh1–14) in A. thaliana present a Sec14 domain fused to a nodulin domain only found in plants (Huang, Ghosh, & Bankaitis, 2016; Montag, Hornbergs, Ivanov, & Bauer, 2020; Vincent et al., 2005). The Sec14-nodulin organization is found in angiosperms and some gymnosperms but is absent from more primitive plants such as moss and fern (Huang, Ghosh, & Bankaitis, 2016; Huang, Ghosh, Tripathi, et al., et al., 2016; Montag et al., 2020). Most of these proteins are highly expressed in tissues where extensive polarized membrane growth is required, such as in pollens or roots (Ghosh et al., 2015; Vincent et al., 2005). It is predicted to adopt a  $\alpha$ -helical coiled-coil structure composed of three antiparallel  $\alpha$ -helices (Ghosh et al., 2015). 3D modeling of AtSfh1 Sec14 domain shows a similar fold as for the yeast Sec14 protein and highlights a conservation of the yeast PI- and PC-binding barcodes among the Sec14-nodulin proteins (Huang, Ghosh, & Bankaitis, 2016; Huang, Ghosh, Tripathi, et al., 2016). The ability of AtSfh1 and other Sec14-nodulin proteins to transport both PI and PC was confirmed by complementation of the yeast sec14 mutant combined with in vitro transfer assays (Böhme et al., 2004; Huang, Ghosh, & Bankaitis, 2016; Huang, Ghosh, Tripathi, et al., 2016; Vincent et al., 2005). These results suggest that this family might also regulate PIPs signaling by stimulating PIPs kinase activity. The occurrence of this mechanism for plant Sec14-nodulin proteins was further supported by

<b>Table 7</b> Genes	s of the Sec14 supe	er-family present in th	ne A. <i>thaliana</i> genome.		
AGI N	ame	Sub-family	Localization	Jomain organization References	
At4g34580 A	tSFH1/COW1	Sec14-nodulin	PM/cytosol	sec14-nodulin Böhme et al. (2004) and	Vincent et al. (2005)
At4g39180 A	tSFH2		Cytosol (Prediction)	Sec14	
At2g21540 A	tSFH3		Cytosol (Prediction)	sec14-nodulin	
At1g19650 A	tSFH4		Cytosol (Prediction)	sec14-nodulin	
At1g75370 A	tSFH5		Cytosol (Prediction)	sec14-nodulin	
At4g39170 A	ttSFH6		Cytosol (Prediction)	sec14-nodulin	
At2g16380 A	tSFH7		Cytosol (Prediction)	sec14-nodulin	
At2g21520 A	tSFH8		Cytosol (Prediction)	sec14-nodulin	
At3g24840 A	tSFH9		Cytosol (Prediction)	sec14-nodulin	
At2g18180 A	tSFH10		Cytosol (Prediction)	sec14-nodulin	
At5g47510 A	tSFH11		Cytosol (Prediction)	Sec14	
At4g36490 A	ttSFH12		PM (Prediction)	sec14-nodulin	
At1g55690 A	tSFH13		Nucleus (Prediction)	sec14-nodulin	
At5g56160 A	tSFH14		Cytosol (Prediction)	sec14	
At1g72150 A	tPATL1	Patellin	Cytosol/Cell plate/PM	Sec14-GOLD Peterman, Sequeira, Sam et al. (2018), and Wu et	ia, and Lunde (2006), Tejos al. (2017)
At1g22530 A	tPATL2		Cytosol/Cell plate/PM	sec14-GOLD Suzuki et al. (2016), Tej (2017)	os et al. (2018), and Wu et al.

Table 7 Genes of the Sec14 super-family present in the A. thaliang gen

At1g72160 AtPATL3	Cell periphery/PM/ cytosol/Cell plate	Sec14-GOLD	Peiro et al. (2014), Tejos et al. (2018), and Wu et al. (2017)
At1g30690 AtPATL4	Cytosol/Cell plate/PM	Sec14-GOLD	Tejos et al. (2018) and Wu et al. (2017)
At4g09160 AtPATL5	PM	Sec14-GOLD	Tejos et al. (2018)
At3g51670 AtPATL6	Cell periphery/PM/ cytosol/Cell plate	Sec14-GOLD	Peiro et al. (2014), Tejos et al. (2018), and Wu et al. (2017)
At4g08690 AtPITP1 Stand-al	one Sec14 Cytosol (Prediction)	Sec14	
At1g22180 AtPITP2	Cytosol (Prediction)	Sec14	
At4g36640 AtPITP3	Cytosol (Prediction)	Sec14	
At1g75170 AtPITP4	Cytosol (Prediction)	Sec14	
At1g05370 AtPITP5	Cytosol (Prediction)	Sec14	
At3g22410 AtPITP6	Cytosol (Prediction)	Sec14	
At5g63060 AtPITP7/CPSFL1	Plastids	Sec14	Hertle et al. (2020)
At1g01630 AtPITP8	Cytosol (Prediction)	Sec14	
At1g14820 AtPITP9	Cytosol (Prediction)	Sec14	
At3g46450 AtPITP10	Cytosol (Prediction)	Sec14	
At1g55840 AtPITP11	Cytosol (Prediction)	Sec14	
At5g47730 AtPITP12	Cytosol (Prediction)	Sec14	
		"IONINAVA UV P	abritan (amalianian) and indiantad /11 and at al 2017. Calarandar at al

Protein localization were determined experimentally or the consensus localizations from SUBA and ARAMEMNON websites (prediction) are indicated (Hooper et al., 2017; Schwacke et al., 2003). The domain organization found for each protein is also indicated. GOLD: Golgi dynamic.

functional analysis of AtSfh1, a protein mainly expressed during and involved in root hair elongation (Böhme et al., 2004; Vincent et al., 2005). During root hair elongation, a huge polarized transport of vesicles to the root tip elongation zone is required and involves the maintenance of a concentration gradient of  $PI(4,5)P_2$  in the PM along the elongating root hair. AtSfh1 associates specifically to  $PI(4,5)P_2$  in PM via a polybasic stretch located at the C-terminal of the nodulin domain (Ghosh et al., 2015; Vincent et al., 2005). The binding of AtSfh1 to PM and its oligomerization via the nodulin domain were suggested to promote the formation of microdomains enriched in  $PI(4,5)P_2$  and to maintain the  $PI(4,5)P_2$  gradient (Ghosh et al., 2015; Huang, Ghosh, & Bankaitis, 2016). In addition, AtSfh1 might also regulate the level of  $PI(4,5)P_2$  by stimulating PI(4) kinase activity and PI(4)P synthesis, which can be subsequently used as a substrate for  $PI(4,5)P_2$  synthesis. Because the Sec14 domain of several Sec14-nodulin proteins are able to complement yeast phenotype and to transport both PI and PC, it is likely that the regulation of PIPs metabolism by the presentation model is a general mechanism of action of Sec14-nodulin proteins (Huang, Ghosh, & Bankaitis, 2016; Huang, Ghosh, Tripathi, et al., 2016; Kf de Campos & Schaaf, 2017).

#### 2.4.2 The Patellin family

Patellins are characterized by the association of the Sec14 domain to a GOLD domain and are present in plants as well as in metazoans. In plants, Patellins are found in angiosperms and gymnosperms and A. thaliana genome encodes six Patellin genes (AtPATL1-6) (Table 7) (Peterman et al., 2006; Zhou et al., 2019). Patellins have a variable N-terminal extension differing in both length and aa composition (Peterman et al., 2006; Peterman, Ohol, McReynolds, & Luna, 2004). They are expressed in many tissues during plant development, in particular in cells with high mitotic activity, and are responsive to biotic and abiotic stress (Hua-Ying et al., 2019; Peterman et al., 2004; Tejos et al., 2018). Patellins do not contain TM domains and are found to be cytosolic and associated to membranes, in particular to PM (Peterman et al., 2004; Suzuki et al., 2016; Tejos et al., 2018; Wu et al., 2017). In dividing cells, AtPATLs are also localized to the cell plate, suggesting a role during cytokinesis (Peterman et al., 2004; Tejos et al., 2018). The PI-binding barcode, but not the PC-binding barcode, is conserved among the A. thaliana Patellins, suggesting they can transport PI and probably other yet unidentified substrates other than PC (Huang, Ghosh, & Bankaitis, 2016; Huang, Ghosh, Tripathi, et al., 2016; Peterman et al., 2004). All Patellins studied until now are able to bind several PIPs in vitro (Montag et al., 2020; Peterman et al., 2004; Suzuki et al., 2016). However, the association of AtPATL2 and AtPATL3 with the PM is mediated by protein-protein interactions via the GOLD domain and not by PIPs binding (Suzuki et al., 2016; Wu et al., 2017). Information concerning the roles of Patellins during plant development and in response to stress is scarce. AtPATL1 is involved in cold and salt stress resistance (Chu et al., 2018; Zhou et al., 2018) whereas AtPATL3 and 6 are involved in plant resistance to Alfalfa mosaic virus (AMV) by interacting with the AMV movement protein, impairing viral particle targeting to PDs and cell-to-cell movement (Peiro et al., 2014). A role in membrane trafficking during cytokinesis, salt stress response, vacuole biogenesis and autophagy is also suspected. AtPATLs are recruited to cell plate during cytokinesis, but their role in the formation or maturation of such structure is unknown (Peterman et al., 2004; Tejos et al., 2018). AtPATL1 is overexpressed in response to high salts and interacts with the PM Na<sup>+</sup> transporter SOS1, reducing Na<sup>+</sup> transport and likely promoting salt stress tolerance (Zhou et al., 2018). Finally, AtPATL1 and 2 interact with AMSH3, a deubiquitinase enzyme involved in vesicular trafficking, vacuole biogenesis and autophogosome degradation (Isono et al., 2010; Katsiarimpa et al., 2013). Because of their ability to bind a wide range of PIPs, roles in cell signaling rather than bulk lipid transport are suspected (Zhou et al., 2019). Unfortunately, the molecular mechanisms involved in the regulation of Patellin function and whether their putative lipid transfer activity is required to sustain this function is still unknown.

#### 2.4.3 Stand-alone Sec14

A. thaliana contains 12 stand-alone Sec14 proteins that can be divided into five clades that might define different biochemical properties and biological functions (Table 7) (Huang, Ghosh, & Bankaitis, 2016). A few proteins of this huge family were characterized and they seem to play a role in chloroplast development as well as in biotic and abiotic stress response (Hertle et al., 2020; Kearns et al., 1998; Kiba et al., 2014). Most of these proteins in plants were first identified by their ability to complement the yeast *sec14* mutant. This includes AtPITP11 (Jouannic et al., 1998), soybean Ssh1 and Ssh2 (Kearns et al., 1998) and *N. benthamiana* NbSec14 (Kiba et al., 2012). AtPITP11 is mainly expressed in leaves and flowers and transport PI *in vitro* (Jouannic et al., 1998), but no further information about its subcellular localization and function are available. Soybean Ssh1 is mainly expressed in leaves Ssh2 shows the highest expression level in developing seeds (Kearns et al., 1998). Both proteins are membrane associated but only Ssh2 is able to transport PI, but not PC, in vitro, whereas Ssh1 was not able transport PI and PC in the tested condition, suggesting it has no lipid transport activity or transports other unidentified molecules (Kearns et al., 1998). Both proteins bind  $PI(4,5)P_2$  and Ssh1 also associates to  $PI(3,5)P_2$  in vitro whereas Ssh2 seems to associate to PS and PA (Kearns et al., 1998). In response to hyperosmotic stress, a rapid increase of Ssh1 phosphorylation mediated by the salt-induced SPK1 and SPK2 kinases was observed, decreasing Ssh1 affinity for membranes (Monks, Aghoram, Courtney, DeWald, & Dewey, 2001). As Ssh1 increases in vitro PI(3) kinase and PI(4) kinase activities, it was proposed that Ssh1 regulates PIPs signaling in response to osmotic stress in soybean via modulation of PI kinases activity (Monks et al., 2001). NbSec14, a stand-alone Sec14 protein able to transfer PI and PC in vitro, is involved in lipid signaling during plant immune response to the bacteria Ralstonia solanacearum (Kiba et al., 2012, 2014). NbSec14 is upregulated during infection and NbSec14 siRNA plants show a higher susceptibility to R. solanacearum. In these lines, no increase in PA and DAG levels, two key lipids involved in plant immune response signaling, was observed upon infection, in accordance with a decreased DAG kinase, phospholipase D and phospholipase C activities in total plant extracts (Kiba et al., 2014). More recently, the first identified chloroplastic Sec14 protein, AtPITP7 (also called CPSFL1), was shown to be involved in chloroplast development and photoautotropic growth (Hertle et al., 2020). AtPITP7 accumulates in plastid stroma vesicles and is involved in vesicular trafficking required for thylakoid biogenesis. In vitro, AtPITP7 binds PA, SQDG and PIPs but is only able to extract and transfer PI(4)P between liposomes (Hertle et al., 2020). Thus, this protein might regulate lipid signaling between the inner envelope of plastids and thylakoids.

#### 2.5 nsLTPs

The non-specific LTP (nsLTP) is a land plant-specific class of LTPs that can bind a wide variety of substrates *in vitro*. They are encoded by large gene families, with 79 and 77 members in *A. thaliana* and rice, respectively (Salminen, Blomqvist, & Edqvist, 2016). NsLTPs are small basic proteins (7–10kDa) characterized by the presence of eight conserved Cys residues. They fold into a compact globular structure composed of four  $\alpha$ -helices with a C-terminal unstructured tail (for detailed reviews, see Carvalho & Gomes, 2007; Salminen et al., 2016). NsLTPs possess an N-terminal peptide sequence that targets proteins to the secretory pathway, most of them being then secreted in the apoplast space. They are soluble but some of them can bind membranes and liposomes *in vitro* while others can be post-translationally associated to a glycosylphosphatidylinositol (GPI) anchor. NsLTPs are able to bind a wide variety of substrates, like phospholipids, galactoglycerolipids, free FAs, acyl-CoA, lyso-lipids or prostaglandin (Kader, 1996; Salminen et al., 2016). Proteins from this family are mainly involved in (1) cell wall synthesis, (2) pollen and seed development, (3) embryogenesis, (4) defense again pathogens, (5) long-distance signaling and (6) stress response (Carvalho & Gomes, 2007; Edqvist, Blomqvist, Nieuwland, & Salminen, 2018; Kader, 1996; Salminen et al., 2016). Most of nsLTPs are likely not involved in the intracellular transfer of lipids dedicated to membrane biogenesis. However, such a role might be supported by two nsLTPs, AsE246 and MtN5, in legumes during nodulation in response to nitrogen starvation and by one nsLTP, GhLTPG1, involved in cotton fiber elongation (Deng et al., 2016; Lei et al., 2014; Pii et al., 2009).

During nitrogen starvation, legume roots are able to establish symbiotic relationships with rhizobial bacteria or arbuscular mycorrhizal fungi that will provide nitrogen to the plant in exchange of carbon (Downie, 2014; Udvardi & Poole, 2013; Verma, 1996). This symbiotic process occurs in special structures formed in roots of infected plants called nodules where bacteria are enclosed in a special cell compartment, the symbiosome, in which they will divide and differentiate into nitrogen-fixing bacteroids. Symbiosome membrane lipids arise from the host plant cell and a tremendous amount of lipid synthesis and transfer is required for nodule formation (Harrison & Ivanov, 2017; Verma, 1996). Symbiosome membranes formation relies on Golgi-derived vesicle trafficking (Harrison & Ivanov, 2017). They are composed of phospholipids (PC, PE, PI and PG) and DGDG (Gaude et al., 2004; Lei et al., 2014), suggesting that chloroplast and likely non-vesicular lipid trafficking might be involved in the biogenesis of these membranes. In line with this, AsE246, a nsLTP specifically expressed during nodulation and located to the symbiosome membrane, is required for nodule formation and symbiosomes development in Astragalus sinicus infected by the rhizobacteria Mesorhizobium huakuii (Chou, 2006; Lei et al., 2014). AsE246 is able to bind different phospholipids and galactoglycerolipids (Lei et al., 2014). However, whether AsE246 promotes lipid transport from cell organelles to symbiosome membranes remains to be defined. Another Medicago truncatula nsLTP, MtN5, plays a role in the regulation of root nodulation during Sinorhizobium meliloti symbiosis (Pii et al., 2009; Pii, Molesini,

Masiero, & Pandolfini, 2012; Pii, Molesini, & Pandolfini, 2013). MtN5 is expressed early during nodule formation and is required for optimal bacterial infection and nodule invasion. In vitro, MtN5 binds to some lyso-PC species (Pii et al., 2009). Lipidomic analyses of roots overexpressing MtN5 highlighted a global increase in lipid content, in particular of DGDG, MGDG, PE and glucosyl-ceramide. This increase is concomitant with the modification of the expression of genes involved in lipid metabolism and lipid transport (Santi et al., 2017). These results suggest that MtN5 might regulate lipid synthesis and/or transport during nodulation. Another role in the regulation of nodulation by lipid signalization has been proposed as (1) MtN5 is able to bind lyso-PC in vitro (Pii et al., 2009), a lipid signal involved in arbuscular mycorrhizal symbiosis (Drissner et al., 2007), and (2) overexpression of MtN5 triggers deregulation of the expression of genes involved in the synthesis of lipid signaling molecules such as PIPs kinases, phospholipases C or Sec14-like proteins (Santi et al., 2017). All these data suggest that non-vesicular lipid transport could also be involved in membrane biogenesis during plant-microorganism symbiosis in response to nitrogen starvation. This might be particularly true for DGDG, as this plastidial lipid has never been shown to be transported *via* vesicular routes.

Cotton fiber synthesis starts from ovule epidermal cells and is divided in four steps: (1) fiber initiation, (2) cell elongation, (3) secondary wall deposition and (4) maturation (Kim & Triplett, 2001). The cell elongation step requires a huge amount of lipids to sustain membrane synthesis in growing cells (Wanjie, Welti, Moreau, & Chapman, 2005). The GPI-anchored nsLTP GhLTPG1 is highly expressed in elongating fibers and is able to specifically bind and transport PI-monophosphates (Deng et al., 2016). GhLTPG1 harbors a peptide signal and a GPI anchor, both required for its localization to the PM (Deng et al., 2016). Silencing of GhLTPG1 slightly reduces cotton fiber length and the expression of cotton-fiber elongation genes. A small decrease in polar lipids, PI in particular, was also observed (Deng et al., 2016), suggesting that GhLTPG1 might be involved in lipid transport to sustain fiber elongation.

# **3. Lipid transport mediated by other proteins** 3.1 NDPK-D

Nucleoside diphosphate kinases (NDPKs) are a family of proteins conserved throughout evolution with a primary role in the exchange of  $\gamma$ -phosphate between nucleosides-tri-phosphate and nucleoside-di-phosphate (Boissan,

Schlattner, & Lacombe, 2018). They act as symmetrical homo-hexamers and are present in different cell compartments where they can play a role in many cellular processes (Boissan et al., 2018; Janin et al., 2000). Interestingly, one well studied homolog in mammals, NDPK-D, is located in mitochondria IMS and is known to bind membranes and transfer CL (Epand, Schlattner, Wallimann, Lacombe, & Epand, 2007; Schlattner et al., 2013; Tokarska-Schlattner et al., 2008). NDPK-D membrane binding is mediated by a cationic motif R89-R90-K91 located at the surface of the hexamer, which associates with negatively charged lipids, in particular CL (Tokarska-Schlattner et al., 2008). The R90 residue, specific to the mitochondrial NDPK-D ortholog in humans, is essential for CL and membrane binding (Tokarska-Schlattner et al., 2008). As one CL binding site is present per monomer, NDPK-D hexamers are able to tether liposomes containing CL in vitro and OM-IM in vivo (Epand et al., 2007; Kagan et al., 2016; Tokarska-Schlattner et al., 2008). Membrane binding inhibits NDPK-D phosphotransferase activity and promotes CL transfer from IM to OM, inducing apoptosis and mitophagy and linking NDPK-D lipid transport function to CL signaling (Kagan et al., 2016; Schlattner et al., 2013, 2018).

Two NDPK-D homologs are found in A. thaliana (NDPK3a and NDPK3b), NDPK3b being expressed at a very low level (Table 8) (Hammargren et al., 2007). Both proteins have been detected in mitochondrial proteome (Heazlewood et al., 2004) and NDPK3a-GFP fusion was found to be located in mitochondria (Carrie et al., 2009) or in mitochondria and plastids (Hammargren et al., 2007; Luzarowski et al., 2017). Interestingly, NDPK3a was also found in proteomic analysis of the MTL complex, a mitochondrial complex involved in lipid transport (see below) (Michaud et al., 2016). However, Western blot analysis suggests that this protein probably co-migrates with the MTL complex on native gel rather than being a real MTL complex component. In addition, the R90 residue required for membrane binding of human NDPK-D is not conserved in A. thaliana, suggesting that NDPK3 could not bind membrane lipids (Fig. 4). However, in pea, NDPK3 is strongly associated with the intermembrane space (IMS) leaflet of the IM and interacts with the ANT carrier (Knorpp, Johansson, & Baird, 2003), suggesting that membrane binding could be mediated by protein-protein interactions in plants (Fig. 3C(i)). Further analyses are required to understand if NDPK3a is a component of the MTL complex and if it transports CL between mitochondrial OM and IM.

AGI	Name	Localization	References
At4g11010	NDPK3a	Mitochondria/ plastids	Carrie et al. (2009), Hammargren, Sundström, Johansson, Bergman, and Knorpp (2007), and Luzarowski et al. (2017)
At4g23900	NDPK3b	Mitochondria (prediction)	
At3g25610	ALA10	PM, ER, ER-plastids MCSs, ER-PM MCSs	Botella et al. (2016) and Poulsen et al. (2015)
At1g19800	TGD1	Chloroplast inner envelope	Xu, Fan, Froehlich, Awai, and Benning (2005)
At3g20320	TGD2	Chloroplast inner envelope	Awai, Xu, Tamot, and Benning (2006)
At1g65410	TGD3	Stroma	Lu, Xu, Awai, Jones, and Benning (2007)
At3g06960	TGD4	Chloroplast outer envelope	Wang, Xu, and Benning (2012)
At1g27695	TGD5	Chloroplast envelopes	Fan, Zhai, Yan, and Xu (2015)
At2g44640	atLPTD1	Chloroplast outer envelope	Hsueh, Ehmann, Flinner, Ladig, and Schleiff (2017)
At4g39690	AtMic60	Mitochondrial inner membrane	Michaud et al. (2016)
At1g22520	AtMic10	Mitochondria (Prediction)	
At3g20000	AtTom40	Mitochondrial outer membrane	Duncan et al. (2011)

 Table 8 Diverse genes playing a role in A. thaliana lipid transport.

Protein localization were determined experimentally or the consensus localizations from SUBA and ARAMEMNON websites (prediction) are indicated (Hooper et al., 2017; Schwacke et al., 2003).



**Fig. 4** Sequence alignment of the mitochondrial NDPK enzymes from human (HsNDPK-D) and *Arabidopsis thaliana* (AtNDPK3a, b) showing the non-conservation in plants of the RRK motif involved in cardiolipin binding in humans.

# 3.2 Enzymes involved in lipid metabolism

In yeast and mammals, several enzymes involved in lipid synthesis are enriched at MCSs, in particular at ER-mitochondria MCSs and at ER-PM MCSs (Gaigg et al., 1995; Pichler et al., 2001; Vance, 1990). In plants, enzymes involved in lipid metabolism have also been detected in ER-PM and in ER-plastids MCSs (Andersson, Goksor, & Sandelius, 2007; Larsson, Kjellberg, Tjellström, & Sandelius, 2007). In addition, during Pi starvation, the phospholipase D PLDζ2 is enriched at vacuolemitochondria and vacuole-chloroplasts MCSs, whereas an even distribution along the vacuole membrane is observed when A. thaliana plants are grown in Pi-sufficient conditions (Yamaryo, Dubots, Albrieux, Baldan, & Block, 2008). An increasing number of evidences suggests that such localization is important to provide an efficient transport of lipids between membranes. This is the case for instance in yeast for the transport of PS at ERmitochondria MCSs, where the localization of PS synthase at this junction increases the efficiency of PS transport to mitochondria (Kannan, Lahiri, Liu, Choudhary, & Prinz, 2017). Several hypotheses can explain why the localization of lipid-synthesizing enzymes at MCSs is important for non-vesicular lipid transfer: (1) local synthesis can create microdomains with special lipid composition facilitating lipid extrusion and transfer, (2) the local enrichment of a lipid at MCSs can favor its transport by energy-independent LTPs following its concentration gradient, (3) the modification of a lipid at MCSs can promote the formation of lipid species more competent for non-vesicular transport (*i.e.*, less hydrophobic), and/or (4) the coupling between synthesis and transfer can be important to rapidly regulate organelle biogenesis during cell division or in response to stress. In addition, several examples have demonstrated that enzymes can also work *in trans* at MCSs, meaning that they can modify a lipid substrate located in the juxtaposed membrane. This is the case for the phosphatidylserine decarboxylase PSD1 in mitochondria IM that can decarboxylate OM PS to produce PE at OM-IM MCSs (Aaltonen et al., 2016). This mechanism is involved in the regulation of lipid composition at MCSs but is not involved in the lipid transport process per se.

# 3.2.1 PC remodeling at ER-plastids MCSs

Lyso-PC can be spontaneously transferred between membranes because of its low hydrophobicity. In plants, lyso-PC synthesis at ER-plastids MCSs might be one of the mechanisms involved in the transport of lipid precursors from ER to chloroplast to fulfill galactoglycerolipid synthesis via the Eukaryotic pathway. This idea was supported by pulse chase experiments and by the presence of several enzymes involved in the synthesis of lyso-PC from PC and in its reacylation at ER-chloroplasts MCSs or in chloroplasts OE (Fig. 3D(i)) (Bessoule, Testet, & Cassagne, 1995; Jessen, Roth, Wiermer, & Fulda, 2015; Mongrand, Cassagne, & Bessoule, 2000; Tan et al., 2011). In Brassica napus, the lipase BnCLIP1 is located at ERchloroplasts MCSs, where it might be involved in lyso-lipid synthesis from ER phospholipids (Tan et al., 2011). Andersson et al. show that cytosolic lipases can generate lyso-PC molecules which are subsequently used for galactoglycerolipid synthesis (Andersson, Kjellberg, & Sandelius, 2004). In A. thaliana, the long-chain acyl-CoA synthetases LACS4 and LASC9 are localized in the ER and the chloroplast OE, respectively, and double lacs 4 lacs 9 mutants show a decrease in the content of galactoglycerolipids arising from the Eukaryotic pathway (Jessen et al., 2015). Thus, these LACSs have been proposed to play a role in the creation of a local pool of acyl-CoA at ER-chloroplasts MCSs that might be used by LPCAT to promote PC synthesis in the chloroplast envelope (Jessen et al., 2015). In addition, LPCAT activity has been detected in the chloroplast envelope of several plant species (Bessoule et al., 1995; Karki, Johnson, & Bates, 2019). However, the analyses of *lpcat1lpcat2* mutants in A. thaliana, the two main enzymes responsible for LPCAT activity, demonstrated that LPCAT activity, and likely lyso-PC transport, is not essential for chloroplastic lipid synthesis via the Eukaryotic pathway (Karki et al., 2019). Interestingly, pulse chase labelling experiments performed in this work support a model in which the LPCAT1/2 enzymes are involved in the incorporation of newly synthesized chloroplastic FAs into PC by lyso-PC acylation. PC is then translocated back to the ER where it can also be synthesized by the *de novo* pathway from glycerol-3-phosphate and acyl-CoA, leading to two distinct pools of PC in the ER (Karki et al., 2019). Thus, this current model suggests that LPCAT1/2 are more likely involved in chloroplast to ER PC transport than in the Eukaryotic pathway of galactoglycerolipid synthesis. Transport of PC via the lyso-PC intermediate could also occur at ER-PM MCSs in plants where LPCAT activity has been detected in pea (Larsson et al., 2007).

#### 3.2.2 DGD1

MGDG synthesis starts by the addition of a galactose residue to a DAG backbone. Bulk MGDG synthesis takes place at the plastid IE and is catalyzed by MGD1, while MGD2/3 operate at the plastid OE in non-photosynthetic tissues or during specific stress conditions, typically Pi

starvation (Awai et al., 2001; Kobayashi et al., 2009). DGDG synthesis requires the addition of another galactose residue moiety to MGDG, a reaction catalyzed by DGD1 and DGD2 at the plastid OE (Fig. 3D(ii)) (Kelly, Froehlich, & Dörmann, 2003). Hence, DGDG synthesis requires the transport of MGDG from the IE to the OE. The N-terminal region of DGD1, NDGD1, has been shown to promote lipid transport between the plastid IE and OE (Kelly et al., 2016). NDGD1 is necessary for DGDG synthesis in the OE. Furthermore, NDGD1 binds PA and enables fusion of PAcontaining liposomes *in vitro* (Kelly et al., 2016). Thus, it has been suggested that NDGD1 could facilitate lipid transport between both chloroplast membranes by triggering membrane-hemifusion (Kelly et al., 2016). Whether DGD1 acts as a tether or as a direct lipid transport protein remains to be determined.

#### 3.3 Enzymes flipping lipids between membrane leaflets

Several recent studies suggest that enzymes involved in lipid translocation between membrane leaflets act as key players in non-vesicular lipid transport between membranes, notably by acting in concert with LTPs. Flippases can flip lipids against their concentration gradient in an ATP-dependent manner to create asymmetric membranes while scramblases can translocate lipids between the two leaflets following their concentration gradient in an ATPindependent manner, thus equilibrating lipids between membrane leaflets (Maxfield & Menon, 2016). The putative roles of these proteins in non-vesicular lipid exchanges might be (1) to ensure the selectivity and directionality of transport, (2) to facilitate the loading of lipids by LTPs, and/or (3) to facilitate the insertion of the transported lipid in the acceptor membrane (Dziurdzik & Conibear, 2021; Reinisch & Prinz, 2021). A good example of this mechanism is found in Gram-negative bacteria: lipopolysaccharide (LPS) is synthetized on the cytosolic leaflet of the IM and is subsequently transported to the extracellular leaflet of the OM (Sperandeo, Martorana, & Polissi, 2017). LPS is first flipped across the IM by the flippase MsbA, then extracted from the IM by the LptB2FGC ABC transporter complex, ensuring the directionality of the transport. LPS transport through the periplasm is facilitated by LptACDFG proteins that form a hollow rod-like structure composed of hydrophobic  $\beta$ -jellyrolls reminiscent of the VPS13 hydrophobic groove (Thélot, Orlando, Li, & Liao, 2020). The insertion of LPS in the OM and its translocation to the outer leaflet is ensured by the LptDE translocon (Sperandeo et al., 2017). A similar mechanism was proposed for lipid transport at ER-autophagosomes MCSs in yeast where the

LTP Atg2 associates with the autophagosome-located Atg9 scramblase and with the ER-located scramblases TMEM41B and VMP1 (Matoba et al., 2020; Noda, 2021; Orii, Tsuji, Ogasawara, & Fujimoto, 2021). Other LTPs, such as the human VPS13A or the yeast Osh6 protein have also been shown to associate with lipid scramblases (D'Ambrosio et al., 2020; Park & Neiman, 2020), suggesting further that lipid-flipping enzymes could play an active role in non-vesicular lipid transfer in association with LTPs.

In plants, lipid scramblases are also conserved but their role in lipid transport has never been investigated. However, in A. thaliana, a P4-ATPase flippase, ALA10, has been shown to regulate plastid MGDG synthesis in leaves (Table 8 and Fig. 3D(iii)) (Botella et al., 2016). ALA10 is located in the ER where it can interact with the ALA-interacting subunits (ALIS) ALIS1 or ALIS5. This association regulates ALA10 subcellular localization: in the ER close to PM when ALA10 is co-expressed with ALIS1 or in the ER close to chloroplasts when co-expressed ALIS5 (Botella et al., 2016). More recently, ALA10 localization was also shown to be impacted by its level of ubiquitination by the E3-Ubiquitin ligase PUB11, showing the complexity of the mechanisms regulating P4-ATPase localization in cells (López-Marqués, Davis, Harper, & Palmgren, 2021; Salvaing et al., 2020). Overexpression of ALA10 increases the ratio MGDG/PC in leaves and the 18:2 content in chloroplastic PC, suggesting it can stimulate MGDG synthesis and enrich 18:2-PC species in chloroplasts (Botella et al., 2016). ALA10 interacts with the ER FA desaturase FAD2, involved in 18:1 acyl chain desaturation to 18:2, but not with FAD3, involved in 18:2 acyl chain desaturation to 18:3 (Botella et al., 2016). Thus, it was proposed that ALA10, by interacting with FAD2, translocates PC species enriched in 18:2 from the lumenal to the cytosolic leaflet of the ER, promoting a local enrichment of this lipid and favoring its transport by an unknown mechanism to chloroplasts. To explain the higher MGDG/PC ratio in ALA10 overexpressing plants, the authors suggested that the translocated PC could be a substrate for phospholipases D to generate PA, a lipid known to activate MGD1 activity (Botella et al., 2016; Dubots et al., 2012). This example illustrates that lipid flipping across membrane leaflets can also be involved in inter-organellar lipid transfer in plants.

# 4. Lipid transport mediated by protein complexes4.1 The TGD complex

An important molecular effector implicated in ER to chloroplast lipid transport is the TGD (Trigalactosyldiacylglycerol) complex composed of at least
five subunits: TGD1–5 (Table 8 and Fig. 3D(iv)). Plants deficient in any of the TGD proteins show substantial trigalactosyldiacylglycerol (TGDG) accumulation (hence their name) (Xu, Fan, Riekhof, Froehlich, & Benning, 2003), reduction in growth and photosynthetic capacity (Li, Gao, Benning, & Sharkey, 2012; Xu, Fan, Cornish, & Benning, 2008), and compromised embryogenesis (Xu et al., 2005). Importantly, they show reduced amounts of C18 acyl chains in *sn*-2 position of chloroplast lipids, consistent with an Eukaryotic lipid import defect into chloroplasts (Xu et al., 2003).

TGD1, TGD2 and TGD3 form a non-canonical ABC transporter system at the chloroplast IE. It probably derives from a Gram-negative bacterial ABC import system that was present in cyanobacteria prior to endosymbiosis (Roston, Gao, Murcha, Whelan, & Benning, 2012). Bacterial ABC importers are divided into two structural categories but show conserved features comprising (1) two TM domains composed of multiple transmembrane helices forming a permease to allow substrate passage, (2) two nucleotide binding domains specialized in ATP hydrolysis that provide energy for substrate transport and (3) a usually periplasmic substrate binding domain (SBD) that captures and delivers the substrate to the transporter subunits (Beis, 2015; Ford & Beis, 2019; Hollenstein, Dawson, & Locher, 2007; Wilkens, 2015). TGD1 structure prediction is reminiscent of a half-permease unit of a bacterial ABC importer harboring six transmembrane helices and is therefore thought to be the permease subunit of the TGD1–3 complex (Xu et al., 2003, 2005). Consistent with its putative role, TGD1 is an integral membrane protein localized in the chloroplast IE (Xu et al., 2005). TGD2 is the SBD-containing subunit of the TGD1-3 complex. In contrast to bacterial SBD subunits, TGD2 is anchored to the chloroplast IE by a N-terminal TM domain while the C-terminal part of the protein is located in the intermembrane space and specifically binds PA (Awai et al., 2006; Lu & Benning, 2009; Roston, Gao, Xu, & Benning, 2011). A mycobacterial cell entry (MCE) domain is juxtaposed to the TM domain (Awai et al., 2006). Interestingly, bacterial MCE proteins are known to assemble into hexamers forming an elongated hydrophobic channel ideal for lipid transport between the bacterial OM and IM (Ekiert et al., 2017). A 25 amino acid sequence partially overlapping the MCE domain is sufficient to mediate PA binding, but the complete TGD2 C-terminus still provides better binding affinity suggesting the existence of multiple binding determinants (Lu & Benning, 2009). TGD3 is a small ATPase that provides the energy for the TGDmediated lipid transport (Lu et al., 2007). TGD3 is a stromal protein that is associated with the chloroplast IE, consistent with its bacterial ABC importer counterparts (Fig. 3D(iv)) (Lu et al., 2007).

TGD1–3 form a highly stable complex of about 500kDa presumably composed of two TGD1 permease subunits, two TGD3 ATPase subunits and between 8 and 12 TGD2 SBD subunits (Roston et al., 2011, 2012). The large number of TGD2 proteins is surprising as it diverges from the canonical bacterial ABC importer architecture that usually contains only one SBD subunit. Why the TGD1-3 complex contains multiple SBD subunits is still unclear. Since TGD2 binds its putative substrate, PA, in a cooperative manner (Lu & Benning, 2009), it has been suggested that an increased amount of SBDs could help to sense and bind PA even at extremely low concentrations (Roston et al., 2012). Alternatively, it has been proposed that the assembly of multiple SBDs could concentrate PA, a non-bilayer forming lipid, and thus facilitate lipid extraction from the donor membrane (LaBrant et al., 2018). Consistent with the latter hypothesis, TGD2 has been found to disrupt membranes in vitro (Roston et al., 2011). Furthermore, the MCE domain contained in TGD2 suggests that its oligomerization might form a lipid transport channel like MlaD in the bacterial non-canonical ABC lipid transporter (Chi et al., 2020; Ekiert et al., 2017; Thong et al., 2016).

The TGD complex also harbors two additional proteins: TGD4 and TGD5. TGD4 is a  $\beta$ -barrel protein that forms homodimers in the chloroplast OE (Fig. 3D(iv)) (Wang et al., 2012; Wang, Anderson, & Benning, 2013; Xu et al., 2008). TGD4 is distantly related to the lipid A transporter D (LptD) subunit that transports LPS from the bacterial IM to the bacterial OM (see above). Even though TGD4 was initially thought to be localized in the ER, further investigations indicate a localization in the chloroplast OE (Wang et al., 2012; Xu et al., 2008). Thus, it has been proposed that TGD4 could be present at ER-chloroplast MCSs, thus providing a direct lipid transport route from the ER to the chloroplast (Wang & Benning, 2012). TGD4 binds PA with two cytosol-facing N-terminal domains that are both necessary and sufficient for lipid import, supporting a lipid transport function within the TGD complex (Wang et al., 2012, 2013). Another  $\beta$ -barrel protein, AtLPTD1, homologous to LptD like TGD4, is present in the chloroplast OE (Fig. 3D(iv)) (Hsueh et al., 2017). Mutants depleted in AtLPTD1 show phenotypic traits similar to tgd mutants, including TGDG accumulation (Hsueh et al., 2017; LaBrant et al., 2018). AtLPTD1 is expressed during plant adaptation to Pi starvation and is presumably implicated in DAG import into chloroplasts (Hsueh et al., 2017). It is presently not known if AtLPTD1 is part of the TGD complex. TGD5 is a small glycine-rich protein interacting with the TGD1–3 complex and TGD4, strongly suggesting that it bridges the TGD complex subunits located in both chloroplast envelope membranes (Fan et al., 2015). A recent study showed that *A. thaliana* TGD5 mutants have a strong increase in plastid stromules (Itoh et al., 2021). Since stromules are known to promote ER-chloroplast contact sites (Schattat, Barton, & Mathur, 2011), it is tempting to hypothesize that stromule formation is the response to a lipid import defect caused by the lack of TGD5 subunit.

The current picture of the TGD complex suggests that ER-synthesized lipids in the chloroplast are directly channeled through the chloroplast OE and IE by TGD4 and TGD1–3, respectively (Fig. 3D(iv)). A big gap of knowledge still concerns the nature of the transported lipid (LaBrant et al., 2018; Michaud & Jouhet, 2019). Since TGD2 and TGD4 have been shown to bind PA (Awai et al., 2006; Wang et al., 2012), it is tempting to speculate that PA is the transported lipid. However, a mathematical model mimicking chloroplast lipid synthesis *via* the Eukaryotic pathway suggests the import of DAG rather than PA (Maréchal & Bastien, 2014). Thus, PA could have a regulatory function of TGD, as it is the case for MGD1 for instance (Dubots et al., 2012). Remaining unanswered questions concern the connection between the TGD complex and the ER and its putative involvement in lipid precursor import from the ER to the OE.

### 4.2 The MTL complex

The MTL complex is a complex involved in mitochondrial lipid transport, notably in response to Pi starvation (Michaud, 2018; Michaud et al., 2016). The MTL complex is a huge lipid-containing entity with roughly 200 proteins and migrating at about 800kDa on native gel (Michaud et al., 2016). Proteomic analysis revealed that the MTL complex contains mainly mitochondrial proteins and also several proteins located in non-mitochondrial compartments, such as the plastid OE, suggesting a possible location at mitochondrial MCSs. Interestingly, all the components of the translocase of the outer membrane (TOM) complex and AtMic60 are present in the MTL complex (Table 8 and Fig. 3C(ii)). AtMic60 is a confirmed functional homolog of yeast Mic60, a core component of the mitochondrial contact site cristae organizing system (MICOS) complex (Pfanner et al., 2014).

MICOS plays a central role in mitochondrial membrane architecture, dynamics of protein import, lipid homeostasis and lipid trafficking (Michaud et al., 2017; Mukherjee et al., 2021; Rampelt, Zerbes, van der Laan, & Pfanner, 2017). In yeast and mammals, Mic60, a core component located in the IM, interacts with multiple partners in the mitochondrial OM, *i.e.*, the TOM complex, SAM50, VDAC and DNAJC11, thus tightly tethering the mitochondrial OM and IM (Mukherjee et al., 2021). AtMic60 interacts with AtTom40 and a decrease in AtTom40 content was observed in the MTL complex of atmic60 mutants, suggesting that AtMic60 also modulates OM-IM tethering (Michaud et al., 2016). Furthermore, the abundance of AtMic60-AtTom40 in the MTL complex increases in Pi deprived conditions, suggesting a role in lipid remodeling (Michaud et al., 2016). Lipid analysis of the MTL complex in *atmic60* KO lines revealed that DGDG accumulation in the MTL complex is compromised whereas PE accumulates during Pi starvation, suggesting that PE export and DGDG import are impaired (Fig. 3C(ii)). A role in intra-mitochondrial lipid trafficking was further supported by mitochondrial lipid analyses showing a decreased DGDG and increased PE levels in *atmic60* compared to WT during Pi starvation (Michaud et al., 2016). AtMic60 also destabilizes liposomes in vitro, suggesting that it could promote lipid desorption from membranes prior to transport (Michaud et al., 2016). Even if AtMic60 and AtTom40 were identified as key players, the exact lipid transport mechanism exerted by the MTL complex as a whole, as well as the role of its partners, remains to be unraveled. Since the MTL complex also encloses several extra-mitochondrial proteins, notably chloroplast OE proteins of unknown function, it is tempting to speculate that the MTL complex might form a structure connecting mitochondrial membranes to other cell compartments such as chloroplasts, promoting direct lipid transport between both compartments (Michaud et al., 2016, 2017). Such a hypothetical structure is reminiscent of the ER-mitochondria organizing network (ERMIONE) that has been suggested to organize ER-mitochondria MCSs functions in yeast and mammals and to be involved in ER to IM lipid transport (Wideman & Muñoz-Gómez, 2016).

## 5. Conclusion and perspectives

The general mechanisms described in mammals and yeast for nonvesicular glycerolipid transport are conserved in plants. For instance, most of the LTP families present in yeast and mammals are also found in plants, even if only a few of them have been characterized in terms of subcellular localization, lipid transport specificities and cellular functions. Peculiarities have to be highlighted concerning non-vesicular lipid transport in plants:

Plants cope with a wide variety of stresses where lipid signaling and membrane remodeling play a crucial role in the adaptation process (Hou et al., 2016; Liu et al., 2019; Michaud & Jouhet, 2019). Thus, specific lipid

signaling and trafficking systems might have been developed during evolution to empower the plant response to these particular situations. In accordance with this, many LTP families are expanded in plants and most of them are highly responsive to biotic and abiotic stresses. In addition, most of the plant LTPs characterized until now have a confirmed role in plant stress susceptibility (Table 1). It also has to be noticed that many *A. thaliana* LTP genes are predicted to be transcribed into several mRNA isoforms in the TAIR database, increasing the putative number of lipid transporter that could co-exist in a cell.

- Plant have a chloroplast which biogenesis requires extensive exchanges of lipids with the ER. Thus, specific lipid transport machineries, such as the TGD complex, have evolved during evolution to ensure chloroplast membranes biogenesis and functions. However, the mechanisms involved in ER-plastids MCSs formation and in the transport of lipids between both organelles remain poorly characterized. In addition, chloroplast lipids can be major contributors for the biogenesis of extraplastidial membranes in stress conditions, such as Pi starvation where DGDG is exported to mitochondria, PM and vacuoles, or nitrogen starvation in legumes where symbiosome membrane formation requires DGDG. Beside the poorly understood mechanisms involved in those transports, the question of whether chloroplast glycerolipids could also be involved in extra-plastidial membrane synthesis in normal growth conditions remains open.
- Finally, the synthesis of cell wall structures in plants, such as cutin, wax or sporopollenin, requires the secretion to the extracellular space of hydrophobic components. To ensure this function, a specific family of LTPs, the nsLTPs, appeared in plants during evolution. In this huge family, some of them are also able to bind glycerolipids and are involved in membrane biogenesis during stress, highlighting that plants have evolved specific pathways for non-vesicular lipid transport.

In summary, the understanding of the processes involved in non-vesicular lipid transfer in plants is in its infancy. Many studies still have to be performed to understand how this process occurs and how it is regulated in response to stress.

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#### References

- Aaltonen, M. J., Friedman, J. R., Osman, C., Salin, B., di Rago, J., Nunnari, J., et al. (2016). MICOS and phospholipid transfer by Ups2–Mdm35 organize membrane lipid synthesis in mitochondria. *The Journal of Cell Biology*, 213(5), 525–534.
- AhYoung, A. P., Jiang, J., Zhang, J., Khoi Dang, X., Loo, J. A., Zhou, Z. H., et al. (2015). Conserved SMP domains of the ERMES complex bind phospholipids and mediate tether assembly. *Proceedings of the National Academy of Sciences of the United States of America*, 112(25), E3179–E3188. https://doi.org/10.1073/pnas.1422363112.
- Andersson, M. X., Goksor, M., & Sandelius, A. S. (2007). Optical manipulation reveals strong attracting forces at membrane contact sites between endoplasmic reticulum and chloroplasts. *Journal of Biological Chemistry*, 282(2), 1170–1174. https://doi.org/ 10.1074/Jbc.M608124200.
- Andersson, M. X., Kjellberg, J. M., & Sandelius, A. S. (2004). The involvement of cytosolic lipases in converting phosphatidyl choline to substrate for galactolipid synthesis in the chloroplast envelope. *Biochimica et Biophysica Acta*, 1684(1–3), 46–53. https://doi.org/ 10.1016/j.bbalip.2004.06.003.
- Andersson, M. X., Larsson, K. E., Tjellström, H., Liljenberg, C., & Sandelius, A. S. (2005). Phosphate-limited oat. *Journal of Biological Chemistry*, 280(30), 27578–27586. https://doi. org/10.1074/jbc.M503273200.
- Awai, K., Marechal, E., Block, M. A., Brun, D., Masuda, T., Shimada, H., et al. (2001). Two types of MGDG synthase genes, found widely in both 16:3 and 18:3 plants, differentially mediate galactolipid syntheses in photosynthetic and nonphotosynthetic tissues in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America*, 98(19), 10960–10965. https://doi.org/10.1073/pnas.181331498.
- Awai, K., Xu, C., Tamot, B., & Benning, C. (2006). A phosphatidic acid-binding protein of the chloroplast inner envelope membrane involved in lipid trafficking. *Proceedings of the National Academy of Sciences of the United States of America*, 103(28), 10817–10822. https:// doi.org/10.1073/pnas.0602754103.
- Bankaitis, V. A., Aitken, J. R., Cleves, A. E., & Dowhan, W. (1990). An essential role for a phospholipid transfer protein in yeast Golgi function. *Nature*, 347(6293), 561–562. https://doi.org/10.1038/347561a0.
- Bankaitis, V. A., Mousley, C. J., & Schaaf, G. (2010). The Sec14 superfamily and mechanisms for crosstalk between lipid metabolism and lipid signaling. *Trends in Biochemical Sciences*, 35(3), 150–160. https://doi.org/10.1016/j.tibs.2009.10.008.
- Barajas, D., Xu, K., de Castro Martin, I. F., Sasvari, Z., Brandizzi, F., Risco, C., et al. (2014). Co-opted oxysterol-binding ORP and VAP proteins channel sterols to RNA virus replication sites via membrane contact sites. *PLoS Pathogens*, 10(10), e1004388. https://doi. org/10.1371/journal.ppat.1004388.
- Bayer, E. M., Sparkes, I., Vanneste, S., & Rosado, A. (2017). From shaping organelles to signalling platforms: The emerging functions of plant ER-PM contact sites. *Current Opinion in Plant Biology*, 40, 89–96. https://doi.org/10.1016/j.pbi.2017.08.006.
- Bean, B. D. M., Dziurdzik, S. K., Kolehmainen, K. L., Fowler, C. M. S., Kwong, W. K., Grad, L. I., et al. (2018). Competitive organelle-specific adaptors recruit Vps13 to membrane contact sites. *The Journal of Cell Biology*, 217(10), 3593–3607. https://doi.org/ 10.1083/jcb.201804111.
- Beis, K. (2015). Structural basis for the mechanism of ABC transporters. *Biochemical Society Transactions*, 43(5), 889–893. https://doi.org/10.1042/BST20150047.
- Bessoule, J. J., Testet, E., & Cassagne, C. (1995). Synthesis of phosphatidylcholine in the chloroplast envelope after import of lysophosphatidylcholine from endoplasmic reticulum membranes. *European Journal of Biochemistry*, 228(2), 490–497.
- Bockelmann, S., Mina, J. G. M., Korneev, S., Hassan, D. G., Müller, D., Hilderink, A., et al. (2018). A search for ceramide binding proteins using bifunctional lipid analogs yields

CERT-related protein StarD7. Journal of Lipid Research, 59(3), 515–530. https://doi.org/10.1194/jlr.M082354.

- Böhme, K., Li, Y., Charlot, F., Grierson, C., Marrocco, K., Okada, K., et al. (2004). The Arabidopsis COW1 gene encodes a phosphatidylinositol transfer protein essential for root hair tip growth. *The Plant Journal: For Cell and Molecular Biology*, 40(5), 686–698. https://doi.org/10.1111/j.1365-313X.2004.02245.x.
- Bohnert, M., & Schuldiner, M. (2018). Stepping outside the comfort zone of membrane contact site research. *Nature Reviews. Molecular Cell Biology*, 19, 483–484. https://doi.org/10.1038/s41580-018-0022-1.
- Boissan, M., Schlattner, U., & Lacombe, M.-L. (2018). The NDPK/NME superfamily: State of the art. *Laboratory Investigation*, *98*(2), 164–174. https://doi.org/10.1038/labinvest. 2017.137.
- Botella, C., Sautron, E., Boudiere, L., Michaud, M., Dubots, E., Yamaryo-Botté, Y., et al. (2016). ALA10, a phospholipid flippase, controls FAD2/FAD3 desaturation of phosphatidylcholine in the ER and affects chloroplast lipid composition in Arabidopsis thaliana. *Plant Physiology*, 170(3), 1300–1314. https://doi.org/10.1104/pp.15.01557.
- Boudière, L., Michaud, M., Petroutsos, D., Rébeillé, F., Falconet, D., Bastien, O., et al. (2014). Glycerolipids in photosynthesis: Composition, synthesis and trafficking. *Biochimica et Biophysica Acta (BBA)*—*Bioenergetics*, 1837(4), 470–480. https://doi.org/ 10.1016/j.bbabio.2013.09.007.
- Browse, J., Warwick, N., Somerville, C. R., & Slack, C. R. (1986). Fluxes through the prokaryotic and eukaryotic pathways of lipid synthesis in the '16:3' plant Arabidopsis thaliana. *The Biochemical Journal*, 235(1), 25–31. https://doi.org/10.1042/bj2350025.
- Buckingham, M., & Relaix, F. (2007). The role of Pax genes in the development of tissues and organs: Pax3 and Pax7 regulate muscle progenitor cell functions. *Annual Review of Cell and Developmental Biology*, 23, 645–673. https://doi.org/10.1146/annurev.cellbio. 23.090506.123438.
- Carrie, C., Kühn, K., Murcha, M. W., Duncan, O., Small, I. D., O'Toole, N., et al. (2009). Approaches to defining dual-targeted proteins in Arabidopsis. *The Plant Journal*, 57(6), 1128–1139. https://doi.org/10.1111/j.1365-313X.2008.03745.x.
- Carvalho, A.d. O., & Gomes, V. M. (2007). Role of plant lipid transfer proteins in plant cell physiology-a concise review. *Peptides*, 28(5), 1144–1153. https://doi.org/10.1016/ j.peptides.2007.03.004.
- Cevher-Keskin, B. (2020). Endomembrane trafficking in plants. In T. Yonar (Ed.), *Electrodialysis* IntechOpen. https://doi.org/10.5772/intechopen.91642.
- Chen, H., Dong, J., & Wang, T. (2021). Autophagy in plant abiotic stress management. International Journal of Molecular Sciences, 22(8), 4075. https://doi.org/10.3390/ijms22084075.
- Chi, X., Fan, Q., Zhang, Y., Liang, K., Wan, L., Zhou, Q., et al. (2020). Structural mechanism of phospholipids translocation by MlaFEDB complex. *Cell Research*, *30*(12), 1127–1135. https://doi.org/10.1038/s41422-020-00404-6.
- Chou, M.-X. (2006). Thirteen nodule-specific or nodule-enhanced genes encoding products homologous to cysteine cluster proteins or plant lipid transfer proteins are identified in Astragalus sinicus L. by suppressive subtractive hybridization. *Journal of Experimental Botany*, 57(11), 2673–2685. https://doi.org/10.1093/jxb/erl030.
- Chu, M., Li, J., Zhang, J., Shen, S., Li, C., Gao, Y., et al. (2018). AtCaM4 interacts with a Sec14-like protein, PATL1, to regulate freezing tolerance in Arabidopsis in a CBF-independent manner. *Journal of Experimental Botany*, 69(21), 5241–5253. https:// doi.org/10.1093/jxb/ery278.
- Chung, J., Torta, F., Masai, K., Lucast, L., Czapla, H., Tanner, L. B., et al. (2015). PI4P/ phosphatidylserine countertransport at ORP5- and ORP8-mediated ER-plasma membrane contacts. *Science*, *349*(6246), 428–432. https://doi.org/10.1126/science. aab1370.

- Clark, B. J. (2012). The mammalian START domain protein family in lipid transport in health and disease. *Journal of Endocrinology*, 212(3), 257–275. https://doi.org/10.1530/JOE-11-0313.
- Clark, B. J. (2020). The START-domain proteins in intracellular lipid transport and beyond. *Molecular and Cellular Endocrinology*, 504, 110704. https://doi.org/10.1016/j.mce.2020. 110704.
- Connerth, M., Tatsuta, T., Haag, M., Klecker, T., Westermann, B., & Langer, T. (2012). Intramitochondrial transport of phosphatidic acid in yeast by a lipid transfer protein. *Science*, 338(6108), 815–818. https://doi.org/10.1126/science.1225625.
- Craxton, M. (2004). Synaptotagmin gene content of the sequenced genomes. BMC Genomics, 5(1), 43. https://doi.org/10.1186/1471-2164-5-43.
- D'Ambrosio, J. M., Albanèse, V., Lipp, N.-F., Fleuriot, L., Debayle, D., Drin, G., et al. (2020). Osh6 requires Ist2 for localization to ER–PM contacts and efficient phosphatidylserine transport in budding yeast. *Journal of Cell Science*, 133(11), jcs243733. https://doi.org/10.1242/jcs.243733.
- De, M., Oleskie, A. N., Ayyash, M., Dutta, S., Mancour, L., Abazeed, M. E., et al. (2017). The Vps13p–Cdc31p complex is directly required for TGN late endosome transport and TGN homotypic fusion. *Journal of Cell Biology*, 216(2), 425–439. https://doi.org/ 10.1083/jcb.201606078.
- de Saint-Jean, M., Delfosse, V., Douguet, D., Chicanne, G., Payrastre, B., Bourguet, W., et al. (2011). Osh4p exchanges sterols for phosphatidylinositol 4-phosphate between lipid bilayers. *Journal of Cell Biology*, 195(6), 965–978. https://doi.org/10.1083/jcb. 201104062.
- de Silva, K., Laska, B., Brown, C., Sederoff, H. W., & Khodakovskaya, M. (2011). Arabidopsis thaliana calcium-dependent lipid-binding protein (AtCLB): A novel repressor of abiotic stress response. *Journal of Experimental Botany*, 62(8), 2679–2689. https:// doi.org/10.1093/jxb/erq468.
- Deng, T., Yao, H., Wang, J., Wang, J., Xue, H., & Zuo, K. (2016). GhLTPG1, a cotton GPI-anchored lipid transfer protein, regulates the transport of phosphatidylinositol monophosphates and cotton fiber elongation. *Scientific Reports*, *6*, 26829. https://doi.org/ 10.1038/srep26829.
- Ding, X., Zhang, X., & Otegui, M. S. (2018). Plant autophagy: New flavors on the menu. *Current Opinion in Plant Biology*, 46, 113–121. https://doi.org/10.1016/j.pbi. 2018.09.004.
- Dong, X., Wang, Z., Ye, S., & Zhang, R. (2020). The crystal structure of ORP3 reveals the conservative PI4P binding pattern. *Biochemical and Biophysical Research Communications*, 529(4), 1005–1010. https://doi.org/10.1016/j.bbrc.2020.06.090.
- Downie, J. A. (2014). Legume nodulation. *Current Biology*, 24(5), R184–R190. https://doi.org/10.1016/j.cub.2014.01.028.
- Drissner, D., Kunze, G., Callewaert, N., Gehrig, P., Tamasloukht, M., Boller, T., et al. (2007). Lyso-phosphatidylcholine is a signal in the arbuscular mycorrhizal symbiosis. *Science*, *318*(5848), 265–268. https://doi.org/10.1126/science.1146487.
- Du, X., Kumar, J., Ferguson, C., Schulz, T. A., Ong, Y. S., Hong, W., et al. (2011). A role for oxysterol-binding protein-related protein 5 in endosomal cholesterol trafficking. *The Journal of Cell Biology*, 192(1), 121–135. https://doi.org/10.1083/jcb.201004142.
- Du, X., Zhou, L., Aw, Y. C., Mak, H. Y., Xu, Y., Rae, J., et al. (2020). ORP5 localizes to ER–lipid droplet contacts and regulates the level of PI(4)P on lipid droplets. *Journal of Cell Biology*, 219(1), e201905162. https://doi.org/10.1083/jcb.201905162.
- Dubots, E., Botté, C., Boudière, L., Yamaryo-Botté, Y., Jouhet, J., Maréchal, E., et al. (2012). Role of phosphatidic acid in plant galactolipid synthesis. *Biochimie*, 94(1), 86–93. https://doi.org/10.1016/j.biochi.2011.03.012.
- Duncan, O., Taylor, N. L., Carrie, C., Eubel, H., Kubiszewski-Jakubiak, S., Zhang, B., et al. (2011). Multiple lines of evidence localize signaling, morphology, and lipid biosynthesis

machinery to the mitochondrial outer membrane of arabidopsis. *Plant Physiology*, 157(3), 1093–1113. https://doi.org/10.1104/pp.111.183160.

- Dziurdzik, S. K., & Conibear, E. (2021). The Vps13 family of lipid transporters and its role at membrane contact sites. *International Journal of Molecular Sciences*, 22, 2905.
- Edqvist, J., Blomqvist, K., Nieuwland, J., & Salminen, T. A. (2018). Plant lipid transfer proteins: Are we finally closing in on the roles of these enigmatic proteins? *Journal of Lipid Research*, 59(8), 1374–1382. https://doi.org/10.1194/jlr.R083139.
- Ekiert, D. C., Bhabha, G., Isom, G. L., Greenan, G., Ovchinnikov, S., Henderson, I. R., et al. (2017). Architectures of lipid transport systems for the bacterial outer membrane. *Cell*, *169*(2), 273–285.e17. https://doi.org/10.1016/j.cell.2017.03.019.
- Elbaz-Alon, Y., Eisenberg-Bord, M., Shinder, V., Stiller, S. B., Shimoni, E., Wiedemann, N., et al. (2015). Lam6 regulates the extent of contacts between organelles. *Cell Reports*, 12(1), 7–14. https://doi.org/10.1016/j.celrep.2015.06.022.
- Elbaz-Alon, Y., Guo, Y., Segev, N., Harel, M., Quinnell, D. E., Geiger, T., et al. (2020). PDZD8 interacts with Protrudin and Rab7 at ER-late endosome membrane contact sites associated with mitochondria. *Nature Communications*, 11(1), 3645. https://doi.org/ 10.1038/s41467-020-17451-7.
- Epand, R. F., Schlattner, U., Wallimann, T., Lacombe, M.-L., & Epand, R. M. (2007). Novel lipid transfer property of two mitochondrial proteins that bridge the inner and outer membranes. *Biophysical Journal*, 92(1), 126–137. https://doi.org/10.1529/biophysj. 106.092353.
- Fan, J., Zhai, Z., Yan, C., & Xu, C. (2015). Arabidopsis TRIGALACTOSY LDIACYLGLYCEROL5 interacts with TGD1, TGD2, and TGD4 to facilitate lipid transfer from the endoplasmic reticulum to plastids. *The Plant Cell*, 27(10), 2941–2955. tpc.15.00394 https://doi.org/10.1105/tpc.15.00394.
- Flores-Martín, J., Reyna, L., Ridano, M. E., Panzetta-Dutari, G. M., & Genti-Raimondi, S. (2016). Suppression of StarD7 promotes endoplasmic reticulum stress and induces ROS production. *Free Radical Biology & Medicine*, 99, 286–295. https://doi.org/10.1016/ j.freeradbiomed.2016.08.023.
- Föller, M., Hermann, A., Gu, S., Alesutan, I., Qadri, S. M., Borst, O., et al. (2012). Chorein-sensitive polymerization of cortical actin and suicidal cell death in choreaacanthocytosis. *The FASEB Journal*, 26(4), 1526–1534. https://doi.org/10.1096/fj.11-198317.
- Ford, R. C., & Beis, K. (2019). Learning the ABCs one at a time: Structure and mechanism of ABC transporters. *Biochemical Society Transactions*, 47(1), 23–36. https://doi.org/10.1042/ BST20180147.
- Gaigg, B., Simbeni, R., Hrastnik, C., Paltauf, F., & Daum, G. (1995). Characterization of a microsomal subfraction associated with mitochondria of the yeast, Saccharomyces cerevisiae. Involvement in synthesis and import of phospholipids into mitochondria. *Biochimica et Biophysica Acta*, 1234(2), 214–220. https://doi.org/10.1016/0005-2736 (94)00287-y.
- Galmes, R., Houcine, A., Vliet, A. R., Agostinis, P., Jackson, C. L., & Giordano, F. (2016). ORP5/ORP8 localize to endoplasmic reticulum–mitochondria contacts and are involved in mitochondrial function. *EMBO Reports*, 17(6), 800–810. https://doi.org/ 10.15252/embr.201541108.
- Gatta, A. T., Sauerwein, A. C., Zhuravleva, A., Levine, T. P., & Matthews, S. (2018). Structural insights into a StART-like domain in Lam4 and its interaction with sterol ligands. *Biochemical and Biophysical Research Communications*, 495(3), 2270–2274. https://doi.org/10.1016/j.bbrc.2017.12.109.
- Gatta, A. T., Wong, L. H., Sere, Y. Y., Calderón-Noreña, D. M., Cockcroft, S., Menon, A. K., et al. (2015). A new family of StART domain proteins at membrane contact sites has a role in ER-PM sterol transport. *eLife*, *4*, e07253. https://doi.org/10.7554/ eLife.07253.

- Gaude, N., Tippmann, H., Flemetakis, E., Katinakis, P., Udvardi, M., & Dörmann, P. (2004). The galactolipid digalactosyldiacylglycerol accumulates in the peribacteroid membrane of nitrogen-fixing nodules of soybean and lotus. *Journal of Biological Chemistry*, 279(33), 34624–34630. https://doi.org/10.1074/jbc.M404098200.
- Ghosh, R., de Campos, M. K. F., Huang, J., Huh, S. K., Orlowski, A., Yang, Y., et al. (2015). Sec14-nodulin proteins and the patterning of phosphoinositide landmarks for developmental control of membrane morphogenesis. *Molecular Biology of the Cell*, 26(9), 1764–1781. https://doi.org/10.1091/mbc.E14-10-1475.
- Goss, V., Hunt, A. N., & Postle, A. D. (2013). Regulation of lung surfactant phospholipid synthesis and metabolism. *Biochimica et Biophysica Acta*, 1831(2), 448–458. https://doi.org/10.1016/j.bbalip.2012.11.009.
- Grabon, A., Bankaitis, V. A., & McDermott, M. I. (2019). The interface between phosphatidylinositol transfer protein function and phosphoinositide signaling in higher eukaryotes. *Journal of Lipid Research*, 60(2), 242–268. https://doi.org/10.1194/jlr. R089730.
- Grabon, A., Khan, D., & Bankaitis, V. A. (2015). Phosphatidylinositol transfer proteins and instructive regulation of lipid kinase biology. *Biochimica et Biophysica Acta (BBA)*— *Molecular and Cell Biology of Lipids*, 1851(6), 724–735. https://doi.org/10.1016/ j.bbalip.2014.12.011.
- Graef, M., Friedman, J. R., Graham, C., Babu, M., & Nunnari, J. (2013). ER exit sites are physical and functional core autophagosome biogenesis components. *Molecular Biology of the Cell*, 24(18), 2918–2931. https://doi.org/10.1091/mbc.e13-07-0381.
- Hammargren, J., Sundström, J., Johansson, M., Bergman, P., & Knorpp, C. (2007). On the phylogeny, expression and targeting of plant nucleoside diphosphate kinases. *Physiologia Plantarum*, 129(1), 79–89. https://doi.org/10.1111/j.1399-3054.2006.00794.x.
- Hanada, K. (2018). Lipid transfer proteins rectify inter-organelle flux and accurately deliver lipids at membrane contact sites. *Journal of Lipid Research*, 59(8), 1341–1366. https://doi.org/10.1194/jlr.R085324.
- Harrison, M. J., & Ivanov, S. (2017). Exocytosis for endosymbiosis: Membrane trafficking pathways for development of symbiotic membrane compartments. *Current Opinion in Plant Biology*, 38, 101–108. https://doi.org/10.1016/j.pbi.2017.04.019.
- Hartel, H., Dormann, P., & Benning, C. (2001). Galactolipids not associated with the photosynthetic apparatus in phosphate-deprived plants. *Journal of Photochemistry and Photobiology B: Biology*, 61, 46–51.
- Heazlewood, J. L., Tonti-Filippini, J. S., Gout, A. M., Day, D. A., Whelan, J., & Millar, A. H. (2004). Experimental analysis of the Arabidopsis mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. *The Plant Cell*, 16(1), 241–256. https://doi.org/10.1105/tpc.016055.
- Hertle, A. P., García-Cerdán, J. G., Armbruster, U., Shih, R., Lee, J. J., Wong, W., et al. (2020). A Sec14 domain protein is required for photoautotrophic growth and chloroplast vesicle formation in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America*, 117(16), 9101–9111. https://doi.org/10.1073/pnas. 1916946117.
- Hirabayashi, Y., Kwon, S. K., Paek, H., Pernice, W. M., Paul, M. A., Lee, J., et al. (2017). ER-mitochondria tethering by PDZD8 regulates Ca(2+) dynamics in mammalian neurons. *Science*, 358(6363), 623–630. https://doi.org/10.1126/science.aan6009.
- Ho, C. M., Paciorek, T., Abrash, E., & Bergmann, D. C. (2016). Modulators of stomatal lineage signal transduction alter membrane contact sites and reveal specialization among ERECTA kinases. *Developmental Cell*, 38(4), 345–357. https://doi.org/10.1016/ j.devcel.2016.07.016.

- Hollenstein, K., Dawson, R. J., & Locher, K. P. (2007). Structure and mechanism of ABC transporter proteins. *Current Opinion in Structural Biology*, 17(4), 412–418. https://doi. org/10.1016/j.sbi.2007.07.003.
- Hooper, C. M., Castleden, I. R., Tanz, S. K., Aryamanesh, N., & Millar, A. H. (2017). SUBA4: The interactive data analysis centre for Arabidopsis subcellular protein locations. *Nucleic Acids Research*, 45(D1), D1064–D1074. https://doi.org/10.1093/nar/gkw1041.
- Horenkamp, F. A., Valverde, D. P., Nunnari, J., & Reinisch, K. M. (2018). Molecular basis for sterol transport by StART-like lipid transfer domains. *The EMBO Journal*, 37(6). https://doi.org/10.15252/embj.201798002.
- Horibata, Y., Ando, H., Zhang, P., Vergnes, L., Aoyama, C., Itoh, M., et al. (2016). StarD7 protein deficiency adversely affects the phosphatidylcholine composition, respiratory activity, and cristae structure of mitochondria. *The Journal of Biological Chemistry*, 291(48), 24880–24891. https://doi.org/10.1074/jbc.M116.736793.
- Horibata, Y., & Sugimoto, H. (2010). StarD7 mediates the intracellular trafficking of phosphatidylcholine to mitochondria. *Journal of Biological Chemistry*, 285(10), 7358–7365. https://doi.org/10.1074/jbc.M109.056960.
- Hou, Q., Ufer, G., & Bartels, D. (2016). Lipid signalling in plant responses to abiotic stress: Lipid signalling in plant responses to abiotic stress. *Plant, Cell & Environment, 39*(5), 1029–1048. https://doi.org/10.1111/pce.12666.
- Hsueh, Y.-C., Ehmann, C., Flinner, N., Ladig, R., & Schleiff, E. (2017). The plastid outer membrane localized LPTD1 is important for glycerolipid remodelling under phosphate starvation: LptD orthologues in *A. thaliana. Plant, Cell & Environment, 40*(8), 1643–1657. https://doi.org/10.1111/pce.12973.
- Huang, J., Ghosh, R., & Bankaitis, V. A. (2016). Sec14-like phosphatidylinositol transfer proteins and the biological landscape of phosphoinositide signaling in plants. *Biochimica et Biophysica Acta*, 1861(9 Pt. B), 1352–1364. https://doi.org/10.1016/ j.bbalip.2016.03.027.
- Huang, J., Ghosh, R., Tripathi, A., Lönnfors, M., Somerharju, P., & Bankaitis, V. A. (2016). Two-ligand priming mechanism for potentiated phosphoinositide synthesis is an evolutionarily conserved feature of Sec14-like phosphatidylinositol and phosphatidylcholine exchange proteins. *Molecular Biology of the Cell*, 27(14), 2317–2330. https://doi.org/ 10.1091/mbc.E16-04-0221.
- Hua-Ying, M., Wen-Ju, W., Wei-Hua, S., Ya-Chun, S., Feng, L., Cong-Na, L., et al. (2019). Genome-wide identification, phylogeny, and expression analysis of Sec14-like PITP gene family in sugarcane. *Plant Cell Reports*, 38(5), 637–655. https://doi.org/ 10.1007/s00299-019-02394-1.
- Hurlock, A. K., Roston, R. L., Wang, K., & Benning, C. (2014). Lipid trafficking in plant cells. *Traffic*, 15(9), 915–932. https://doi.org/10.1111/tra.12187.
- Im, Y. J., Raychaudhuri, S., Prinz, W. A., & Hurley, J. H. (2005). Structural mechanism for sterol sensing and transport by OSBP-related proteins. *Nature*, 437(7055), 154–158. https://doi.org/10.1038/nature03923.
- Ishikawa, K., Tamura, K., Fukao, Y., & Shimada, T. (2020). Structural and functional relationships between plasmodesmata and plant endoplasmic reticulum–plasma membrane contact sites consisting of three synaptotagmins. *New Phytologist*, 226(3), 798–808. https://doi.org/10.1111/nph.16391.
- Isono, E., Katsiarimpa, A., Müller, I. K., Anzenberger, F., Stierhof, Y.-D., Geldner, N., et al. (2010). The deubiquitinating enzyme AMSH3 is required for intracellular trafficking and vacuole biogenesis in Arabidopsis thaliana. *The Plant Cell*, 22(6), 1826–1837. https://doi. org/10.1105/tpc.110.075952.
- Itoh, R. D., Nakajima, K. P., Sasaki, S., Ishikawa, H., Kazama, Y., Abe, T., et al. (2021). TGD5 is required for normal morphogenesis of non-mesophyll plastids, but not

mesophyll chloroplasts, in Arabidopsis. The Plant Journal, 107, 237–255. https://doi.org/10.1111/tpj.15287.

- Janin, J., Dumas, C., Moréra, S., Xu, Y., Meyer, P., Chiadmi, M., et al. (2000). Three-dimensional structure of nucleoside diphosphate kinase. *Journal of Bioenergetics* and Biomembranes, 32(3), 215–225. https://doi.org/10.1023/a:1005528811303.
- Jeong, H., Park, J., Jun, Y., & Lee, C. (2017). Crystal structures of Mmm1 and Mdm12-Mmm1 reveal mechanistic insight into phospholipid trafficking at ER-mitochondria contact sites. *Proceedings of the National Academy of Sciences of the United States of America*, 114(45), E9502– E9511. https://doi.org/10.1073/pnas.1715592114.
- Jessen, D., Roth, C., Wiermer, M., & Fulda, M. (2015). Two activities of long-chain acyl-coenzyme A synthetase are involved in lipid trafficking between the endoplasmic reticulum and the plastid in Arabidopsis. *Plant Physiology*, *167*(2), 351–366. https://doi.org/10.1104/pp.114.250365.
- Jeyasimman, D., & Saheki, Y. (2020). SMP domain proteins in membrane lipid dynamics. Biochimica et Biophysica Acta (BBA)—Molecular and Cell Biology of Lipids, 1865(1), 158447. https://doi.org/10.1016/j.bbalip.2019.04.007.
- Jouannic, N., Lepetit, M., Vergnolle, C., Cantrel, C., Gardies, A. M., Kader, J. C., et al. (1998). Isolation of a cDNA from Arabidopsis thaliana that complements the sec14 mutant of yeast. *European Journal of Biochemistry*, 258(2), 402–410. https://doi.org/10.1046/j.1432-1327.1998.2580402.x.
- Jouhet, J., Maréchal, E., Baldan, B., Bligny, R., Joyard, J., & Block, M. A. (2004). Phosphate deprivation induces transfer of DGDG galactolipid from chloroplast to mitochondria. *Journal of Cell Biology*, 167(5), 863–874. https://doi.org/10.1083/jcb.200407022.
- Jouhet, J., Maréchal, E., Bligny, R., Joyard, J., & Block, M. A. (2003). Transient increase of phosphatidylcholine in plant cells in response to phosphate deprivation. *FEBS Letters*, 544(1–3), 63–68. https://doi.org/10.1016/S0014-5793(03)00477-0.
- Kader, J.-C. (1996). Lipid-transfer proteins in plants. Annual Review of Plant Physiology and Plant Molecular Biology, 47(1), 627–654. https://doi.org/10.1146/annurev.arplant.47.1.627.
- Kagan, V. E., Jiang, J., Huang, Z., Tyurina, Y. Y., Desbourdes, C., Cottet-Rousselle, C., et al. (2016). NDPK-D (NM23-H4)-mediated externalization of cardiolipin enables elimination of depolarized mitochondria by mitophagy. *Cell Death and Differentiation*, 23(7), 1140–1151. https://doi.org/10.1038/cdd.2015.160.
- Kang, H. W., Wei, J., & Cohen, D. E. (2010). PC-TP/StARD2: Of membranes and metabolism. Trends in Endocrinology and Metabolism: TEM, 21(7), 449–456. https://doi.org/ 10.1016/j.tem.2010.02.001.
- Kannan, M., Lahiri, S., Liu, L.-K., Choudhary, V., & Prinz, W. A. (2017). Phosphatidylserine synthesis at membrane contact sites promotes its transport out of the ER. *Journal of Lipid Research*, 58(3), 553–562. https://doi.org/10.1194/jlr.M072959.
- Kanno, K., Wu, M. K., Agate, D. S., Fanelli, B. J., Wagle, N., Scapa, E. F., et al. (2007). Interacting proteins dictate function of the minimal START domain phosphatidylcholine transfer protein/StarD2. *Journal of Biological Chemistry*, 282(42), 30728–30736. https://doi.org/10.1074/jbc.M703745200.
- Karki, N., Johnson, B. S., & Bates, P. D. (2019). Metabolically distinct pools of phosphatidylcholine are involved in trafficking of fatty acids out of and into the chloroplast for membrane production. *The Plant Cell*, 31, 2768–2788. https://doi.org/10.1105/tpc. 19.00121.
- Katsiarimpa, A., Kalinowska, K., Anzenberger, F., Weis, C., Ostertag, M., Tsutsumi, C., et al. (2013). The deubiquitinating enzyme AMSH1 and the ESCRT-III subunit VPS2.1 are required for autophagic degradation in *Arabidopsis*. *The Plant Cell*, 25(6), 2236–2252. https://doi.org/10.1105/tpc.113.113399.
- Kearns, M. A., Monks, D. E., Fang, M., Rivas, M. P., Courtney, P. D., Chen, J., et al. (1998). Novel developmentally regulated phosphoinositide binding proteins from

soybean whose expression bypasses the requirement for an essential phosphatidylinositol transfer protein in yeast. *The EMBO Journal*, 17(14), 4004–4017. https://doi.org/10.1093/emboj/17.14.4004.

- Kelly, A. A., Froehlich, J. E., & Dörmann, P. (2003). Disruption of the two digalactosyldiacylglycerol synthase genes DGD1 and DGD2 in Arabidopsis reveals the existence of an additional enzyme of galactolipid synthesis. The Plant Cell, 15(11), 2694–2706. https://doi.org/10.1105/tpc.016675.
- Kelly, A. A., Kalisch, B., Hölzl, G., Schulze, S., Thiele, J., Melzer, M., et al. (2016). Synthesis and transfer of galactolipids in the chloroplast envelope membranes of *Arabidopsis* thaliana. Proceedings of the National Academy of Sciences of the United States of America, 113(38), 10714–10719. https://doi.org/10.1073/pnas.1609184113.
- Kf de Campos, M., & Schaaf, G. (2017). The regulation of cell polarity by lipid transfer proteins of the SEC14 family. *Current Opinion in Plant Biology*, 40, 158–168. https://doi.org/ 10.1016/j.pbi.2017.09.007.
- Khafif, M., Balague, C., Huard-Chauveau, C., & Roby, D. (2017). An essential role for the VASt domain of the Arabidopsis VAD1 protein in the regulation of defense and cell death in response to pathogens. *PLoS One*, 12(7), e0179782. https://doi.org/10.1371/ journal.pone.0179782.
- Khafif, M., Cottret, L., Balague, C., & Raffaele, S. (2014). Identification and phylogenetic analyses of VASt, an uncharacterized protein domain associated with lipid-binding domains in Eukaryotes. *BMC Bioinformatics*, 15, 222. https://doi.org/10.1186/1471-2105-15-222.
- Khosla, A., Paper, J. M., Boehler, A. P., Bradley, A. M., Neumann, T. R., & Schrick, K. (2014). HD-zip proteins GL2 and HDG11 have redundant functions in Arabidopsis trichomes, and GL2 activates a positive feedback loop via MYB23. *The Plant Cell*, 26(5), 2184–2200. https://doi.org/10.1105/tpc.113.120360.
- Kiba, A., Galis, I., Hojo, Y., Ohnishi, K., Yoshioka, H., & Hikichi, Y. (2014). SEC14 phospholipid transfer protein is involved in lipid signaling-mediated plant immune responses in Nicotiana benthamiana. *PLoS One*, 9(5), e98150. https://doi.org/10.1371/journal. pone.0098150.
- Kiba, A., Nakano, M., Vincent-Pope, P., Takahashi, H., Sawasaki, T., Endo, Y., et al. (2012). A novel Sec14 phospholipid transfer protein from Nicotiana benthamiana is up-regulated in response to Ralstonia solanacearum infection, pathogen associated molecular patterns and effector molecules and involved in plant immunity. *Journal of Plant Physiology*, 169(10), 1017–1022. https://doi.org/10.1016/j.jplph.2012.04.002.
- Kim, H., Kwon, H., Kim, S., Kim, M. K., Botella, M. A., Yun, H. S., et al. (2016). Synaptotagmin 1 negatively controls the two distinct immune secretory pathways to powdery mildew fungi in Arabidopsis. *Plant & Cell Physiology*, 57(6), 1133–1141. https://doi.org/10.1093/pcp/pcw061.
- Kim, H. J., & Triplett, B. A. (2001). Cotton fiber growth in planta and in vitro. Models for plant cell elongation and cell wall biogenesis. *Plant Physiology*, 127(4), 1361–1366.
- Knorpp, C., Johansson, M., & Baird, A.-M. (2003). Plant mitochondrial nucleoside diphosphate kinase is attached to the membrane through interaction with the adenine nucleotide translocator. *FEBS Letters*, 555(2), 363–366. https://doi.org/10.1016/ s0014–5793(03)01288-2.
- Kobayashi, K., Awai, K., Nakamura, M., Nagatani, A., Masuda, T., & Ohta, H. (2009). Type-B monogalactosyldiacylglycerol synthases are involved in phosphate starvation-induced lipid remodeling, and are crucial for low-phosphate adaptation. *The Plant Journal*, 57(2), 322–331. https://doi.org/10.1111/j.1365-313X.2008.03692.x.
- Kopec, K. O., Alva, V., & Lupas, A. N. (2010). Homology of SMP domains to the TULIP superfamily of lipid-binding proteins provides a structural basis for lipid exchange between ER and mitochondria. *Bioinformatics*, 26(16), 1927–1931. https://doi.org/ 10.1093/bioinformatics/btq326.

- Kornmann, B., Currie, E., Collins, S. R., Schuldiner, M., Nunnari, J., Weissman, J. S., et al. (2009). An ER-mitochondria tethering complex revealed by a synthetic biology screen. *Science*, 325(5939), 477–481. https://doi.org/10.1126/science.1175088.
- Kumar, N., Leonzino, M., Hancock-Cerutti, W., Horenkamp, F. A., Li, P., Lees, J. A., et al. (2018). VPS13A and VPS13C are lipid transport proteins differentially localized at ER contact sites. *Journal of Cell Biology*, 217(10), 3625–3639. https://doi.org/10.1083/jcb. 201807019.
- LaBrant, E., Barnes, A. C., & Roston, R. L. (2018). Lipid transport required to make lipids of photosynthetic membranes. *Photosynthesis Research*, 138(3), 345–360. https://doi.org/ 10.1007/s11120-018-0545-5.
- Larsson, K. E., Kjellberg, J. M., Tjellström, H., & Sandelius, A. (2007). LysoPC acyltransferase/PC transacylase activities in plant plasma membrane and plasma membrane-associated endoplasmic reticulum. *BMC Plant Biology*, 7(1), 64. https:// doi.org/10.1186/1471-2229-7-64.
- Lee, J.-Y., Colinas, J., Wang, J. Y., Mace, D., Ohler, U., & Benfey, P. N. (2006). Transcriptional and posttranscriptional regulation of transcription factor expression in Arabidopsis roots. *Proceedings of the National Academy of Sciences of the United States of America*, 103(15), 6055–6060. https://doi.org/10.1073/pnas.0510607103.
- Lee, I., & Hong, W. (2006). Diverse membrane-associated proteins contain a novel SMP domain. *The FASEB Journal*, 20(2), 202–206. https://doi.org/10.1096/fj.05-4581hyp.
- Lee, E., Santana, B. V. N., Samuels, E., Benitez-Fuente, F., Corsi, E., Botella, M. A., et al. (2020). Rare earth elements induce cytoskeleton-dependent and PI4P-associated rearrangement of SYT1/SYT5 endoplasmic reticulum–plasma membrane contact site complexes in Arabidopsis. *Journal of Experimental Botany*, 71(14), 3986–3998. https:// doi.org/10.1093/jxb/eraa138.
- Lee, E., Vanneste, S., Perez-Sancho, J., Benitez-Fuente, F., Strelau, M., Macho, A. P., et al. (2019). Ionic stress enhances ER-PM connectivity via phosphoinositide-associated SYT1 contact site expansion in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 116(4), 1420–1429. https://doi.org/10.1073/ pnas.1818099116.
- Lei, L., Chen, L., Shi, X., Li, Y., Wang, J., Chen, D., et al. (2014). A nodule-specific lipid transfer protein AsE246 participates in transport of plant-synthesized lipids to symbiosome membrane and is essential for nodule organogenesis in chinese milk vetch. *Plant Physiology*, 164(2), 1045–1058. https://doi.org/10.1104/pp.113.232637.
- Levy, A., Zheng, J. Y., & Lazarowitz, S. G. (2015). Synaptotagmin SYTA forms ER-plasma membrane junctions that are recruited to plasmodesmata for plant virus movement. *Current Biology*, 25(15), 2018–2025. https://doi.org/10.1016/j.cub.2015.06.015.
- Li, Z., Gao, J., Benning, C., & Sharkey, T. D. (2012). Characterization of photosynthesis in Arabidopsis ER-to-plastid lipid trafficking mutants. *Photosynthesis Research*, 112(1), 49–61. https://doi.org/10.1007/s11120-012-9734-9.
- Li, D. Y., Inoue, H., Takahashi, M., Kojima, T., Shiraiwa, M., & Takahara, H. (2008). Molecular characterization of a novel salt-inducible gene for an OSBP (oxysterolbinding protein)-homologue from soybean. *Gene*, 407(1–2), 12–20. https://doi.org/ 10.1016/j.gene.2007.02.029.
- Li, Y., Kabbage, M., Liu, W., & Dickman, M. B. (2016). Aspartyl protease-mediated cleavage of BAG6 Is necessary for autophagy and fungal resistance in plants. *Plant Cell*, 28(1), 233–247. https://doi.org/10.1105/tpc.15.00626.
- Li, P., Lees, J. A., Lusk, C. P., & Reinisch, K. M. (2020). Cryo-EM reconstruction of a VPS13 fragment reveals a long groove to channel lipids between membranes. *Journal* of Cell Biology, 219(5), e202001161. https://doi.org/10.1083/jcb.202001161.
- Li, X., Routt, S. M., Xie, Z., Cui, X., Fang, M., Kearns, M. A., et al. (2000). Identification of a novel family of nonclassic yeast phosphatidylinositol transfer proteins

whose function modulates phospholipase D activity and Sec14p-independent cell growth. *Molecular Biology of the Cell*, 11(6), 1989–2005. https://doi.org/10.1091/mbc. 11.6.1989.

- Li, N., Xu, C., Li-Beisson, Y., & Philippar, K. (2016). Fatty acid and lipid transport in plant cells. *Trends in Plant Science*, 21(2), 145–158. https://doi.org/10.1016/j.tplants.2015.10.011.
- Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M. X., Arondel, V., Bates, P. D., et al. (2013). Acyl-lipid metabolism. *The Arabidopsis Book*, *11*, e0161. https://doi.org/10.1199/tab.0161.
- Lin, S., Ikegami, M., Moon, C., Naren, A. P., & Shannon, J. M. (2015). Lysophosphatidylcholine acyltransferase 1 (LPCAT1) specifically interacts with phospholipid transfer protein StarD10 to facilitate surfactant phospholipid trafficking in alveolar type II cells. *Journal of Biological Chemistry*, 290(30), 18559–18574. https://doi. org/10.1074/jbc.M115.666701.
- Lindquist, E., & Aronsson, H. (2018). Chloroplast vesicle transport. *Photosynthesis Research*, 138(3), 361–371. https://doi.org/10.1007/s11120-018-0566-0.
- Lipp, N.-F., Ikhlef, S., Milanini, J., & Drin, G. (2020). Lipid exchangers: cellular functions and mechanistic links with phosphoinositide metabolism. *Frontiers in Cell and Development Biology*, 8, 663. https://doi.org/10.3389/fcell.2020.00663.
- Liu, L., & Li, J. (2019). Communications between the endoplasmic reticulum and other organelles during abiotic stress response in plants. *Frontiers in Plant Science*, 10(749), 1–14. https://doi.org/10.3389/fpls.2019.00749.
- Liu, L.-K., Choudhary, V., Toulmay, A., & Prinz, W. A. (2017). An inducible ER–Golgi tether facilitates ceramide transport to alleviate lipotoxicity. *Journal of Cell Biology*, 216(1), 131–147. https://doi.org/10.1083/jcb.201606059.
- Liu, X., Ma, D., Zhang, Z., Wang, S., Du, S., Deng, X., et al. (2019). Plant lipid remodeling in response to abiotic stresses. *Environmental and Experimental Botany*, 165, 174–184. https://doi.org/10.1016/j.envexpbot.2019.06.005.
- López-Marqués, R. L., Davis, J. A., Harper, J. F., & Palmgren, M. (2021). Dynamic membranes: The multiple roles of P4 and P5 ATPases. *Plant Physiology*, 185(3), 619–631. https://doi.org/10.1093/plphys/kiaa065.
- Lorrain, S., Lin, B., Auriac, M. C., Kroj, T., Saindrenan, P., Nicole, M., et al. (2004). Vascular associated death1, a novel GRAM domain-containing protein, is a regulator of cell death and defense responses in vascular tissues. *Plant Cell*, *16*(8), 2217–2232. https://doi.org/10.1105/tpc.104.022038.
- Lu, B., & Benning, C. (2009). A 25-amino acid sequence of the Arabidopsis TGD2 protein is sufficient for specific binding of phosphatidic acid. *Journal of Biological Chemistry*, 284(26), 17420–17427. https://doi.org/10.1074/jbc.M109.016014.
- Lu, B., Xu, C., Awai, K., Jones, A. D., & Benning, C. (2007). A small ATPase protein of Arabidopsis, TGD3, involved in chloroplast lipid import. *Journal of Biological Chemistry*, 282(49), 35945–35953. https://doi.org/10.1074/jbc.M704063200.
- Luzarowski, M., Kosmacz, M., Sokolowska, E., Jasińska, W., Willmitzer, L., Veyel, D., et al. (2017). Affinity purification with metabolomic and proteomic analysis unravels diverse roles of nucleoside diphosphate kinases. *Journal of Experimental Botany*, 68(13), 3487–3499. https://doi.org/10.1093/jxb/erx183.
- Maeda, K., Anand, K., Chiapparino, A., Kumar, A., Poletto, M., Kaksonen, M., et al. (2013). Interactome map uncovers phosphatidylserine transport by oxysterol-binding proteins. *Nature*, 501(7466), 257–261. https://doi.org/10.1038/nature12430.
- Maréchal, E., & Bastien, O. (2014). Modeling of regulatory loops controlling galactolipid biosynthesis in the inner envelope membrane of chloroplasts. *Journal of Theoretical Biology*, 361, 1–13. https://doi.org/10.1016/j.jtbi.2014.07.013.
- Mathur, J. (2020). Review: Morphology, behaviour and interactions of organelles. *Plant Science*, 301, 110662. https://doi.org/10.1016/j.plantsci.2020.110662.

- Matoba, K., Kotani, T., Tsutsumi, A., Tsuji, T., Mori, T., Noshiro, D., et al. (2020). Atg9 is a lipid scramblase that mediates autophagosomal membrane expansion. *Nature Structural & Molecular Biology*, 27(12), 1185–1193. https://doi.org/10.1038/s41594-020-00518-w.
- Maxfield, F. R., & Menon, A. K. (2016). Intramembrane and intermembrane lipid transport. In Biochemistry of lipids, lipoproteins and membranes (pp. 415–436). Elsevier. https://doi.org/ 10.1016/B978-0-444-63438-2.00014-6.
- McFarlane, H. E., Lee, E. K., van Bezouwen, L. S., Ross, B., Rosado, A., & Samuels, A. L. (2017). Multiscale structural analysis of plant ER-PM contact sites. *Plant & Cell Physiology*, 58(3), 478–484. https://doi.org/10.1093/pcp/pcw224.
- Meï, C., Michaud, M., Cussac, M., Albrieux, C., Gros, V., Maréchal, E., et al. (2015). Levels of polyunsaturated fatty acids correlate with growth rate in plant cell cultures. *Scientific Reports*, 5, 15207. https://doi.org/10.1038/srep15207.
- Michaud, M. (2018). Analysis of the MTL supercomplex at contact sites between mitochondria and plastids. *Methods in Molecular Biology*, 1829, 173–188. https://doi.org/ 10.1007/978-1-4939-8654-5\_12.
- Michaud, M., Gros, V., Tardif, M., Brugière, S., Ferro, M., Prinz, W. A., et al. (2016). AtMic60 Is involved in plant mitochondria lipid trafficking and is part of a large complex. *Current Biology*, 26(5), 627–639. https://doi.org/10.1016/j.cub.2016.01.011.
- Michaud, M., & Jouhet, J. (2019). Lipid trafficking at membrane contact sites during plant development and stress response. *Frontiers in Plant Science*, 10, 2. https://doi.org/10.3389/ fpls.2019.00002.
- Michaud, M., Prinz, W. A., & Jouhet, J. (2017). Glycerolipid synthesis and lipid trafficking in plant mitochondria. *The FEBS Journal*, 284(3), 376–390. https://doi.org/10.1111/febs. 13812.
- Miliara, X., Tatsuta, T., Berry, J.-L., Rouse, S. L., Solak, K., Chorev, D. S., et al. (2019). Structural determinants of lipid specificity within Ups/PRELI lipid transfer proteins. *Nature Communications*, 10(1), 1130. https://doi.org/10.1038/s41467-019-09089-x.
- Miyata, N., Watanabe, Y., Tamura, Y., Endo, T., & Kuge, O. (2016). Phosphatidylserine transport by Ups2-Mdm35 in respiration-active mitochondria. *The Journal of Cell Biology*, 214(1), 77–88. https://doi.org/10.1083/jcb.201601082.
- Moellering, E. R., & Benning, C. (2011). Galactoglycerolipid metabolism under stress: A time for remodeling. *Trends in Plant Science*, 16(2), 98–107. https://doi.org/10.1016/ j.tplants.2010.11.004.
- Mongrand, S., Bessoule, J.-J., Cabantous, F., & Cassagne, C. (1998). The C16:3\C18:3 fatty acid balance in photosynthetic tissues from 468 plant species. *Phytochemistry*, 49(4), 1049–1064. https://doi.org/10.1016/S0031-9422(98)00243-X.
- Mongrand, S., Cassagne, C., & Bessoule, J. J. (2000). Import of lyso-phosphatidylcholine into chloroplasts likely at the origin of eukaryotic plastidial lipids. *Plant Physiology*, 122(3), 845–852. https://doi.org/10.1104/pp.122.3.845.
- Monks, D. E., Aghoram, K., Courtney, P. D., DeWald, D. B., & Dewey, R. E. (2001). Hyperosmotic stress induces the rapid phosphorylation of a soybean phosphatidylinositol transfer protein homolog through activation of the protein kinases SPK1 and SPK2. *The Plant Cell*, 13(5), 1205–1219. https://doi.org/10.1105/tpc.13.5.1205.
- Montag, K., Hornbergs, J., Ivanov, R., & Bauer, P. (2020). Phylogenetic analysis of plant multi-domain SEC14-like phosphatidylinositol transfer proteins and structure-function properties of PATELLIN2. *Plant Molecular Biology*, 104(6), 665–678. https://doi.org/ 10.1007/s11103-020-01067-y.
- Morris, J. S., Caldo, K. M. P., Liang, S., & Facchini, P. J. (2021). PR10/Bet v1-like proteins as novel contributors to plant biochemical diversity. *Chembiochem*, 22(2), 264–287. https://doi.org/10.1002/cbic.202000354.
- Moser von Filseck, J., & Drin, G. (2016). Running up that hill: How to create cellular lipid gradients by lipid counter-flows. *Biochimie*, 130, 115–121. https://doi.org/10.1016/j.biochi.2016.08.001.

- Moser von Filseck, J., opi, A., Delfosse, V., Vanni, S., Jackson, C. L., Bourguet, W., et al. (2015). Phosphatidylserine transport by ORP/Osh proteins is driven by phosphatidylinositol 4-phosphate. *Science*, 349(6246), 432–436. https://doi.org/10.1126/ science.aab1346.
- Mueller-Schuessele, S. J., & Michaud, M. (2018). Plastid transient and stable interactions with other cell compartments. *Methods in Molecular Biology*, *1829*, 87–109. https://doi.org/10.1007/978-1-4939-8654-5\_6.
- Mukherjee, I., Ghosh, M., & Meinecke, M. (2021). MICOS and the mitochondrial inner membrane morphology—When things get out of shape. *FEBS Letters*, 595(8), 1159–1183.
- Muñoz-Braceras, S., Calvo, R., & Escalante, R. (2015). TipC and the chorea-acanthocytosis protein VPS13A regulate autophagy in *Dictyostelium* and human HeLa cells. *Autophagy*, 11(6), 918–927. https://doi.org/10.1080/15548627.2015.1034413.
- Murley, A., Sarsam, R. D., Toulmay, A., Yamada, J., Prinz, W. A., & Nunnari, J. (2015). Ltc1 is an ER-localized sterol transporter and a component of ER-mitochondria and ER-vacuole contacts. *Journal of Cell Biology*, 209(4), 539–548. https://doi.org/ 10.1083/jcb.201502033.
- Murley, A., Yamada, J., Niles, B. J., Toulmay, A., Prinz, W. A., Powers, T., et al. (2017). Sterol transporters at membrane contact sites regulate TORC1 and TORC2 signaling. *The Journal of Cell Biology*, 216(9), 2679–2689. https://doi.org/10.1083/jcb.201610032.
- Nagy, P. D. (2015). Viral sensing of the subcellular environment regulates the assembly of new viral replicase complexes during the course of infection. *Journal of Virology*, 89(10), 5196–5199. https://doi.org/10.1128/JVI.02973-14.
- Nagy, P. D., Barajas, D., & Pogany, J. (2012). Host factors with regulatory roles in tombusvirus replication. *Current Opinion in Virology*, 2(6), 691–698. https://doi.org/ 10.1016/j.coviro.2012.10.004.
- Nakatogawa, H. (2020). Mechanisms governing autophagosome biogenesis. Nature Reviews Molecular Cell Biology, 21(8), 439–458. https://doi.org/10.1038/s41580-020-0241-0.
- Nicholls, H. T., Hornick, J. L., & Cohen, D. E. (2017). Phosphatidylcholine transfer protein/StarD2 promotes microvesicular steatosis and liver injury in murine experimental steatohepatitis. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 313(1), G50–G61. https://doi.org/10.1152/ajpgi.00379.2016.
- Noda, N. N. (2021). Atg2 and Atg9: Intermembrane and interleaflet lipid transporters driving autophagy. *Biochimica et Biophysica Acta (BBA)—Molecular and Cell Biology of Lipids*, 1866(8), 158956. https://doi.org/10.1016/j.bbalip.2021.158956.
- Olayioye, M. A., Buchholz, M., Schmid, S., Schoöffler, P., Hoffmann, P., & Pomorski, T. (2007). Phosphorylation of StarD10 on serine 284 by casein kinase II modulates its lipid transfer activity. *Journal of Biological Chemistry*, 282(31), 22492–22498. https://doi.org/10.1074/jbc.M701990200.
- Olayioye, M. A., Vehring, S., Müller, P., Herrmann, A., Schiller, J., Thiele, C., et al. (2005). StarD10, a START domain protein overexpressed in breast cancer, functions as a phospholipid transfer protein. *Journal of Biological Chemistry*, 280(29), 27436–27442. https:// doi.org/10.1074/jbc.M413330200.
- Orii, M., Tsuji, T., Ogasawara, Y., & Fujimoto, T. (2021). Transmembrane phospholipid translocation mediated by Atg9 is involved in autophagosome formation. *Journal of Cell Biology*, 220(3), e202009194. https://doi.org/10.1083/jcb.202009194.
- Osawa, T., Kotani, T., Kawaoka, T., Hirata, E., Suzuki, K., Nakatogawa, H., et al. (2019). Atg2 mediates direct lipid transfer between membranes for autophagosome formation. *Nature Structural & Molecular Biology*, *26*(4), 281–288. https://doi.org/10.1038/s41594-019-0203-4.
- Osman, C., Haag, M., Potting, C., Rodenfels, J., Dip, P. V., Wieland, F. T., et al. (2009). The genetic interactome of prohibitins: Coordinated control of cardiolipin and

phosphatidylethanolamine by conserved regulators in mitochondria. Journal of Cell Biology, 184(4), 583-596. https://doi.org/10.1083/jcb.200810189.

- Park, J.-S., & Neiman, A. M. (2012). VPS13 regulates membrane morphogenesis during sporulation in Saccharomyces cerevisiae. Journal of Cell Science, 125(12), 3004–3011. https://doi.org/10.1242/jcs.105114.
- Park, J.-S., & Neiman, A. M. (2020). XK is a partner for VPS13A: A molecular link between Chorea-Acanthocytosis and McLeod Syndrome. *Molecular Biology of the Cell*, 31(22), 2425–2436. https://doi.org/10.1091/mbc.E19-08-0439-T.
- Peiro, A., Izquierdo-Garcia, A. C., Sanchez-Navarro, J. A., Pallas, V., Mulet, J. M., & Aparicio, F. (2014). Patellins 3 and 6, two members of the Plant Patellin family, interact with the movement protein of Alfalfa mosaic virus and interfere with viral movement. *Molecular Plant Pathology*, 15(9), 881–891. https://doi.org/10.1111/mpp.12146.
- Perez-Sancho, J., Tilsner, J., Samuels, A. L., Botella, M. A., Bayer, E. M., & Rosado, A. (2016). Stitching organelles: Organization and function of specialized membrane contact sites in plants. *Trends in Cell Biology*, 26(9), 705–717. https://doi. org/10.1016/j.tcb.2016.05.007.
- Perez-Sancho, J., Vanneste, S., Lee, E., McFarlane, H. E., Esteban Del Valle, A., Valpuesta, V., et al. (2015). The Arabidopsis synaptotagmin1 is enriched in endoplasmic reticulum-plasma membrane contact sites and confers cellular resistance to mechanical stresses. *Plant Physiology*, 168(1), 132–143. https://doi.org/10.1104/pp.15.00260.
- Peterman, T. K., Ohol, Y. M., McReynolds, L. J., & Luna, E. J. (2004). Patellin1, a novel Sec14-like protein, localizes to the cell plate and binds phosphoinositides. *Plant Physiology*, 136(2), 3080–3094. discussion 3001-3002 https://doi.org/10.1104/pp.104. 045369.
- Peterman, T. K., Sequeira, A. S., Samia, J. A., & Lunde, E. E. (2006). Molecular cloning and characterization of patellin1, a novel sec14-related protein, from zucchini (Cucurbita pepo). *Journal of Plant Physiology*, 163(11), 1150–1158. https://doi.org/10.1016/ j.jplph.2006.01.009.
- Petit, J. D., Immel, F., Lins, L., & Bayer, E. M. (2019). Lipids or proteins: who is leading the dance at membrane contact sites? *Frontiers in Plant Science*, 10, 198. https://doi.org/10.3389/fpls.2019.00198.
- Petrungaro, C., & Kornmann, B. (2019). Lipid exchange at ER-mitochondria contact sites: A puzzle falling into place with quite a few pieces missing. *Current Opinion in Cell Biology*, 57, 71–76. https://doi.org/10.1016/j.ceb.2018.11.005.
- Pfanner, N., van der Laan, M., Amati, P., Capaldi, R. A., Caudy, A. A., Chacinska, A., et al. (2014). Uniform nomenclature for the mitochondrial contact site and cristae organizing system. *Journal of Cell Biology*, 204(7), 1083–1086. https://doi.org/10.1083/jcb. 201401006.
- Pfisterer, S. G., Bakula, D., Frickey, T., Cezanne, A., Brigger, D., Tschan, M. P., et al. (2014). Lipid droplet and early autophagosomal membrane targeting of Atg2A and Atg14L in human tumor cells. *Journal of Lipid Research*, 55(7), 1267–1278. https://doi. org/10.1194/jlr.M046359.
- Pichler, H., Gaigg, B., Hrastnik, C., Achleitner, G., Kohlwein, S. D., Zellnig, G., et al. (2001). A subfraction of the yeast endoplasmic reticulum associates with the plasma membrane and has a high capacity to synthesize lipids. *European Journal of Biochemistry*, 268(8), 2351–2361. https://doi.org/10.1046/j.1432-1327.2001.02116.x.
- Pietrangelo, A., & Ridgway, N. D. (2018). Bridging the molecular and biological functions of the oxysterol-binding protein family. *Cellular and Molecular Life Sciences*, 75(17), 3079–3098. https://doi.org/10.1007/s00018-018-2795-y.
- Pii, Y., Astegno, A., Peroni, E., Zaccardelli, M., Pandolfini, T., & Crimi, M. (2009). The Medicago truncatula N5 gene encoding a root-specific lipid transfer protein is required for the symbiotic interaction with Sinorhizobium meliloti. *Molecular Plant-Microbe Interactions*, 22(12), 1577–1587.

- Pii, Y., Molesini, B., Masiero, S., & Pandolfini, T. (2012). The non-specific lipid transfer protein N5 of Medicago truncatula is implicated in epidermal stages of rhizobiumhost interaction. BMC Plant Biology, 12(233), 1–13.
- Pii, Y., Molesini, B., & Pandolfini, T. (2013). The involvement of Medicago truncatula non-specific lipid transfer protein N5 in the control of rhizobial infection. *Plant Signaling & Behavior*, 8(7), e24836. https://doi.org/10.4161/psb.24836.
- Plaxton, W. C., & Tran, H. T. (2011). Metabolic adaptations of phosphate-starved plants. *Plant Physiology*, 156(3), 1006–1015. https://doi.org/10.1104/pp.111.175281.
- Poirier, Y., Thoma, S., Somerville, C., & Schiefelbein, J. (1991). A mutant of Arabidopsis deficient in xylem loading of phosphate. *Plant Physiology*, *97*(7), 1087–1093.
- Ponting, C. P., & Aravind, L. (1999). START: A lipid-binding domain in StAR, HD-ZIP and signalling proteins. *Trends in Biochemical Sciences*, 24(4), 130–132. https://doi.org/ 10.1016/S0968-0004(99)01362-6.
- Potting, C., Tatsuta, T., König, T., Haag, M., Wai, T., Aaltonen, M. J., et al. (2013). TRIAP1/PRELI complexes prevent apoptosis by mediating intramitochondrial transport of phosphatidic acid. *Cell Metabolism*, 18(2), 287–295. https://doi.org/10.1016/ j.cmet.2013.07.008.
- Potting, C., Wilmes, C., Engmann, T., Osman, C., & Langer, T. (2010). Regulation of mitochondrial phospholipids by Ups1/PRELI-like proteins depends on proteolysis and Mdm35. *The EMBO Journal*, 29(17), 2888–2898. https://doi.org/10.1038/emboj. 2010.169.
- Poulsen, L. R., López-Marqués, R. L., Pedas, P. R., McDowell, S. C., Brown, E., Kunze, R., et al. (2015). A phospholipid uptake system in the model plant Arabidopsis thaliana. *Nature Communications*, 6(1), 7649. https://doi.org/10.1038/ncomms8649.
- Prinz, W. A., Toulmay, A., & Balla, T. (2020). The functional universe of membrane contact sites. *Nature Reviews. Molecular Cell Biology*, 21, 7–24. https://doi.org/10.1038/s41580-019-0180-9.
- Pulli, I., Lassila, T., Pan, G., Yan, D., Olkkonen, V. M., & Törnquist, K. (2018). Oxysterol-binding protein related-proteins (ORPs) 5 and 8 regulate calcium signaling at specific cell compartments. *Cell Calcium*, 72, 62–69. https://doi.org/10.1016/j.ceca. 2018.03.001.
- Rabitsch, K. P., Tóth, A., Gálová, M., Schleiffer, A., Schaffner, G., Aigner, E., et al. (2001). A screen for genes required for meiosis and spore formation based on wholegenome expression. *Current Biology*, 11(13), 1001–1009. https://doi.org/10.1016/ S0960-9822(01)00274-3.
- Rampelt, H., Zerbes, R. M., van der Laan, M., & Pfanner, N. (2017). Role of the mitochondrial contact site and cristae organizing system in membrane architecture and dynamics. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1864(4), 737–746. https:// doi.org/10.1016/j.bbamcr.2016.05.020.
- Reinisch, K. M., & De Camilli, P. (2016). SMP-domain proteins at membrane contact sites: Structure and function. *Biochimica et Biophysica Acta (BBA)*—Molecular and Cell Biology of Lipids, 1861(8), 924–927. https://doi.org/10.1016/j.bbalip.2015.12.003.
- Reinisch, K. M., & Prinz, W. A. (2021). Mechanisms of nonvesicular lipid transport. *Journal* of Cell Biology, 220(3), e202012058. https://doi.org/10.1083/jcb.202012058.
- Ren, J., Pei-Chen Lin, C., Pathak, M. C., Temple, B. R. S., Nile, A. H., Mousley, C. J., et al. (2014). A phosphatidylinositol transfer protein integrates phosphoinositide signaling with lipid droplet metabolism to regulate a developmental program of nutrient stress-induced membrane biogenesis. *Molecular Biology of the Cell*, 25(5), 712–727. https://doi.org/10.1091/mbc.e13-11-0634.
- Rochin, L., Sauvanet, C., Jääskeläinen, E., Houcine, A., Kivelä, A. M., Ma, X., et al. (2020). ORP5 transfers phosphatidylserine to mitochondria and regulates mitochondrial calcium uptake at endoplasmic reticulum—Mitochondria contact sites. *Preprint*, 84. https://doi. org/10.1101/695577.

- Roderick, S. L., Chan, W. W., Agate, D. S., Olsen, L. R., Vetting, M. W., Rajashankar, K. R., et al. (2002). Structure of human phosphatidylcholine transfer protein in complex with its ligand. *Nature Structural Biology*, 9(7), 507–511. https://doi.org/10.1038/nsb812.
- Rodriguez-Peña, R., Mounadi, K. E., & Garcia-Ruiz, H. (2021). Changes in subcellular localization of host proteins induced by plant viruses. *Viruses*, 13(4), 677. https://doi.org/10.3390/v13040677.
- Roston, R. L., Gao, J., Murcha, M. W., Whelan, J., & Benning, C. (2012). TGD1, -2, and -3 proteins involved in lipid trafficking form ATP-binding cassette (ABC) transporter with multiple substrate-binding proteins. *Journal of Biological Chemistry*, 287(25), 21406–21415. https://doi.org/10.1074/jbc.M112.370213.
- Roston, R., Gao, J., Xu, C., & Benning, C. (2011). Arabidopsis chloroplast lipid transport protein TGD2 disrupts membranes and is part of a large complex: TGD2 involved in chloroplast lipid transport. *The Plant Journal*, 66(5), 759–769. https://doi.org/10.1111/ j.1365-313X.2011.04536.x.
- Ruiz-Lopez, N., Pérez-Sancho, J., del Valle, Haslam, R. P., Vanneste, S., ... Botella, M. A. (2020). Synaptotagmins maintain diacylglycerol homeostasis at endoplasmic reticulumplasma membrane contact sites during abiotic stress [preprint]. *BioRxiv*. https://doi.org/ 10.1101/2020.07.28.222919.
- Saheki, Y., Bian, X., Schauder, C. M., Sawaki, Y., Surma, M. A., Klose, C., et al. (2016). Control of plasma membrane lipid homeostasis by the extended synaptotagmins. *Nature Cell Biology*, 18(5), 504–515. https://doi.org/10.1038/ncb3339.
- Saheki, Y., & De Camilli, P. (2017a). Endoplasmic reticulum-plasma membrane contact sites. Annual Review of Biochemistry, 86, 659–684. https://doi.org/10.1146/annurevbiochem-061516-044932.
- Saheki, Y., & De Camilli, P. (2017b). The extended-synaptotagmins. *Biochimica et Biophysica Acta*, 1864(9), 1490–1493. https://doi.org/10.1016/j.bbamcr.2017.03.013.
- Saita, S., Tatsuta, T., Lampe, P. A., Konig, T., Ohba, Y., & Langer, T. (2018). PARL partitions the lipid transfer protein STARD7 between the cytosol and mitochondria. *The EMBO Journal*, 37(4). https://doi.org/10.15252/embj.201797909.
- Salminen, T. A., Blomqvist, K., & Edqvist, J. (2016). Lipid transfer proteins: Classification, nomenclature, structure, and function. *Planta*, 244(5), 971–997. https://doi.org/ 10.1007/s00425-016-2585-4.
- Salvaing, J., Botella, C., Albrieux, C., Gros, V., Block, M. A., & Jouhet, J. (2020). PUB11-dependent ubiquitination of the phospholipid flippase ALA10 modifies ALA10 localization and affects the pool of linolenic phosphatidylcholine. *Frontiers in Plant Science*, 11, 1070. https://doi.org/10.3389/fpls.2020.01070.
- Sandhu, J., Li, S., Fairall, L., Pfisterer, S. G., Gurnett, J. E., Xiao, X., et al. (2018). Aster proteins facilitate nonvesicular plasma membrane to er cholesterol transport in mammalian cells. *Cell*, 175(2), 514–529.e20. https://doi.org/10.1016/j.cell.2018.08.033.
- Santi, C., Molesini, B., Guzzo, F., Pii, Y., Vitulo, N., & Pandolfini, T. (2017). Genome-wide transcriptional changes and lipid profile modifications induced by medicago truncatula N5 overexpression at an early stage of the symbiotic interaction with sinorhizobium meliloti. *Genes (Basel)*, 8(12), 396. https://doi.org/10.3390/genes8120396.
- Santos, N. C., Girik, V., & Nunes-Hasler, P. (2020). ORP5 and ORP8: Sterol sensors and phospholipid transfer proteins at membrane contact sites? *Biomolecules*, 10(6), 928. https://doi.org/10.3390/biom10060928.
- Saravanan, R. S., Slabaugh, E., Singh, V. R., Lapidus, L. J., Haas, T., & Brandizzi, F. (2009). The targeting of the oxysterol-binding protein ORP3a to the endoplasmic reticulum relies on the plant VAP33 homolog PVA12. *The Plant Journal*, 58(5), 817–830. https://doi.org/10.1111/j.1365-313X.2009.03815.x.
- Satheesh, V., Chidambaranathan, P., Jagannadham, P. T., Kumar, V., Jain, P. K., Chinnusamy, V., et al. (2016). Transmembrane START domain proteins: In silico

identification, characterization and expression analysis under stress conditions in chickpea (Cicer arietinum L.). *Plant Signaling & Behavior*, 11(2), e992698. https://doi.org/ 10.4161/15592324.2014.992698.

- Schaaf, G., Ortlund, E. A., Tyeryar, K. R., Mousley, C. J., Ile, K. E., Garrett, T. A., et al. (2008). Functional anatomy of phospholipid binding and regulation of phosphoinositide homeostasis by proteins of the sec14 superfamily. *Molecular Cell*, 29(2), 191–206. https:// doi.org/10.1016/j.molcel.2007.11.026.
- Schapire, A. L., Voigt, B., Jasik, J., Rosado, A., Lopez-Cobollo, R., Menzel, D., et al. (2008). Arabidopsis synaptotagmin 1 is required for the maintenance of plasma membrane integrity and cell viability. *Plant Cell*, 20(12), 3374–3388. https://doi.org/10.1105/tpc.108.063859.
- Schattat, M., Barton, K., & Mathur, J. (2011). Correlated behavior implicates stromules in increasing the interactive surface between plastids and ER tubules. *Plant Signaling & Behavior*, 6(5), 715–718. https://doi.org/10.4161/psb.6.5.15085.
- Schauder, C. M., Wu, X., Saheki, Y., Narayanaswamy, P., Torta, F., Wenk, M. R., et al. (2014). Structure of a lipid-bound extended synaptotagmin indicates a role in lipid transfer. *Nature*, 510(7506), 552–555. https://doi.org/10.1038/nature13269.
- Schlattner, U., Tokarska-Schlattner, M., Epand, R. M., Boissan, M., Lacombe, M.-L., & Kagan, V. E. (2018). NME4/nucleoside diphosphate kinase D in cardiolipin signaling and mitophagy. *Laboratory Investigation: A Journal of Technical Methods and Pathology*, 98(2), 228–232. https://doi.org/10.1038/labinvest.2017.113.
- Schlattner, U., Tokarska-Schlattner, M., Ramirez, S., Tyurina, Y. Y., Amoscato, A. A., Mohammadyani, D., et al. (2013). Dual function of mitochondrial Nm23-H4 protein in phosphotransfer and intermembrane lipid transfer. *Journal of Biological Chemistry*, 288(1), 111–121. https://doi.org/10.1074/jbc.M112.408633.
- Schrick, K., Bruno, M., Khosla, A., Cox, P. N., Marlatt, S. A., Roque, R. A., et al. (2014). Shared functions of plant and mammalian StAR-related lipid transfer (START) domains in modulating transcription factor activity. *BMC Biology*, 12, 70. https://doi.org/ 10.1186/s12915-014-0070-8.
- Schrick, K., Nguyen, D., Karlowski, W. M., & Mayer, K. F. X. (2004). START lipid/ sterol-binding domains are amplified in plants and are predominantly associated with homeodomain transcription factors. *Genome Biology*, 5(R41).
- Schulz, T. A., & Prinz, W. A. (2007). Sterol transport in yeast and the oxysterol binding protein homologue (OSH) family. *Biochimica et Biophysica Acta (BBA)*—Molecular and Cell Biology of Lipids, 1771(6), 769–780. https://doi.org/10.1016/j.bbalip.2007.03.003.
- Schwacke, R., Schneider, A., van der Graaff, E., Fischer, K., Catoni, E., Desimone, M., et al. (2003). ARAMEMNON, a novel database for Arabidopsis integral membrane proteins. *Plant Physiology*, 131(1), 16–26. https://doi.org/10.1104/pp.011577.
- Scorrano, L., De Matteis, M. A., Emr, S., Giordano, F., Hajnoczky, G., Kornmann, B., et al. (2019). Coming together to define membrane contact sites. *Nature Communications*, 10(1), 1287. https://doi.org/10.1038/s41467-019-09253-3.
- Selitrennik, M., & Lev, S. (2016). The role of phosphatidylinositol-transfer proteins at membrane contact sites. *Biochemical Society Transactions*, 44(2), 419–424. https://doi.org/10.1042/BST20150182.
- Senkler, J., Senkler, M., Eubel, H., Hildebrandt, T., Lengwenus, C., Schertl, P., et al. (2017). The mitochondrial complexome of *Arabidopsis thaliana*. *The Plant Journal*, 89(6), 1079–1092. https://doi.org/10.1111/tpj.13448.
- Shirane, M., Wada, M., Morita, K., Hayashi, N., Kunimatsu, R., Matsumoto, Y., et al. (2020). Protrudin and PDZD8 contribute to neuronal integrity by promoting lipid extraction required for endosome maturation. *Nature Communications*, 11(1), 4576. https://doi.org/10.1038/s41467-020-18413-9.
- Skirpan, A. L., Dowd, P. E., Sijacic, P., Jaworski, C. J., Gilroy, S., & Kao, T. H. (2006). Identification and characterization of PiORP1, a Petunia oxysterol-binding-protein

related protein involved in receptor-kinase mediated signaling in pollen, and analysis of the ORP gene family in Arabidopsis. *Plant Molecular Biology*, *61*(4–5), 553–565. https://doi.org/10.1007/s11103-006-0030-y.

- Slee, J. A., & Levine, T. P. (2019). Systematic prediction of FFAT motifs across eukaryote proteomes identifies nucleolar and eisosome proteins with the predicted capacity to form bridges to the endoplasmic reticulum. *Contact*, 2, 251525641988313. https://doi.org/ 10.1177/2515256419883136.
- Somerharju, P. (2015). Is spontaneous translocation of polar lipids between cellular organelles negligible? *Lipid Insights*, 8s1, LPI.S31616. https://doi.org/10.4137/LPI.S31616.
- Sperandeo, P., Martorana, A. M., & Polissi, A. (2017). The lipopolysaccharide transport (Lpt) machinery: A nonconventional transporter for lipopolysaccharide assembly at the outer membrane of Gram-negative bacteria. *The Journal of Biological Chemistry*, 292(44), 17981–17990. https://doi.org/10.1074/jbc.R117.802512.
- Stoica, R., De Vos, K. J., Paillusson, S., Mueller, S., Sancho, R. M., Lau, K.-F., et al. (2014). ER-mitochondria associations are regulated by the VAPB-PTPIP51 interaction and are disrupted by ALS/FTD-associated TDP-43. *Nature Communications*, *5*, 3996. https://doi. org/10.1038/ncomms4996.
- Suzuki, K., Akioka, M., Kondo-Kakuta, C., Yamamoto, H., & Ohsumi, Y. (2013). Fine mapping of autophagy-related proteins during autophagosome formation in *Saccharomyces cerevisiae. Journal of Cell Science*, 126(11), 2534–2544. https://doi.org/ 10.1242/jcs.122960.
- Suzuki, T., Matsushima, C., Nishimura, S., Higashiyama, T., Sasabe, M., & Machida, Y. (2016). Identification of phosphoinositide-binding protein PATELLIN2 as a substrate of Arabidopsis MPK4 MAP kinase during septum formation in cytokinesis. *Plant & Cell Physiology*, 57(8), 1744–1755. https://doi.org/10.1093/pcp/pcw098.
- Tan, X., Wang, Q., Tian, B., Zhang, H., Lu, D., & Zhou, J. (2011). A Brassica napus lipase locates at the membrane contact sites involved in chloroplast development. *PLoS One*, 6(10), e26831. https://doi.org/10.1371/journal.pone.0026831.
- Tejos, R., Rodriguez-Furlán, C., Adamowski, M., Sauer, M., Norambuena, L., & Friml, J. (2018). PATELLINS are regulators of auxin-mediated PIN1 relocation and plant development in Arabidopsis thaliana. *Journal of Cell Science*, 131(2), jcs204198. https://doi.org/10.1242/jcs.204198.
- Thélot, F., Orlando, B. J., Li, Y., & Liao, M. (2020). High-resolution views of lipopolysaccharide translocation driven by ABC transporters MsbA and LptB2FGC. *Current Opinion in Structural Biology*, *63*, 26–33. https://doi.org/10.1016/j.sbi.2020.03.005.
- Thong, S., Ercan, B., Torta, F., Fong, Z. Y., Wong, H. Y. A., Wenk, M. R., et al. (2016). Defining key roles for auxiliary proteins in an ABC transporter that maintains bacterial outer membrane lipid asymmetry. *eLife*, *5*, e19042. https://doi.org/10.7554/eLife.19042.
- Tjellström, H., Andersson, M. X., Larsson, K. E., & Sandelius, A. S. (2008). Membrane phospholipids as a phosphate reserve: The dynamic nature of phospholipid-to-digalactosyl diacylglycerol exchange in higher plants. *Plant, Cell & Environment, 31*(10), 1388–1398.
- Tokarska-Schlattner, M., Boissan, M., Munier, A., Borot, C., Mailleau, C., Speer, O., et al. (2008). The nucleoside diphosphate kinase D (NM23-H4) binds the inner mitochondrial membrane with high affinity to cardiolipin and couples nucleotide transfer with respiration. *The Journal of Biological Chemistry*, 283(38), 26198–26207. https://doi.org/ 10.1074/jbc.M803132200.
- Tong, J., Manik, M. K., & Im, Y. J. (2018). Structural basis of sterol recognition and nonvesicular transport by lipid transfer proteins anchored at membrane contact sites. *Proceedings of the National Academy of Sciences of the United States of America*, 115(5), E856–E865. https://doi.org/10.1073/pnas.1719709115.

- Tripathi, A., Martinez, E., Obaidullah, A. J., Lete, M. G., Lönnfors, M., Khan, D., et al. (2019). Functional diversification of the chemical landscapes of yeast Sec14-like phosphatidylinositol transfer protein lipid-binding cavities. *Journal of Biological Chemistry*, 294(50), 19081–19098. https://doi.org/10.1074/jbc.RA119.011153.
- Udvardi, M., & Poole, P. S. (2013). Transport and metabolism in legume-rhizobia symbioses. *Annual Review of Plant Biology*, 64(1), 781–805. https://doi.org/10.1146/annurev-arplant-050312-120235.
- Umate, P. (2011). Oxysterol binding proteins (OSBPs) and their encoding genes in Arabidopsis and rice. *Steroids*, 76(5), 524–529. https://doi.org/10.1016/j.steroids.2011.01.007.
- Upchurch, R. G. (2008). Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnology Letters*, 30(6), 967–977. https://doi.org/10.1007/s10529-008-9639-z.
- Valverde, D. P., Yu, S., Boggavarapu, V., Kumar, N., Lees, J. A., Walz, T., et al. (2019). ATG2 transports lipids to promote autophagosome biogenesis. *Journal of Cell Biology*, 218(6), 1787–1798.
- Vance, J. E. (1990). Phospholipid synthesis in a membrane fraction associated with mitochondria. *The Journal of Biological Chemistry*, 265(13), 7248–7256.
- Velayos-Baeza, A., Vettori, A., Copley, R. R., Dobson-Stone, C., & Monaco, A. P. (2004). Analysis of the human VPS13 gene family. *Genomics*, 84(3), 536–549. https://doi.org/10. 1016/j.ygeno.2004.04.012.
- Verma, D. (1996). Biogenesis of the peribacteriod membrane in root nodules. Trends in Microbiology, 4(9), 364–368. https://doi.org/10.1016/0966-842X(96)10053-6.
- Vincent, P., Chua, M., Nogue, F., Fairbrother, A., Mekeel, H., Xu, Y., et al. (2005). A Sec14p-nodulin domain phosphatidylinositol transfer protein polarizes membrane growth of Arabidopsis thaliana root hairs. *Journal of Cell Biology*, 168(5), 801–812. https://doi.org/10.1083/jcb.200412074.
- Vorwerk, S., Schiff, C., Santamaria, M., Koh, S., Nishimura, M., Vogel, J., et al. (2007). EDR2 negatively regulates salicylic acid-based defenses and cell death during powdery mildew infections of Arabidopsis thaliana. *BMC Plant Biology*, 7(1), 35. https://doi.org/ 10.1186/1471-2229-7-35.
- Wang, Z., Anderson, N. S., & Benning, C. (2013). The phosphatidic acid binding site of the arabidopsis trigalactosyldiacylglycerol 4 (TGD4) protein required for lipid import into chloroplasts. *Journal of Biological Chemistry*, 288(7), 4763–4771. https://doi.org/10. 1074/jbc.M112.438986.
- Wang, Z., & Benning, C. (2012). Chloroplast lipid synthesis and lipid trafficking through ER-plastid membrane contact sites. *Biochemical Society Transactions*, 40(2), 457–463. https://doi.org/10.1042/BST20110752.
- Wang, H., Han, S., Siao, W., Song, C., Xiang, Y., Wu, X., et al. (2015). Arabidopsis synaptotagmin 2 participates in pollen germination and tube growth and is delivered to plasma membrane via conventional secretion. *Molecular Plant*, 8(12), 1737–1750. https://doi.org/10.1016/j.molp.2015.09.003.
- Wang, Z., Xu, C., & Benning, C. (2012). TGD4 involved in endoplasmic reticulum-tochloroplast lipid trafficking is a phosphatidic acid binding protein: TGD4 involvement in ER-to-chloroplast trafficking. *The Plant Journal*, 70(4), 614–623. https://doi.org/ 10.1111/j.1365-313X.2012.04900.x.
- Wanjie, S. W., Welti, R., Moreau, R. A., & Chapman, K. D. (2005). Identification and quantification of glycerolipids in cotton fibers: Reconciliation with metabolic pathway predictions from DNA databases. *Lipids*, 40(8), 773–785. https://doi.org/10.1007/ s11745-005-1439-4.
- Watanabe, Y., Tamura, Y., Kawano, S., & Endo, T. (2015). Structural and mechanistic insights into phospholipid transfer by Ups1–Mdm35 in mitochondria. *Nature Communications*, 6(1), 7922. https://doi.org/10.1038/ncomms8922.

- Wideman, J. G., & Muñoz-Gómez, S. A. (2016). The evolution of ERMIONE in mitochondrial biogenesis and lipid homeostasis: An evolutionary view from comparative cell biology. *Biochimica et Biophysica Acta*, 1861(8 Pt. B), 900–912. https://doi.org/10.1016/ j.bbalip.2016.01.015.
- Wilkens, S. (2015). Structure and mechanism of ABC transporters. F1000Prime Reports, 7, 14. https://doi.org/10.12703/P7-14.
- Wirtz, K. W. (1991). Phospholipid transfer proteins. Annual Review of Biochemistry, 60, 73–99. https://doi.org/10.1146/annurev.bi.60.070191.000445.
- Wong, L. H., Gatta, A. T., & Levine, T. P. (2019). Lipid transfer proteins: The lipid commute via shuttles, bridges and tubes. *Nature Reviews Molecular Cell Biology*, 20(2), 85–101. https://doi.org/10.1038/s41580-018-0071-5.
- Wong, L. H., & Levine, T. P. (2016). Lipid transfer proteins do their thing anchored at membrane contact sites. But what is their thing? *Biochemical Society Transactions*, 44(2), 517–527. https://doi.org/10.1042/BST20150275.
- Wong, L. H., & Levine, T. P. (2017). Tubular lipid binding proteins (TULIPs) growing everywhere. Biochimica et Biophysica Acta (BBA)—Molecular Cell Research, 1864(9), 1439–1449. https://doi.org/10.1016/j.bbamcr.2017.05.019.
- Wu, C., Tan, L., van Hooren, M., Tan, X., Liu, F., Li, Y., et al. (2017). Arabidopsis EXO70A1 recruits Patellin3 to the cell membrane independent of its role as an exocyst subunit: A subunit-specific role of EXO70A1. *Journal of Integrative Plant Biology*, 59(12), 851–865. https://doi.org/10.1111/jipb.12578.
- Xu, C., Fan, J., Cornish, A. J., & Benning, C. (2008). Lipid trafficking between the endoplasmic reticulum and the plastid in *Arabidopsis* requires the extraplastidic TGD4 protein. *The Plant Cell*, 20(8), 2190–2204. https://doi.org/10.1105/tpc.108.061176.
- Xu, C., Fan, J., Froehlich, J. E., Awai, K., & Benning, C. (2005). Mutation of the TGD1 chloroplast envelope protein affects phosphatidate metabolism in *Arabidopsis*. *The Plant Cell*, 17(11), 3094–3110. https://doi.org/10.1105/tpc.105.035592.
- Xu, C., Fan, J., Riekhof, W., Froehlich, J. E., & Benning, C. (2003). A permease-like protein involved in ER to thylakoid lipid transfer in Arabidopsis. *The EMBO Journal*, 22(10), 2370–2379. https://doi.org/10.1093/emboj/cdg234.
- Yamaryo, Y., Dubots, E., Albrieux, C., Baldan, B., & Block, M. A. (2008). Phosphate availability affects the tonoplast localization of PLDζ2, an *Arabidopsis thaliana* phospholipase D. *FEBS Letters*, 582(5), 685–690. https://doi.org/10.1016/j.febslet.2008.01.039.
- Yamazaki, T., Kawamura, Y., Minami, A., & Uemura, M. (2008). Calcium-dependent freezing tolerance in Arabidopsis involves membrane resealing via synaptotagmin SYT1. *Plant Cell*, 20(12), 3389–3404. https://doi.org/10.1105/tpc.108.062679.
- Yamazaki, T., Takata, N., Uemura, M., & Kawamura, Y. (2010). Arabidopsis synaptotagmin SYT1, a type I signal-anchor protein, requires tandem C2 domains for delivery to the plasma membrane. *The Journal of Biological Chemistry*, 285(30), 23165–23176. https:// doi.org/10.1074/jbc.M109.084046.
- Yang, L., Na, C.-L., Luo, S., Wu, D., Hogan, S., Huang, T., et al. (2017). The phosphatidylcholine transfer protein Stard7 is required for mitochondrial and epithelial cell homeostasis. *Scientific Reports*, 7, 46416. https://doi.org/10.1038/srep46416.
- Ye, H., Ji, C., Guo, R., & Jiang, L. (2020). Membrane contact sites and organelles interaction in plant autophagy. *Frontiers in Plant Science*, 11, 477. https://doi.org/10.3389/fpls.2020. 00477.
- Zhang, H., Zhang, L., Gao, B., Fan, H., Jin, J., Botella, M. A., et al. (2011). Golgi apparatus-localized synaptotagmin 2 is required for unconventional secretion in arabidopsis. *PLoS One*, 6(11), e26477. https://doi.org/10.1371/journal.pone.0026477.

- Zhou, H., Duan, H., Liu, Y., Sun, X., Zhao, J., & Lin, H. (2019). Patellin protein family functions in plant development and stress response. *Journal of Plant Physiology*, 234-235, 94–97. https://doi.org/10.1016/j.jplph.2019.01.012.
- Zhou, H., Wang, C., Tan, T., Cai, J., He, J., & Lin, H. (2018). Patellin1 negatively modulates salt tolerance by regulating PM Na+/H+ antiport activity and cellular redox homeostasis in arabidopsis. *Plant and Cell Physiology*, *59*(8), 1630–1642. https://doi. org/10.1093/pcp/pcy081.

# 4 Non-vesicular lipid transport at endoplasmic reticulum – mitochondria and intramitochondrial contact sites

#### 4.1 Functions of endoplasmic reticulum – mitochondria contact sites

ER-mitochondria contact sites are the first MCSs that have been discovered (Bernhard & Rouiller, 1956; Copeland & Dalton, 1959). In the following decades, mitochondria have been found to interact with virtually all organelles and to fulfill numerous cellular functions (Harper et al., 2020; Lackner, 2019). One particularly important mitochondrial contact site is the interface between the ER and mitochondria. The functions of ER-mitochondria contact sites (EMCSs) have already been extensively reviewed (Prinz et al., 2020; Rowland & Voeltz, 2012; Sassano et al., 2022; Wenzel et al., 2022) and a short, nonexhaustive overview is given hereafter.

Ca<sup>2+</sup> signaling is a well-known function of EMCSs and is facilitated by the physical coupling of IP3R and VDAC ion channels (Szabadkai et al., 2006). ER-mitochondria Ca<sup>2+</sup> exchange contributes to ROS signaling response, regulation of mitochondrial energy metabolism (Silva-Pavez et al., 2021), apoptosis and its deregulation is the cause of several human diseases (Sassano et al., 2022). EMCSs regulate mitochondria biogenesis and abundance. Notably, they mark sites of mitochondrial fission and fusion by regulating the fission/fusion machinery (Abrisch et al., 2020; Friedman et al., 2011). EMCSs also regulate mitophagy during nutrient stress (Böckler & Westermann, 2014). The tight link between ER and mitochondria favors the exchange and channeling of metabolites. Beside Ca<sup>2+</sup> and iron import, EMCSs are lipid exchange platforms at which extensive lipid transport takes place. EMCSs are highly enriched in lipid synthesis enzymes, foreshowing their importance for cell lipid homeostasis (Vance, 1990). The following sections address lipid metabolism, lipid trafficking and the associated lipid transport proteins at EMCSs. Given the rather low amount of data on these topics in plants, the general concepts will be reviewed for yeast and mammals and compared or inferred to plants with emphasis on *A. thaliana*.

#### 4.2 Glycerolipid synthesis and trafficking to and in plant mitochondria

Glycerolipid trafficking to yeast and mammalian mitochondria has already been extensively studied and reviewed (Horvath & Daum, 2013; Petrungaro & Kornmann, 2019; Y. Tamura et al., 2020; Tatsuta et al., 2014; Tatsuta & Langer, 2017). In contrast, much less is known about glycerolipid trafficking to plant mitochondria, even though the main concepts are presumably similar (Michaud et al., 2017). This section focuses on glycerolipid synthesis in plant mitochondria and the trafficking routes enabling their import into plant mitochondria. The proteins involved in mitochondrial glycerolipid transport will be described in **section 4.3**.

Mitochondrial membranes are composed of the same variety of phosphoglycerolipids than the ER. In *A. thaliana*, the most abundant glycerolipid species in mitochondria are PC and PE, representing about

80% of the total lipid content (Jouhet et al., 2004). PI and PG are both present in smaller quantities while DPG makes up about 10% of total lipids. Like plastids, plant mitochondria have retained the ability to synthesize fatty acids. Mitochondrial fatty acid synthesis is carried out by a type II FAS that uses imported malonate as precursor for malonyl-ACP and acetyl-ACP to initiate the acyl chain elongation cycles (Li-Beisson et al., 2013). The elongation cycles are terminated to generate C8:0-ACP and C14:0-ACP that are used for lipoylation of proteins important for mitochondrial metabolism and for mitochondrial lipopolysaccharide synthesis, respectively (C. Li et al., 2011; Li-Beisson et al., 2013; Wada et al., 1997). Despite the presence of a FAS in mitochondria, the vast majority of the acyl chains used for *de novo* glycerolipid synthesis in mitochondria are provided by the plastid (Li-Beisson et al., 2013).

Plant mitochondria have the capacity to synthetize only a small subset of the lipids required for their biogenesis and function. They likely contain the six required enzymes for *de novo* PG and DPG synthesis (Figure 3). In A. thaliana, five out of six enzymes have been characterized. Like in plastids and the ER, de novo glycerolipid synthesis starts with the successive acylation of glycerol-3-phosphate (G3P) by a glycerol-3-phosphate acyltransferase (GPAT) and a lysophosphatidic acid acyltransferase (LPAAT) to form PA. While the mitochondrial GPAT remains elusive, the LPAAT is found in soluble form in mitochondria (Ghosh et al., 2009). Five CDP-DAG synthetases that convert PA to CDP-DAG have been identified in A. thaliana. CDP-DAG is then converted to phosphatidylglycerol phosphate (PGP) by the PGP synthase 1 (PGP1) that is dually targeted to mitochondria and plastids. The conversion of PGP to PG is catalyzed by a PGP phosphatase (PGPP). PGPP1 has been characterized in Chlamydomonas reinhardtii and its putative homolog has been inferred in A. thaliana (Hung et al., 2015). Lin et al. characterized PGPP1 in A. thaliana and show that it is involved in PG synthesis and that it is localized in mitochondria in root cells (Lin et al., 2018). The final conversion of PG to DPG is catalyzed by a DPG synthase (DPGS or cardiolipin synthase, CLS). DPGS is localized at the mitochondrial IM. Thus, plant mitochondria seem to be able to synthesize PA, PG and DPG on their own, even though it cannot be excluded that intermediates like CDP-DAG and PG are transported to mitochondria as a complement (Figure 3). Beside PA, PG and DPG, mitochondria are not known to neosynthetize any other glycerolipids. The fact that not all the enzymes required for *de novo* DPG synthesis have been identified in A. thaliana raises questions on whether and which precursor lipids are transported to mitochondria. In yeast, PA is transported from the mitochondrial OM to the IM and is integrated into the synthesis of DPG (Connerth et al., 2012). In humans, DPG synthesis is thought to be mainly driven by PA import (Horvath & Daum, 2013; Potting et al., 2013). It is not known if a similar transport exists in plants. The deletion of PGP1 in A. thaliana only had minor effects on DPG synthesis, suggesting that PG is efficiently transported from extramitochondrial membranes to mitochondria to sustain DPG synthesis, even though this pathway might play a minor role in normal conditions (Babiychuk et al., 2003).



Figure 3 Representation of lipid synthesis and trafficking pathways in and to plant mitochondria (adapted from Michaud et al., 2017). Enzymatic reactions for PE, PC and DPG are represented with black arrows. Black question marks represent the uncertainty over the existence of an enzyme. Orange arrows represent the action of enzymes in trans. The transport of lipids is represented by green arrows. Dashed arrows indicate processes that are thought to be minor in plants. IM: mitochondrial inner membrane, OM: mitochondrial outer membrane, DAG: diacylglycerol, PC: phosphatidylcholine, PE: phosphatidylethanolamine, PS: phosphatidylserine, PA: phosphatidic acid, PGP: phosphatidylglycerol phosphate, PG: phosphatidylglycerol, DPG: diphosphatidylglycerol (cardiolipin), ACP: acyl carrier protein, G3P: glycerol-3-phosphate, P-Etn: phospho-ethanolamine, P-Cho; phospho-choline, CDP: cytidine diphosphate, PECT: phospho-ethanolamine phospho-choline cytidyltransferase, PEAMT: phospho-ethanolamine methyltransferase, CCT: cvtidyltransferase, AAPT: aminoalcohol phosphotransferase, PSD: phosphatidylserine decarboxylase, GPAT: glycerol-3-phopshate acyltransferase, LPAAT: lysophosphatidic acid acyltransferase, CDP-DAGS: CDP-DAG synthase, PGP: phosphatidylglycerol phosphate, PGP1: PGP synthase, PGPP1: PGP phosphatase, DPGS: DPG synthase.

Plant mitochondria have also the capacity to synthesize a fraction of PE. In yeast, mammals and plants, PE can be synthetized through two different main pathways: the CDP-ethanolamine (CDP-Etn) pathway and the decarboxylation pathway (Horvath & Daum, 2013) (**Figure 3**). In the CDP-Etn pathway, ethanolamine (Etn) is first phosphorylated by an Etn kinase to form phospho-Etn. Phospho-Etn is then activated to CDP-Etn by a phospho-Etn cytidyltransferase. CDP-Etn is finally transferred to a DAG backbone by a phospho-Etn transferase to form PE in the ER. In the decarboxylation pathway, PE is directly formed by the decarboxylation of PS by a PS decarboxylase (PSD). In yeast, the major PE synthesis pathway is the decarboxylation pathway (Horvath & Daum, 2013). PSD1 is dually localized to the mitochondrial inner membrane and the ER (Friedman et al., 2018) while PSD2 is Golgi and vacuole-localized (Trotter & Voelker, 1995). Since PSD2 accounts for a negligible part of the decarboxylase activity (Trotter & Voelker, 1995), a substantial transport of PS takes place from the ER

to mitochondria to sustain PE synthesis. A part of the synthetized PE is then transported back to extramitochondrial compartments. In *A. thaliana*, PSD1, PSD2 and PSD3 respectively localize to the mitochondrial IM, the vacuole and the ER (Nerlich et al., 2007). The *psd1 psd2 psd3* triple mutants present no PE synthesis defect (Nerlich et al., 2007), showing that plants mainly use the CDP-Etn pathway for PE synthesis. Interestingly, PECT1, the only phospho-Etn cytidylyltransferase in *A. thaliana*, is associated to mitochondria (Duncan et al., 2011; Mizoi et al., 2007), while both phospho-Etn transferases AAPT1/2 are localized in the ER (Sutoh et al., 2010). This strongly suggests that PE synthesis resides at ER-mitochondria contact sites.

Since PC, PE and PI cannot be synthetized in mitochondria, they rely entirely on the import of these lipids from other compartments to sustain their membrane biogenesis (Li-Beisson et al., 2013). Furthermore, mitochondria being of endosymbiotic origin, these lipids have to be imported by non-vesicular routes at MCSs. Because the ER is the main site of lipid synthesis, ER-mitochondria contact sites (EMCSs) are known to harbor the vast majority of lipid trafficking activity to mitochondria. While this is an established fact in yeast and humans, experimental evidence is lacking to confirm a similar extensive role in plants. The current knowledge on glycerolipid trafficking routes to plant mitochondria is summarized in **Figure 3**.

Even though it is clear that intense lipid trafficking occurs at EMCSs, it is interesting to note that several enzymes have been reported to function *in trans*, that is carrying out their catalytic function on a closely apposed membrane different from the one they are localized in (**Figure 3**, orange arrows). This is the case for PSD1 in yeast (Aaltonen et al., 2016). Since PE synthesis likely occurs at EMCSs in plants, Michaud et al. hypothesized that AAPT1/2 could perform its activity *in trans* on the mitochondrial OM (Michaud et al., 2017). All the aforementioned glycerolipid trafficking steps and the functioning of lipid synthesis enzymes *in trans* highlight the necessity for a tight ER-mitochondria association as well as the requirement for facilitated lipid transport.

# 4.3 Proteins involved in ER-mitochondria and intramitochondrial contact site tethering and lipid transport

The ER – mitochondria membrane contact sites (EMCSs), initially named mitochondria associated membranes (MAMs), are the first contact sites at which non-vesicular lipid transport has been observed (Vance, 1990). In yeast and mammals, the EMCS and the intramitochondrial membrane contact site have already been extensively studied and many tethers and LTPs are known to allow non-vesicular lipid transport to sustain mitochondrial membrane biogenesis and maintenance (Y. Tamura et al., 2020; Tatsuta et al., 2014). The aim of this section is to depict the current knowledge in yeast and mammals and to assess the conservation of the lipid transport processes as well as the tethering proteins in plants with a focus on *A. thaliana*. The known tethers and LTPs at the ER-mitochondria and intramitochondrial

contact sites in mammals, yeast and plants are listed in Table 1, Table 2 and Table 3 and represented

#### in Figure 4 and Figure 5.

**Table 1** Proteins implied in tethering and/or lipid transport identified at ER-mitochondria and intramitochondrial contact sites in mammals, mostly *Homo sapiens*. For each tethering protein, tethering complex or lipid transport protein, the relevant literature, main known functions and putative homologs in *Arabidopsis thaliana* are indicated. Putative homologs have been determined by protein sequence BLAST and the results with an E-value lower than 0.01 are indicated. The cited proteins are represented in the corresponding figure.

Protein name	References	Function at EMCSs	Putative homologs in <i>A</i> . <i>thaliana</i>
VDAC1 GRP75 IP3R DJ-1	(Szabadkai et al., 2006) (Y. Liu et al., 2019) (Basso et al., 2020)	Tethering ER-OM Ca <sup>2+</sup> transport	VDAC1 (At3g01380), VDAC2 (At5g67500), VDAC3 (At5g15090), VDAC4 (At5g57490) mtHSP70-1 (At4g37910), mtHSP70-2 (At5g09590) No DJ-1A-F
FIS1 BAP31	(Iwasawa et al., 2011)	Tethering ER-OM Mitochondrial fission	FIS1A (At3g57090), FIS1B (At5g12390) At5g42570, At1g11905
MFN1 MFN2	(de Brito & Scorrano, 2008) (Hernández-Alvarez et al., 2019)	Tethering ER-OM Mitochondrial fusion Lipid transport	FZL (At1g03160) FZL (At1g03160)
PTPIP51 VAPB	(De Vos et al., 2012) (Stoica et al., 2014) (Yeo et al., 2021) (Di Mattia et al., 2018)	Tethering ER-OM PA transport	No VAP27-1 (At3g60600), VAP27-2 (At1g08820), PVA12 (At2g45140), At5g47180, At4g00170, At2g23830 Ves (>10 including VAP27
MOSPD2 BAP31			and Patellins) $\Delta t5 \sigma / 2570$ $\Delta t1 \sigma 11905$
TOM40	(Namba, 2019)	Tethering ER-OM	AtTOM40 (At3g20000)
ORP5, ORP8	(Galmes et al., 2016) (Monteiro-Cardoso et al., 2022)	PS transport	Yes (>10)
MIGA2 VAPB	(Freyre et al., 2019) (Kim et al., 2022)	Tethering ER-OM Lipid (PS) transport	No VAP27-1 (At3g60600), VAP27-2 (At1g08820), PVA12 (At2g45140), At5g47180, At4g00170, At2g23830
PDZD8	(Hirabayashi et al., 2017) (Shirane et al., 2020)	Tethering ER-OM Lipid transport	No
E-Syt1 PERK SYNJ2BP	(Sassano et al., 2023) (Janer et al., 2022)	Lipid transport	Syt1-7 Yes (>10) No
HsVPS13A VAPA	(Kumar et al., 2018) (Muñoz-Braceras et al., 2019) (Yeshaw et al., 2019) (Guillén-Samander et al., 2022)	Bulk lipid transport	AtVPS13M1 (At4g17140), AtVPS13M2 (At1g48090), AtVPS13S (At5g24740), AtVPS13X (At3g50380)
HsVPS13D MIRO	(Guillén-Samander et al., 2021)	Bulk lipid transport	AtVPS13M1 (At4g17140), AtVPS13M2 (At1g48090), AtVPS13S (At5g24740), AtVPS13X (At3g50380)

Table 1 continued

Protein name	References	Function at EMCSs	Putative homologs in <i>A</i> . <i>thaliana</i>
MIC60	(Xie et al., 2007)	Tethering OM-IM	AtMic60 (At4g39690)
SAM50	(Ott et al., 2012)	Mitochondrial protein	SAM50.1 (At3g11070),
SANDO	(J. Tang et al., 2020)	import	SAM50.2 (At5g05520)
			VDAC1 (At3g01380),
VDAC	(Reutner et al. 1006)		VDAC2 (At5g67500),
VDAC	(Boutner et al., 1990) (Boutner et al., 1990)	Tethering OM-IM	VDAC3 (At5g15090),
	(Beutilei et al., 1998)		VDAC4 (At5g57490)
ANT			Yes (>10)
PRELID1	(Potting et al., 2013)	PA transport OM-IM	At5g13070
TRIAP1		Chaperone	At4g33100
SLMO1	(Miliara et al., 2015)	PA transport OM-IM	At5g13070
SLMO2	(Aaltonen et al., 2016)	PS transport OM-IM	At5g13070
	(Horibata at al. 2016)		At4g14500, CP5
StARD7	(1010ata et al., 2010)	PC transport OM-IM	(At1g64720), At3g23080,
	(Sana et al., 2018)		At5g54170, At3g13062
mtCK	(Epand et al., 2007)	DPG transport	No
NDPK-D	(Epand et al., 2007) (Schlattner et al., 2013)	DPG transport	NDPK3 (At4g11010)

**Table 2** Proteins implied in tethering and/or lipid transport identified at ER-mitochondria and intramitochondrial contact sites in yeast (*Saccharomyces cerevisiae*). For each tethering protein, tethering complex or lipid transport protein, the relevant literature, main known functions and putative homologs in *Arabidopsis thaliana* are indicated. Putative homologs have been determined by protein sequence BLAST and the results with an E-value lower than 0.01 are indicated. The cited proteins are represented in the corresponding figure.

Protein name	References	Function at EMCSs	Putative homologs in <i>A</i> . <i>thaliana</i>
Mmr1	(Swayne et al., 2011)	Tethering ER-OM Mitochondria inheritance	No
Mmm1 Mdm12 Mdm34 Mdm10 Gem1 Lam6 Tom7	(Kornmann et al., 2009) (Yamano et al., 2010) (Kornmann et al., 2011) (Elbaz-Alon et al., 2015) (John Peter, Petrungaro, et al., 2022)	ERMES complex Tethering ER-OM Lipid transport	No No No MIRO1 (At5g27540), MIRO2 (At3g63150) MIRO3 (At3g05310) VAD1 (At1g02120), At3g59660, At1g03370 No
EMC1 EMC2 EMC3 EMC4 EMC5 EMC6 Tom5	(Lahiri et al., 2014)	Tethering ER-OM Transmembrane protein insertion Lipid transport	At5g11560 No At4g12590 No No At5g08040
Lam6 Tom70/71	(Murley et al., 2015)	Tethering ER-OM	VAD1 (At1g02120), At3g59660, At1g03370 No
Hob1/Fmp27	(Toulmay et al., 2022)	Lipid transport	SABRE (At1g58250), KIP (At5g49680)
Hob2	(Toulmay et al., 2022)	Lipid transport	SABRE (At1g58250), KIP (At5g49680)
Tweek/Csf1	(Toulmay et al., 2022)	Lipid ransport	No

Protein name	References	Function at EMCSs	Putative homologs in <i>A</i> . <i>thaliana</i>
Mic60	(von der Malsburg et al.,	Tethering OM-IM	AtMic60 (At4g39690)
Tom40	2011) (Bohnert et al., 2012)	Protein import	AtTOM40 (At3g20000)
Mic60	(Bohnert et al., 2012)	Tethering OM-IM	AtMic60 (At4g39690)
SAM50	(Körner et al., 2012)	Protein import	SAM50.1 (At5g11070), SAM50.2 (At5g05520)
Mic60 Ugo1	(Harner et al., 2011)	Tethering OM-IM	AtMic60 (At4g39690) No
Mic60 VDAC	(Hoppins et al., 2011)	Tethering OM-IM	AtMic60 (At4g39690) VDAC1 (At3g01380), VDAC2 (At5g67500), VDAC3 (At5g15090), VDAC4 (At5g57490)
Tom22 Tim21	(Chacinska et al., 2005)	Tethering OM-IM	Tom9.1 (At1g04070), Tom9.2 (At5g43970) Tim21 (At4g00026)
Ugo1 Mgm1	(Sesaki & Jensen, 2004)	Tethering OM-IM Mitochondrial fusion	No Yes (>10)
Ups1	(Y. Tamura et al., 2009) (Connerth et al., 2012)	PA transport OM-IM	At5g13070
Ups2	(Y. Tamura et al., 2009) (Aaltonen et al., 2016) (Miyata et al., 2016)	PS transport OM-IM	At5g13070
Mdm35	(Connerth et al., 2012) (Miyata et al., 2016)	Chaperone, Ups1/2 stabilization	At4g33100
Mdm31 VDAC1	(Miyata et al., 2017) (Miyata et al., 2018)	PA transport OM-IM	No VDAC1 (At3g01380), VDAC2 (At5g67500), VDAC3 (At5g15090), VDAC4 (At5g57490)

Table 2 continued

**Table 3** Proteins implied in tethering and/or lipid transport identified at ER-mitochondria and intramitochondrial contact sites in *Arabidopsis thaliana* known to date. For each protein or protein complex, the corresponding literature is cited, the known functions are indicated and the TAIR accession numbers are given. MELL1 has been discovered in *Physcomitrella patens* and its putative homologs in *Arabidopsis thaliana* are indicated in the right column.

Protein name	References	Function at EMCSs	TAIR accession
MELL1	(Mueller et al., 2014) (Mueller & Reski, 2015)	Tethering ER-OM	At5g44310, At4g21020, At1g72100
TIM17-2	(Murcha et al., 2005)	Tethering OM-IM	At2g37410
MIRO2	(White et al., 2020)	Tethering ER-OM	At3g63150
TRB1	(C, Li at al. 2022)	Tethering ER-OM	At1g05270
VAP27	(C. LI et al., 2022)	Mitophagy	At3g60600
AtMIC60 (Michaud et al., 2016)	(Michaud et al. 2016)	Tethering OM-IM	At4g39690
	Lipid transport	At3g20000	

#### 4.3.1 Tethering at ER-mitochondria contact sites

In yeast and mammals, EMCSs are considered to be among the most stable contact sites between organelles, which has greatly facilitated their study (Rowland & Voeltz, 2012; Subra et al., 2023). A multitude of ER-mitochondria tethers have already been described (**Table 1** & **Table 2**). As mentioned

above, it is often difficult to clearly identify the tethering function of a contact site protein as the overall MCS tethering force might result from a combination of multiple tethers and as a tethering protein might also perform other functions at the MCS (Eisenberg-Bord et al., 2016).

In mammals, four protein complexes have been classically described to mediate EMCS tethering (Prinz, 2014): the IP3R-GRP75-VDAC-DJ-1 complex (Y. Liu et al., 2019; Szabadkai et al., 2006), the BAP31-FIS1 complex (Iwasawa et al., 2011), the MFN1/2-MFN2 complex (de Brito & Scorrano, 2008) and the VAPB-PTPIP51 complex (De Vos et al., 2012). Most proteins present in these complexes are known to perform other functions than tethering. The IP3R-GRP75-VDAC-DJ-1 complex is known to facilitate channeling of  $Ca^{2+}$  into mitochondria (Szabadkai et al., 2006). FIS1 and MFN1/2 both function in mitochondrial fission and fusion (van der Bliek et al., 2013) while PTPIP51 was shown to be a lipid transporter (Yeo et al., 2021). Since then, numerous additional proteins spanning through EMCSs have been discovered (Figure 4, Table 1). While a proper role as tethers has not been confirmed for all of these new EMCS effectors, it is probable that they all contribute to tethering to some extent since they are in contact with both the ER membrane and the mitochondrial OM either directly, or as part of a complex. PDZD8 and MIGA2 have both been described to function as tethers at EMCSs (Freyre et al., 2019; Hirabayashi et al., 2017), while neither ORP5/8 nor E-Syt1 seem to substantially contribute to tethering (Monteiro-Cardoso et al., 2022; Sassano et al., 2023). New protein-protein interactions have also been discovered. BAP31 was found to physically interact with TOM40 (Namba, 2019) and ORP5/8 interact with SAM50 and MIC60 (Monteiro-Cardoso et al., 2022). In yeast, only two EMCS tethers are classically described (Prinz, 2014): Mmr1 (Swayne et al., 2011) and the ERMES (ER-mitochondria encounter structure) complex (Kornmann et al., 2009). Like in mammals, both tethers accomplish other cellular functions, namely mitochondria inheritance (Itoh et al., 2004) and lipid transport (John Peter, Petrungaro, et al., 2022), respectively. Interestingly, all the endoplasmic reticulum membrane protein complex (EMC) subunits interact with Tom5 and form an efficient EMCS tethering complex (Lahiri et al., 2014). Lam6, a MCS regulator and sterol transporter present at EMCSs and mitochondria-vacuole contact sites in yeast, interacts with Tom70/71 (Murley et al., 2015).

Surprisingly, few tethering proteins are conserved in *A. thaliana* (**Table 1, Table 2 & Table 3**). A high number of VAPs are present in the *A. thaliana* genome, suggesting that VAP-FFAT motif interactions are conserved in plants. Accordingly, the VAP27-TRB1 EMCS tether has been identified (C. Li et al., 2022). TRB1 belongs to the TraB protein family implied in mitochondrial movement. Its association with VAP27 is important during mitophagy. Lam6 has four homologs in plants (Leterme & Michaud, 2022). Except for VAD1, the localization of *A. thaliana* plant homologs is unknown and none has been found to play a role at EMCSs (see Leterme & Michaud, 2022, section 2.1.4).



Figure 4 Tethers and lipid transport proteins present at ER-mitochondria and intramitochondrial membrane contact sites in mammals and yeast.



Figure 5 Tethers and lipid transport proteins present at ER-mitochondria and intramitochondrial membrane contact sites in *Arabidopsis thaliana*.

MELL1 has been identified as a plant specific EMCS tether in the moss *Physcomitrella patens* (Mueller et al., 2014; Mueller & Reski, 2015) and uncharacterized homologs are also present in *A. thaliana* (Michaud et al., 2017). The effect of MIRO2 in *A. thaliana* ER-mitochondria tethering has also only been observed in plants (White et al., 2020). However, it is not clear how and with what partners MIRO2 contacts the ER.

#### 4.3.2 Tethering at intramitochondrial contact sites

Most intramitochondrial tethers were discovered through co-immunoprecipitation. Since the mitochondrial membranes are densely packed with proteins and protein complexes, the risk of false positives is high. Nevertheless, several interactions could be determined.

Interestingly, the interaction between MIC60 and TOM complexes seems to be conserved in mammals, yeast and plants (**Figure 4 & Figure 5**) (Bohnert et al., 2012; Michaud et al., 2016; von der Malsburg et al., 2011). Similarly, the MIC60-SAM50 interaction is found in mammals and yeast (Bohnert et al., 2012; Körner et al., 2012). In yeast, Mic60 additionally interacts with Ugo1 and VDAC (Harner et al., 2011; Hoppins et al., 2011). Thus, the MICOS (mitochondrial contact site and cristae organizing system) complex plays a central role in intramitochondrial contact site formation and can be regarded as a mitochondrial IM interaction hub (Mukherjee et al., 2021). The MICOS complex is known to be important for mitochondrial membrane architecture, protein import and lipid homeostasis and trafficking (Rampelt et al., 2017), which is consistent with its broad interactome. In plants, only the AtMIC60-AtTOM40 interaction has been shown (Michaud et al., 2016). However, it is likely that other AtMIC60 interactors will be discovered in the future since the mitochondrial complexes are well conserved throughout evolution.

In mammals, VDAC in the OM and ANT (adenylate translocator) in the IM are also tethering both mitochondrial membranes (Beutner et al., 1996, 1998). Multiple homologs of VDAC and ANT are present in *A. thaliana*, suggesting that a similar interaction could take place in plants (**Table 1**). In yeast, beside the Ugo1-Mgm1 complex important for OM-IM coupling during mitochondrial fusion (Sesaki & Jensen, 2004), another interaction implying VDAC and the IM protein Mdm31 was discovered (Miyata et al., 2018). However, neither Ugo1 nor Mdm31 have a sequence homolog in *A. thaliana* (**Table 2**). This does not rule out the possibility that structural homologs or functional analogs exist in plants. Interestingly, the IM-located TIM17-2 protein has been found to mediate OM-IM tethering in *A. thaliana* as its C-terminus remains sensitive to proteases applied on intact mitochondria (Murcha et al., 2005). How TIM17-2 is inserted in both the OM and IM and the role of such a peculiar topology remains to be clarified.

#### 4.3.3 Phosphatidylserine transport to the mitochondrial inner and outer membranes

Mitochondria do not possess the enzymatic machinery to neosynthetize PS and therefore must at least import the PS present in the mitochondrial membranes. PS is also imported into mitochondria and channeled to the IM-localized PS decarboxylase to generate PE. Human, yeast and *A. thaliana* cells all have different needs for PS import mostly depending on their ability to synthetize PE through the mitochondrial PS decarboxylase pathway and the CDP-Etn pathway (see section 4.2). This section focuses on the known components of the PS import machinery into mitochondria.

In mammals, three potential PS transporters have been identified at EMCSs: ORP5, ORP8 and MIGA2. ORP5/8 function as a duo and have initially been identified as a PS/PI counter-exchanger at ER-plasma membrane contact sites (see Leterme & Michaud, 2022, section 2.2). Recently, ORP5/8 have been found to be EMCS residents in physiological conditions (Monteiro-Cardoso et al., 2022). Interestingly, the authors found that ORP5/8 knock down lines had decreased PE amounts while not being needed for ERmitochondria tethering, suggesting that they are effectively transporting PS from the ER to mitochondria. ORP5/8 were found to interact with members of the MIB (mitochondrial intermembrane space bridging) complex and ORP5/8 or MIC60 knock down lines presented a similar PE decrease (Monteiro-Cardoso et al., 2022). This led the authors to hypothesize that ORP5/8 and the MIB/MICOS complexes function in tandem to channel PS directly to the mitochondrial IM. MIGA2 is inserted in the mitochondrial OM by a transmembrane helix, interacts with ER-localized VAPs and contacts lipid droplets with a hydrophobic helix in human adipocytes (Freyre et al., 2019). MIGA2 depleted adipocytes had reduced TAG production, suggesting a function in lipid metabolism (Freyre et al., 2019). Furthermore, MIGA2 preferentially binds and transports PS between liposomes (Kim et al., 2022). Therefore, it has been proposed that MIGA2 transports PS at EMCSs. It is interesting to note that the MIGA2 lipid transport domain folds as a coiled coil forming a hydrophobic pocket, thus forming an atypical lipid transporting fold (Kim et al., 2022). The exact mechanism of transport, however, remains to be determined. MFN2, which is part of the MFN1/2-MFN2 tethering complex, was also found to
promote PS transport from the ER to mitochondria since MFN2-depleted mice had reduced PE amounts in liver cell mitochondria (Hernández-Alvarez et al., 2019). Furthermore, MFN2 is able to bind, extract and concentrate PS in membrane subdomains in vitro (Hernández-Alvarez et al., 2019). Whether MFN2 has a direct or indirect lipid transport function remains to be clarified. In yeast, the ERMES complex has been proposed to transport PS between ER and mitochondria. The ERMES complex is anchored in the ER membrane by Mmm1 and in the mitochondrial membrane by Mdm10. Mmm1, Mdm12 and Mdm34 harbor a SMP lipid transport domain, presumably assembling to form a hydrophobic cleft allowing the passage of lipids. Several structural studies proposed different ERMES subunit configurations (Jeong et al., 2016, 2017; Kawano et al., 2018), but only recently a native cryo-EM subtomogram averaging study concluded that ERMES most likely forms a continuous conduit from the ER to mitochondria (Wozny et al., 2022). ERMES was found to substantially contribute to lipid transport to mitochondria (John Peter, Petrungaro, et al., 2022). The same in vivo study reveals that ERMES has no obvious glycerolipid class transport specificity, but an *in vitro* assay using purified yeast membranes showed that ERMES is required for PS transport to mitochondria (Kojima et al., 2016). Beside the ERMES complex, the EMC has been shown to impact lipid transport to mitochondria (Lahiri et al., 2014). The EMC seems to have three different functions: 1) protein insertion into the ER, 2) ERmitochondria tethering by interaction with Tom5 and 3) lipid transport (Chitwood & Hegde, 2019; Lahiri et al., 2014). A set of *in vitro* and *in vivo* studies showed that the EMC likely plays an important role in PS transport to mitochondria (Lahiri et al., 2014). However, the absence of lipid transport domain in the EMC subunits and its supposed primary role in transmembrane protein insertion raises questions on the direct implication of the EMC in lipid trafficking. In fact, the EMC may merely act on lipid transport by promoting the insertion of lipid transporters in the ER membrane or of EMCS regulation proteins or by regulating the proximity between ER and mitochondria. However, expression of an artificial ER-mitochondria tether restored the PS transfer defect in EMC defective cells, suggesting that the EMC tethering function is also important (Lahiri et al., 2014).

Interestingly, neither MIGA2 nor ERMES have sequence homologs in *A. thaliana* (**Table 1**). Many ORPs and SMP-domain proteins are present in *A. thaliana* (Leterme & Michaud, 2022), but none has been characterized nor localized at EMCSs. Of the six EMC subunits present in yeast, only two are conserved in plants. Since all the EMC subunits were found to interact with Tom5 (Lahiri et al., 2014), a similar tethering and lipid transport function might exist in plants as well, which remains to be demonstrated. Since *A. thaliana* produces the vast majority of its PE by the CDP-Etn pathway in the ER (Nerlich et al., 2007), it is possible that PS transporters at EMCSs are scarce. Nevertheless, plant mitochondria do contain PS, which has to be transported.

Intramitochondrial PS transport seems conserved in mammals and yeast. SLMO2/PRELID3b in humans and Ups2 in yeast are both LTPs from the Ups/PRELI family known to transport PS from the OM to the IM (see Leterme & Michaud, 2022, section 2.1.5). SLMO2/PRELID3b and Ups2 associate to the

chaperone TRIAP1 in humans and Mdm35 in yeast. *A. thaliana* possesses only one Ups/PRELI and one TRIAP1/Mdm35 homolog, suggesting a broader lipid class specificity for lipid transport (**Table 1** & **Table 2**).

#### 4.3.4 Phosphatidic acid transport to the mitochondrial inner and outer membranes

While it is commonly accepted that mitochondria are able to neosynthetize PA, DPG synthesis requires additional amounts of PA that has to be imported from the ER.

In mammals, the only characterized PA transporter at EMCSs is PTPIP51. PTPIP51 uses its tetratricopeptide repeats as an atypical lipid binding domain and was shown to bind and transport PA *in vitro* (Yeo et al., 2021). Furthermore, PTPIP51 depleted cells presented reduced DPG amounts in mitochondria, suggesting a PA supply role for PTPIP51 (Yeo et al., 2021). No specific PA transporter has been characterized in yeast. It is probable that PA is supplied by other non-specific glycerolipid transporters or by not yet characterized LTPs at EMCSs or other MCSs. *A. thaliana* is lacking a PTPIP51 homolog and to date, no other EMCS-residing plant specific PA transporter has been identified (**Table 1**).

As for intramitochondrial PS transport, members of the Ups/PRELI family function in PA transport in mitochondria. In mammals, SLMO1/PRELID3a and PRELID1 combined to the chaperone TRIAP1 mediate PA transport from the OM to the IM (Miliara et al., 2015; Potting et al., 2013). In yeast, Ups1 combined to the chaperone Mdm35 performs the same function (Connerth et al., 2012; Y. Tamura et al., 2009). As mentioned above, only one Ups/PRELI homolog is found in *A. thaliana* and putatively performs both PA and PS transport. An additional putative PA transporter has been identified in the yeast mitochondrial IM. The Chorein\_N domain-containing protein Mdm31 has been shown to interact with VDAC and has been proposed to mediate Ups-independent PA transport (Levine, 2019; Miyata et al., 2017, 2018). In fact, yeast cells depleted in both Ups1 and Ups2 were still capable of synthetizing DPG and even showed an increase in DPG content (Miyata et al., 2017). This shows that an alternative PA import route exists in mitochondria, which is likely mediated by Mdm31 (Miyata et al., 2017, 2018). However, whether the observed PA transport is due to other transport routes in absence of Ups1 and Ups2 remains to be clarified. No Mdm31 sequence homolog has been detected in *A. thaliana* (**Table 2**).

4.3.5 EMCS lipid transport proteins with general or unknown glycerolipid specificity Beside PS and PA, mitochondria have to import a wide variety of glycerolipid species to sustain their membrane biogenesis. Several LTPs of general or unknown specificity have been discovered and are susceptible to fulfill this crucial import function.

In mammals, PDZD8, E-Syt1 and HsVPS13A/D have been reported to function as LTPs at EMCSs (**Figure 4**). PDZD8 is an SMP-containing protein initially discovered as an EMCS tether (Hirabayashi et al., 2017), but was later shown to be present at other contact sites (Elbaz-Alon et al., 2020; Shirane et

al., 2020). The suspected lipid transport activity of PDZD8 was confirmed by in vitro lipid extraction experiments (Y. Gao et al., 2022; Shirane et al., 2020). An in vivo study in C. elegans suggests that PDZD8 contributes to PI(4,5)P<sub>2</sub> transport from the ER to endosomes (Jeyasimman et al., 2021). To date, no study showed PDZD8-mediated lipid transport to mitochondria in vivo. The currently available in vitro data rather suggest a broad glycerolipid transport specificity for the PDZD8 SMP domain. However, further investigation is required to clarify the nature of the transported lipids and whether PDZD8 affects the mitochondrial lipidome. E-Syt1 is a well-known ER-plasma membrane resident harboring a SMP lipid transport domain (see Leterme & Michaud, 2022, section 2.1.1). E-Syt1 has recently been identified at EMCSs where it interacts with PERK and mediates phospholipid transport from the ER to mitochondria (Sassano et al., 2023). Cells lacking E-Syt1 presented a delay in NBD-PS transport to mitochondria and their mitochondria had reduced amounts of the major mitochondrial phospholipids, suggesting an important role in ER to mitochondria lipid transport (Sassano et al., 2023). HsVPS13A and HsVPS13B are localized at EMCSs and are known to perform bulk lipid transport (Guillén-Samander et al., 2021, 2022; Kumar et al., 2018; Muñoz-Braceras et al., 2015; Yeshaw et al., 2019). VPS13 proteins will be extensively described in **part 2** of this manuscript. Three proteins of the VPS13-like/repeating  $\beta$ -groove family are also present at EMCSs in yeast: Hob1, Hob2 and Csf1 (Toulmay et al., 2022). They also seem to play a role in lipid transport at various MCSs (see part 2 of this manuscript).

While no PDZD8 homolog is found in plants (**Table 1**), *A. thaliana* has multiple copies of E-Syt and VPS13 proteins. The *A. thaliana* genome encodes at least seven E-Syts mostly located at ER-plasma membrane contact sites (see Leterme & Michaud, 2022, section 2.1.1). No E-Syt protein has been found at EMCSs in plants yet. However, several E-Syts have been located at ER-PM contact sites in *A. thaliana*. Thus, it is possible that a fraction of those proteins can also be recruited to EMCSs as it has been shown recently for E-Syt1 in human cells (Sassano et al., 2023). Four VPS13 proteins are found in *A. thaliana*, which will be detailed in **part 2** of this manuscript. Similarly, Hob1 and Hob2 are conserved in plants (SABRE and KIP) while no Csf1 homolog has been detected.

Lipid transport of other lipids than PS and PA at intramitochondrial contact sites has not been well studied. In mammals, StARD7 is known to transport PC from the OM to the IM (Horibata et al., 2016; Saita et al., 2018). Five StARD7 *A. thaliana* homologs have been found but none of them is predicted to localize to mitochondria. NDPK-D/Nm23-H4 is a human OM-IM contact site resident that transports DPG from the IM to the OM (Epand et al., 2007; Schlattner et al., 2013). *A. thaliana* has two NDPK homologs, NDPK3a/b, which may be localized in mitochondria (see Leterme & Michaud, 2022, section 3.1). Finally, in *A. thaliana*, the MTL complex is a unique lipoprotein complex that promotes PE export and DGDG import to *A. thaliana* mitochondria (see Leterme & Michaud, 2022, section 4.2; Michaud et al., 2016).

#### 4.3.6 Concluding remarks

When observing the known tethers and lipid transport proteins and complexes at EMCSs in mammals, yeast and plants, the absence of conservation is striking. While the general lipid transport and tethering mechanisms are conserved, the protein effectors are highly divergent. The reasons for this are probably multiple. As stated above, different organisms (and cell types) have different lipid transport requirements that vary in quantity and in nature and are dependent on the mitochondrial lipid synthesis capacity. Furthermore, mitochondrial contact sites perform various functions in the cell that may vary from species to species. For example, Ca<sup>2+</sup> exchange is an important function of EMCSs in mammals but not in yeast. In addition, the tethering and lipid transport functions may be more important than the structure of the EMCS effectors, favoring evolutionary convergence based on protein function rather than sequence or tertiary structure. A striking example are the SMP domain-containing proteins in yeast and mammals. The ERMES complex has no mammalian homologs which nevertheless harbor other SMP domain proteins like PDZD8 or E-SYT1. Finally, multiple redundant pathways exist for lipid transport to mitochondria and may not be the same in different organism. In yeast, vacuole-mitochondria contact sites will be shortly addressed in **part 2** of this manuscript.

The small number of EMCS tethering and lipid transport effectors identified and their apparent low conservation in plants highlights the gap of knowledge that persists in the field. Much remains to be discovered and investigated from the plant perspective. This work is aiming to contribute to uncover new actors and mechanisms of non-vesicular lipid transport to mitochondria.

### 5 Phosphate starvation-induced membrane lipid remodeling in *Arabidopsis thaliana*

Plants are sedentary organisms that have to cope with biotic and abiotic stresses by exerting molecular, cellular and systemic responses using locally available resources. Phosphate (Pi) starvation is a nutritional scarcity commonly encountered by plants in nature. Pi is an elementary building block found in a huge variety of vital molecules such as nucleic acids, adenosine triphosphate (ATP), lipids and many more metabolites (Lambers, 2022). To overcome Pi shortage, plants trigger mechanisms that facilitate Pi uptake from the environment like changing root architecture, developing longer root hairs or excreting organic acids and phosphatases (Dissanayaka et al., 2021; Plaxton & Tran, 2011). Plants also experience important gene regulation and metabolic changes to remobilize the Pi present in intracellular reserves (Dissanayaka et al., 2021; Plaxton & Tran, 2011; P. Wu et al., 2003). Membrane phospholipids retain approximately one third of the intracellular Pi, constituting an enormous Pi reserve (Poirier et al., 1991) and thus make them a valuable source of intracellular Pi.

Upon Pi starvation, plants degrade up to 30% of the phospholipids of various cell compartments to recycle the Pi contained in their headgroup (Jouhet et al., 2004). To date, three major phospholipid degradation pathways have been described and are reviewed in detail elsewhere (Y. Nakamura, 2013; Zhu et al., 2022) (Figure 6). In the phospholipase C (PLC) pathway, phospholipids are degraded to DAG by specific PLCs like PI-PLCs that remove the phosphoinositol moiety from phosphoinositides (Mueller-Roeber & Pical, 2002) or by non-specific PLCs (NPCs) that are active on a broader phospholipid spectrum and degrade PC, PE and PS. While PI-PLCs are important for plant stress signaling (Sagar & Singh, 2021), their impact on phospholipid degradation upon Pi starvation is negligible. In contrast, NPCs account for the majority of the Pi starvation-induced PLC activity (Y. Nakamura, 2013). In Arabidopsis, NPC4 expression is strongly induced upon Pi starvation and is the most active PLC as the npc4 KO mutants only retain basal PLC activity (Y. Nakamura et al., 2005). NPC5, a close paralog of NPC4, is also induced during Pi starvation but exhibits a 40-fold smaller PLC activity than NPC4 in vitro (Gaude et al., 2008). Interestingly, NPC4 and NPC5 differ in localization as NPC4 is localized at the plasma membrane (Y. Nakamura et al., 2005) while NPC5 is soluble and is presumably recruited to membranes that require DAG for downstream metabolic purposes (Gaude et al., 2008). In the phospholipase D (PLD) pathway, phospholipids are degraded to PA, which can be used as signaling molecule or be further degraded to DAG by phosphatidic acid phosphatases (PAPs) (Y. Nakamura, 2013). Among the 12 PLDs known in Arabidopsis, only PLDζ1 and PLDζ2 are upregulated upon Pi starvation (Cruz-Ramírez et al., 2006; M. Li, Qin, et al., 2006). Upon Pi starvation, pldζ2 KO mutants exhibit lower PC and PE degradation rates and accumulate less DGDG, showing its importance in Pi starvation-membrane remodeling (Cruz-Ramírez et al., 2006). While PLDζ1 localizes to the trans-Golgi network, PLDζ2 has been reported to localize to the vacuole membrane, multivesicular bodies and the trans-Golgi network (Shimamura et al., 2022; Yamaryo et al., 2008). The third and last known phospholipid degradation pathway includes a lipid acyl hydrolase (LAH) that removes the fatty acyl chains from the glycerol backbone to form glycerophosphodiester (GDP) (Y. Nakamura, 2013; Zhu et al., 2022). GDP can then be further degraded to glycerol-3-phosphate (G3P) by a glycerophosphodiester phosphodiesterase (GDPD). While no Pi is directly released by this process, the generated G3P can be used for further anabolism.

The degradation of extraplastidial phospholipids during Pi starvation could lead to the destabilization of intracellular membranes. To maintain membrane integrity, a huge export of DGDG takes place from the chloroplast to the tonoplast, mitochondria and plasma membrane and dynamically replaces the degraded phospholipids (Andersson et al., 2005; Hartel et al., 2001; Jouhet et al., 2003; Moellering & Benning, 2011; Tjellström et al., 2008) (**Figure 6**). Similarly, in chloroplasts, the degraded PG is replaced by the glycolipid sulfoquinovosyldiacylglycerol (SQDG) (Essigmann et al., 1998; Yu et al., 2002). In Pi starved calli, the DGDG content increases by a factor four compared to Pi replete conditions and attains up to 30% of the total lipid content (Jouhet et al., 2004). Whether these lipids are transported by vesicular

or non-vesicular routes is unknown. However, since neither chloroplasts nor mitochondria are connected to the endomembrane system, it is reasonable to hypothesize that DGDG uses non-vesicular lipid transport routes at least to exit the chloroplast and to enter mitochondria. DGDG transport between the vacuole and the plasma membrane during Pi limitation, however, could take place by both vesicular



Figure 6 Phospholipid degradation and recycling into galactolipids in plant cells upon phosphate (Pi) starvation. To allow Pi recycling during Pi starvation, phospholipids can be degraded by three different pathways, two of which are represented. In the phospholipase C pathway, phospholipids are degraded to DAG. NPC4 operates at the plasma membrane while NPC5 cytoplasmic and likely operates at different degradation sites. PI-PLC converts phosphoinositides to DAG. In the phospholipase D pathway, phospholipids are first converted to PA and can be further converted to DAG by PAPs. PLDZ2 operates at the vacuole membrane in close proximity of mitochondria and chloroplasts. PLD(1 (not represented) operates at the Golgi membrane. The DAG and/or PA generated during Pi starvation are imported in the chloroplast or on the chloroplast surface by unknown pathways (dotted arrows and question marks). These precursors are used for *de novo* galactolipid (MGDG and DGDG) and sulfolipid (SQDG, not represented) synthesis by plastidial lipid synthesis enzymes. Note that MGD2/3 and DGD1/2 might also function in trans. DGDG is exported to extrachloroplastic membranes to compensate for the loss of phospholipids. NPC4/5: non-specific phospholipase C 4/5, PI-PLC: phosphoinositides-specific phospholipase C, PLD(2; phospholipase D (2, PAP: phosphatidic acid phosphatase, MGD1/2/3: monogalactosyl diacylglycerol synthase 1/2/3, DGD1/2: digalactosyl diacylglycerol synthase 1/2, TGD: trigalactosyldiacylglycerol complex, MTL: mitochondrial transmembrane lipoprotein complex, PC: phosphatidylcholine, PE: phosphatidylethanolamine, DAG: diacylglycerol, PA: phosphatidic acid, MGDG: monogalactosyl diacylglycerol, DGDG: digalactosyl diacylglycerol.

and/or non-vesicular pathways. Interestingly, the number of contact sites between chloroplasts and mitochondria significantly increases during phosphate starvation (Jouhet et al., 2004), suggesting that DGDG could be directly channeled from chloroplasts to mitochondria. The mitochondrial transmembrane lipoprotein (MTL) complex spans through both mitochondrial membranes and its key component, AtMIC60, is implied in DGDG import and PE export to mitochondria upon Pi starvation (Michaud et al., 2016). The MTL complex might also contact other organelles, among which the chloroplast, thus providing a direct transport route between the chloroplast and mitochondria.

Concomitantly with the huge DGDG export from the chloroplasts, the MGDG and DGDG synthesis genes MGD2, MGD3 and DGD2 are upregulated upon Pi starvation (Awai et al., 2001; Kelly & Dörmann, 2002; Kobayashi et al., 2009). De novo galactolipid biogenesis necessitates DAG molecules to condensate with UDP-galactose (Li-Beisson et al., 2013). Since Pi starvation-induced phospholipid degradation generates high amounts of DAG, it is tempting to hypothesize that the degradation-linked DAG pool could be re-channeled to the chloroplast to promote galactolipid synthesis (Figure 6). The fatty acid composition of DGDG upon Pi starvation suggested that it was derived from DAG generated by the degradation of PC, thus supporting the re-channeling hypothesis (Jouhet et al., 2003; Michaud & Jouhet, 2019). Furthermore, deletion of NPC5, PLD $\zeta$ 2 as well as the concomitant deletion of PLD $\zeta$ 1/2 decreased DGDG accumulation upon Pi starvation, further suggesting that phospholipid degradation is directly linked to galactolipid synthesis (Cruz-Ramírez et al., 2006; Gaude et al., 2008; M. Li, Welti, et al., 2006). How the excess DAG molecules are transported to the chloroplast and how the sites of phospholipid degradation are determined and regulated is unknown. Interestingly, PLD<sup>2</sup> has been observed at sites of close proximity between the tonoplast and mitochondria and between the tonoplast and chloroplasts, suggesting that PA can be generated in close proximity of these organelles (Yamaryo et al., 2008). Similarly, cytosolic NPC5 might be directed to specific sites to degrade phospholipids close to chloroplasts or lipid trafficking hubs (Gaude et al., 2008). The trigalactosyldiacylglycerol (TGD) complex is supposed to drive unidirectional transport of lipids from the ER to chloroplasts and could therefore provide a convenient galactolipid precursor import route (Leterme & Michaud, 2022; C. Xu et al., 2010). However, disruption of the TGD complex did not affect lipid remodeling upon Pi starvation (C. Xu et al., 2010). Interestingly, the pore-forming protein LPTD1, a close homolog of TGD4, is highly expressed upon Pi starvation and its deletion alters Pi starvation-induced lipid remodeling (Hsueh et al., 2017). It is not known whether LPTD1 is part of the TGD complex, but it is tempting to speculate that it could promote import of galactolipid precursors into the chloroplast in response to Pi starvation by associating with the canonical TGD import machinery (Hsueh et al., 2017; Michaud & Jouhet, 2019). It is however important to mention that the galactolipid precursors might not need to be transported to the plastid inner envelope to undergo processing. A. thaliana possesses three MGDs: MGD1, 2 and 3. MGD1 is localized at the plastid inner envelope and is responsible for bulk MGDG synthesis (Awai et al., 2001; Miège et al., 1999). MGD2 and 3 are localized in the outer leaflet of the plastid outer envelope and are thought to mediate MGDG synthesis upon phosphate starvation (**Figure 6**) (Awai et al., 2001; Kobayashi et al., 2009). This suggests that the transport of galactolipid precursors to the outer envelope could be sufficient. This hypothesis is supported by the fact that plastids increase their contacts with other organelles in Pi limited conditions. Furthermore, it cannot be excluded that MGD2/3 and DGD1/2 could perform their galactosyltransferase activity *in trans*, thus drastically reducing the need for extensive lipid transport to plastids.

The amount of lipids being transported between compartments during Pi starvation-induced lipid remodeling is enormous and most likely requires intense non-vesicular lipid transport mediated by LTPs. Since DGDG is exclusively synthetized in the chloroplast and since extra-plastidial compartments contain very low galactolipid amounts in Pi replete conditions, the artificial induction of Pi starvation in *Arabidopsis thaliana* provides a convenient method to study the effect of LTPs in lipid movements between compartments, in particular, the transport of lipids between chloroplasts and mitochondria and the degradation of phospholipids. In this study, induction of Pi starvation is used to characterize the effect of different LTP KO mutants by comparing the lipidomes of Pi starved plants to Pi replete plants. This work is divided in two parts: 1) the structural characterization of the MTL complex and 2) the characterization of a VPS13 homolog (AtVPS13M1) in the context of Pi starvation in *A. thaliana*.

## **Materials and methods**

#### 1 Biological material and culture conditions

#### 1.1 Arabidopsis thaliana

*Arabidopsis thaliana* (*A. thaliana*), also called mouse-ear cress or thale cress, is a small angiosperm belonging to the *Brassicaceae* family. All the *A. thaliana* plants used for experiments or for transformation in this study are from the Columbia-0 (Col0) ecotype. Four T-DNA insertion mutants were used (**Table 4**) (Alonso et al., 2003).

**Table 4** Review of the T-DNA insertion lines identified as AtVPS13M1 KO lines. The SALK code is indicated in the left column. The T-DNA experimentally determined insertion site is indicated in the right column for each SALK line.

SALK line	Given name	gDNA insertion site
SALK_128148	atvps13m1.1	Exon 7; between nt 2317-2318
SALK_019531	atvps13m1.2	Intron 14; between nt 4199-4200
SALK_021158	atvps13m1.3	Exon 17; between nt 4985-4990
SALK_096315	atvps13m1.4	Exon 25; between nt 7761-7768

#### 1.1.1 Arabidopsis thaliana plants

#### 1.1.1.1 Seed sterilization, stratification and vernalization

To avoid unwanted pests from spreading in the phytotrons, the seeds are subjected to a  $-80^{\circ}$ C temperature for a minimum of 2h and then sterilized using an aqueous solution of 0.4% (v/v) bleach and 0.01% (v/v) Triton X100. Under a sterile laminar flow hood, the seeds to be sown are immerged in 1 mL of sterilization solution and spun for 15 min on a rotative wheel. After seed sedimentation, the supernatant is replaced by fresh sterilization solution and spun again for 15 min. The seeds are washed four times in 1 mL of sterile water and put at 4°C for a minimum of 48 h to allow seed vernalization. After 48 h, the seeds were sown on soil or on an agar-containing medium.

#### 1.1.1.2 Plants grown on soil

Plants were grown in long day (16h-8h light-dark photoperiod) or short day (8h-16h light-dark photoperiod) conditions in phytotrons maintaining the temperature at 22°C, the hygrometry at 60-70% and a photon flux density of 100  $\mu$ mol/m<sup>2</sup>/s. Plants were grown on soil containing 35 L of soil, 7 L vermiculite and 10 g of biological fungicide (Prestop, Lallemand). Plants were watered 3 times a week.

#### 1.1.2 Arabidopsis thaliana calli

#### 1.1.2.1 Callus generation from Arabidopsis thaliana seedlings

*Arabidopsis* seeds are sown and grown on sterile Murashige & Skoog (MS)-agar medium (**Table 5**) at 22C° under long day conditions. After two weeks of growth, the cotyledons of the seedlings are cut in four pieces using a scalpel and placed on solid adapted Murashige & Skoog (MS) medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) (**Table 5**) that induces plant cell dedifferentiation and maintains cells undifferentiated. After approximately 3 weeks of growth in continuous light at 22°C, the

dedifferentiated cells form calli that can be transferred on new solid MS medium. Calli are then passed to new plates once per month.

**Table 5** Murashige & Skoog solid media for *A. thaliana* seedlings and callus growth. In both cases, pH was adjusted to 5.7 with KOH. N/A: not applicable.

Component	Pant seedling growth	Callus growth
MS with vitamins	4.41 g/L	4.41 g/L
Sucrose	10 g/L	30 g/L
MES	0.5 g/L	N/A
2,4-D	N/A	1.2 g/L
Agar	0.8% (w/v)	0.8% (w/v)

#### 1.1.2.2 Callus growth in liquid Murashige & Skoog media

Murashige & Skoog liquid callus growth medium was prepared with or without phosphate as described in **Table 6**. Gamborg's Vitamin Mixture was added (GBL01, Caisson Labs). This medium contains 2,4-D to maintain the cells undifferentiated. pH was adjusted to 5.7 with NaOH. Calli were grown in round flasks containing 200 mL of respective MS medium under 125 rpm agitation and constant illumination. Cells were passed to new medium once a week.

Table 6 Murashige & Skoog liquid medium composition for callus growth. pH was adjusted to 5.7 using NaOH.

Component	Concentration in +Pi medium	Concentration in -Pi medium
MS –Pi (MSP11, Caisson Labs)	4.41 g/L	4.41 g/L
Sucrose	15 g/L	15 g/L
Vitamins	1000X dilution	1000X dilution
2,4-D	1.2 mg/L	1.2 mg/L
Phosphate	0.5 mmol	0 mmol

#### 1.2 Nicotiana benthamiana plants

*Nicotiana benthamiana* is an angiosperm from the *Solanaceae* family. All the plants used in this study are wild type *Nicotiana benthamiana* and were cultured in the same conditions as *Arabidopsis thaliana* plants.

#### 1.3 Escherichia coli

*Escherichia coli* (*E. coli*) is a Gram-negative enterobacterium naturally occurring in the human gut. For standard plasmid transformations, the Escherichia coli DH5 $\alpha$  strain (60602 Lucigen) with the genotype fhuA2 $\Delta$ (argF-lacZ)U169 phoA glnV44  $\Phi$ 80  $\Delta$ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 was used. To increase transformation efficiency for big plasmids, the NEB 5 $\alpha$  strain with the genotype fhuA2 $\Delta$ (argF-lacZ)U169 phoA glnV44  $\Phi$ 80  $\Delta$ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 was used. NEB 5 $\alpha$  strain with the genotype fhuA2 $\Delta$ (argF-lacZ)U169 phoA glnV44  $\Phi$ 80  $\Delta$ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 was used. NEB 5 $\alpha$  bacteria are derived from the DH5 $\alpha$  strain but have an increased transformation efficiency.

*E. coli* bacteria were cultured on solid or in liquid medium containing the appropriate antibiotics concentration (**Table 7**). Solid culture medium is composed of 2% agar dissolved in lysogeny broth

(LB) medium poured in dishes. Bacteria streaked or spread on solid LB medium were grown at 37°C for at least 8h. Liquid cultures are realized in liquid LB at 37°C under constant shaking at 180 rpm for at least 8h.

**Table 7** Standard antibiotics used for bacterial selection purposes. Final concentrations used in the growth medium are indicated. Antibiotics were prepared at a 1000x concentration and diluted directly in the medium before use.

Antibiotic	Concentration in LB medium	
Spectinomycin	50 µg/mL	
Streptomycin	50 µg/mL	
Kanamycin	50 µg/mL	
Carbenicillin	$100 \mu g/mL$	
Rifampicin	50 µg/mL	
Gentamycin	30 µg/mL	
Chloramphenicol	30 µg/mL	

#### 1.4 Agrobacterium tumefaciens

Agrobacterium tumefaciens (A. tumefaciens) is a phytopathologic Gram-negative bacterium. For plasmid transformation, transient expression in *Nicotiana benthamiana* and floral dips, we used the C58C1 strain with the genotype strR RifR pRiA4b (agropine type). *A. tumefaciens* are cultured on the same media as *E. coli* but at 28°C for 24h for liquid cultures and for 72h for growth on solid medium. *A. tumefaciens* liquid cultures grown for subsequent agroinfiltration (section 2.5) or floral dip (section 2.8) also contain acetosyringone (20  $\mu$ M). Acetosyringone is a phenolic compound produced by wounded plants that has been shown to enhance the production of virulence factors in *A. tumefaciens* (Engström et al., 1987) and to dramatically increase plant transformation rate (Sheikholeslam & Weeks, 1987).

#### 1.5 Saccharomyces cerevisiae

The yeast *Saccharomyces cerevisiae* strain BY4741 with the genotype MATa his $3\Delta 1 \ leu 2\Delta 0 \ met 15\Delta 0$ ura $3\Delta 0$  was used for protein expression. This strain is deficient for *de novo* synthesis of the essential nucleobase uracil. The uracil auxotrophy was used for selection purposes.

Yeast strains were cultured in solid or liquid Yeast Extract–Peptone–Dextrose (YPD, MP Biomedicals) medium or in the minimal medium complete supplement mixture (CSM) devoid of uracil (–URA).

#### 2 Molecular biology

#### 2.1 Arabidopsis thaliana genomic DNA extraction from callus

The method used to prepare genomic *Arabidopsis thaliana* genomic DNA from leaves or callus was adapted from (Dellaporta et al., 1983).

A small piece of leave or callus (100 mg) is collected and put in a 2 mL bead-beater tissue homogenizer tube (PreCellys® 2 mL Empty Reinforced Tubes, Ozyme). Samples can be frozen in liquid nitrogen and

stored at -80°C before further manipulation. Approximately 200  $\mu$ L of 425-600  $\mu$ m acid-washed glass beads (Sigma-Aldrich) and 200  $\mu$ L of CTAB (cetyltrimethylammonium bromide) buffer (CTAB 2% (w/v), NaCl 1.4 M, Tris-HCl 100 mM pH 7.4, EDTA 20 mM, dithiothreitol 10 mM). Cells are grounded in a bead-beater tissue homogenizer (PreCellys® Evolution, Ozyme) at 4°C with 3 rounds of 20 s agitation at 10 000 rpm separated by pauses of 30 s. 800  $\mu$ L of CTAB buffer are added, cells are heated at 60°C for 30 min and centrifuged for 10 min at 20 000 x g at room temperature. The supernatant (usually 500  $\mu$ L) is collected and thoroughly mixed for 10 s with an equivalent volume of chloroformisoamylalcohol (24:1). The samples are centrifuged 10 min at 20 000 x g, the upper phase (usually 400  $\mu$ L) is collected and mixed with an equivalent volume of cold isopropanol. The mixture is left 1 h at -20°C and centrifuged for 15 min at 20 000 x g at 4°C to pellet genomic DNA which is then washed with cold 70% ethanol. Genomic DNA is dried, resuspended in sterile water and stored at -20°C.

#### 2.2 Extraction of total RNAs from Arabidopsis thaliana calli

To guarantee a successful RNA extraction, all the solutions are prepared with 0,1% (v/v) diethyl pyrocarbonate (DEPC)-treated water prior to autoclaving. Additionally, to guarantee the integrity of long RNA's, samples are mixed by hand by multiple inversions.

Calli are collected by filtering and grounded in liquid nitrogen in a mortar using a pestle until a fine powder is obtained. Approximately 100 mg of callus powder are collected in 2 mL microcentrifuge tubes and maintained in liquid nitrogen. Immediately after removing the samples from liquid nitrogen, 1.5 mL of TRI Reagent® (Sigma-Aldrich) is added to process the biological material and enable RNA, DNA and protein solubilization. The samples are agitated for 10 min on a rotative wheel at room temperature to enable complete sample processing. The samples are centrifuged at 12 000 x g at 4°C for 10 min to eliminate cell debris and polysaccharides. 300 µL of chloroform (200 µL chloroform / 1 mL of TRI reagent) are added to the supernatant in a fresh 2 mL microcentrifuge tube to separate RNA from DNA and proteins. To do so, the samples are mixed by 10 inversions, left at room temperature for 15 min and centrifuged at 12 000 x g at  $4^{\circ}$ C for 10 min. This results in the formation of a triple phase separation : an upper aqueous phase containing RNAs, a lower organic phase containing proteins and an intermediate phase containing genomic DNA. The upper phase is transferred to a fresh 2 mL microcentrifuge tube and for each volume of aqueous phase, 0.4 volumes of isopropanol and 0.4 volumes of high salt solution (1.2 M NaCl, 0.8 M sodium citrate) are added. Samples are gently mixed by 5-6 inversions and incubated for 1h at room temperature to precipitate RNAs. To complete RNA precipitation, samples are centrifuged at 12 000 x g at 4°C for 15 min. A maximum of supernatant is discarded and pellets are washed in 1 mL of cold (4°C) 75% (v/v) ethanol without disturbing the pellet. Samples are centrifuged at 7 500 x g at 4°C for 10 min and the supernatant is carefully removed. Pellets are air-dried at room temperature (usually 30 min) and resuspended in 50 µL of RNAse free (DEPCtreated) water.

To check the concentration and quality of the extracted RNAs, samples are subjected to spectrophotometry analysis at 260 nm, 230 nm and 280 nm on a Nanodrop (NanoDrop 2000, Thermo Fisher). An absorbance ratio 260/280 of 2 and 260/230 of 1.9 indicates good RNA quality. Additionally, 1  $\mu$ g of RNAs are run on a 2% (w/v) agarose gel at 100 V for 15 min. The presence of two bands corresponding to 28S and 18S RNAs as well as a brightness ratio 28S/18S of approximately 2 indicate good RNA quality.



Figure 7 Map of the pFP108 plasmid used for constitutive protein overexpression in plants.



Figure 8 Map of the pMM100 (pFP108-GFP) plasmid used to constitutively overexpress GFP-fused proteins for localization studies.

#### 2.3 Generation of cDNA from Arabidopsis thaliana calli

We used the SuperScript IV VILO reverse transcription kit (Invitrogen N°11766050) to generate cDNAs from the extracted RNAs. Briefly, 1  $\mu$ g of RNAs are subjected to a DNAse (ezDNAse) treatment to avoid gDNA contamination in downstream applications. Reverse transcription is realized using the SuperScript IV VILO Master Mix that contains degenerate primers. Reverse transcription occurs by annealing the degenerate primers at 25°C for 1 min, elongation by the reverse transcriptase at 50°C for 10 min and enzyme inactivation at 85°C for 5 min. The final cDNAs have a theoretical concentration of 50 ng/ $\mu$ L and are diluted 10 times before use.

#### 2.4 Molecular cloning

#### 2.4.1 Plasmids

#### 2.4.1.1 Plant expression vectors pFP108 and pMM100

The plant vector pFP108 was used as basis to construct the pFP108-GFP plasmid, which was renamed pMM100. pFP108 is destined for plant protein overexpression (**Figure 7**). It contains a Cauliflower Mosaic Virus 35S promoter (CaMV 35S) repeated in tandem to constitutively drive strong protein expression. Transcription is terminated by the nopaline synthase terminator (tnos). A multiple cloning site allows efficient cloning between the CaMV promoter and tnos terminator. The promoter, multiple cloning site and nos terminator are inserted between the T-DNA left and right borders.



Figure 9 Map of the pUC-L1L4 plasmid used as entry vector for Gateway cloning into the pBiFC plasmids.

The pMM100 corresponds to the pFP108 plasmid in which the EGFP gene preceded with a custom multiple cloning site was cloned (**Figure 8**). Coding sequences to be C-terminally fused with EGFP were cloned in the multiple cloning site according to the Gibson cloning method downstream of the inducible CaMV 35S promoter and using SpeI and BamHI restriction sites to linearize the plasmid backbone. Both plasmids are selected with spectinomycin in bacteria and Basta in plants.

#### 2.4.1.2 Plasmids for ratiometric bimolecular fluorescence complementation (rBiFC)

Ratiometric bimolecular fluorescence complementation (rBiFC) allows to express both the bait and the prey on the same plasmid as well as a cytosolic RFP that enables the standardization of the rBiFC signal between different cells and different constructs (Grefen & Blatt, 2012).



Figure 10 Map of the pUC-L3L2 plasmid used as entry vector for Gateway cloning into the pBiFC plasmids.

The coding sequence of interest was cloned by the Gibson method in pUC-L1L4 or pUC-L3L2 entry vectors (**Figure 9** and **Figure 10**) using SalI and PstI restriction sites to linearize the plasmid backbone. Both entry vectors contain a bacterial origin of replication, a carbenicillin resistance gene and two Gateway recombination sites, L1 and L4 for pUC-L1L4 and L2 and L3 for pUC-L3L2. A chloramphenicol resistance gene is inserted between the Gateway recombination sites allowing the selection of correct transgene insertion.



**Figure 11** Top: Representation of the four possible pBiFC plasmid configurations. Note that the expression of all transgenes is driven by the same CaMV 35S promoter. Bottom: Map of one of the four pBiFC plasmids allowing to perform rBiFC experiments by transient expression in *N. benthamiana*.

The entry vectors are then used to insert the sequence present between the attL sites between the attR sites of a pBiFC plasmid (**Figure 11**) in a Gateway recombination reaction (see section 2.4.5). The attL1/4 recombine with attR1/4 and the attL2/3 sites recombine with the attR2/3 sites. The pBiFC vector exists in four different versions to allow all the possible prey and bait split YFP fusions (**Figure 11**). Note the presence of already incorporated antigenic epitopes (HA and Myc) to allow easy Western blot analysis.

#### 2.4.1.3 Plasmid for in vitro protein expression

The pIVEX2.3d plasmid was used for the overexpression of proteins in a bacterial in vitro translation system (Imbert et al., 2021) (**Figure 12**). It contains a bacterial origin of replication, an ampicillin/carbenicillin resistance encoding gene allowing molecular cloning in *E. coli*. The transgene was cloned in the multiple cloning site with the Gibson cloning method downstream of the constitutive T7 bacteriophage promoter and using SalI and SacI as restriction sites to linearize the plasmid backbone. The transgene was cloned in frame with a His<sub>6</sub> tag.



Figure 12 Map of the pIVEX2.3d plasmid was used for bacterial cell-free in vitro protein expression.

#### 2.4.1.4 Protein expression vector for Saccharomyces cerevisiae

The YEplac195 plasmid was used to drive protein expression in yeast (**Figure 13**). It contains a bacterial origin of replication allowing cloning in *E. coli* and a yeast origin of replication. The ampicillin/carbenicillin resistance gene allows selection in *E. coli* for cloning purposes. The URA3 gene allows to alleviate the uracil auxotrophy of the yeast strain used allowing selection of transformed yeast. The transgene was cloned in the multiple cloning site according to the Gibson cloning method downstream of the inducible *lac* promoter and using PstI and BamHI restriction sites to linearize the plasmid backbone.



Figure 13 Map of the YEplac195 plasmid was used for constitutive and efficient protein expression in *Saccharomyces cerevisiae*.

#### 2.4.2 Polymerase chain reaction

Different commercial DNA polymerases were used for polymerase chain reaction (PCR) depending on the nature of the template.

For accurate template amplification for molecular cloning purposes, the Phusion DNA Polymerase (New England Biolabs) was used. The PCR reaction contains 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP (dTTP, dATP, dCTP, dGTP), 0.5  $\mu$ M of forward and reverse primers, 0.02 U/ $\mu$ L Phusion DNA polymerase and an adequate template DNA concentration for a final reaction volume of 25 or 50  $\mu$ L. The samples are put in a thermocycler and are denatured for 5 min at 98°C. Each cycle starts with a denaturing step at 98°C for 10 sec followed by a 30 sec primer annealing step at a temperature depending on the nature of the primer pair used (3°C below the calculated melting temperature) and ends with an amplification step at 72°C for a duration depending on the template length (30 sec/kb). The PCR cycle is repeated 35 times.

For the amplification of long fragments necessitating high accuracy and processivity, the Platinum SuperFi II DNA polymerase (Thermo Fisher) was used. The PCR reaction contains 1.75 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP (dTTP, dATP, dCTP, dGTP), 0.5  $\mu$ M of forward and reverse primers, 0.02 U/ $\mu$ L Platinum SuperFi II DNA polymerase and an adequate template DNA concentration for a final reaction volume of 25 or 50  $\mu$ L. The samples are put in a thermocycler and are denatured for 5 min at 98°C. Each cycle starts with a denaturing step at 98°C for 10 sec followed by a 30 sec primer annealing step at 60°C and ends with an amplification step at 72°C for a duration depending on the template length (30 sec/kb). The PCR cycle is repeated 35 times unless indicated otherwise. Long fragments for Nanopore long-read sequencing were obtained by amplification of cDNA with the Platinum SuperFi II DNA polymerase protocol. The amplification step was realized at 72°C for 8 min and 5 sec were added per PCR cycle to compensate for the possible loss of polymerase activity from cycle to cycle.

To verify the presence of a plasmid in a *E. coli* colony grown on LB-agar, PCRs were performed directly on the colony using the Taq DNA polymerase (Euromedex). The colony PCR reaction contains 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP (dTTP, dATP, dCTP, dGTP), 0.5  $\mu$ M of forward and reverse primers, 0.02 U/ $\mu$ L Taq DNA polymerase for a final reaction volume of 15  $\mu$ L. The bacterial colony is added using a pipette tip. The samples are put in a thermocycler and are denatured for 5 min at 95°C. Each cycle starts with a denaturing step at 95°C for 10 sec followed by a 30 sec primer annealing step at a temperature on the nature of the primer pair used (3°C below the calculated melting temperature, usually around 56°C) and ends with an amplification step at 72°C for a duration depending on the template length (1 min/kb). The PCR cycle is repeated 40 times since the first 5 PCR cycle are necessary to disrupt the bacterial cells.

The primer melting temperatures were calculated using the ThermoFisher primer Tm calculator last accessed online on 02/01/2023: https://www.thermofisher.com/fr/fr/home/brands/thermo-

scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html

#### 2.4.3 DNA fragment purification on agarose gel

PCR fragments are run on 1% agarose (w/v) gels for 35 min at 100V. The fragments of interest are cut out of the gel using a scalpel and extracted with a Monarch DNA gel extraction kit (New England Biolabs N° T1020L) according to the manufacturer's protocol. For fragments longer than 8 kb, 1.5 volumes of water are added to the dissolved fragment to facilitate DNA-agarose separation.

#### 2.4.4 DNA fragment assembly by the Gibson method

The Gibson assembly method allows to directionally assemble multiple overlapping DNA fragments together based on sequence homology (Gibson et al., 2009). The DNA fragments are generated by PCR and by digesting the backbone plasmid. About 15 fmol of each fragment to be assembled are added to a Gibson reaction mix containing 100 mM Tris-HCl pH 7.5, 10 mM MgCl<sub>2</sub>, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 10 mM dithiothreitol, 0.05 g/mL polyethylene glycol (PEG)-8000, 1 mM NAD, 2.5 mU/µL T5 exonuclease (New England Biolabs N°M0363S), 25 mU/µL Phusion High fidelity DNA polymerase (New England Biolabs N°M0530S) and 4 U/µL TAQ DNA ligase (New England Biolabs N°M0208S) in a final volume of 20 µL. The reaction mix incubated at 50°C for 1h is ready to be used for transformation.

#### 2.4.5 Gateway recombinational cloning

The Gateway cloning method uses site-specific tyrosine recombinase activity to directionally assemble DNA fragments flanked with recombinase recognition sites into DNA backbones carrying complementary recombinase recognition sites. The DNA fragment of interest is cloned into a donor vector by Gibson assembly. 200 ng of destination vector is mixed with donor vector in a molecular ration of 1:3 (destination:donor) in a final volume of 8  $\mu$ L according to Grefen & Blatt, 2012. 2  $\mu$ L of commercial Gateway LR clonase mix II (Invitrogen N° 11791020) is added to the DNA mix and the recombination reaction is carried out at room temperature over 8h. After incubation, the reaction mix can be used for transformation.

#### 2.4.6 Escherichia coli transformation and selection of transformants

To generate transformation competent *E. coli* cells, the commercially available DH5 $\alpha$  strain (60602 Lucigen) was used (see section 1.3). 150 mL of LB medium were inoculated with 5 mL of overnight cultures and grown at 37°C until an optical density at 600 nm of approximately 0.4 is reached. The cells were centrifuged for 10 min at 5 000 x g at 4°C and the pellet is gently resuspended in 15 mL of cold sterile MgCl<sub>2</sub> 100 mM while being maintained on ice. After 30 min incubation on ice at 4°C, the cells were centrifuged 10 min at 5 000 x g at 4°C and the pellet is gently resuspended in 3 mL of cold sterile CaCl<sub>2</sub> 100 mM. The cells are incubated on ice at 4°C for 90 min and glycerol is added to a final

concentration of 25%. 100  $\mu$ L aliquots of competent cells are frozen in liquid nitrogen and stored at - 80°C.

Competent cells are thawed on ice and the DNA to be transformed is added. As a negative control, the appropriate digested DNA backbone to which no insert was added is incubated with competent cells. After a gentle mix, the cells are incubated on ice for 20 min before undergoing a heat shock at 42°C for 45 sec. After heat shock, the transformed cells are put back on ice for 5 min and 900  $\mu$ L of LB medium is added. Cells are grown for 1 h at 37°C for recovery before being spread on LB-agar containing the appropriate selection pressure. The transformed bacteria are grown at least 12 h at 37°C. The bacteria colonies that grew on the selection medium are selected and screened by PCR to confirm the presence of the desired construct and thus their successful transformation.

#### 2.4.7 Plasmid DNA preparation from *E. coli*

Plasmid DNA preparation was accomplished using commercial Miniprep and Midiprep kits (Macherey-Nagel NucleoSpin/NucleoBond) according to the manufacturer's protocol. The technique is based on the alkaline denaturation of chromosomal DNA while plasmid DNA is retained on an anion exchange column (Birnboim & Doly, 1979). Minipreps and Midipreps differ by the initial volume of culture used for cell lysis. For Minipreps, 10 mL of liquid overnight cultures is used while for Midipreps, 200 mL of culture is used. The purity of the plasmids is tested by running a 1% agarose (w/v) gel and plasmid quantity is assessed by spectrophotometry (NanoDrop 2000, Thermo).

#### 2.4.8 Sanger sequencing of plasmids and DNA fragments

Sanger sequencing was performed by the Macrogen sequencing service according to the Sanger sequencing technique (Sanger et al., 1977).

#### 2.4.9 Cryopreservation of Escherichia coli and Agrobacterium tumefaciens strains

A single colony of *E. coli* or *A. tumefaciens* is scratched off an LB-agar plate containing the appropriate selection pressure and inoculated in 10 mL liquid LB medium containing the same selection pressure. *E. coli* and *A. tumefaciens* strains are cultured at  $37^{\circ}$ C under 180 rpm agitation for 16 hours or 24h respectively. Bacteria are mixed with sterile glycerol to a final concentration of 20% (v/v) glycerol and flash-frozen in liquid nitrogen prior to storage at -80°C.

#### 2.5 Agrobacterium tumefaciens transformation

To produce transformation competent *A. tumefaciens* C58C1 cells, 5 mL of overnight liquid cultures are used to seed 200 mL of LB containing the appropriate antibiotics mix. Cells are grown at 28°C with 180 rpm agitation until the optical density measured at 600 nm reaches 0.5. The cells are incubated on ice for 5 min at 4°C and centrifuged for 20 min at 5 000 x g at 4°C. The pellets are gently resuspended in 4 mL of cold sterile CaCl<sub>2</sub> 20 mM, frozen in liquid nitrogen and stored at -80°C.

To transform *A. tumefaciens* C58C1, 600 ng of plasmid DNA were added to 100  $\mu$ L of competent cells, which are then incubated for 5 min at 37°C. 900  $\mu$ L of LB are added and the cells are incubated 2 h at 28°C for recovery. The cells are spread on LB-agar containing the appropriate selection pressure for plasmid selection. The bacteria are grown at 28°C for 2-3 days until colonies appear.

#### 2.6 Transient transgene expression in *Nicotiana benthamiana* by agroinfiltration

Approximately five colonies of *A. tumefaciens* C58C1 transformed with the desired construct are used to seed 10 mL of LB medium containing the appropriated antibiotics mix supplemented with acetosyringone (20  $\mu$ M) and the appropriate antibiotic for plasmid selection. In addition to the strains transformed with the constructs of interest, the *A. tumefaciens* strain expressing P19 protein, a suppressor of post-transcriptional gene silencing that enhances transient transgene expression (Voinnet et al., 2003, retracted), is grown in LB medium containing the appropriate antibiotics mix supplemented with acetosyringone (20  $\mu$ M). All the strains are grown for 24 h at 28°C with 180 rpm agitation, then centrifuged for 10 min at 4 000 rpm. The pellets are resuspended in 5 mL of agro-infiltration buffer (MgCl<sub>2</sub> 1 mM, acetosyringone 200 mM) and further diluted to obtain between 5mL or 10 mL of cell suspensions with an optical density measured at 600 nm of one. The different Agrobacterium strains to be agroinfiltrated are then mixed in a ratio of 1:1.

Using a needle, a small superficial laceration is performed on top of young leafs of 3-4 weeks old *Nicotiana benthamiana* grown in controlled conditions (see **section 1.1.1.2**). An appropriate volume of bacterial suspension is gently injected in the leaf through the laceration with a 1 mL syringe until the whole surface of the leaf is soaked. The agroinfiltrated plants are put back in controlled growth conditions and grown for at least 2 days. The expression of the transgene usually takes place between 2 and 4 days after agroinfiltration.

#### 2.7 Saccharomyces cerevisiae transformation

To generate heat shock transformation-competent yeast, 3 mL of YPD medium inoculated with the desired strain to be transformed is incubated at 30°C for 16h under 200 rpm agitation. The culture is diluted 10 times in a final YPD volume of 10 mL and incubated at 30°C under 200 rpm agitation until the exponential phase is attained (OD600 of 1-1.5). The cells are pelleted by centrifugation at 3000 rpm for 3min and washed in 10 mL of sterile water. Then he cells are washed in 1 mL of lithium acetate (LiAc)-sorbitol (Sorb) solution (LiAc 100mM, 10mM Tris-Acetate pH8, 1mM EDTA, 1M Sorbitol)) and centrifuged at 3000 rpm for 3min. LiAc is thought to favor transformation because of its chaotropic effect (Kawai et al., 2010). The pellet is resuspended in 250  $\mu$ L of LiAc-Sorb. The competent cells can be stored at 4°C for a few hours.

 $50\mu$ L of competent cells are mixed with  $5\mu$ L of ssDNA 10mg/mL,  $500\mu$ g of plasmid and  $300\mu$ L  $\mu$ L of LiAc-polyethylene glycol (PEG) solution (LiAc 100mM, 10mM Tris-Acetate pH8, 1mM EDTA, PEG3350 40% (w/v)), Transformation is achieved by incubating the cells at 30°C for 30 min under 400

rpm agitation followed by a heat shock allowing plasmid DNA entry at 42°C for 15 min under 400 rpm agitation. Cells are centrifuged at 3000 rpm for 3 min and the pellet is resuspended in 100  $\mu$ L of sterile water. Cells are spread on solid CSM –URA medium for selection. Transformed yeast cells are grown for 48h at 30°C and growing colonies are selected for genotyping.

#### 2.8 Arabidopsis thaliana stable line generation by floral dip

Arabidopsis thaliana plants are grown for approximately 6 weeks in a 16h-8h light-dark photoperiod until the inflorescences contain a high number of floral buds. To increase the number of inflorescencebearing stalks, the first stalk rising above the rosette is cut to allow multiple secondary stalks to grow, which increases the number of inflorescences. 24h before the floral dip, 2 mL pre-cultures are started from a single colony on a plate in LB medium containing the appropriate antibiotics mix. Pre-cultures are grown for 8 h at 28°C under 180 rpm agitation. After 8 h, the pre-cultures are transferred to 100 mL of liquid LB medium containing the same antibiotics cocktail supplemented with acetosyringone (50 µM) and grown for 16 h at 28°C under 180 rpm agitation. After 16 h of growth, the OD600 of the cultures are measured and 140 OD equivalents are collected in 50 mL Falcon tubes. Bacteria are centrifuged at 5000 x g for 20 min and the pellets are resuspended in 200 mL of floral dip buffer (Murashige & Skoog with vitamins [MSP09, Caisson Labs] 2.1 g/L, 5% (w/v) sucrose, 0.05% (v/v) Silwet-77 adjuvant, 200 µM acetosyringone). All the inflorescences are dipped in the Agrobacterium solution (floral dip) for 30 sec and the treated plants isolated in a humid environment to favor the activity of the bacterium. The plants are then kept in the dark for 48 h to avoid the light to burn the parts that have been in contact with the agroinfiltration solution. After 48 h, the treated plants are put back to normal growth conditions.

The treated plants are grown until siliques appear, then let dried to recover the seeds. To select for transformed seeds, approximately 500  $\mu$ L of seeds are sown on soil and germinated. Two-three weeks after sowing, the seedlings are sprayed with an aqueous solution containing 0.1% (v/v) of Basta herbicide containing glufosinate-ammonium (Basta®, Profert). The seedlings effectively transformed with the transgene carry a gene conferring resistance to the herbicide, thus allowing their selection and sub-culturing.

#### 3 Plant phenotype analysis

#### 3.1 Cold stress

To detect a potential phenotype in cold growth conditions, *Arabidopsis thaliana* Col0 or mutant lines were sown and grown on soil for two weeks in short days conditions as described in **section 1.1.1.2**. While one half was kept in standard conditions, the other half was put at 4°C with the same light conditions. Pictures of each plant were taken every week.

#### 3.2 Arabidopsis thaliana pollen collection and pollen tube germination

*A. thaliana* pollen grains were collected according to Dumont et al., 2015. Pollens were obtained from *A. thaliana* plants grown on soil for six weeks in long day conditions. For each *A. thaliana line*, 35 newly opened flowers were collected using a scalpel and tweezers and put in a 2 mL microcentrifuge tube. 1 mL of pollen germination medium (PGM) (5 mM CaCl<sub>2</sub>, 0.01% (w/v) H<sub>3</sub>BO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 5 mM KCl, 10% (w/v) sucrose, pH adjusted to 7.5) was added and samples were vigorously shaken with a mechanical shaker at maximum speed for 5 min. Samples were centrifuged at 3 200 x g for 7 min to pellet the pollen grains that appear as a yellowish pellet. The remaining floral parts are removed using tweezers and the supernatant is removed. 500 µL of PGM are added and pollen grains are carefully resuspended with mild pipette strokes.

To follow pollen tube germination and growth, an appropriate amount of freshly collected pollen grains were immediately put in 6 well plates and volume was adjusted to 1 mL with PGM medium. Pollen tube germination was followed over time with an inverted microscope (Olympus CKX53). At each time point, six images were taken and analyzed.

#### 4 Subcellular fractionation

#### 4.1 Mitochondria purification on a discontinuous Percoll gradient

Mitochondria from calli were purified according to well established protocols in our laboratory (Jouhet et al., 2004; Leterme et al., 2021). The purification process is illustrated in **Figure 14**.

Mitochondria are fragile organelles that have to be manipulated with great care. To preserve their integrity and function, each purification step (grinding, centrifugation, fractionation) has to be performed at 4°C and all the material used (mortars, tubes, rotors, buffers) has to be pre-chilled at 4°C before use. Very importantly, the material used for mitochondria purification has to be free of detergent.

*Arabidopsis thaliana* calli are cultured for 4 days in liquid medium are filtered through a glass fiber filter (Merck, AP1509000) using a vacuum filtration unit. Cells are weighed and immediately placed in a pre-chilled mortar. At this step, a small aliquot can be collected and stored at -80°C for further analysis of the whole cell fraction. In the cold room, adequate volumes of grinding buffer (0.3 M mannitol, 15 mM MOPS pH 8, 2 mM EGTA, 0.6% (w/v) polyvinylpyrrolidone K 25, 0.5% (w/v) bovine serum albumin, 10 mM DTT, 1 mM PMSF, 5 mM 6-aminocaproic acid, 1 mM benzamidine) (30 mL for 64 g of callus) and sand (15 mL for 64 g of callus) are added to the cells in the mortar. Cells are grounded for 7 min with a pestle until a completely homogenized lysate is obtained. 20 mL of grinding buffer are added to the lysate to facilitate its transfer to 250 mL centrifuge bottles that fit in the JA10 (Beckman Coulter) rotor. The residual lysate is washed out and the pestle rinsed with an additional 30 mL of grinding buffer. The lysate is centrifuged at 700 x g for 5 min at 4°C in an ultracentrifuge (Optima LE-80K, Beckman Coulter) using a JA10 rotor to pellet unbroken cells, heavy material and sand. The

supernatant is collected with a pipette and distributed in 35 mL centrifuge tubes that fit in a JA25.50 rotor (Beckman Coulter). The supernatant is centrifuged at 3000 x g for 5 min at 4°C in an ultracentrifuge (Optima LE-80K, Beckman Coulter) using a JA25.50 rotor. The supernatant is transferred to fresh 35 mL tubes and the centrifugation is repeated. The supernatant is transferred to fresh 35 mL tubes and centrifuged at 20 000 x g for 15 min at 4°C in a JA25.50 rotor to pellet the crude mitochondria. The supernatant is discarded and the crude mitochondria are gently resuspended in 1 mL of washing buffer (300 mM mannitol, 15 mM MOPS pH 8, 1 mM PMSF, 5 mM 6-aminocaproic acid, 1 mM benzamidine) using a soft paintbrush. The suspension is transferred to a Potter-Elvehjem tissue grinder using a cut pipette tip. Crude mitochondria are homogenized by three gentle strokes and transferred to a 15 mL Falcon tube. An appropriate volume of washing buffer is used to complete the crude mitochondria fraction to a total volume of 2 mL and is previously used to wash the paintbrush, the 35 mL centrifuge tube and the Potter-Elvehjem tissue grinder.

Prepare discontinuous gradients by layering 5 mL of 40 % Percoll, 20 mL of 23 % Percoll and 10 mL of 18 % Percoll on top of each other in a 40 mL ultracentrifuge tube using a peristaltic pump. The three layers should be deposited with a very slow flow rate and should be clearly visible. 2 mL of crude mitochondria are carefully added on top of a discontinuous gradient and centrifuged at 50 000 x g for 45 min at 4°C in a Beckman Coulter centrifuge SW32-Ti swing-out rotor (Beckman Coulter) with maximum acceleration and a slow deceleration. After centrifugation, mitochondria appear at the 23%-40% Percoll interface. The upper part of the gradient is removed by aspiration and 4 mL of mitochondrial fraction is retrieved with a pipette and placed in a 35 mL centrifuge tube. The tube is filled with washing buffer and centrifuged at 20 000 x g for 15 min at 4°C in a JA25.50 rotor. The supernatant is carefully discarded and the tube is filled again with washing buffer. The centrifugation is repeated and the supernatant removed. The pellet containing the purified mitochondria is resuspended in 1 mL of washing buffer using a pipette with a cut tip.

At this step, mitochondria can be used in different manners. If a single-gradient purification is sufficient, mitochondria can be transferred to an Eppendorf tube and centrifuged at 20 000 x g for 8 min at 4°C in a microcentrifuge. The pellet can either be stored dry at -80°C for further lipid analysis or be resuspended in an appropriate volume of buffer for subsequent protein and/or complex analysis. If a second purification on a continuous Percoll gradient is necessary, the mitochondria suspension is transferred to a 4 mL Potter-Elvehjem cell grinder and homogenized by 3 gentle strokes. The homogenized mitochondrial fraction can then be loaded on top of a continuous 28% (v/v) Percoll gradient.



**Figure 14** Flow chart of the mitochondria purification process using a discontinuous Percoll gradient (adapted from Leterme et al. 2021). The major steps are indicated and the pictures illustrate the result of the concerned step.

# 5 Methods for protein and protein complex purification, analysis and quantification

#### 5.1 Total protein extraction from Arabidopsis thaliana calli

A small amount of callus is collected with a spatula and put in a resistant 2 mL bead-beater tissue homogenizer tube (PreCellys® 2 mL Empty Reinforced Tubes). 200  $\mu$ L of protein buffer (urea 8 M, Tris-HCl 50 mM pH 6.8, EGTA 1 mM, DTT 10 mM) and about 200  $\mu$ L of 425-600  $\mu$ m acid-washed glass beads (Sigma-Aldrich) are added. The sample is heated at 70°C for 5 min with manual agitation every minute. The cells are grounded in a bead-beater tissue homogenizer (PreCellys® Evolution, Ozyme) at 4°C with 3 rounds of 20 s agitation at 10 000 rpm separated by pauses of 30 s. The heating and grinding steps are repeated before centrifugation for 5 min at 5 000 x g at 4°C. The supernatant contains the total protein extract. At this step, the sample can be frozen and stored at -20°C or put on ice for further manipulations.

## 5.2 Protein quantity determination by a colorimetric protein assay with Coomassie blue

The protein content of a sample is determined by a colorimetric protein assay with Coomassie blue (Bradford, 1976). The protein assay dye (Bio-Rad, Protein Assay Dye Reagent Concentrate, undisclosed proprietary composition) is diluted 5 times in water and distributed in spectrophotometry cuvettes. An appropriate quantity (between 1  $\mu$ L and 50  $\mu$ L) of sample is diluted in the dye, thoroughly mixed and incubated for 5 min before absorbance values are read at 595 nm on a spectrophotometer (Eppendorf Biophotometer D30). Protein concentration is determined using a BSA standard curve as a reference and Lambert-Beer's law. If detergent is present in the protein samples, the Pierce Detergent compatible Bradford assay (Invitrogen) is used following similar procedure.

#### 5.3 Protein sample concentration through a centrifugable molecular cutoff filter

If protein concentration has to be increased for further use, the protein-containing sample is loaded on a centrifugable molecular cutoff filter. Depending on the protein size and sample volume, different centrifugable molecular cutoff filters were used (Amicon centrifugal filter units). The samples contained in the centrifugable molecular cutoff filter are centrifuged at a maximum speed of 3 000 x g to prevent proteins from sedimenting and agglomerate on the filter membrane. Samples are centrifuged until the desired volume is obtained. Samples are removed from the centrifugable molecular cutoff filter with a micropipette after washing off the filter membrane with the sample.

#### 5.4 Protein expression systems

#### 5.4.1 Protein expression in the yeast *Saccharomyces cerevisiae*

Yeast strains are inoculated in 10 mL of CSM –URA medium and cultured overnight for 24h at 30°C. After 24h, the OD600 is measured and an appropriate volume of culture is placed in 100 mL of –URA medium so as to obtain an OD600 of 0.025. The culture is grown 16h (overnight) at 30°C under 160 rpm agitation. When the OD600 is between 1 and 2, which corresponds to the exponential phase, the yeast is grown for 2h at 17°C under 160 rpm agitation. Recombinant protein production under the control of pGAL1 promoter is induced by adding galactose to a final concentration of 2% (v/v). The yeast is grown overnight at 17°C until the culture reaches stationary phase at an OD600 of approximately 4-5. The recombinant proteins are natively extracted using a cell disruption method. The equivalent of 100 OD600 of yeast culture is centrifuged for 3 min at 3000 x g at 4°C. The pellet is resuspended in 1 mL of NaCl 0.9% (w/v), placed in a bead beater tube (PreCellys® 2 mL Empty Reinforced Tubes, Ozyme) and maintained on ice for the rest of the process. The cells are centrifuged for 3 min at 3000 x g and washed 2 times with NaCl 0.9% (w/v). The cells are eventually resuspended in lysis buffer (20 mM Tris-HCl pH8, 150 mM Nacl, 1 mM EDTA pH8, 1 mM PMSF, protease inhibitor (Roche Complete, 1 tablet for 50 mL, added just before experiments)) and 425-600  $\mu$ m acid-washed glass beads (Sigma-Aldrich, 100  $\mu$ L for 100 OD) are added. Cells are broken using a bead beater tissue homogenizer

(PreCellys® Evolution, Ozyme) at 4°C with 3 rounds of 20 sec agitation at 5 500 rpm followed by a pause of 30 sec to avoid heat. The disrupted cells are centrifuged 3 min at 3 000 x g at 4°C to pellet intact cells, cell debris and glass beads. The supernatant that contains the soluble recombinant protein is placed in fresh 1.5 mL microcentrifuge tubes and centrifuged at 16 000 x g for 20 min at 4°C to further eliminate insoluble material. The supernatant is collected and used for the protein purification process. The pellet is conserved for analysis.

#### 5.4.2 Cell-free protein expression

The sequence corresponding to the protein of interest was cloned into a pIVEX 2.3d vector with an His<sub>6</sub> tag (see **section 2.4.1.3**). The protein of interest is synthesized *in vitro* using a cell-free expression system under the supervision of Lionel Imbert (ISBG, Grenoble). Briefly, the protein is expressed under RNAse-free conditions in batch mode, in a volume of 15 mL of reaction mixture, for 3 h at 27 °C under gentle agitation. The cell-free mixture (Imbert et al., 2021) contained 16  $\mu$ g/mL of pIVEX 2.3d plasmid encoding the protein sequence, 1 mM of 20 essential amino acids protonated, 0.8 mM of each rNTPs (guanosine-, uracil-, and cytidine-5'-triphosphate), 55 mM HEPES (pH 7.5), 68  $\mu$ M folinic acid, 0.64 mM cyclic adenosine monophosphate, 3.4 mM dithiothreitol, 27.5 mM ammonium acetate, 2 mM spermidine, 80 mM creatine phosphate, 200 mM potassium acetate, 18 mM magnesium acetate, 250  $\mu$ g/mL creatine kinase, 27  $\mu$ g/mL T7 RNA polymerase, 0.175  $\mu$ g/mL tRNA and 400  $\mu$ L/mL S30 *E. coli* bacterial extract.

#### 5.5 Protein purification by nickel affinity chromatography

The His-tagged proteins produced by a cell-free or yeast expression system are centrifuged at 16 000 x g at 4°C for 30 min to pellet any insoluble material. The supernatant is mixed with 20 mL of solubilization buffer (Tris-HCl pH8, NaCl 0.15 M) and loaded by gravity flow on 2 mL of Ni-Sepharose resin (Ni-Sepharose Fast Flow, Cytiva) previously equilibrated with 10 mL of solubilization buffer. The flow through is collected and passed a second time through the Ni-NTA resin. To eliminate contaminants retained on the column, the resin is consecutively washed with 15 mL of three washing buffers containing increasing concentrations of imidazole (washing buffers 1, 2 and 3 : Tris-HCl pH8, NaCl 0.15 M, 0.025, 0.05 and 0.075 M imidazole respectively). The protein of interest is eluted by affinity competition with 5 mL of a buffer containing a high imidazole concentration (Tris-HCl pH8, NaCl 0.15 M, imidazole 0.2 M) and fractions of 1.5 mL are collected. The fractions containing the purest form of the protein of interest is determined by SDS-PAGE followed by Coomassie blue staining (see section 5.8). Protein concentrations are determined by colorimetric protein assay with Coomassie Blue (see section 5.3).

To remove imidazole that might interfere with downstream measures, the concentrated fraction was then subjected to desalting through a PD10 desalting column (Cytiva) according to the manufacturers recommendations.

#### 5.6 MTL complex purification and analysis

#### 5.6.1 Extraction of mitochondrial membrane complexes

Crude mitochondria are obtained by the standard protocol detailed in a previous section (section 4.1). The following steps have been adapted from a protocol previously established in our laboratory (Michaud, 2018).

#### 5.6.1.1 Mitochondrial membranes purification

After resuspension, mitochondrial fractions are centrifuged at 11 000 x g at 4°C for 5 min to pellet mitochondria. Mitochondrial membranes are obtained by bursting mitochondria by osmotic choc. The pellet is resuspended in 1 mL of swelling buffer (10 mM MOPS pH 7.4. Just before use, 1 mM PMSF, 5 mM 6-aminocaproic acid and 1 mM benzamidine are added) and incubated on ice for 5 min. Mitochondrial integrity is broken by vigorously shaking the suspension for 10 s using a shaker at. Mitochondrial membranes are pelleted with a centrifugation at 100 000 x g at 4°C for 20 min using an ultracentrifuge (Sorvall Discovery M150 SE) with the S55A2 rotor. The membranes are resuspended in 150  $\mu$ L of purification buffer (30 mM HEPES pH7, 1 mM EDTA, NaCl variable concentration) of varying NaCl concentration depending on the type of experiment that has to be performed subsequently. For subsequent size exclusion chromatography or sucrose gradient ultracentrifugation, the mitochondrial membranes were resuspended in a buffer containing 100 mM NaCl. For anion exchange chromatography, the mitochondrial membranes were resuspended in a buffer containing 5 mM NaCl.

#### 5.6.1.2 Crosslinking

The crosslinking step has only been realized during the MTL complex stability tests. To crosslink the mitochondrial membrane complexes, the equivalent of 300  $\mu$ g of mitochondrial membrane proteins are resuspended in 150  $\mu$ L of washing buffer (300 mM mannitol, 15 mM MOPS pH 8, 1 mM PMSF, 5 mM 6-aminocaproic acid, 1 mM benzamidine). 1.5  $\mu$ L of DSP (3,3'-dithiobis(succinimidyl propionate)) crosslinking reagent freshly dissolved in DMSO is added to a final concentration of 0.4  $\mu$ g/ $\mu$ L. The mixture is gently shaken and incubated on ice for 15 min. DSP is a bifunctional crosslinking reagent that reacts with the terminal amines situated on the lysine side chains and on the N-terminal end of the protein backbone (Hermanson, 2013). The crosslinking reaction is stopped by adding 15  $\mu$ L of Tris-HCl 1 M pH 7.4 and by gently mixing before another 15 min incubation on ice. The sample is centrifuges at 11 000 x g for 5 min at 4°C and the supernatant is discarded. To wash off all the DSP, the washing step is repeated with Tris-HCl 1 M pH 7.4. The crosslinked mitochondrial complexes still linked to membranes are resuspended in purification buffer of varying NaCl concentration depending on the type of experiment that has to be performed subsequently.

#### 5.6.1.3 Solubilization of mitochondrial complexes

The solubilized mitochondrial membranes are placed in 1.5 mL ultracentrifuge tubes (Beckman Coulter Ref 357448). To extract and solubilize the mitochondrial membrane complexes from the membranes, 1.5  $\mu$ g of DDM was added per  $\mu$ g of mitochondrial membrane proteins. Typically, 13  $\mu$ L of n-dodecyl- $\beta$ -D-maltoside (DDM) 20% (w/v) were added to 2600  $\mu$ g of membrane proteins for a final volume of 520  $\mu$ L and a final concentration of 0.5% (w/v) DDM. The complexes are solubilized by slowly pipetting the mixture up and down 30 times (pipette set on 150  $\mu$ L). After 5 min incubation on ice, the sample is centrifuged at 100 000 x g for 20 min at 4°C with an ultracentrifuge (Sorvall Discovery M150 SE) to pellet insoluble materials. The supernatant that contains the solubilized mitochondrial membrane complexes is transferred to a fresh 1.5 mL tube for further downstream applications.

#### 5.6.2 Size exclusion chromatography

To separate the mitochondrial complexes according to their size, we chose a size exclusion chromatography (SEC) approach. Given the uncertainty concerning the real size of the MTL complex, we chose a SEC column with a broad separation range. We used a Superose 6 Increase 10/300 GL (GE Healthcare) composed of crosslinked agarose beads with an average diameter of 8.6  $\mu$ L and a separation range ranging from 5 kDa to 5 000 kDa for globular proteins. The exclusion volume, which corresponds to the volume outside the beads, was determined experimentally at 8.2 mL by running dextrans. The column has a total volume of 24 mL. Samples are run on the column with a fast protein liquid chromatography (FPLC) system (ÄKTA pure) equipped with an injection loop, a UV (280 nm) spectrophotometer, a conductivity sensor, a hydrostatic pressure sensor and an automated fraction collector.

The whole SEC process is performed at 4°C and samples are kept on ice. The SEC column is equilibrated with 2 column volumes of water and 2 column volumes of purification buffer (30 mM HEPES pH7, 1 mM EDTA, 100 mM NaCl) supplemented with 0.05% (w/v) DDM to maintain a detergent environment. The flow rate is set so as to keep the hydrostatic pressure below 1.5 MPa, typically between 0.3 mL/min and 0.4 mL/min for aqueous solutions. The concentrated sample containing the solubilized mitochondrial membrane complexes is injected in the injection loop with a syringe. The sample volume should not exceed 500  $\mu$ L to increase the SEC resolution. The sample is injected on the column with 2.5 mL of purification buffer. The sample is eluted with 1.5 column volumes (36 mL) of purification buffer at a constant flow rate of 0.3 mL/min. During all the elution, 1 mL fractions are collected and conserved for further analysis. The protein content in the eluted fractions is followed by measuring the absorbance at 280 nm. Additionally, the total protein content is determined by a colorimetric protein assay (see **section 5.2**). The presence of the MTL complex in the fractions is detected by CN-PAGE (see **section 5.6.5**).

#### 5.6.3 Sucrose density gradient ultracentrifugation

Gradient ultracentrifugation allows to separate particles in solution according to their hydrodynamic radius and density (Fernandez-Martinez et al., 2016; Voet & Voet, 2011). We used a pre-formed sucrose density gradient to try to isolate the MTL complex. The sucrose density gradient is generated using a 15 mL gradient mixer (Interchim), a peristaltic pump and two buffers containing different sucrose concentrations. The whole sucrose gradient ultracentrifugation process is realized at 4°C and samples are kept on ice. 5 mL of heavy buffer (30 mM HEPES pH7, 1 mM EDTA, 100 mM NaCl, 30% (w/v) sucrose, 0.05% (w/v) DDM) is placed in the downstream compartment of the gradient mixer and 5 mL of light buffer (30 mM HEPES pH7, 1 mM EDTA, 100 mM NaCl, 5% (w/v) sucrose, 0.05% (w/v) DDM) is placed in the upstream compartment separated by a valve. Both buffers are supplemented with a protease inhibitor cocktail (Roche Complete, 1 tablet for 50 mL, added just before experiments). The gradient mixer is connected to the peristaltic pump that allows a flow rate of 1 mL/min. The output is carefully poured in an ultra-clear ultracentrifuge tube (Beckman Coulter, ref. 344059). The valve between the compartments is opened so that the heavy buffer is progressively diluted, hence forming a bottom-up 30%-5% sucrose density gradient in the ultracentrifuge tube. The solubilized mitochondrial membrane complexes are carefully placed on top of the density gradient with pipette. The sample volume should not exceed 500  $\mu$ L to enhance the separation resolution. The gradient is placed in a SW41Ti rotor (Beckman Coulter) and is centrifuged at 200 000 x g for 20h at 4°C in an ultracentrifuge (Beckman Coulter Optima LE-80K). The centrifuge tube is punctured at the bottom and the centrifuged sucrose gradient is slowly removed with a peristaltic pump at a flow rate of 1 mL/min. The sucrose gradient is divided into 500  $\mu$ L fractions that are kept for further analysis and purification steps. The total protein content is determined by a colorimetric protein assay (see section 5.2). The presence of the MTL complex in the fractions is detected by CN-PAGE (see section 5.6.5).

#### 5.6.4 Anion exchange chromatography

To separate proteins and complexes according to their charge, we used a 1 mL Q-sepharose anion exchange chromatography (AEC) column and applied a NaCl gradient. Samples are run on the column with the same fast protein liquid chromatography (FPLC) system as for SEC (ÄKTA Pure). The AEC column is equilibrated with 8 column volumes of water followed by 10 column volumes of low salt buffer (30 mM HEPES pH7, 1 mM EDTA, 5 mM NaCl, 0.05% (w/v) DDM) at a flow rate of 0.5 mL/min. 1.5 mL of concentrated sample containing the solubilized mitochondrial membrane complexes is loaded in the injection loop with a syringe. The sample is injected on the column with 5 mL of low salt buffer. The column is washed with 2 mL of low salt buffer (30 mM HEPES pH7, 1 mM EDTA, 1 M NaCl, 0.05% (w/v) DDM) is progressively injected so as to reach 40% after 15 column volumes effectively increasing the NaCl concentration from 5 mM to 400 mM. 500  $\mu$ L fractions are collected during the whole elution process and conserved for further analysis and purification steps. To wash off

the remaining proteins, the NaCl concentration is increased to 1 M. As for the SEC and sucrose gradient ultracentrifugation, the total protein content in the fractions is determined by a colorimetric protein assay (see section 5.2). The presence of the MTL complex in the fractions is detected by CN-PAGE (see section 5.6.5).

5.6.5 Clear native polyacrylamide gel electrophoresis for MTL complex visualization Clear native polyacrylamide gel electrophoresis (CN-PAGE) allows the separation of proteins in a complex sample according to their net charge and size. To generate the separation clear native gradient gel, two different mixtures are realized (**Table 8**). Mix 1 contains a high acrylamide concentration (12%) as well as glycerol. Mix 2 has a lower acrylamide concentration (3.5%) and no glycerol. The APS (ammonium persulfate, the electron donor) and TEMED (tetramethylethylenediamine, the catalyst for the polymerization reaction) are added at the last moment. 2 mL of Mix 1 is loaded on the downstream compartment of a 15 mL gradient mixer that contains a magnetic stirrer and 2.5 mL of mix 2 is loaded in the upstream compartment separated by a valve. The gradient mixer is connected to a peristaltic pump to regulate the flow. The peristaltic pump is activated at a rate of approximately 3 mL/min and the valve separating both compartments is opened to allow the progressive mixing of both acrylamide solutions, hence generating an acrylamide and glycerol gradient. The mix is progressively poured between two 1 mm glass plates and 1 mL of water is carefully placed at the top of the gel to generate a straight interface. The gel is left to polymerize for at least 1h at room temperature. After removal of the water on top of the separation gel, the stacking gel (Table 9) is poured and left to polymerize for at least 1h at room temperature.

20 µL of sample usually containing about 5 µg of proteins is mixed with 5 µL of native loading buffer (300 mM Tris-HCl pH 6.8, 50% (v/v) glycerol, 0.5% (w/v) bromophenol blue in water) and loaded on the stacking gel. Molecular size is assessed using a native protein molecular weight ladder (Native Mark unstained protein standard, Thermo Fisher). CN-PAGE is run in native migration buffer (50 mM Tris, 384 mM glycine, pH 8.3 adjusted with HCl) at 80V until the bromophenol blue reaches the bottom of the gel (approximately 2h15min). The proteins on the clear-native gel can be stained by Coomassie blue (InstantBlue® Coomassie Protein Stain (ISB1L), ABCAM), by NoStain protein labeling reagent (Thermo Fisher) or by the silver nitrate staining method (Silver Stain, Bio-Rad).

**Table 8** Composition of the separation clear native gradient gel. The highest acrylamide concentration is at the bottom of the gel. Note the double gradient in acrylamide and glycerol concentration. N/A: not applicable.

Component	Concentrations in 12% gel (Mix 1)	Concentrations in 3.5% gel (Mix 2)
Acryl/Bis 37.5/1 30%	12% (v/v)	3.5% (v/v)
Tris pH 8.8	0.4 M	0.4 M
Glycerol	10% (v/v)	N/A
APS	0.1% (w/v)	0.1% (w/v)
TEMED	0.04% (v/v)	0.04% (v/v)
Table 9 Composition of the stacking gel.

Component	Concentration in stacking gel	
Acryl/Bis 37.5/1 30%	3% (v/v)	
Tris pH 6.8	0.125 M	
APS	0.1% (w/v)	
TEMED	0.04% (v/v)	

#### 5.6.6 Cryo-electron microscopy

Negative staining electron microscopy, sample vitrification and cryo-electron microscopy was realized by Irina Gutsche (IBS, MICA) and Maria Bacia-Verloop (IBS, MICA).

Briefly, for negative stain, sample was applied to the clear side of carbon on a carbon-mica interface, stained with 2% (w/v) uranyl acetate and floated on a copper grid. Images were recorded with a ThermoFisher Scientific T12 microscope at 120kV with a LaB6 filament, at a nominal 23,000× magnification. Freshly purified protein samples that have not been frozen were used in cryo-EM experiments. For cryo-EM, 3 microliter of sample was put on a Quantifoil 2:1 grid, vitrified with a ThermoFisher Scientific Vitrobot IV as described in Dubochet et al., 1988 and observed with a ThermoFisher Scientific Glacios 300 kV Field Emission Gun transmission electron microscope.

#### 5.7 *In vitro* lipid binding assay

To test the ability of a polypeptide to bind to lipids, an in vitro binding assay was performed. Different quantities of protein of interest (between 5µg and 15 µg) were incubated with 1 µg of commercial nitrobenzoxadiazole (NBD)-conjugated lipids in lipid binding buffer (50 mM Tris pH 8, 300 mM NaCl, 10% (v/v) glycerol) at 22°C for 1h. The NBD-conjugated lipids were NBD-PC, NBD-PE, NBD-PS and NBD-PA (Avanti polar lipids). The samples were run on a commercial CN-PAGE gel (Mini-Protean TGX, native gel, Bio-Rad) at 90v until bromophenol blue reached the bottom of the gel. NBD fluorescence was read directly in-gel using a fluorescence imager (Amersham Typhoon) using a 488 nm excitation laser and a Cy2 emission filter. Protein content was read by Coomassie blue staining (InstantBlue® Coomassie Protein Stain (ISB1L), ABCAM).

#### 5.8 Polyacrylamide gel electrophoresis in denaturing conditions (SDS-PAGE)

Polyacrylamide gel electrophoresis in denaturing conditions using sodium dodecyl sulfate (SDS) as denaturing agent enables to separate proteins contained in a complex protein sample according to their molecular mass only and independently from their intrinsic electric charge. An appropriate quantity of proteins, typically between 5 µg and 40 µg, are mixed with a four times concentrated denaturing buffer (LDS (4X) NuPAGE<sup>TM</sup>, Life technologies) and a ten times concentrated reducing buffer (Sample reducing agent (10X) NuPAGE<sup>TM</sup>, Life technologies) to charge proteins negatively and suppress their tertiary structure. Samples are heated at 95°C for 5 min to finalize their denaturation. Proteins are loaded on a polyacrylamide gel (NuPAGE<sup>TM</sup> 4 to 12 % Bis-Tris minigel, Life Technologies) and their migration

is carried out in a Mini Gel Tank (Life Technologies) first at 100V for 5 min, then at 80V for 1h45min. Proteins are co-migrated with a molecular weight marker (Precision Plus Protein<sup>™</sup> Dual Color Standards, BioRad) enabling to assess the molecular masses of the proteins contained in the samples.

If the proteins are not meant to undergo Western blotting after SDS-PAGE, the gel is colored by Coomassie blue (InstantBlue<sup>®</sup> Coomassie Protein Stain (ISB1L), ABCAM) for 30 min and washed three times in water prior to photography.

#### 5.9 Protein immunodetection by immunoblotting (Western Blot)

Immunoblotting enables to specifically identify a protein of interest in a complex protein sample previously separated by SDS-PAGE or CN-PAGE using antibody specificity. A nitrocellulose membrane (Amersham Protran 0.2 µm) and Whatman papers cut to the same size as the polyacrylamide gel are plunged into Laemmli 1X (Glycine 192 mM, Tris-HCl 25 mM, pH 8.3) containing 20% ethanol. A standard Western blot sandwich is assembled so as to place the polyacrylamide gel on the negatively charged pole and in direct apposition with the nitrocellulose membrane to enable the migration of negatively charged proteins from the gel to the membrane. Transfer was carried out at 90V and 300 mA for 1h with agitation and cooling. After transfer, the membrane is washed three times in water and reversibly stained with Ponceau red (0.2% w/v Ponceau red, 3% (v/v) trichloroacetic acid) to verify the presence of proteins on the membrane. The membrane is destained by three washes in Tris buffer saline (TBS; Tris-HCl 20 mM, NaCl 150 mM, pH7.6) and saturated in TBS-milk 5% (w/v) for at least 2h under agitation. Primary antibodies were applied on the membrane by dilution in TBS-milk; the dilution factor depends on the antibody (Table 10). The primary antibodies were applied on the membrane overnight at 4°C under mild agitation. If the primary antibodies are not coupled to the horseradish peroxidase (HRP), secondary antibodies coupled to the aforementioned enzyme have to be applied. In that case, the membrane was washed three times in TBS-milk and the secondary antibodies (Table 10) were diluted to 0.01% (v/v) and incubated 3h at room temperature under mild agitation. The membrane was washed three times 10 min in TBS to remove the unbound antibodies. The detection of HRPconjugated antibodies was achieved by applying Clarity<sup>™</sup> or Clarity Max Western ECL Substrate (BioRad) that contains luminol. The HRP catalyzes luminol oxidation, which generates light at 428 nm that can be detected using a luminescence detection system (Amersham ImageQuant 800).

Antibody name	Туре	Dilution used	Host	Reference
Anti-AtMIC60	Polyclonal	1/5000	Rabbit	(Michaud et al., 2016)
Anti-AtTOM40	Polyclonal	1/2000	Rabbit	(Michaud et al., 2016)
Anti-RISP	Polyclonal	1/10000	Rabbit	(Michaud et al., 2016)
Anti-COXII	Polyclonal	1/1000	Rabbit	Agrisera Ref. AS04053A
Anti-VDAC	Polyclonal	1/1000	Rabbit	(Michaud et al., 2016)
Anti-NDPK3	Polyclonal	1/5000	Rabbit	Agrisera Ref. AS08302
Anti-DGDG	Polyclonal	1/1000	Rabbit	(Maréchal et al., 2002)
Anti-His <sub>6</sub> Cter	Monoclonal	1/5000	Mouse	Life technologies Ref. 46-0693
Anti-RFP	Polyclonal	1/1000	Rabbit	Invitrogen Ref. R10367
Anti-HA	Monoclonal	1/5000	Mouse	Sigma Ref. H9658
Anti-Myc	Monoclonal	1/500	Mouse	Invitrogen Ref. MA1-980
Anti-Rabbit (2°)	Polyclonal	1/10000	Goat	Interchim Ref. 115-035-003
Anti-Mouse (2°)	Polyclonal	1/10000	Goat	Interchim Ref. 111-035-003

Table 10 List of primary and secondary (2°) antibodies used in this study.

#### 6 Lipid analysis

#### 6.1 Glycerolipid extraction

#### 6.1.1 Glycerolipid extraction from biological samples by the Folch method

The Folch method (Folch et al., 1957) was used to extract glycerolipids from solid biological samples. Lipids were extracted according to a modified Folch method (Simionato et al., 2013). Typically, approximately 0.5 g of callus or a quantity of isolated mitochondria equivalent to 600  $\mu$ g of mitochondrial proteins were used for lipid extraction. Calli are collected and dried using a Whatman filter and a vacuum filtration unit. Living plant material is either collected from a specific part of the plant (leaves, roots, hypocotyl) or from a whole seedling. Mitochondria are obtained as described in **section 4.1**. The collected biological material is immediately flash-frozen in liquid nitrogen and stored at -80°C to prevent lipase activity. Before extraction, the biological material is lyophilized overnight using a lyophilizator (Christ Alpha 2-4 LDplus) to remove water from the samples and prevent lipase activity.

The lyophilized samples, which are constantly maintained in liquid nitrogen to prevent lipase activity, are transferred to 35 mL Corex tubes pre-chilled in liquid nitrogen. Samples are then grounded using a

Potter-Elvehjem pestle to allow maximal surface contact with the reagents. 4 mL of boiling ethanol are added to the samples which are then incubated 5 min at 90°C to inhibit lipase activity. 2 mL of methanol and 8 mL of ethanol-stabilized chloroform (chloroform, Puriss. p.a., Reag. ISO, Reag. Ph. Eur., 99.0-99.4% (GC), Honeywell) are added and the samples are homogenized by bubbling argon through them which additionally protects the lipids from oxidation. Samples are incubated 1 h at room temperature under an argon atmosphere before being filtrated through quartz wool into another Corex tube to remove cellular debris. The organic phase containing the lipids is separated from the hydrophilic phase by adding 3 mL of chloroform/methanol (2:1 v/v) and 5 mL of NaCl 1% (w/v). After mixing with bubbled argon, the samples are centrifuged 10 min at 2000 rpm at room temperature and the inferior organic phase is collected. The lipid extracts are retrieved by evaporating the organic phase under argon. Lipids can be stored at -20°C for several months without degradation if maintained in an argon atmosphere.

#### 6.2 Methanolysis of total lipid extracts

Methanolysis is a nucleophilic transesterification reaction in which the acyl groups are detached from the glycerol skeleton and esterified to methanol thus forming fatty acid methyl esters (FAMEs) which can be used to determine the total lipid content of the lipid extracts. FAMEs were obtained according to (Jouhet et al., 2003). The lipid extracts are resuspended in 1 mL of ethanol-stabilized chloroform (chloroform, Puriss. p.a., Reag. ISO, Reag. Ph. Eur., 99.0-99.4% (GC), Honeywell) and 50  $\mu$ L are mixed with 5  $\mu$ g of internal standard (C15:0, a saturated odd chain fatty acid containing 15 carbons) and 3 mL of 2.5% sulfuric acid in methanol in sealable glass tubes. The methanolysis reaction takes place at 100°C for 1 h. While the acidic environment and the high temperature catalyze the transesterification reaction, the excess of methanol and absence of water displace the reaction equilibrium towards FAMEs formation. The tubes are left to cool for 5 min at room temperature before 3 mL of water are added to stop the reaction. To retrieve the hydrophobic FAMEs, 3 mL of hexane are added, the tubes are thoroughly mixed and the phases are allowed to form for at least 20 min. The upper organic phase containing the FAMEs is collected and the phase partition step is iterated. The collected hexane phases are evaporated under argon and the FAMEs can be stored at -20°C.

#### 6.3 Fatty acid and lipid quantification

### 6.3.1 Quantification of fatty acid methyl esters by gas chromatography coupled to ion flame detection

The FAMEs were resuspended in 50  $\mu$ L of hexane analyzed by gas chromatography-flame ionization detector (GC-FID) (Perkin Elmer Clarus 580) on a 30-m long cyanopropyl polysilphenesiloxane column (SGE BPX70) with a diameter of 0.22 mm and a film thickness of 0.25  $\mu$ m. The GC column is heated at 180°C and nitrogen is used as vector gas. FAMEs were identified by comparison of their retention times with those of standards (Sigma-Aldrich) and quantified by the surface peak method using the

C15:0 fatty acid internal standard for calibration. The measured FAMEs quantity allows to determine the glycerolipid concentration in the initial sample.

6.3.2 Quantification of glycerolipids by high performance liquid chromatography coupled to triple quadrupole mass spectrometry (HPLC-MS/MS)

Lipid analysis by high performance liquid chromatography (HPLC)-MS/MS was performed on the LIPANG (Lipid Analysis in Grenoble) platform directed by Juliette Jouhet. Quantification experiments were carried out by Juliette Jouhet, Mathilde Louwagie and Morgane Michaud.

The lipid extracts corresponding to 25 nmol of total fatty acids were dissolved in 100  $\mu$ L of chloroform/methanol [2/1, (v/v)] containing 125 pmol of each internal standard. Internal standards used were PE 18:0-18:0 and DAG 18:0-22:6 from Avanti Polar Lipid and SQDG 16:0-18:0 extracted from spinach thylakoid (Demé et al., 2014) and hydrogenated as described in Buseman et al., 2006. Lipids were then separated by HPLC and quantified by MS/MS.

The HPLC separation method was adapted from (Rainteau et al., 2012). Lipid classes were separated using an Agilent 1200 HPLC system using a 150 mm×3 mm (length × internal diameter) 5 µm diol 40°C. column (Macherey-Nagel), at The mobile phases consisted of hexane/isopropanol/water/ammonium acetate 1M, pH5.3 [625/350/24/1, (v/v/v/v)] (A) and isopropanol/water/ammonium acetate 1M, pH5.3 [850/149/1, (v/v/v)] (B). The injection volume was 20 µL. After 5 min, the percentage of B was increased linearly from 0% to 100% in 30 min and stayed at 100% for 15 min. This elution sequence was followed by a return to 100% A in 5 min and an equilibration for 20 min with 100% A before the next injection, leading to a total runtime of 70 min. The flow rate of the mobile phase was 200  $\mu$ L/min. The distinct glycerophospholipid classes were eluted successively as a function of the polar head group.

Mass spectrometric analysis was done on a 6460 triple quadrupole mass spectrometer (Agilent) equipped with a Jet stream electrospray ion source under following settings: Drying gas heater: 260°C, Drying gas flow 13 L/min, Sheath gas heater: 300°C, Sheath gas flow: 11L/min, Nebulizer pressure: 25 psi, Capillary voltage: ± 5000 V, Nozzle voltage ± 1000. Nitrogen was used as collision gas. The quadrupoles Q1 and Q3 were operated at widest and unit resolution respectively. PC analysis were carried out in positive ion mode by scanning for precursors of m/z 184 at a collision energy (CE) of 34 eV. SQDG analysis was carried out in negative ion mode by scanning for precursors of m/z -225 at a CE of -56eV. PE, PI, PS, PG, PA, MGDG and DGDG measurements were performed in positive ion mode by scanning for neutral losses of 141 Da, 277 Da, 185 Da, 189 Da, 115 Da, 179 Da and 341 Da at CEs of 20 eV, 12 eV, 20 eV, 16 eV, 16 eV, 8 eV and 8 eV, respectively. Quantification was done by multiple reaction monitoring (MRM) with 30 ms dwell time. DAG and TAG species were identified and quantified by MRM as singly charged ions [M+NH4]+ at a CE of 16 and 22 eV respectively with 30 ms dwell time. CL species were quantified by MRM as singly charged ions [M-H]- at a CE of -45 eV with

50 ms dwell time. Mass spectra were processed by MassHunter Workstation software (Agilent) for identification and quantification of lipids. Lipid amounts (pmol) were corrected for response differences between internal standards and endogenous lipids and by comparison with a quality control (QC). QC extract correspond to a known lipid extract from *Arabidopsis* cell culture qualified and quantified by TLC and GC-FID as described by (Jouhet et al., 2017).

#### 7 Imaging techniques

#### 7.1 Laser scanning confocal microscopy

Laser scanning confocal microscopy was performed on a microscope Zeiss LSM880 equipped with a 40x and a 63x/1.4 oil-immersed Plan-Apochromat objective, running Zen 2.3 SP1 acquisition software (Platform µLife, IRIG, LPCV). For rBiFC experiments, the 40x objective was used for acquisitions. The organelle markers used during confocal fluorescence microscopy are listed in Table 11. RFP was excited at 561 nm at 2.8% laser capacity and its fluorescence was detected between 582 nm and 622 nm with a photomultiplier tube detector set to 750V. YFP was excited at 514 nm at 2.8% laser capacity and its fluorescence was detected between 519 nm and 550 nm with a photomultiplier tube detector set to 750V. At least 8 images were taken randomly over the whole sample in two leaves and in three biological replicates. AtVPS13M1 localization experiments were performed using the 63x/1.4 oil-immersed Plan-Apochromat objective. eGFP was excited at 488 nm at laser capacities between 1.5% and 10% and its fluorescence was detected between 493 nm and 524 nm with a photomultiplier tube detector set between 700V and 800V. RFP was excited at 561 nm at laser capacities between 1.5% and 10% and its fluorescence was detected between 583 nm and 625 nm with a photomultiplier tube detector set between 600V and 800V. YFP was excited at 514 nm at laser capacities between 1.5% and 10% and its fluorescence was detected between 519 nm and 570 nm with a photomultiplier tube detector set between 600V and 800V. Chlorophyll was excited at 633 nm at laser capacities between 1.5% and 5% and autofluorescence was detected between 642 nm and 698 nm with a photomultiplier tube detector set between 600V and 700V. At least 8 images were taken randomly over the whole sample in two leaves and in three biological replicates. Images were visualized and treated with the ImageJ 1.53a software running the image processing package Fiji.

Name	Construct	Target organelle
ER-RFP	From Anne-Marie Duchene, IBMP, Strasbourg	ER
Mito-YFP	pFP108-ctYFP-TOM20.2 (split YFP C-terminus) pFP108-ntYFP-TOM20.3 (split YFP N-terminus)	Mitochondria
Mito-RFP	pFP108-CYS4-RFP	Mitochondria

Table 11 List of the constructs used as organelle markers during confocal fluorescence microscopy experiments.

## Part 1

# Characterization of the mitochondrial transmembrane lipoprotein complex

## 1 The discovery of the mitochondrial transmembrane lipoprotein (MTL) complex

The MTL complex has already been introduced in section 4.2 of Leterme & Michaud, 2022. A more detailed description of its discovery by Michaud et al. and further experimental characteristics are given in this introductory section.

The MTL complex was initially found during a radiolabeling experiment designed to identify complexes implied in chloroplast to mitochondria DGDG transport during Pi starvation (Michaud et al., 2016). During this experiment, the authors took advantage of the fact that when mitochondria are isolated on a Percoll cushion, fragments of plastid membranes remain attached and co-purify with mitochondria, probably due to mitochondria-chloroplast MCSs. Mitochondria from calli grown in presence or absence of Pi were isolated and incubated with radiolabeled UDP-galactose. UDP-galactose is used by MGDG and DGDG synthases to form their respective products, thus radiolabeling DGDG, the final product. The radiolabeled DGDG is then transported to mitochondria and tags the complexes through which it is transported. The mitochondrial membrane complexes were extracted and run on CN-PAGE which revealed the existence of a single radioactive band in both Pi replete and deplete conditions. The proteins associated to the radioactive band form a wavy band at an apparent size of 800 kDa.

The protein content corresponding to this wavy band was determined by mass spectrometry. The results reveal that the MTL complex contains about 200 proteins distributed mostly in both mitochondrial membranes (**Figure 15A.1**), but also in other membranes like the chloroplast OE or the ER. A protein was considered to be part of the MTL complex if it was retrieved in the wavy band on CN-PAGE in all the analyzed conditions, that is when the mitochondrial membrane complexes were solubilized using 1.5  $\mu$ g or 10  $\mu$ g of DDM per gram of mitochondrial complexes and in phosphate replete and deplete conditions. The mitochondrial components of the MTL complex are distributed among multiple mitochondrial complexes. Notably, all the components of the TOM complex are present in the MTL complex and AtMIC60 from the MICOS complex have also been identified. Furthermore, AtMIC60 and TOM40 have been shown to interact with each other (**Figure 15A.2**). Thus, the MTL complex spans through both mitochondrial membranes and forms an intramitochondrial contact site tether. The MTL complex is enriched in lipids and was shown to contain mostly DGDG, PE and PC. The lipid content might explain the wavy aspect of the band as well as the mismatch between the protein content and the apparent size of the complex.

During Pi starvation, Col0 presented an increase of DGDG content following its import in mitochondria and a decrease of PE content following its degradation, as expected (**Figure 15A.3**). Interestingly, the same changes in lipid content were reflected in the MTL complex (**Figure 15A.4**). Furthermore, the amount of AtMIC60 and TOM40 proteins both increased during Pi starvation while their total quantity

remained unchanged in whole mitochondria, suggesting that these proteins are relocalized to MTL complexes upon Pi starvation (**Figure 15A.5**, green arrows). In the *atmic60* KO mutant (**Figure 15B**), the amount of PE inside mitochondria and the MTL complex increase compared to Col0, suggesting a PE export defect (**Figure 15B.1**). A lack of DGDG was also observed in mitochondria and the MTL complex of *atmic60* KO mutants upon Pi starvation, suggesting a defect in DGDG import (**Figure 15B.2**). AtMIC60 has also been shown to destabilize liposomes *in vitro*. This led to hypothesize that AtMIC60 could promote lipid extrusion or insertion from or into membranes, hence facilitating lipid transport.

Altogether, these data support a model in which the MTL complex, and particularly AtMIC60, promotes PE and DGDG transport between both mitochondrial membranes (**Figure 15C**). Notably, its implication in Pi starvation-induced lipid remodeling strongly suggests a function in PE export to promote its degradation and DGDG import to replace the degraded phospholipids (**Figure 15C**). The position of the MTL complex in mitochondria suggests that it is implied in intramitochondrial lipid trafficking. However, the presence of extramitochondrial proteins in the MTL complex suggests that it could be implicated in the direct lipid channeling from other cell compartments, notably chloroplasts, to mitochondria, but the transport mechanism allowing the suspected lipid movements remain to be discovered.



in both mitochondrial membranes, but some are found in extramitochondrial compartments, notably in chloroplasts. ② AtMIC60 and AtTOM40 physically interact with abundance remains constant in whole mitochondria. (B) MTL complex and lipid movements in atmic60 KO mutant plants during phosphate starvation. ① PE content Figure 15 (A) MTL complex and lipid movements in Col0 plants during phosphate starvation. ① The MTL complex is composed of about 200 proteins. Most are distributed (4) Lipid remodeling is also observed inside the MTL complex. ③ AtMIC60 and AtTOM40 amounts both increase in the MTL complex upon phosphate starvation while their Model for the role of the MTL complex, and in particular AtMIC60, in intramitochondrial lipid trafficking. The MTL complex is likely implicated in mitochondrial DGDG increases in mitochondria and inside the MTL complex in absence of AtMIC60. (2) The quantity of imported DGDG decreases in *atmic60* mutants compared to Col0. (C) each other, forming an intramitochondrial tether. ③ The mitochondrial PE content decreases while the DGDG content increases upon phosphate starvation. mport and PE export upon phosphate starvation.

#### 2 Results

#### 2.1 Rationale

The MTL complex is an intriguing molecular arrangement between proteins and lipids, presumably spanning through the two mitochondrial membranes, at least. The initial determination of its composition from CN-PAGE revealed that it contained over 200 proteins belonging to different already known complexes mainly located in mitochondria, but also in other, and dispatched through multiple cell compartments, including the chloroplast. Given its number and variety of components as well as its enrichment in lipids, the MTL complex cannot be considered as a complex in the first biochemical sense of the word, but rather as a supercomplex. Even though the MTL complex might regroup a huge number of proteins, it is hard to imagine that all the identified components are permanent members of the complex. In this context, we wanted to get more insight on the composition, architecture and structure of the MTL complex. To this end, we decided to try to further purify the MTL complex using different biochemical and biophysical approaches to get access to a refined protein composition by proteomic analysis and to structural information by cryo-electron microscopy (cryo-EM).

After mitochondria purification by our standard protocol (see materials and methods), mitochondrial membranes were obtained by centrifugation after osmotic shock and mitochondrial complexes were extracted using different buffers complemented with  $1.5 \ \mu g$  DDM per  $\mu g$  of complexes (Figure 16A). The MTL complex presents multiple physico-chemical characteristics important for potential purification processes. First, the MTL complex is a huge complex enclosing multiple sub-complexes. This huge size is important to consider biochemical isolation methods based on the size of particle hydrodynamic radius like size exclusion chromatography or density gradient ultracentrifugation. Second, when the crude mitochondrial complexes are run on CN-PAGE, the MTL complex appears as a wavy band around 800 kDa (Figure 16B). On CN-PAGE, particles migrate according to their global charge, size and propensity to interact with the polyacrylamide matrix. The fact that the MTL complex is able to enter the clear native gel shows that it is carrying a net negative charge. Thus, it might be reasonable to attempt techniques based on electrostatic interactions like anion exchange chromatography. Finally, the MTL complex is enriched in lipids, namely PE and PC, the major mitochondrial lipids, and DGDG when cells are subjected to phosphate starvation. The presence of hydrophobic components in the MTL complex influences its separation on CN-PAGE as well as its charge, compaction and propensity to interact with diverse matrices, which is difficult to predict.

Therefore, to attempt the MTL complex purification, the crude complexes were subjected to different purification approaches, namely size exclusion chromatography (SEC), sucrose gradient ultracentrifugation followed by SEC and anion exchange chromatography (AEC) followed by SEC (**Figure 16C**). Since the MTL complex has no measurable activity, its characteristic migration pattern as a wavy band on CN-PAGE is the only way to detect its presence. This pattern will be used to



**Figure 16** Workflow of the MTL complex purification attempts. (A) Mitochondria were purified on a discontinuous Percoll gradient and their membranes were purified after breaking the mitochondria by osmotic shock. Mitochondrial complexes were extracted and solubilized with dodecyl- $\beta$ -D-maltoside (DDM) in a 1.5µg/1µg DDM/mitochondrial complexes ratio. (B) When the crude mitochondrial complexes are run on clear native polyacrylamide gel electrophoresis (CN-PAGE), the MTL complex appears as a wavy band around 800 kDa (red star). (C) Different biochemical purification techniques were attempted to purify the MTL complex. The final goal is to refine the protein composition of the MTL complex by proteomic analysis and to acquire structural information by cryo-electron microscopy.

determine the presence of the MTL complex and the amount of contamination by other complexes along the purification process. The integrity of the MTL complex will be tested by analyzing the presence of different known subunits by immunoblotting on CN-PAGE.

#### 2.2 MTL complex stability in solution

For its initial characterization, the MTL complex was kept in a buffer containing 50 mM imidazole pH 7, 0.5 M aminocaproic acid, 1mM EDTA (**Figure 17A**; buffer B1). Since most protein purification methods necessitate a different buffer composition with a particular ionic strength to prevent unwanted protein interactions, we set out to determine buffer compositions suitable to maintain MTL complex integrity (**Figure 17A**). In standard conditions (buffer B1), the MTL complex is recognizable by its migration pattern as a wavy band around 800 kDa on CN-PAGE (**Figure 17B**). To test the stability of the MTL complex in variable ionic strength environments, different NaCl concentrations were tested. While the buffers containing 50 mM NaCl (buffer B2) and 150 mM NaCl (buffer B4) caused the wavy band to appear at higher molecular masses, the buffer containing 100 mM NaCl (buffer B3) generated a MTL complex migration pattern similar to the original conditions (**Figure 17B**). Thus, we decided to keep the native complexes in buffer 3 containing 100 mM NaCl. To know if the MTL complex can be crosslinked to facilitate downstream applications, we added the crosslinking agent dithiobis-succinimidyl propionate (DSP) to buffer 1. In these conditions, the MTL complex loses its wavy shape and migrates at a higher apparent molecular mass on CN-PAGE (**Figure 17B**). Since the MTL complex

A		<b>B</b> 50 5 10 10 10 10 10 10 10 10 10 10 10 10 10
Buffer N°	Composition	
B1	50mM immidazole pH 7, 0.5M aminocaproic acid	
	(ACA), 1mM EDTA	* *
B2	30mM HEPES pH 7, 50mM NaCl, 1mM EDTA	/20 -
B3	30mM HEPES pH 7, 100mM NaCl, 1mM EDTA	480 -
B4	30mM HEPES pH 7, 150mM NaCl, 1mM EDTA	242
B5	50mM immidazole pH 7, 0.5M aminocaproic acid	242 -
	(ACA), 1mM EDTA, DSP crosslinked	66 -
		20 -

Α

CN-PAGE; Coomassie

Figure 17 Buffer selection for MTL complex solubilization in stable conditions and enabling downstream purification steps. (A) Composition of the envisaged buffers for complex solubilization. Buffer B1 corresponds to the buffer initially used (Michaud et al. 2016). Buffers 2-4 are classic protein buffers buffered by HEPES and containing increasing amounts of NaCl to verify the influence of different ionic strength environments on the stability and behavior of the MTL complex. Buffer B5 is the same as buffer B1 but with the addition of the crosslinking agent dithiobis-succinimidyl propionate (DSP). (B) Crude mitochondrial complexes were extracted and solubilized in the respective buffers described before. The complexes were run on CN-PAGE and proteins were stained by Coomassie. Red stars indicate the MTL complex on the CN-PAGE. ACA: aminocaproic acid.

is still visible in buffer 3 and since DSP seems to modify its aspect on CN-PAGE, we decided to use buffer 3 containing 100 mM NaCl to attempt the native purification of the MTL complex.

#### 2.3 MTL complex purification by size exclusion chromatography

When run on CN-PAGE, the MTL complex has an apparent molecular mass of 800 kDa (Michaud et al., 2016). The plant mitochondrial complexes have molecular masses ranging from 100 kDa to 1500 kDa and can be distinguished on two dimensional BN-PAGE/SDS-PAGE (Klodmann et al., 2011; Senkler et al., 2017). Thus, we tried to isolate the MTL complex from the other mitochondrial complexes by size exclusion chromatography (SEC). Given the huge size of the MTL complex and the uncertainty around its true molecular mass and hydrodynamic radius, we used a Superose 6 Increase 10/300 column that is able to discriminate between molecular masses ranging from 5 kDa to 5000 kDa.

A total of 520 µg of solubilized mitochondrial complexes were loaded on the SEC column. Elution was realized with a buffer containing 0.05% (w/v) of DDM to maintain a detergent environment and 1 mL fractions were collected over 1.5 column volumes. Since the 5 first collected fractions correspond to the column void volume (8.2 mL in our case), we monitored the elution on CN-PAGE beginning at the 6<sup>th</sup> fraction (Figure 18A). The CN-PAGE was stained using the silver nitrate method to maximize the sensibility of detection. The fractions 6-8 contain almost no protein complexes, indicating that our sample does not contain complexes with an apparent hydrodynamic radius bigger than 5000 kDa. The wavy band corresponding to the MTL complex appeared in fractions 11-16. Intriguingly, this wavy band

is observed at multiple apparent molecular masses ranging from 800 kDa to 1000 kDa depending on the fraction (**Figure 18A**). The wavy band size seems to follow a bell-shaped distribution with higher apparent molecular masses observed at the beginning and at the end of its appearance while the size closest to the one observed for the MTL complex in previous experiments is found in fraction 14 (**Figure 18A**). The broad distribution of the band of interest in the elution fractions suggests that either the complex sticks to the column due to its physico-chemical properties, or that it exists in multiple versions with different compositions and properties. Given the concordance between the wavy band observed in fraction 14 and the usual MTL complex. Fraction 14 also corresponds to the fraction with the highest protein content (**Figure 18B**). Interestingly, a complex with an apparent molecular mass of 720 kDa is eluted in fractions 10 and 11, thus removing this contaminant from the fraction of interest. However, multiple other contaminants of lower apparent molecular masses co-elute with the complex of interest in fraction 14, which probably contributes to the high protein concentration.

The UV absorbance diagram following the elution reveals the existence of multiple peaks (**Figure 18B**). Consistent with the CN-PAGE results, the absorbance starts to increase in fractions 10 and 11 to attain a first peak in fraction 12, which may correspond to the 720 kDa contaminant. Interestingly, the elution of the complex of interest in fraction 14 corresponds to a single, narrow and intense peak appearing after 16.3 mL of elution volume (**Figure 18B**). However, the high peak intensity is likely due to the high amount of contaminants observed in this fraction.

To verify that the biochemical integrity of the MTL complex is conserved during the SEC process, we checked for the presence of AtTOM40, AtMIC60, RISP (OXPHOS complex III) and COXII (OXPHOS complex IV) proteins, which are known to be components of the MTL complex (Michaud et al., 2016), in the complex of interest found in fraction 14. The immunoblots reveal that AtTOM40, AtMIC60 and RISP are indeed present in the fraction 14 MTL complex (**Figure 18C**). COXII is also partly present in the complex, but forms a smear beneath the wavy band, suggesting that it might be partly dissociated from the main complex. Such smears are also observed for AtMIC60 and RISP, further suggesting that a small subset of MTL complexes might be partly dissociated during the SEC process. However, all four proteins have been observed associated to the MTL complex, showing that it is resistant to harsh biochemical and biophysical manipulations even though a small population might lose some of its components.

Altogether, our data suggest that the MTL complex can be subjected to a purification step by SEC without losing its biochemical integrity. However, SEC alone is not sufficient to eliminate a big part of the contaminants that co-elute in the MTL complex-enriched fraction. Other biochemical methods will have to be tested and combined to isolate the MTL complex.



**Figure 18** MTL complex purification by size exclusion chromatography. (A) Size exclusion chromatography fractions (1 mL) run on CN-PAGE. Red asterisks indicate the band corresponding to the MTL complex. The input corresponds to the mitochondrial complexes loaded on the SEC column. About 2500  $\mu$ g of complexes were loaded on the SEC column. Each fraction has a volume of 1 mL. The pictures in red squares correspond to the areas of the clear native gels indicated with red dashed boxes with enhanced contrast. (B) Absorbance at 280 nm measured on the SEC column elution fractions as a function of the elution time. The elution flow was set to 0.4 mL/min. Collected fractions are indicated on the x axis in red. The fraction containing the biggest amount of MTL complex is highlighted in orange (fraction 14). (C) Western blot analysis of the fraction containing the MTL complex showing the presence of the indicated proteins. Red stars indicate the MTL complex.

#### 2.4 Redefining the apparent size and hydrodynamic radius of the MTL complex

The elution profile of the SEC revealed that the wavy band, defined as the marker for the presence of the MTL complex, is found at different apparent molecular sizes on CN-PAGE. This raises the question how the same complex can generate bands at different apparent molecular sizes. Whether this is due to differences in composition (and thus size) of the complex or to an effect of concentration is not known. We already observed that the higher the total protein content in a fraction, the smaller the apparent molecular size of the MTL complex on CN-PAGE (**Figure 18A**). Thus, we hypothesized that the MTL complex concentration has a direct effect on its apparent molecular size on CN-PAGE. To test this, we ran different quantities of SEC elution fraction containing the MTL complex on CN-PAGE and revealed with NoStain rather than by the silver staining method (**Figure 19A**). The results clearly show that the



**Figure 19** (A) 32  $\mu$ g, 16  $\mu$ g and 8  $\mu$ g of proteins from fraction 14 obtained by size exclusion chromatography (SEC) were run on CN-PAGE. Proteins were visualized with NoStain reagent. Red stars indicate the MTL complex. The higher the MTL complex quantity, the lower the apparent molecular mass of the wavy band. (B) Calibration curve of the Superose 6 Increase 10/300 GL size exclusion chromatography column used for the MTL complex purifications. Commercial pure proteins of different known molecular masses were run on the SEC column and their respective elution volumes (V<sub>e</sub>) were determined. The partition coefficient was calculated using the total column volume (V<sub>c</sub>; 24 mL) and the void volume (V<sub>0</sub>; 8.2 mL) as follows: (V<sub>e</sub>-V<sub>0</sub>)/(V<sub>c</sub>-V<sub>0</sub>). When the partition coefficient is plotted as a function of the base 10 logarithm of the molecular mass (MW) in Daltons, the linear regression allows to infer molecular masses of particles eluting with a discrete V<sub>e</sub>. The linear regression parameters are indicated on the graph. The red arrows represent the elution volumes of the MTL complex according to three independent SEC runs.

apparent molecular size of the wavy band increases as the quantity of elution fraction applied on CN-PAGE decreases. Protein and complex size determination from CN-PAGE data is most often misleading and inaccurate as the particles migrate according to their global charge, size and propensity to interact with the polyacrylamide matrix. The observed effect of complex concentration could be due to the modification of the overall particle size or charge by agglomeration, even though this remains to be confirmed.

To get more insight on the size of the MTL complex, we used our SEC data to infer the apparent molecular size of the MTL complex based on the hydrodynamic radius. The SEC column was calibrated according to the manufacturer's recommendations by plotting the partition coefficient of different standard globular proteins of known molecular weight as a function of the decimal logarithm of the molecular weight (**Figure 19B**). The SEC has been performed three times and the elution of the peak at which the MTL complex is observed in its most native state is comprised between 14.3 mL and 16.3 mL. By reporting these data on the SEC calibration curve, we obtained a theoretical molecular weight comprised between 87 kDa and 329 kDa for the MTL complex (**Figure 19B**). This calculated molecular mass is highly divergent from the one initially determined on CN-PAGE (800 kDa). This is not surprising, since the calibration curve has been set with soluble globular proteins which gives an

assessment of their apparent hydrodynamic radius on the column. Given the hydrophobic and insoluble nature of the MTL complex, the measure of its hydrodynamic radius is most likely erroneous. Furthermore, it is not known if the MTL complex can be considered as globular and as stated above, the apparent particle size on CN-PAGE depends on more than just the particle size.

### 2.5 Sucrose gradient ultracentrifugation combined with size exclusion chromatography

To try to eliminate other contaminants that could not be removed by size exclusion chromatography, we set out to try other purification methods. Purifying native membrane complexes has often been achieved by applying gradient ultracentrifugation techniques. Determining the right parameters for a particular purification, however, is a long and often empirical process implying many trials even though some general rules can be followed (Fernandez-Martinez et al., 2016; Voet & Voet, 2011). Since the MTL complex is supposed to have a molecular mass comprised between 100 kDa and 800 kDa, we chose to use a 5%-30% sucrose gradient in a buffer containing 0.05% DDM to maintain a detergent environment. Given the long ultracentrifugation time, we added a protein inhibitor cocktail in the buffer.

The crude mitochondrial complex extracts were loaded on the gradient from the top and centrifuged for 20h at 200 000 x g. The sucrose gradient was sampled from the bottom. The MTL complex appears in multiple fractions located at about 2/3 from the top, meaning that the complex crossed the gradient over a substantial distance which can be considered enough to enable a satisfactory separation (**Figure 20A & B**). The broad distribution of the MTL complex in the sucrose gradient fractions again suggests that the complex might exist in multiple states or versions with different compositions and/or different physico-chemical properties (**Figure 20B**). The differential centrifugation allowed to eliminate a contaminant with an apparent molecular mass of 720 kDa, probably the same that was eliminated by SEC alone (**Figure 20B** and **Figure 18B**). However, the MTL complex still co-purifies with several other particles of lower apparent molecular masses. This suggests that multiple complexes with similar hydrodynamic properties are present in the mitochondrial complex extracts and that additional purification steps have to be added.

In order to further separate the MTL complex from lower molecular weight complexes, we combined the sucrose gradient ultracentrifugation with an additional SEC step. Since the sucrose gradient fractions 6 to 9 seemed to have less low molecular mass contaminants, they were pooled, concentrated and loaded on a Superose 6 Increase 10/300 SEC column. The SEC elution UV profile reveals a much more clean peak compared to the elution profile of the SEC alone (**Figure 20C**). This peak, situated in fraction 14 and appearing after 15.7 mL elution volume, corresponds to the fraction that contains the highest amount of MTL complex (**Figure 20D**) and is consistent with the results obtained for SEC alone. Once again, the MTL complex is found in multiple SEC fractions, namely fractions 12-17 (**Figure 20D**).



Figure 20 MTL complex purification attempt by sucrose gradient ultracentrifugation and subsequent size exclusion chromatography. (Figure legend continued on next page).

Unfortunately, SEC following sucrose gradient ultracentrifugation was not sufficient to remove the low molecular mass contaminants and hardly improves the MTL complex purity compared to SEC alone (compare **Figure 20B** and **Figure 20D**).

**Figure 20** MTL complex purification attempt by sucrose gradient ultracentrifugation and subsequent size exclusion chromatography. (A) Illustration of the 10 mL 5%-30% sucrose gradient and the fraction distribution. Fractions were collected from the bottom after ultracentrifugation. (B) Sucrose gradient fractions run on CN-PAGE and stained using the silver nitrate method. The fraction size is 500  $\mu$ L. Fractions 6 to 9 were pooled and concentrated before loading on the size exclusion chromatography column. (C) Size exclusion chromatography UV profile. A single peak is observed, corresponding to fraction 14 (F14), indicating that the eluted particles have comparable sizes. The MTL complex is most abundant in fraction 14. (D) Size exclusion chromatography fractions run on CN-PAGE and stained using the silver nitrate method. The fractions 13 to 16 were individually analyzed by Western blot. (E) Western blot of the size exclusion chromatography fractions run on CN-PAGE.

To verify the biochemical integrity of the MTL complex after these consecutive treatments, we checked the presence of AtMIC60, AtTOM40, RISP and COXII in the SEC fractions 12-16 by immunoblotting (**Figure 20E**). AtMIC60, AtTOM40, RISP and COXII were all present in the MTL complex in the SEC fraction 14. A very strong signal can be observed in fraction 13 but it seems to contain a degraded version of the MTL complex since a smear appears below the expected MTL complex size for all four tested proteins. Only faint bands could be observed in faction 15 and no signal could be detected in fraction 16. The higher apparent molecular mass of the MTL complex in fractions 15 and 16 suggest that the complex quantity is lower in these fractions. However, the characteristic wavy band can still clearly be seen in fraction 15, suggesting that the MTL complex might have a different composition.

Altogether, our data show that the MTL complex conserves its biochemical integrity throughout complex manipulations, but that its purity is difficult to achieve as only few differences between the SEC alone and following sucrose gradient ultracentrifugation have been observed. Since the MTL complex appears to be quite tolerant to harsh biochemical manipulations, we set out to try other purification methods.

#### 2.6 Anion exchange chromatography combined with size exclusion chromatography The fact that the MTL complex can be observed on CN-PAGE shows that it is influenced by the electric field and hence has a negative net charge. Therefore, we hypothesized that the MTL complex could be separated from the other mitochondrial complexes using anion exchange chromatography (AEC). To test this, we loaded the solubilized mitochondrial complexes on a 1 mL Q-sepharose AEC column and applied NaCl gradient ranging from 5 mM to 400 mM. The AEC UV elution profile revealed five peaks distributed over the whole elution process, probably corresponding to different complex populations (Figure 21A). The AEC fractions run on CN-PAGE reveal a very broad distribution of the MTL complex over almost all the elution process (Figure 21B). The first six fractions contain almost no proteins except for fraction 6 in which very small concentrations of MTL complex can be seen. In fractions 7 and 8, the wavy band corresponding to the MTL complex appears isolated with almost no contaminants (Figure 21B). These fractions correspond to the shoulder appearing just before the first peak on the UV profile (see fractions 7 and 8 on Figure 21A). Peak 1 is distributed over fractions 10 and 11 in which the MTL complex co-purifies with high amounts of contaminants. It is not known what part of peak 1 can be attributed to the MTL complex or to the contaminants. However, comparing the



**Figure 21** MTL complex purification attempt by anion exchange chromatography (AEC). (**A**) Anion exchange chromatography UV absorbance profile. Multiple peaks appear during the elution and are assigned numbers (in red). (**B**) Anion exchange chromatography fractions run on CN-PAGE and stained using the silver nitrate method. The MTL complex appears in multiple fractions after fraction 7 (red stars). Fractions 1-6 (green box) and 7+8 (red box) were pooled together, concentrated and loaded on a size exclusion chromatography column.

intensity of the UV signal in fractions 7 and 8 in which the MTL complex is almost isolated with peak 1 suggests that the contaminants account for the majority of peak 1 intensity. Strikingly, the MTL complex can be found in all the remaining fractions. Whether the UV absorbance peaks 2 and 3 are caused by variations in the MTL complex or by the contaminants is not known. Such a broad distribution of the MTL complex in the AEC elution fractions once again suggests that the MTL complex exists in multiple states or versions with different compositions and/or different physico-chemical properties inducing its dissociation from the Q-sepharose matrix at different ion forces.

The level of isolation of the wavy band in fractions 7 and 8 and presumably corresponding to the MTL complex obtained by AEC was already a big achievement. But since cryo-electron microscopy requires samples uniform in particle size, SEC had to be applied on the AEC fractions of interest. This gave us the opportunity to add a supplementary purification step to try to further improve the purification. The AEC fractions 7 and 8 contained very low amounts of contaminants. Therefore, they were pooled and concentrated before being subjected to SEC. Fractions 1-6 contained low amounts of MTL complex, but were very pure and were therefore also pooled and subjected to SEC. The UV absorbance profiles of



**Figure 22** (A) Size exclusion chromatography UV absorbance profiles for the pooled AEC fractions 1-6 and 7+8. A single peak is observed indicating that the eluted particles have comparable sizes. Both peaks are eluted in fraction 14. (B) Size exclusion chromatography fractions run on CN-PAGE and stained using the silver nitrate method. Red stars indicate the presence of the MTL complex. The MTL complex is mostly found in fractions 14.

both SEC elutions exhibit a single peak (**Figure 22A**). These peaks both appear after 16.4 mL (AEC fractions 1-6, hereafter F1-6) and 16.1 mL (AEC fractions 7 and 8, hereafter F7+8) of elution volume and correspond to the elution of the MTL complex in the SEC fraction 14, which is consistent with previous SEC results (**Figure 22A & B**). In both cases, the MTL complex wavy band has been obtained at a purity never achieved before (**Figure 22B**).

To verify the presence of key MTL complex components in the purified fractions, we performed Western blot analysis on SEC fraction 14 run on CN-PAGE and SDS-PAGE (**Figure 23A**). Surprisingly, neither AtMIC60 nor AtTOM40 have been detected in the wavy band, showing that the MTL complex lost its two putative key components during the purification process (**Figure 23A**, CN-PAGE). Their loss has been confirmed by SDS-PAGE immunoblotting (Figure 23A, SDS-PAGE). While both AtMIC60 and AtTOM40 are present in total mitochondria and in the solubilized mitochondrial

complexes, neither can be found in the purified fractions except for a faint band corresponding to AtTOM40 in the F7+8 fraction, confirming that their loss occurred during the purification process (**Figure 23A**, SDS-PAGE). We further looked for MTL complex components and found that multiple VDACs were retained in the MTL complex. NDPK-3, which is known to migrate just beneath the MTL complex on CN-PAGE but is not part of it, is also found in the purified fractions. Interestingly, even though the putative key components AtMIC60 and AtTOM40 have been lost, the galactolipid DGDG is maintained in the MTL complex (**Figure 23A**). The MTL complex is known to contain lipids which are thought to play a structural role (Michaud et al., 2016). The presence of DGDG in an incomplete MTL complex is consistent with the idea that even though the MTL complex composition may vary, its components are held together by lipids.

That the wavy band corresponding to the MTL complex can exist without AtMIC60 and AtTOM40, which we thought were key components, is puzzling and raises the question how and when they have been lost. Western blot analysis on SDS-PAGE clearly show that both proteins were present in the mitochondria and mitochondrial complex extracts (Figure 23A). To answer this question, we analyzed the 28 first AEC elution fractions by Western blot following SDS-PAGE (Figure 23B). Interestingly, AtMIC60 is totally absent in the 13 first AEC fractions and starts to appear in fractions 14-16. AtTOM40 is already present in small amounts in the fractions 5-7, which is consistent with the previous Western blot results (Figure 23A). The amounts of AtTOM40 and AtMIC60 proteins increase gradually and are most abundant in fractions 20-22, which correspond to the second AEC UV absorbance peak (Figure 23B). The similar distribution profile of AtMIC60 and AtTOM40 is in accordance with previous results showing that both proteins interact together (Michaud et al., 2016). VDACs have a broad distribution and are found in variable amounts in all elution fractions (Figure 23B). Interestingly, VDACs are less abundant in the fractions 20-22 that contain high amounts of AtMIC60, which might indicate that both proteins are present in two different MTL complex sub-populations. Nevertheless, AtMIC60, AtTOM40 and VDAC are all three found in fractions 23-25. NDPK-3, which is not in the MTL complex, is highly abundant in the first AEC fractions and gradually decreases during the elution (Figure 23B).

Altogether, our data strongly suggest the existence of several sub-populations of MTL complexes that differ in protein composition. This modular complex organization is reflected by the broad distribution of the MTL complex in the AEC fractions and by the peculiar distribution of the putative key components AtMIC60 and AtTOM40. AtMIC60 and AtTOM40 seem to be characteristic only for a MTL complex sub-population while another is characterized by the presence of VDAC and/or other MTL complex components that have not been detected by immunoblotting. Interestingly, we also show that neither AtMIC60 nor AtTOM40 seem to be necessary to retain DGDG in the MTL complex. This huge diversity of MTL complex populations raises questions about the real identity of the MTL complex.



**Figure 23** (A) Western blot analysis of the size exclusion chromatography fractions after CN-PAGE (left column) and after SDS-PAGE (right column). (B) Western blot analysis of the anion exchange fractions. The corresponding UV absorbance profile of the elution is indicated above. The thee visible peaks are numbered as in figure 6A.

The MTL complex sub-population containing both AtMIC60 and AtTOM40 co-elutes with high amounts of contaminants that do not allow downstream applications (see fractions 20-22 in **Figure 21B**). The high amounts of contaminants found to co-purify with the MTL complex and the MTL complex sub-population diversity makes the MTL complex purification even more complex. It is unclear what techniques could be used to further improve the purification process.

#### 2.7 Proteomic analysis and cryo-electron microscopy

The fact that the MTL complex could retain DGDG without AtMIC60 or AtTOM40 but in presence of VDAC raised the possibility that this sub-complex population could also play a role in lipid transport. To gain more insight into the composition and structure of this MTL complex sub-population, proteomic analysis and cryo-EM imaging were performed on the pooled SEC fractions 14 and 15 originating from the AEC fractions 7 and 8 (**Figure 21** and **Figure 22**).

Proteomic analysis identified a total of 604 proteins in our sample. Among the five most abundant proteins, only two are part of the MTL complex (**Table 12**). Both proteins are also found at high abundance values in the original MTL composition dataset (Michaud et al., 2016). AtMIC60 and AtTOM40 have been detected in the fraction, but with a very low abundance, which is consistent with the anti-AtMIC60 and anti-AtTOM40 immunoblots (**Figure 23A & B**). Interestingly, all four *Arabidopsis* VDACs are detected with high abundance, especially VDAC1, VDAC2 and VDAC3 (**Table 12**). This is consistent with the results obtained in the anti-VDAC immunoblot and shows that VDACs are among the most abundant proteins composing the MTL complex sub-population we analyzed. NDPK3 is also found at high abundance, which corroborates with the immunoblot results

(**Table 12**). Unfortunately, none of the other MTL complex components confirmed by immunoblotting by Michaud et al. have been detected in the sample. Our proteomics results suggest that the purified fraction contains a MTL complex sub-population showing a substantially different composition than the one initially described. Notably, it is lacking most of the TOM complex components. Future research should focus on determining the composition of other AEC fractions or other purification methods.

**Table 12** Partial results of the proteomic analysis performed on the size exclusion chromatography fraction 14 originating from the pooled AEC fractions 7+8. The rank is determined based on the calculated protein abundances. The presence of a protein in the MTL complex is determined by the original list of components by Michaud et al. 2016. n.d.: not detected.

Rank	Accession	Protein name	In MTL complex	Abundance
1	AT3G08580	AAC1	No	1332698821
2	AT4G28390	AAC3	No	500324842
3	AT5G63400	ADK1	Yes	497123100
4	AT5G13490	AAC2	No	436164207
5	AT1G14980	CPN10	Yes	375124032
13	AT4G11010	NDPK3	No	127365606
15	AT3G01280	VDAC1	Yes	114772680
28	AT5G15090	VDAC3	Yes	47402153
36	AT5G67500	VDAC2	Yes	36027545
106	AT5G57490	VDAC4	Yes	6828034
180	AT2G37410	TIM17.2	Yes	2329245
266	AT4G39690	AtMIC60	Yes	1024495
495	AT3G20000	AtTOM40	Yes	87341
n.d.	AT3G27080	TOM20.3	Yes	n.d.
n.d.	AT2G42210	B14.7	Yes	n.d.
n.d.	ATMG00160	COXII	Yes	n.d.
n.d.	AT1G06530	PMD2	Yes	n.d.
n.d.	AT5G13430	RISP	Yes	n.d.
n.d.	AT3G52230	OMP24	Yes	n.d.

Even if the fractions did not contain the previously described version of the MTL complex, we decided to collect structural information by cryo-EM of a MTL complex sub-population in collaboration with Irina Gutsche (IBS, Grenoble). Before turning to cryo-EM, we observed the purified fraction by negative stain EM to verify the homogeneity of the particle size (**Figure 24A**). Uranyl acetate stain EM revealed that the particles were concentrated and presented a slight heterogeneity in size and shape, suggesting that the purification method was not sufficient to select a single complex. However, the quality of the grids was not sufficient to draw a definitive conclusion. Therefore, we set out to generate cryo-EM grids and observe our sample with a Glacios cryo-microscope (see materials and methods). Surprisingly, the sample was less heterogeneous than expected and ring-like particles of approximately 7 nm could be observed (**Figure 24B**). Such a size is probably insufficient for a complex like the MTL, with an expected molecular mass between 100 kDa and 1000 kDa. We hypothesized that these ring-like particles may correspond to VDAC since they are highly abundant in the fraction (**Table 12**). However, plant VDACs are proteins of approximately 30 kDa and with a diameter of 3.5 nm estimated by cryo-EM

(Homblé et al., 2012), which is too small to fit the observed particles. However, it cannot be excluded that the observed particles are VDAC oligomers. These particles may also correspond to the particular MTL complex sub-population we were hoping to see. Given the quality and the numerous uncertainties concerning the biochemical characterization of the sample, no further acquisitions were realized.



**Figure 24** (A) Negative stain electron microscopy on the size exclusion chromatography fraction 14 originating from the pooled AEC fractions 7+8. The fraction was deposited on EM grids and stained with uranyl acetate to increase the contrast. (B) Cryo-electron microscopy on the same fraction than in (A). The fractions was deposited on cryo-EM grids and imaged using a ThermoFisher Scientific Glacios cryo-electron microscope. Electron dense ring-like structures with a 7 nm diameter can be seen. Scale bars are indicated in red.

#### 3 Discussion and perspectives

The MTL complex is a huge mitochondrial complex containing approximately 200 proteins, spanning through both mitochondrial membranes and is implied in DGDG import and PE export to/from mitochondria upon phosphate starvation (Michaud et al., 2016). The goal of this study is to further investigate the composition, architecture and structure of the MTL complex, which requires its isolation from other mitochondrial membrane complexes. Here, we took advantage of the known physicochemical characteristics of the MTL complex to attempt its purification using different combinations of biochemical techniques. Using the presumably high molecular mass of the MTL complex as discrimination factor, we conducted size exclusion chromatography and density gradient ultracentrifugation experiments to separate it from the other mitochondrial membrane complexes. Both processes illustrate that the MTL complex is resistant to harsh biochemical treatments and that it occupies a large range of hydrodynamic radiuses, suggesting that sub-complexes varying in size and composition might co-exist. We next exploited the presumed negative charge of the MTL complex to isolate it by anion exchange chromatography. We show that AtMIC60 and AtTOM40, the proteins previously suggested to be the MTL complex key components, are not present in all the purification fractions in which the presence of the MTL complex has been identified. Additionally, neither AtMIC60 nor AtTOM40 seem to be important to retain DGDG in the complex and can be lost without affecting the migration pattern of the MTL complex. Taking all our data in consideration, we propose that different MTL complex sub-populations characterized by different protein and maybe lipid compositions or sub-complex arrangements co-exist.

#### 3.1 Size of the MTL complex

In the initial discovery of the MTL complex, about 200 proteins were considered to be part of the MTL complex. A complex containing that many proteins is expected to have a proportionally large molecular mass. However, the estimation of the MTL complex molecular mass using its electrophoretic mobility on CN-PAGE (800 kDa) was found to be inconsistent for a complex of that size (Michaud et al., 2016). This discrepancy may be explained by the lipid enrichment of the MTL complex that could modify its migration pattern. This inconsistency could also be explained by the way the MTL complex protein composition was determined in the first place. In fact, a protein was considered to be part of the MTL complex if it was retrieved in the wavy band on CN-PAGE in all the analyzed conditions, that is when the mitochondrial membrane complexes were solubilized using 1.5  $\mu$ g or 10  $\mu$ g of DDM per gram of mitochondrial complexes and in phosphate replete and deplete conditions (Michaud et al., 2016). Despite the stringent protein selection, it cannot be excluded that some proteins or complexes simply co-migrate with the actual MTL complex and hence artificially increase the number of components. In fact, immunoblots performed on CN-PAGEs showed that over twelve MTL complex components tested, two were actually not in the MTL complex but in a complex close to it or having a smeary migration pattern overlapping it (Michaud et al., 2016). Furthermore, since proteins and protein complexes migrate

according to their charge, size and shape on CN-PAGE, inaccuracies in molecular size assessment are a well-known phenomenon. In addition, the mitochondrial membrane complexes were solubilized with DDM, which is a non-ionic detergent that does not add electrical charges to the proteins it associates to.

Size exclusion chromatography separates particles contained in a complex sample according to their hydrodynamic radius, which is correlated to the molecular mass of a globular protein or protein complex. We found that the MTL complex was predominantly eluted in one particular fraction (**Figure 18**). The elution volume of the fraction of interest was between 14.3 and 16.3 mL, which allowed to calculate the theoretical molecular mass of the MTL complex based on the SEC column calibration. We found that the MTL complex has a theoretical molecular mass between 87 kDa and 329 kDa. This result is surprisingly low and remains difficult to explain. First, soluble and globular proteins were used to calibrate the SEC column. However, we expected the MTL complex to have a bigger hydrodynamic radius than most of the protein standards. We suspected that the lipid-rich nature of the MTL complex substantially contributes to the separation pattern we observed during SEC. On one hand, the hydrophobicity might lead to the agglomeration of the MTL complex, but this should result in an even bigger apparent hydrodynamic radius. On the other hand, the particular physico-chemical properties of the MTL complex might enhance interactions with the SEC column, resulting in a reduced apparent hydrodynamic radius. A third possibility is that the MTL complex adopts a completely unknown conformation due to the detergents added to the elution or to its intrinsic properties.

We further used sucrose density gradient ultracentrifugation to separate particles according to their density and/or sedimentation speed (**Figure 20**). The movement of a centrifuged particle in a viscous environment depends on two opposing forces: the sedimentation force and the friction force (Voet & Voet, 2011). The sedimentation force can be decomposed in two opposing components: the centrifugal force that depends on the mass of the particle and the buoyancy that depends on the volume and density of the particle (**Figure 25**). There are two ways to separate particles by centrifugation. In isopycnic centrifugation, particles are separated using their difference in density by centrifuging them until both components of the sedimentation force equilibrate each other. In zonal centrifugation, particles are



**Figure 25** Mathematical expressions of the sedimentation force ( $F_{sedimentation}$ ) and friction force ( $F_{friction}$ ) acting on the movement of a centrifuged particle in a viscous environment. m: particle mass,  $\omega$ : angular speed of the centrifuge, *r*: centrifugation radius,  $V_p$ : particle volume,  $\rho$ : particle density. Parameters highlighted in red vary according to the particle.

separated using the difference in sedimentation speed after a certain amount of centrifugation time. We currently do not know what type of centrifugation was performed on our sucrose gradient, but given the low centrifugation time (20h), it is probable that the ultracentrifugation was zonal. Surprisingly, the MTL complex was found in multiple fractions in the bottom half of the gradient. Such a broad distribution suggests that the MTL complex sedimented according to variable particle masses and/or particle volumes. It is therefore probable that the MTL complex does not represent a population uniform in molecular mass and/or particle volume.

Despite our attempts to assess the molecular mass and size of the MTL complex, the inconsistencies and uncertainties encountered during our experiments do not allow to give a precise answer. Further characterizations will be necessary. One way to assess the MTL complex size would be to include particles/molecules of known sedimentation parameters in the sample run on density gradient ultracentrifugation. Alternatively, if the MTL complex can be isolated with satisfactory purity, a multi-angle laser light scattering (MALLS) approach could be attempted.

#### 3.2 The MTL complex is divided into different sub-populations

Density gradient ultracentrifugation suggested a disparity in the MTL complex molecular mass and particle volume. Interestingly, anion exchange chromatography aiming to separate mitochondrial complexes according to their propensity to interact with a positively charged matrix revealed that the MTL complex displays a broad range of affinities for the Q-sepharose resin (**Figure 21**). Furthermore, the protein content analysis by immunoblotting of the AEC elution fractions unveils that the presumable MTL key components AtMIC60 and AtTOM40 are unevenly distributed in these fractions even though the MTL complex-specific wavy band can be seen in all of them (**Figure 23**). Taken together, these data strongly suggest that different MTL complex sub-populations differing in size and composition co-exist.

The MTL complex is an assembly of several mitochondrial but also extramitochondrial complexes. Proteins from the TOM, TIM, MICOS, respiratory chain complexes I-V and TIC complexes have been found in the MTL complex (Michaud et al., 2016). Notably, all the components of the TOM complex are present. Given the high number of functional complexes that compose the MTL complex, it is hard to imagine that all the MTL sub-complexes interact with each other at all times. For example, in yeast, Mic60 interacts with both TOM and SAM complexes while another MICOS component, Mic26, only interacts with the SAM complex (Bohnert et al., 2012). Another study in *A. thaliana* shows that AtMIC60 and DGS1 co-migrate with several mitochondrial complexes also present in the MTL complex on BN-PAGE, namely the TOM and respiratory complex III (L. Li et al., 2019). Interestingly, the amount of AtMIC60 associated to the respective complexes was not evenly distributed, suggesting that MICOS associates to different complexes with variable affinities. Furthermore, the fact that all these complexes can be separated on BN-PAGE and do not migrate together indicates that their interaction inside the MTL complex is either week or transient and that they might associate only when needed. A

recent study analyzing the mitochondrial proteome and lipidome in *A. thaliana* leaves retrieved 183 out of the 216 proteins initially identified in the MTL complex (Y.-T. Liu et al., 2023). While this shows that most MTL complex components are conserved across different plant tissues, this also suggests that the MTL composition may vary.

If multiple MTL complex sub-population co-exist, the question of their respective biological roles is an outstanding one. On one hand, it cannot be excluded that the biochemical techniques used to attempt the MTL complex purification partly dissociate some of its components, hence artificially generating variability in the physico-chemical properties of the MTL complex. On the other hand, these potential artefacts may help to reveal the MTL complex sub-population diversity that would otherwise go unnoticed. The MICOS complex is considered to be an interaction hub at the mitochondrial IM and its huge known interactome in yeast and human is consistent with its diverse functions in mitochondria (Mukherjee et al., 2021; Rampelt et al., 2017). Thus, the association of AtMIC60 with different partners is a convenient way to regulate the MTL complex functions according to the cellular and/or mitochondrial needs. It is interesting to note that a wavy band characteristic of the MTL complex could still be observed even in AEC elution fractions in which neither AtMIC60 nor AtTOM40 could be found (Figure 23A). This corroborates the finding that *atmic60* KO mutants still had a visible MTL complex on CN-PAGE (Michaud et al., 2016). Nonetheless, another MTL complex component, VDAC, was highly abundant in these fractions (Figure 23A) as confirmed by proteomic analysis (Table 12). This suggests that the MTL complex can exist even without its putative key components and that MICOS might just be one of the multiple MTL sub-complexes.

Overall, these rather speculative considerations suggest that the MTL complex exists in different configurations, each characterized by a specific arrangement of components. Such a modular complex arrangement is reminiscent of the well-documented case of the mitochondrial respiratory chain complexes. In fact, the respiratory chain complexes are known to assemble into plastic higher-order supercomplexes in various combinations to respond to physiological needs or stresses (Guan et al., 2022; Vercellino & Sazanov, 2022). For example, complex I and complex III assemble in response to ROS increase to ameliorate substrate channeling between both sub-complexes (Lopez-Fabuel et al., 2016). Complexes I and III can also be found in association with complex IV to form respirasomes (M. Wu et al., 2016). Interestingly, TIM23-2 (TIM17:23 complex) and B14.7 (complex I) are both dually localized in both TIM17:23 and B14.7 complexes in *A. thaliana* and seem to regulate the abundance of both complexes (Murcha et al., 2012; Y. Wang et al., 2012). Thus, the dynamic arrangements of complexes into higher-order structures and the exchange of components between complex sub-populations seem to be common features in mitochondrial membrane complexes. However, much remains to be done to determine the exact composition of the different MTL sub-populations and their biological relevance remains to be investigated.

#### 3.3 Lipids might hold the MTL complex components together

As mentioned above, the characteristic MTL complex migration pattern could still be observed in absence of AtMIC60 and AtTOM40. This suggests that the proteins initially defined as the MTL complex key components might not be necessary to maintain its migration pattern on CN-PAGE, and more speculatively, that other components play a scaffolding role for the MTL complex. Interestingly, the fractions without AtMIC60 and AtTOM40 still contained DGDG. Thus, neither of them are required to maintain DGDG in the complex. This is in accordance with previous lipidomic analyses performed on the MTL complex of atmic60 KO calli showing that the level of DGDG is similar to wild type in Pirich media, but that AtMIC60 is required to increase the MTL complex DGDG content in response to Pi starvation (Michaud et al., 2016). The MTL complex is known to be enriched in lipids, notably PE and PC as well as DGDG (Michaud et al., 2016). Even though the other lipid species have not been quantified in the AEC elution fraction of interest, it is possible that the lipid content provides the required environment to allow the interaction of different MTL complex components. This is consistent with the fact that the peculiar migration pattern has been attributed to the MTL complex lipid enrichment (Michaud et al., 2016). It is interesting to note that phospholipids like DPG and PE, play an important role in respiratory chain supercomplex stability by taking part in protein-protein interactions (Guan et al., 2022; M. Wu et al., 2016). Similarly, the chloroplast specific lipids MGDG, DGDG and SQDG have been found to be important for the stabilization of photosynthetic complexes and to play a role as cofactors for the photosystems (Boudière et al., 2014). These examples illustrate the more general concept that lipids play a crucial role in the stability, maintenance and function of complexes in densely packed membranes and that we are just beginning to understand the role of lipids in the MTL complex.

#### 3.4 Conclusions and perspectives

We took a biochemical approach to attempt the purification of the previously identified MTL complex with the aim to refine its composition, architecture and structure. We show that the biochemical manipulations used for the MTL complex purification conserve the MTL complex-specific migration pattern on CN-PAGE. We further show that the size and composition of the MTL complex are not homogenous and that multiple MTL complex sub-populations co-exist. Finally, we suggest that the lipid environment provides a scaffold for the association of the MTL complex components and promote the stability of different MTL complex sub-populations.

Much remains to be done to confirm and refine our conclusions. Testing other purification techniques by exploiting other physico-chemical parameters could be useful. To separate the mitochondrial membrane complexes according to their hydrophobicity, a hydrophobic interaction chromatography (HIC) approach could be tested. The MTL complex is enriched in lipids and therefore could be retained on the HIC column. Further characterizing the different MTL complex sub-populations would be another important step. Each AEC fraction could be analyzed for the presence of multiple MTL complex components to reveal the compositional variability. A similar analysis could also be performed on the density gradient ultracentrifugation fractions to ask whether the distribution in complex sedimentation correlates with its composition. We hypothesized that the different MTL complex sub-populations might have different biological roles. Future work should also concentrate on determining the functions of the sub-complexes if these can be identified.

Interestingly, proteins localized in the chloroplast outer and inner envelope have been identified in the MTL complex proteome (Michaud et al., 2016). Since the number of mitochondria-chloroplast contact sites significantly increases upon phosphate starvation (Jouhet et al., 2004) and given the role of the MTL complex in intramitochondrial lipid transport (Michaud et al., 2016), it is tempting to speculate that some MTL complex components might directly contact chloroplasts and maybe other compartments to create a direct lipid exchange hub. In yeast, genetic interactions between ERMES and MICOS components led to hypothesize that MICOS and its interacting partners like the TOM and SAM translocases may be part of a much larger supercomplex spanning through both mitochondrial membranes and maybe reaching the ER membrane and hence was called ER-mitochondria organizing network (ERMIONE) (Hoppins et al., 2011; Jin et al., 2015). ERMIONE provides a convenient communication system between ER and mitochondria and is thought to regulate multiple functions like lipid transport, mitochondrial protein import or mitochondrial membrane architecture (Wideman & Muñoz-Gómez, 2016). A similar network has also been described in mammals. The mitochondrial intermembrane space bridging (MIB) complex, which classically includes MICOS, the SAM complex and other components of the mitochondrial OM (Huynen et al., 2016), could also expand to the ER membrane using the multiple identified EMCS tethers and/or effectors like ORP5/8 (Monteiro-Cardoso et al., 2022). In plants, further investigating the links between the MTL complex and partners located in other compartments is assuredly an interesting and important prospect. Future studies will focus on promising potential chloroplastic interaction partners like OMP24, which is already under investigation in our laboratory.

The MTL complex is an atypical supercomplex whose peculiar properties and seemingly plastic nature make its structure and composition difficult to grasp. We propose that the MTL complex is composed of not yet identified core components that dynamically interact with other mitochondrial and/or extramitochondrial complexes/components depending on the physiological needs. The lipid enrichment likely plays an important role in stabilizing the interactions between the different sub-complexes. Future work should allow to better define and understand the identity and functions of the MTL complex.

### Part 2

## Identification and characterization of AtVPS13M1, a VPS13 homolog in *Arabidopsis thaliana*

### 1 VPS13 proteins – hydrophobic tunnels for bulk lipid transport between membranes

#### 1.1 Initial discovery and historical overview

Vacuolar protein sorting (VPS) 13 proteins have gained a lot of attention in the last years due to their widespread cellular and physiological effects as well as their peculiar biochemical properties. The original discovery, however, goes back to 1977 during a screen for the loss of intracellular carboxypeptidase Y (CPY) activity in yeast and the mutant corresponding to mutations in yeast VPS13 was initially named PEP9 (Jones, 1977). CPY is a vacuole-localized peptidase that transits from the Golgi complex through multivesicular bodies before attaining the vacuole (S. C. Li & Kane, 2009). Defects in the vacuolar protein sorting machinery leads to CPY secretion. This characteristic mistargeting was further used in several genetic screens in yeast to identify proteins involved in vacuolar protein sorting which were named either vpt mutants (Bankaitis et al., 1986; Robinson et al., 1988) or vpl mutants (Rothman et al., 1989; Rothman & Stevens, 1986). Both vpt and vpl mutants lists were later fused together and the protein initially named VPT2 was renamed VPS13 (Raymond et al., 1992). Another study aiming to identify the localization determinants of Kex2p in the trans-Golgi network (TGN) identified SOI1 (Redding et al., 1996) which was later characterized as a 3144 amino acid (aa) residues long protein (Brickner & Fuller, 1997). Thus, PEP9, VPT2, SOI1 and VPS13 are the same protein.

While the context of VPS13 discovery rather suggested a role in protein sorting and vesicular trafficking (De et al., 2017), an increasing number of studies found that VPS13 proteins in diverse organisms are required for a huge variety of cellular and organellar processes like meiosis and sporulation (Muñoz-Braceras et al., 2015; Park & Neiman, 2012; Stege et al., 1999), actin skeleton maintenance and cell morphology (Föller et al., 2012; Park et al., 2015; Rzepnikowska et al., 2017), mitochondrial function (Anding et al., 2018; Lesage et al., 2016; Park et al., 2016; Seong et al., 2018) cellular phosphatidylinositol phosphates level regulation (Park et al., 2015; Park & Neiman, 2012) and autophagosome and acrosome formation (Anding et al., 2018; Da Costa et al., 2020; Muñoz-Braceras et al., 2015). Their absence or mutation in humans lead to neurodegenerative diseases (Gauthier et al., 2018; Kolehmainen et al., 2003; Lesage et al., 2016; Rampoldi et al., 2001; Seong et al., 2018).

The variety of cellular and physiological effects of VPS13 proteins is astonishing and make it hard to define a particular function for these proteins. The progressive discovery of their localizations and biochemical properties paved the way for further investigations towards the understanding of the role played by these unusual proteins.
1.2 VPS13s are huge proteins localized at membranes and membrane contact sites and conserved throughout eukaryotic evolution

VPS13 proteins are huge molecules constituted of usually more than 3000 aa residues. VPS13 proteins are evolutionarily conserved among eukaryotes (Levine, 2022; Neuman, Levine, et al., 2022; Velayos-Baeza et al., 2004). While the general protein structure and length are conserved, the number of VPS13 paralogues varies in different eukaryotes. *Saccharomyces cerevisiae* has only one VPS13 protein called ScVPS13 while humans have four (HsVPS13A, B, C, D) (Velayos-Baeza et al., 2004), *Dictyostelium discoideum* has six (Leiba et al., 2017) and *Arabidopsis thaliana* has four (AtVPS13M1, AtVPS13M2, AtVPS13S) (Levine, 2022).

Given the huge size of the VPS13 proteins, the study of their subcellular localization has been challenging. However, in the last decades, the localizations of VPS13 proteins have been reported in several organisms and reviewed by Dziurdzik & Conibear, 2021 as well as in the present study. VPS13 proteins localize to a huge variety of membranes and MCSs in *Saccharomyces cerevisiae* (**Table 13**), *Homo sapiens* (**Table 14**), *Mus musculus*, *Drosophila melanogaster*, *Tetrahymena thermophila* and *Arabidopsis thaliana* (**Table 15**). This broad distribution on organelles and MCSs of various functions foreshows the importance of VPS13 proteins in multiple cellular processes.

Protein name	Organelle / MCS	References	
	Mitochondria – vacuole (vCL AMPs)	(Lang et al., 2015)	
	whiteholidina – vacuole (velawii s)	(Park et al., 2016)	
		(Lang et al., 2015)	
	Nucleus – vacuole (NVJ)	(Park et al., 2016)	
		(Bean et al., 2018)	
	Endosome – mitochondria	(Park et al., 2016)	
	ER – prospore membrane	(T. S. Nakamura et al., 2021)	
		(Park & Neiman, 2012)	
	Endosome	(Lang et al., 2015)	
ScVPS13		(Park et al., 2016)	
		(Huh et al., 2003)	
		(Park & Neiman, 2012)	
	Prospore membrane	(Park et al., 2013)	
		(Park et al., 2016)	
		(T. S. Nakamura et al., 2021)	
	Lipid droplets	(Grippa et al., 2015)	
	Peroxisome	(John Peter et al., 2017)	
	Golgi complex	(Kolakowski et al., 2021)	
	Autophagosome	(Dabrowski et al., 2022)	

 
 Table 13 Known localizations of ScVPS13 in the yeast Saccharomyces cerevisiae.
 vCLAMP: vacuolemitochondria patch, NVJ: nucleus-vacuole junction.

Protein name	Organelle / MCS	References	
	ER – mitochondria	(Kumar et al., 2018) (Muñoz-Braceras et al., 2019) (Yeshaw et al., 2019) (Guillén-Samander et al., 2022)	
HsVPS13A	ER – lipid droplet	(Kumar et al., 2018) (Park & Neiman, 2020)	
	Mitochondria – endolysosome ER – plasma membrane Golgi complex	(Muñoz-Braceras et al., 2019) (Guillén-Samander et al., 2022) (Hayashi et al., 2012)	
	Lipid droplet	(Yeshaw et al., 2019) (Baldwin et al., 2021)	
HsVPS13B	Golgi complex	(Seifert et al., 2011) (Seifert et al., 2015)	
	Endosome	(Koike & Jahn, 2019)	
	ER – endolysosome	(Kumar et al., 2018) (Cai et al., 2022)	
	ER – lipid droplet	(Kumar et al., 2018)	
	Lysosome	(Yang et al., 2016)	
HsVPS13C	Lipid droplet	(Yang et al., 2016) (Baldwin et al., 2021)	
	Mitochondria	(Lesage et al., 2016)	
	Phagophore	(Rogers & Foster, 2007) (Shui et al., 2008) (Trost et al., 2009)	
	Mitochondria – lipid droplet	(J. Wang et al., 2021)	
	ER – mitochondria	(Guillén-Samander et al., 2021)	
HeVPS13D	ER – peroxisome	(Guillén-Samander et al., 2021)	
110 11 0150	Golgi complex	(Guillén-Samander et al., 2021) (Baldwin et al., 2021) (Fasimoye et al., 2022)	

 Table 14 Known localizations of VPS13 proteins (HsVPS13A-D) in Homo sapiens.

 Table 15 Known localizations of VPS13 proteins in diverse organisms. N/A: not applicable, unknown.

Protein name	Organelle / MCS	References			
Mus musculus (4 VPS13 proteins)					
MmVPS13A	Golgi	(Hayashi et al., 2012)			
MmVPS13B	Acrosome	(Da Costa et al., 2020)			
		(Rogers & Foster, 2007)			
MmVPS13C	Phagosome	(Shui et al., 2008)			
		(Trost et al., 2009)			
Drosophila melanoga	ster (2 VPS13 proteins)				
DmVPS13A/C	ER – plasma membrane	(Faber et al., 2020)			
DmVPS13D	Lysosome	(Anding et al., 2018)			
Tetrahymena thermophila					
TtVPS13A	Phagosome	(Jacobs et al., 2006)			
		(Samaranayake et al., 2011)			
TtVPS13C Phagosome		(Jacobs et al., 2006)			
Arabidopsis thaliana (4 VPS13 proteins)					
AtVPS13M1	N/A	N/A			
AtVPS13M2	Pollen tube tip	(Tangpranomkorn et al., 2022)			
AtVPS13S	N/A	N/A			
AtVPS13X	N/A	N/A			

### 1.3 VPS13 proteins are lipid transport proteins

Even though the direct or indirect implication of VPS13 proteins in lipid transport has long been suspected, proving their function as LTPs remained a challenging task. Physiological, biochemical and structural data gathered over more than a decade were necessary to affirm that the primary biochemical function of VPS13 proteins is indeed to transport lipids between membranes (Leonzino et al., 2021; Melia & Reinisch, 2022).

#### 1.3.1 Insight on VPS13 lipid transport function from physiological data

#### 1.3.1.1 VPS13 proteins modify membrane lipid compositions

The first hints on a potential role of VPS13 proteins in lipid transport came from their effect on the cellular distribution and abundance of phosphatidylinositol phosphates (PIPs). PIPs levels in biological membranes play crucial roles as signaling molecules, in the recruitment of proteins at specific locations, in vesicular trafficking and many more (Balla, 2013). Yeast and human knock out mutants of ScVPS13 and HsVPS13A, respectively, caused a reduction in PIP pools (Park et al., 2015; Park & Neiman, 2012). The absence of ScVPS13 in yeast decreased PI(4)P and PI(4,5)P<sub>2</sub> levels while the absence of HsVPS13A decreased PI(4,5)P<sub>2</sub> levels in the plasma membrane and in the Golgi complex, suggesting a conserved role of VPS13 proteins in PIPs distribution. Even though these effects may be indirect, these data constitute the first evidence that VPS13 proteins have an impact on their phospholipid environment. Apart from modifications in the PIPs cellular distribution and quantity, no other lipid class was shown to be impacted by the absence of VPS13 proteins.

#### 1.3.1.2 VPS13 proteins play a role in membrane and organelle biogenesis and maintenance

Membrane and organelle biogenesis require huge quantities of lipids that have to be transported from a site of synthesis to their target membrane. Strikingly, VPS13 proteins are required at multiple membranes to sustain their rapid growth and/or maintenance.

During the process of sporulation in the yeast *Saccharomyces cerevisiae*, the four sets of chromosomes generated by meiosis are separated into individual cells by the fast-growing prospore membrane (Neiman, 2011). The absence of ScVPS13 in yeast leads to severe defects in prospore membrane expansion, closure and capture of nuclei (Enyenihi & Saunders, 2003; Nakanishi et al., 2007; Park et al., 2013; Park & Neiman, 2012). Similarly, the absence of DdVPS13C (TipC) in *Dictyostelium discoideum* leads to reduced spore formation, a process that also requires extensive membrane biogenesis (Muñoz-Braceras et al., 2015; Stege et al., 1999). Like the prospore membrane formation, the acrosome biogenesis, which is a male gamete-specific organelle formed *de novo* in spermatids during spermiogenesis, requires huge amounts of lipids to sustain its fast expansion (Khawar et al., 2019). The spermatids of mice devoid of MmVPS13B, a mouse homolog of human HsVPS13B, fail to form a normal acrosome and leads to infertility suggesting a role of MmVPS13B in lipid delivery to the acrosome (Da Costa et al., 2020).

The autophagosome is also a fast-growing membrane requiring the supply of lipids. Interestingly, ScVPS13 localizes to the phagophore rim, contributes to phagophore expansion together with ATG2 (Dabrowski et al., 2022) and is required for efficient autophagy (Lei et al., 2022). ScVPS13 likely functions in the late stages of autophagy as the autophagosomes did not fail to close in absence of ScVPS13 but rather to be cleared from the cytoplasm (Lei et al., 2022). Consistently, the autophagic flux was impaired in absence of DdVPS13C (TipC) and HsVPS13A (Muñoz-Braceras et al., 2015). Furthermore, the absence of HsVPS13A in humans and of DmVPS13D in *Drosophila* leads to mitophagy defects (Anding et al., 2018; Insolera et al., 2021; Yeshaw et al., 2019). As for the prospore membrane and acrosome membrane formation, the defects in autophagy observed in absence of VPS13 proteins strongly suggest a role in membrane expansion-associated lipid transport.

Peroxisomes are small organelles that rely on exogenous lipid supply for their biogenesis and expansion (Carmichael & Schrader, 2022). While the necessary lipid supply mainly relies on vesicular routes, it has been shown that peroxisome biogenesis can be carried out using non-vesicular pathways only (Raychaudhuri & Prinz, 2008). Interestingly, the absence of HsVPS13D causes the loss of peroxisomes in human cells (Baldwin et al., 2021). Similarly, ScVPS13 is required for peroxisome expansion in yeast since its loss leads to a reduction in peroxisome numbers (Yuan et al., 2022). Furthermore, HsVPS13D localizes to peroxisome membrane contact sites by interacting with Miro (Guillén-Samander et al., 2021). Thus, it is likely that HsVPS13D contributes to non-vesicular lipid transport to peroxisomes.

Pollen tubes are one of the fastest elongating structures in plants. Thus, during pollen tube growth, huge quantities of membranes have to be generated in a very short time and transported to the pollen tube tip, implying efficient transport mechanisms (Adhikari et al., 2020). In absence of AtVPS13M2 in *Arabidopsis thaliana*, pollen germination is abolished *in vivo* and drastically reduced *in vitro* (Tangpranomkorn et al., 2022). Furthermore, AtVPS13M2 localizes to the tip of growing pollen tubes. Even though non-vesicular lipid transport probably plays a major role in the previously cited processes, they are all known to heavily rely on vesicular trafficking. Since VPS13 proteins have been discovered in the context of and are known to play a role in vesicular transport, it cannot be ruled out that they act in membrane and organelle biogenesis in an indirect manner by regulating other processes.

Mitochondria have a limited lipid synthesis capacity and have to import most of their membrane lipids by non-vesicular transport since they are not connected to the endomembrane system as a result of their endosymbiotic origin. Multiple studies reported that the absence of ScVPS13 in yeast, of HsVPS13A, HsVPS13C or HsVPS13D in human and of DmVPS13D in *Drosophila* leads to mitochondrial phenotypes like membrane destabilization, altered mitochondrial morphology (round and enlarged), defects in transmembrane potential and respiration and impaired fission and elongation (Anding et al., 2018; Gauthier et al., 2018; Lesage et al., 2016; Park et al., 2016; Seong et al., 2018; Shen et al., 2021; Yeshaw et al., 2019). Some of these observed phenotypes could be attributed to defects in autophagy but the general mitochondrial dysfunction and elongation impairment suggest a general disturbance of membrane homeostasis.

Taken together, these data show that VPS13 proteins are generally associated to membranes and organelles necessitating the supply of huge amounts of lipids for their biogenesis and growth. However, it is very likely that VPS13 proteins also perform other cellular functions necessitating smaller lipid fluxes as suggested by their widespread cellular distribution.

#### 1.3.1.3 The ERMES bypass

The endoplasmic reticulum-mitochondria encounter structure (ERMES) is a well-studied EMCS tether and effector in yeast. Among its numerous attributed functions (Kornmann, 2020; Kundu & Pasrija, 2020), ERMES is known to mediate lipid transport between the ER and mitochondria (John Peter, Petrungaro, et al., 2022) (see general introduction). Yeast strains depleted for ERMES ( $\Delta ermes$ ) grow slowly, have enlarged and round mitochondria and have a decreased PE and DPG content (Kornmann et al., 2009). However, despite the mitochondrial and lipid phenotype, lipids were still delivered to mitochondria indicating that lipid transport to mitochondria is still occurring through other alternative pathways.

With the aim to identify possible pathways redundant with ERMES, genetic screens based on the identification of genes synthetic lethal with ERMES unveiled VPS39, a member of the HOPS (homotypic fusion and protein sorting) complex (Elbaz-Alon et al., 2014; Hönscher et al., 2014). This was particularly interesting as VPS39 overexpression increased the extent of vacuole-mitochondria contact sites (vCLAMPs) (Elbaz-Alon et al., 2014; Hönscher et al., 2014) and as VPS39 was shown to interact with both Ypt7 and Tom40 to link vacuoles to mitochondria (González Montoro et al., 2018). This suggested that VPS39-mediated vCLAMPs might be responsible for an alternative lipid transport route to mitochondria, especially because a conditional  $\Delta ermes \Delta vps39$  mutant had a severe defect in mitochondrial phospholipid delivery while  $\Delta ermes \Delta vps39$  was synthetic lethal (Elbaz-Alon et al., 2014). However, disruption of the Tom40 binding site in VPS39 almost abolishes vCLAMP formation, but did not affect VPS39 function in the HOPS complex and was not synthetic lethal with ERMES (González Montoro et al., 2018). Thus, VPS39 synthetic lethality with ERMES is rather caused by VPS39 dysfunction in the HOPS complex rather than in vCLAMPs.

Further forward genetics screens identified point mutations in the ScVPS13 gene suppressing the  $\Delta ermes$  growth and mitochondrial phenotypes (Lang et al., 2015; Park et al., 2016). Furthermore, ScVPS13 localizes to multiple MCSs including vacuole-mitochondria contact sites and is synthetic lethal with ERMES, suggesting a functional redundancy (Lang et al., 2015; Park et al., 2016). ScVPS13 is recruited to mitochondrial membranes by Mcp1 (Bean et al., 2018; John Peter et al., 2017) (see section 1.5.3). Interestingly, Mcp1 was initially identified as a high-copy suppressor of  $\Delta ermes$  phenotype (hence its name Mdm10 complementing protein 1, Mcp1) (Tan et al., 2013). This finding was further

corroborated by the fact that overexpression of Mcp1 in  $\Delta ermes$  background rescued the growth and mitochondrial phenotypes (John Peter et al., 2017). Furthermore, like ScVPS13, Mcp1 is synthetic lethal with ERMES (Lang et al., 2015) and expression of Mcp1 partially rescues the  $\Delta ermes$  lipid phenotype. These data suggest that Mcp1 recruits ScVPS13 to mitochondria to promote lipid transport from vacuole to mitochondria. That ScVPS13 or VPS39 overexpression alone are not sufficient to rescue the  $\Delta ermes$ phenotype reflects the fact that in both cases, rescue is limited by the amount of available Mcp1 to recruit ScVPS13 (John Peter et al., 2017). This, in turn, strongly suggests a lipid transport function for ScVPS13.

1.3.2 Insight on VPS13 lipid transport function from in vitro and in vivo biochemical data Given the difficulty of analyzing lipid binding and lipid fluxes directly in vivo, in vitro studies set out to tackle the question whether VPS13 proteins are able to bind and transport lipids in an artificial setup. The first 1350 aa residues of ScVPS13 were shown to bind glycerophospholipids in vitro with no selectivity apart from a slight enrichment in PC (Kumar et al., 2018). Further analysis on the same protein fragment revealed that a single protein molecule binds to approximately 10 glycerophopholipid molecules. To test whether ScVPS13 is able to transfer lipids, a FRET-based in vitro assay to detect lipid transport activity was carried out using the first 1350 aa residues of ScVPS13 (Kumar et al., 2018). Similar results were obtained during *in vitro* binding and transfer assays for full length human ATG2A protein that harbors extensive sequence and structural homology with VPS13 proteins, especially in its N-terminal region (Valverde et al., 2019). The results clearly show that the polypeptide is indeed able to transfer lipids between liposomes, strongly suggesting that VPS13 proteins are indeed able to transfer lipids at membrane contact sites. The low binding selectivity and the high number of bound lipids per protein further suggests that VPS13 proteins unspecifically transport large amounts of lipids, even though no impact on the cellular distribution or quantity of major lipids has been reported yet.

Even though *in vitro* data suggest that VPS13 proteins bind and transport lipids between membranes, a direct proof of lipid transport by VPS13 proteins *in vivo* was still missing. An elegant method enabling the tracking of mass tagged lipids in cells (Mass tagging-Enabled TrAcking of Lipids In Cells; METALIC) finally allowed to visualize direct transport of lipids between compartments in living cells (John Peter, Petrungaro, et al., 2022). Using this approach in cells lacking ERMES and ScVPS13 demonstrated that ScVPS13 is indeed transporting lipids between ER and mitochondria.

#### 1.3.3 Insight on VPS13 lipid transport function from structural data

Even though the lipid transport function of VPS13 proteins had already been inferred and hypothesized based on physiological and biochemical data, structural data was still missing to gain insight in the mechanistic details of lipid transport.

A 3D reconstruction of purified ScVPS13 based on negative stain electron microscopy (EM) at an estimated resolution of 30 Å showed that ScVPS13 forms a long rod-shaped structure with a length of

20 nm and a diameter of 4 nm. (De et al., 2017). The dimensions of the elongated structure are in accordance with the standard distance of an MCS where VPS13 proteins have been extensively located. The crystal structure of the first 335 aa residues of CtVPS13 from Chaetomium thermophilum shows that the polypeptide forms a hydrophobic pocket that can accommodate lipids (Kumar et al., 2018). Furthermore, a 3.75 Å resolution cryo-EM structure of the first 1390 aa residues of CtVPS13 revealed that the hydrophobic cavity previously observed is extended through most of the rod-like structure and is lined with hydrophobic residues (P. Li et al., 2020). This data suggests that VPS13 proteins transport lipids between membranes by forming a long hydrophobic cavity that can accommodate a huge number of lipids at once. The question asking whether VPS13 proteins transport lipids by forming a hydrophobic tunnel between two membranes or by acting as a shuttle was addressed by mutating a ring of hydrophobic aa residues in the hydrophobic cavity to hydrophilic ones in ScVPS13 guided by the CtVPS13 structure (P. Li et al., 2020). Since a non-functional ScVPS13 protein results in sporulation defects (see above), the mutated ScVPS13 was introduced into a  $vps13\Delta$  yeast background and checked for sporulation phenotype rescue. The mutated ScVPS13 failed to rescue sporulation, thus supporting the hypothesis that VPS13 proteins form hydrophobic tunnels between membranes to allow lipid transport. The tunnel hypothesis is further corroborated by the fact that human HsVPS13C forms 29 nmlong rod-like structures at ER – lysosome MCSs after cryo-ET observations (Cai et al., 2022). The advent of artificial intelligence (AI)-driven protein structure prediction tools like RoseTTAFold and AlphaFold2 enabled to get insight in the molecular architecture of VPS13 proteins (Baek et al., 2021; Jumper et al., 2021). Notably, the full length predictions of yeast ScVPS13 (Toulmay et al., 2022) and human HsVPS13A-C (Cai et al., 2022; Dall'Armellina et al., 2023; Guillén-Samander et al., 2022) reveal that VPS13 proteins form a continuous hydrophobic tunnel enriched in β-sheets and lined with hydrophobic residues forming a suitable environment to accommodate lipids and allow their transport between membranes (see below).

#### 1.3.4 Emerging model for VPS13 bulk lipid transport

The large amount of physiological, biochemical and structural evidence gathered over the last decade allowed to identify VPS13 proteins as functional lipid transport proteins operating at a huge diversity of MCSs. Currently, the widely accepted model proposes that VPS13 proteins form long hydrophobic tunnels spanning through the MCS to nonspecifically transport large amounts of lipids directly from one membrane to another (Leonzino et al., 2021; P. Li et al., 2020; Neuman, Levine, et al., 2022; Reinisch & Prinz, 2021). Other proteins and complexes have been reported to use a continuous hydrophobic groove to transport lipids between membranes. The three soluble ERMES complex subunits Mmm1, Mdm12 and Mdm34 each harbor a SMP domain and likely assemble head to tail to form a continuous hydrophobic conduit to mediate lipid transport between the ER and mitochondria in yeast (Jeong et al., 2017; John Peter, Petrungaro, et al., 2022; Kawano et al., 2018; Kornmann, 2020; Wozny et al., 2022). Similarly, the bacterial lipopolysaccharide (LPS) transport system (LPT) connects the inner and outer

membranes with multiple β-jellyroll fold-containing subunits that form a hydrophobic groove enabling LPS transport through the periplasm (Botos et al., 2016; Hicks & Jia, 2018; Luo et al., 2017). However, in contrast to VPS13 proteins, both ERMES and LPT complexes function by only binding the fatty acids of the transported lipids leaving the polar headgroup out of the cavity, are composed of multiple subunits and in the case of the LPT system is ATP-driven. VPS13 proteins are able to accommodate multiple lipids at the same time inside of their huge hydrophobic cavity that stretches through only one single protein. In that sense, VPS13 proteins are the first members of a new type of LTPs that perform bulk lipid transport at MCSs called bridge-like LTPs (Leonzino et al., 2021; Reinisch & Prinz, 2021). However, even though it has become clear that VPS13 are indeed LTPs, the mechanistic and thermodynamic details of this bulk lipid transport including lipid loading and unloading into the hydrophobic tunnel, directionality of the lipid flow and binding of lipid molecules inside the tunnel remain largely unknown but will be discussed below.

#### 1.4 Organization and structure of VPS13 proteins

# 1.4.1 Structure of the hydrophobic tunnel and general domain organization of VPS13 proteins

Experimental and predicted structures of VPS13 proteins show that they form elongated rods containing a hydrophobic tunnel decorated with additional functional domains (Cai et al., 2022; Dall'Armellina et al., 2023; De et al., 2017; Guillén-Samander et al., 2022; Kumar et al., 2018; P. Li et al., 2020). The main part of the structure is occupied by the hydrophobic tunnel, which is composed of tens of apposed  $\beta$ -strands that eventually form an enormous  $\beta$ -sheet continuous from one protein end to the other (**Figure 26**). The  $\beta$ -sheets form the bottom of the cavity and are lined with hydrophobic residues. Each  $\beta$ -strand is divided in two parts that form an angle to each other (>100° in VPS13 proteins, 30-60° in other VPS13-like proteins) and are separated by strand-braking residues like glycines or prolines (Neuman, Levine, et al., 2022) (**Figure 26**). Thus, the apposition of the  $\beta$ -strands generates a U-shaped tunnel. The continuous  $\beta$ -sheet does not form a planar surface but instead harbors an internal twist resulting in a  $\beta$ sheet superhelix (Cai et al., 2022; Neuman, Levine, et al., 2022). Loops,  $\alpha$ -helices as well as complete functional domains are inserted between the beta-strands without disturbing the continuity of the hydrophobic  $\beta$ -sheets. Some of these loops reach from one side of the hydrophobic tunnel to the other, thus roofing the hydrophobic  $\beta$ -surface.

VPS13 proteins usually harbor several canonical domains inserted between the  $\beta$ -strands or at their Cterminus (**Figure 26**). The most conserved domain among VPS13 proteins is the Chorein\_N domain that carries the name of the N-terminal part of the protein in which it was first described: Chorein/HsVPS13A (Rampoldi et al., 2001). This domain forms the N-terminal entrance of the hydrophobic tunnel. It is composed of an amphipathic helix followed by four hydrophobic, antiparallel  $\beta$ -strands. Sequence alignment of VPS13 proteins from various clades shows a high degree of conservation in structure and hydrophobic residues. The Chorein\_N domain is not only found in VPS13 proteins but also in ATG2, SHIP164 as well as Mdm31-related proteins (Levine, 2019). Another functional domain characteristic of VPS13 proteins is the VPS13 adaptor binding (VAB) domain. The VAB domain is constituted of about 600 aa residues and is inserted in the middle of the  $\beta$ -sheet sequence. However, structure predictions show that despite this huge insertion, continuity of the hydrophobic surface is assured by the apposition of both parts of the  $\beta$ -sheets (Levine, 2022). The VAB domain is composed of six repeated structural modules assembled end to end that form a crown around the hydrophobic tunnel according to a low resolution cryo-EM structure and structure predictions (De et al., 2017) (see section 1.5.3). The C-terminal end of the protein is composed of two domains. The ATG2\_C domain, standing for ATG2\_C-terminus homology domain, is composed of a bundle of long amphipathic helices (usually four) and is supposedly implied in membrane binding (see section 1.5.4). The last canonical domain usually located at the very C-terminal end of the VPS13 proteins is a PH-like domain, which is implied in protein-protein as well as protein-lipid interactions (see section 1.5.5).



RoseTTAFold prediction of Chaetomium thermophilum VPS13

**Figure 26** General domain organization and structure of VPS13 proteins. VPS13 proteins are composed of several functional domains. The linear layout of the *Chaetomium thermophilum* CtVPS13 protein is displayed on top. The structure of CtVPS13 has been predicted using RoseTTAFold and assembled with PyMol. The hydrophobic tunnel, Chorein\_N, VAB, ATG2\_C and PH domains constitute so-called canonical additional functional domains of VPS13 proteins found in almost all VPS13 proteins. The hydrophobic tunnel is composed of repeated tunnel units called repeating  $\beta$ -groove (RBG) domains (Neuman et al., 2022; Levine, 2022). The individual RBG domains are depicted by grey squares on the linear protein layout.



**Figure 27** On scale representation of VPS13 proteins from *Saccharomyces cerevisiae* (ScVPS13), *Homo sapiens* (HsVPS13A-D) and *Arabidopsis thaliana* (AtVPS13M1, M2, S & X) and their known domains. Domain and motif borders were extracted from HHpred, PHMMER and Levine 2022. Respective protein sizes are indicated on the left. The additional functional domains are indicated on scale on the protein maps and categorized in the legend below. PH: pleckstrin homology domain, FFAT: Two phenylalanines in an acidic tract motif, ATG2\_C: Autophagy-related protein 2 C-terminus homology domain.

Altogether, the hydrophobic tunnel, the Chorein\_N, the VAB, the ATG2\_C and the PH-like domains constitute the canonical functional domains of VPS13 proteins.

A more detailed remote homology and structural analysis of VPS13 proteins allowed the discovery of several non-canonical functional domains in human and plant VPS13 proteins (Levine, 2022) (**Figure 27**). These domains include ubiquitination-associated domains like the UBA or WWE domains. They also include domains forming  $\beta$ -helices,  $\beta$ -sandwiches and ricin-type lectin  $\beta$ -trefoils, which presumably bind diverse membrane- or protein-bound carbohydrate moieties. Finally, plant VPS13 proteins harbor domains that are generally known to associate to membranes like C2 domains,  $\beta$ -tripods or additional bundles of amphipathic helices. It is noteworthy that AtVPS13M1 and M2 have an additional PH domain

in their N-terminal part and that the C-terminal PH domain of AtVPS13X is absent but replaced by a bundle of amphipathic helices.

1.4.2 VPS13 proteins belong to the repeating  $\beta$ -groove lipid transport protein family VPS13 and ATG2 proteins are known to harbor the previously described elongated hydrophobic tunnel structure composed of a continuous  $\beta$ -sheet surface. Three other protein types, namely Hobbit (Hob), Tweek, and SHIP164, have been identified for presenting a similar topology (Neuman, Levine, et al., 2022).

The detailed analysis of the hydrophobic tunnel structure lead to the discovery of a new protein domain that forms a structural unit of the tunnel and was called repeating  $\beta$ -groove (RBG) domain (Neuman, Levine, et al., 2022) (**Figure 28A**). A RBG domain is composed of five antiparallel  $\beta$ -strands lined with hydrophobic residues on their concave face that form a hydrophobic  $\beta$ -sheet surface. The  $\beta$ -strands are followed by a variable loop that can be disorganized or harbor  $\alpha$ -helices. This structural motif is repeated a variable number of times depending on the protein type or paralog to form the full-length hydrophobic tunnel. In VPS13 proteins, the additional functional domains are usually inserted between two apposed RBG domains. While most RBG domains follow the standard topology, some have a slightly different structural arrangement (**Figure 28B**). Notably, the RBG domains forming the N-terminal and C-terminal ends of the hydrophobic tunnels are shortened in RBG proteins (Neuman, Levine, et al., 2022). Some RBG domains positioned in the middle of the RBG repeats can also be shorter (three  $\beta$ -strands; in Tweek) or longer (seven  $\beta$ -strands; in Tweek and AtVPS13X) (Levine, 2022; Neuman, Levine, et al., 2022) (**Figure 28B**).

In VPS13 proteins, the central RBG domains are either homologous to the N-terminal penultimate RBG domain or to the C-terminal penultimate RBG domain suggesting that the contemporary RBG proteins evolved from smaller RBG proteins by duplication and apposition of the RBG domains (Levine, 2022). Interestingly, RBG-like proteins harboring a similar fold have been described in the bacterial proteins AsmA, TamB and YicH, further suggesting that RBG proteins evolved from very simple bacterial proteins (Josts et al., 2017; Levine, 2019; Neuman, Levine, et al., 2022). These bacterial proteins could then have been passed on to eukaryotes by horizontal gene transfer as suggested by the presence of AsmA/TamB homologs in chloroplasts (TIC236) and mitochondria (Mdm31), two endosymbiotic organelles (Chen et al., 2018; Levine, 2019; Neuman, Levine, et al., 2022).

The different types of RBG proteins are conserved in diverse organisms however with some missing types in some clades (**Table 16**). To standardize RBG protein names in various organisms, a new nomenclature has been proposed (Braschi et al., 2022), but is not used here. Strikingly, some orthologues of the same RBG type dramatically vary in size between organisms. This is especially true for Tweek-like proteins as in yeast Csf1 has 2958 aa residues while in humans or *Drosophila* they contain more than 5000 aa residues. A substantial variability in size is also observed among VPS13 protein orthologs and paralogs. The only yeast VPS13 protein contains 3145 aa residues and 12 RBG repeats which is

ganisms. Proteins were retrieved from	SHIP164	Not conserved	SHIP164 UHRF1BP1	Dme1	G5EE16	Q54XA9	Q84R14 (At3g20720)
	Tweek	Csf1	KIAA1109 / BLTP1	Tweek	LPD-3	KIAA1109	Not detected
pe in various model or	Hobbit	Hob1 / Fmp27 Hob2 / Ypr117w	KIAA0100 / BCOX1	Hobbit	062191	Not detected	SABRE KIP
proteins classed by typ. AST search.	ATG2	Atg2	ATG2A ATG2B	Atg2	ATG2	ATG2	ATG2 / PEUP1
erent repeating β-groove an et al., 2022) or by BL	VPS13	ScVPS13	HsVPS13A HsVPS13B HsVPS13C HsVPS13D	DmVPS13A/C DmVPS13D	Q94010 Q9BIA3 / Q18183	DdVPS13A DdVPS13B DdVPS13C / TipC DdVPS13D DdVPS13D DdVPS13E DdVPS13F	AtVPS13M1 AtVPS13M2 AtVPS13S AtVPS13S
Table 16 List of the difflitterature (mostly Neum)	Organism	Saccharomyces cerevisiae	Homo sapiens	Drosophila melanogaster	Caenorhabditis elegans	Dictyostelium discoideum	Arabidopsis thaliana



**Figure 28** Repeating beta-groove (RBG) proteins are unified by their fundamental RBG building block (Neuman et al.,2022; Levine, 2022). (A) RBG domain organization in VPS13 proteins. (1) RoseTTAFold prediction of *Chaetomium thermophilum* VPS13 protein as described in **Figure 1**. (2) Hydrophobic tunnel of CtVPS13 stripped from the other functional domains. (3) Division of the hydrophobic tunnel in its individual RBG domain components. CtVPS13 is composed of 12 apposed RBG domains. Note that The central RBG domains all adopt a canonical RBG domain structure. The N-terminal RBG1 and C-terminal RBG12 domains have a different topology. (B) Observed variations in the RBG domain structure. While a canonical RBG domain is composed of 5 apposed  $\beta$ -strands followed by one loop usually carrying an  $\alpha$ -helix, some RBG domains can be bigger or smaller depending on the number of  $\beta$ -strands. The VPS13 N-terminal and C-terminal RBG domains have a different topology notably implying amphipathic helices.

comparable to human HsVPS13A that contains 3174 aa residues and also 12 RBG repeats. The other human VPS13 proteins are substantially bigger reaching up to 4388 aa residues and 15 RBG repeats for HsVPS13D. Some VPS13 proteins in *Chlamydomonas* and *Plasmodium* have been reported to respectively contain 7192 and 9307 aa residues and more than 30 RBG repeats (Levine, 2022). Therefore, it seems that RBG proteins are highly plastic, maybe to adapt to the huge variety of MCSs between all types of organelles and in differently ultrastructurally shaped organisms.

Deeper analysis of the VPS13 protein RBG domains also contributed to gain insight in the evolutionary history of VPS13 proteins (Levine, 2022). Since the N-terminal and C-terminal RBG domains are conserved in all VPS13 proteins and since the central RBG domains are derived from both penultimate domains, the contemporary VPS13 proteins likely originated from an ancestor harboring only these four domains. The ancestral human VPS13 protein HsVPS13A/B/C/D likely diverged at the six or seven RBG domains stage to give rise to HsVPS13A/C/D and HsVPS13B, confirmed by the structural divergence of the latter. According to phylogenetic analysis, the last eukaryotic common ancestor (LECA) expressed both VPS13B and VPS13A/C/D (Levine, 2022). A new divergence gave rise to HsVPS13D and HsVPS13A/C and finally the latter formed HsVPS13A and HsVPS13C. Interestingly, an internal three RBG domain repeat took place independently in HsVPS13C and D that both have 15 RBG repeats instead of 12. In *Arabidopsis thaliana*, AtVPS13M1, M2 and S have a standard RBG domain arrangement resembling the one in yeast ScVPS13 and human HsVPS13A, consistent with the hypothesis that VPS13A/C/D was expressed in LECA. AtVPS13X, however, harbors 9 RBG repeats only six of which are homologous to other VPS13 RBG domains, thus blurring its evolutionary origin.

Given the high structure similarity between RBG proteins, it is expected that they all function as bridgelike LTPs to promote bulk lipid transport between membranes like VPS13 and ATG2 proteins. Interestingly, all the RBG proteins are localized at MCSs (reviewed in (Neuman, Levine, et al., 2022)). In addition, all five RBG proteins have been identified as being located at yeast MCSs by a new high throughput MCS screening approach in yeast (Castro et al., 2022). Hobbit influences the cellular distribution of PI(4,5)P<sub>2</sub> in *Drosophila melanogaster* (Neuman, Jorgensen, et al., 2022) and regulates intracellular trafficking (Neuman & Bashirullah, 2018). In Arabidopsis thaliana, the Hob-like proteins SABRE and KIP mutants both present defects in pollen tube elongation (Procissi et al., 2003), consistent with the APT1 mutant phenotype in Zea mays (Z. Xu & Dooner, 2006). SABRE mutants are also defective in root hair patterning (Pietra et al., 2015) maybe caused by a defect in cell plate deposition leading to anarchic epidermal cell arrangement like observed in Physcomitrella patens (Cheng & Bezanilla, 2021). Pollen tube growth, root hair growth and cell plate deposition both heavily rely on vesicular trafficking (Adhikari et al., 2020; Hochholdinger et al., 2018; Smertenko et al., 2017), suggesting that regulation of vesicular trafficking might be a conserved function for Hob-like proteins. However, the mechanistic link between this regulation and a putative function as lipid transporter remains unknown. Csf1, a Tweek-like RBG protein in Saccharomyces cerevisiae, is implicated in the channeling of GPI-anchor lipid precursors at ER contact sites (Toulmay et al., 2022). Csf1 is also essential for the homeoviscous adaptation during cold stress and becomes necessary for lipid homeostasis in rewired conditions (John Peter, Cheung, et al., 2022; John Peter, Schie, et al., 2022; Tokai et al., 2000). Tweek was also reported to be required for phospholipid trafficking and cold resilience in Caenorhabditis elegans (C. Wang et al., 2022). SHIP164 has been shown to transport lipids between liposomes in vitro and to regulate endosome to Golgi retrograde trafficking (Hanna et al., 2022).

Consistently, SHIP164 has been proposed to promote early endosome buds biogenesis by acting as a lipid transporter as part of a complex at early endosome – Golgi complex contact sites (J. Wang et al., 2022).

Despite their differences in functional domain organization, size and localization, RBG proteins are linked by their unique RBG domain, by their putative primary function as lipid transporters and by their likely common evolutionary origin. Dissecting the molecular details enabling their vast array of cellular functions and localizations will be a challenging guideline for future research.

# 1.5 VPS13 hydrophobic tunnels are decorated by functional domains that determine their localization

#### 1.5.1 The N-terminal domain anchors VPS13 proteins to the endoplasmic reticulum

The Chorein\_N domain is highly conserved among VPS13, ATG2 and SHIP164 proteins. The first 57 aa residues of yeast ScVPS13 corresponding to the very N-terminal end of the Chorein N domain localizes to the ER in vegetative cells (T. S. Nakamura et al., 2021). Similarly, the first 47 aa residues of yeast ATG2 were sufficient to target the ER membrane (Kotani et al., 2018). Both these peptides start with an  $\alpha$ -helix that replace the first  $\beta$ -strand of the first RBG repeat (Levine, 2022) (Figure 28B & Figure 29B). In VPS13 and SHIP164 proteins, this first helix is amphipathic. This helix, which contains mostly uncharged residues and has a wide hydrophobic face, has been proposed to associate to less packed membranes that contain few anionic lipids like the ER membrane (Giménez-Andrés et al., 2018; Levine, 2022). ATG2 proteins, on the other hand, start with a hydrophobic loop followed by a short amphipathic helix (Levine, 2022). Interestingly, the mutation of three conserved residues (K-Q-R) in this helix to aspartic acid resulted in the loss of ER localization, suggesting that the rather positively charged motif is important for membrane interaction (Kotani et al., 2018). While data tends to indicate that the N-terminal part of the Chorein N domain is specifically targeted to the ER membrane by an amphipathic helix-lipid interaction, it cannot be excluded that some type of ER-associated proteins contribute to the binding as hinted by the implication of a conserved protein motif in ATG2 (Figure 29B).

#### 1.5.2 The VPS13 FFAT motifs interact with VAPs in the endoplasmic reticulum

In most VPS13 proteins analyzed so far, two phenylalanines (FF) in an acidic tract (hereafter FFAT motif) can be found inserted between the  $\beta$ -strands in the first half of the hydrophobic tunnel (Slee & Levine, 2019). The FFAT motifs are known to interact with vesicle-associated membrane protein (VAMP)-associated protein (VAP), which reside in the ER (Murphy & Levine, 2016). Accordingly, the FFAT motifs of human HsVPS13A and HsVPS13C are targeted to the ER membrane (Kumar et al., 2018). VAPs bind FFAT motifs on a positively charged patch of their MSP (major sperm protein) domain (Murphy & Levine, 2016) (**Figure 29C**). According to NMR data, the acidic residues around the conserved residues are first non-specifically recognized by the electro-positive patch allowing the

phenylalanines to engage into specific hydrogen bonds (Furuita et al., 2010) (**Figure 29C**). FFAT motifs are often found in combination with other domains that target the protein to a different membrane than the ER, making them ideal MCS tethers or/and effectors (Murphy & Levine, 2016). Consistently, cooverexpression of human HsVPS13C, which harbors a FFAT motif, and VAP resulted in ER-lysosome contact sites extension (Cai et al., 2022). Some VPS13 proteins like human HsVPS13D harbor a modified FFAT motif allowing its phosphorylation called phospho-FFAT (pFFAT) (Di Mattia et al., 2020; Guillén-Samander et al., 2021). pFFAT motifs harbor a phosphorylable residue that, if phosphorylated, creates specific interactions with the VAP electro-positive surface that differ from those formed with a standard phenylalanine (Di Mattia et al., 2020). Phosphorylation/dephosphorylation of the pFFAT motif is an easy and efficient way to rapidly regulate the binding of a protein to the ER (Kors et al., 2022). The pFFAT motif of HsVPS13D enables interaction with VAP-B and its recruitment to the ER as the mutation of the phosphorylable threonine abolished HsVPS13D recruitment (Guillén-Samander et al., 2021).

#### 1.5.3 The VAB domain interacts with multiple proteins at organelle surfaces

The VAB domain was first identified as DUF1162 in human VPS13 proteins (Velayos-Baeza et al., 2004). The VAB domain was also identified in the *Arabidopsis thaliana* VPS13 homolog AtVPS13S/SHRUBBY and renamed SHORT-ROOT binding (SHR-BD) as AtVPS13S was shown to interact with SHR (Koizumi & Gallagher, 2013; Rzepnikowska et al., 2017). However, the initial SHR-BD/DUF1162 domain was just approximately half the size of the actual VAB domain. Bean et al. determined that the ScVPS13 VAB domain extends over approximately 600 aa residues and is composed of six repeated conserved regions separated by small, less conserved sequences (Bean et al., 2018). Each repeat harbors an invariant asparagine residue towards the beginning of the sequence. The VAB domain was initially thought to fold into a 6 or 12 bladed  $\beta$ -propeller similar to a WD40-like fold (Dziurdzik et al., 2020; Dziurdzik & Conibear, 2021). The X-ray structure of the VAB domain of *Chaetomium thermophilum* CtVPS13 revealed that it is composed of six repeated  $\beta$ -sandwich-like folds taking the general shape of a fishhook (Adlakha et al., 2022) (**Figure 29D**). Each  $\beta$ -sandwich is composed of nine  $\beta$ -strands arranged in two  $\beta$ -sheets facing each other. The conserved asparagine is invariably situated at the end of the first (N-terminal)  $\beta$ -strand.

The ScVPS13 VAB domain has been found to interact with protein partners containing a proline-xproline (PxP) motif (Bean et al., 2018). The PxP motif is bound at the interface between the fifth and the sixth VAB repeat (Adlakha et al., 2022) (**Figure 29D**), which is consistent with the observation that these repeats are sufficient for adaptor protein binding (Dziurdzik et al., 2020). The PxP motifcontaining proteins known to be bound by the ScVPS13 VAB domain are Ypt35, Spo71 and Mcp1, which are located on different cellular membranes and direct ScVPS13 to the required location by competing at the PxP binding site (Bean et al., 2018). Ypt35 directs ScVPS13 to endosomes of NVJs depending on the yeast growth conditions (Bean et al., 2018). Spo71 directs ScVPS13 to the prospore membrane where it is needed for rapid membrane expansion (Park et al., 2013). Mcp1 recruits ScVPS13 to the mitochondrial membrane (John Peter et al., 2017).

The fact that, in yeast, only the fifth and sixth VAB repeats are required for PxP motif-containing proteins binding and that Spo71 and Mcp1 are found only in fungi raises questions about the utility of



Figure 29 VPS13 localization determinants. (Figure legend continued on next page)

Figure 29 VPS13 localization determinants. (A) Typical canonical domain organization of a VPS13 protein. All the represented domains have been found to be implied in VPS13 localization. The organelles, proteins or molecules that are contacted by the respective domains are indicated below the domains. ER: endoplasmic reticulum, Mito: mitochondria, LD: lipid droplet, PIPs: phosphatidylinositol phosphates, PM: plasma membrane. (B) The N-terminus of VPS13 and ATG2 proteins target the ER. Protein model: PDB ID 6CBC (Kumar et al., 2018), VPS13 Chorein N domains have an N-terminal amphipathic helix that might directly interact with ER membranes with not vet identified partners. The amphipathic helix is colored half red, half green. Note that the hydrophobic side of the helix (green) is turned towards the basket interior. (C) Canonical FFAT (two phenylalanines in an acidic tract) motif general mode of binding. Here, the interactions between the OSBP FFAT motif and the MSP (major sperm protein) domain of VAP-A is represented. On the left: model showing the interaction of acidic residues composing the acidic tract with the basic (negatively charged) MSP surface while the first phenylalanine and another hydrophobic residue interact inside hydrophobic pockets. On the right: molecular model of the same interaction (PDB ID 2RR3; Furuita et al., 2010). (D) Structure of the VPS13 adaptor binding (VAB) domain and its interaction with a PxP motif-containing peptide (PDB ID 7U8T; Adlakha et al., 2022). The VAB domain is divided into six successive  $\beta$ -sandwich-like repeats arranged as a fishhook. The PxP motif is bound in a cleft formed between the repeats 5 and 6. (E) Potential modes of binding of the ATG2\_C domain. On the left: molecular dynamics simulation of the interaction between HsVPS13B ATG2\_C domain and a lipid bilayer (taken from Dall'Armellina et al., 2023). This could be the non-specific mode of binding to lipid droplets or other membranes. On the right: ATG2\_C domains of CtVPS13 modelled with RoseTTAFold. The ATG2\_C domain might also engage into specific protein-protein contacts. (F) Structure of HsVPS13A C-terminal PH domain (model retrieved from Guillén-Samander et al., 2022). Interaction with mitochondria is mediated by the seventh  $\beta$ -strand ( $\beta$ 7) and the C-terminal  $\alpha$ -helix of the PH domain. Interaction with XK (X-linked Kx blood group antigen) at the plasma membrane is mediated by the same seventh  $\beta$ -strand ( $\beta$ 7) and the third  $\beta$ -strand ( $\beta$ 3). (G) Predicted structure of the CtVPS13 APT1 (Aberrant Pollen Transmission 1) domain. The APT1 domain has been found to bind PIPs with a yet to be discovered mechanism.

the rest of the VAB repeats (Dziurdzik & Conibear, 2021). Interestingly, the proteins MIRO1 and MIRO2 have been reported to recruit HsVPS13D to mitochondria or peroxisomes in humans by direct interaction with the VAB domain without the help of a PxP motif (Guillén-Samander et al., 2021). This suggests that the VAB domain is able to bind to adaptor proteins using different regions or motifs that have not yet been identified. For example, the ESCRT component TSG101 binds HsVPS13D by interacting with the VAB domain, presumably through a Pro-Ser-Ala-Pro motif (J. Wang et al., 2021).

#### 1.5.4 The ATG2\_C domain forms a bundle of amphipathic helices

The ATG2\_C domain, which was also recently nicknamed the "gondola" domain (Dall'Armellina et al., 2023), is a region with a high degree of homology to the ATG2 C-terminus and present in all known VPS13 proteins (Levine, 2022). Structure predictions show that the ATG2\_C domain folds into four main  $\alpha$ -helices that form two bundles of antiparallel helices (Dall'Armellina et al., 2023; Levine, 2022) (**Figure 26 & Figure 29E**). The number of  $\alpha$ -helices present in the domain seem to vary from species to species. While in yeast ScVPS13 presents only four helices, human VPS13 proteins seem to harbor six (Dall'Armellina et al., 2023).

The very long helices composing the ATG2\_C domain are amphipathic and resemble domains found in lipid droplet (LD)-binding proteins (Dall'Armellina et al., 2023; Kumar et al., 2018) (**Figure 29E**). Consistently, protein fragments from HsVPS13A, C and D containing the complete ATG2\_C domain or only some of its amphipathic helices localized to LDs (Kumar et al., 2018; J. Wang et al., 2021). The ATG2\_C domain of ATG2 proteins have also been shown to associate to LDs. Protein fragments

containing the two C-terminal amphipathic helices of human ATG2A (1723-1829) and ATG2B (1863-1982) as well as a single ATG2A amphipathic helix (1830-1938) were sufficient to localize to LDs (N. Tamura et al., 2017; Velikkakath et al., 2012).

The ATG2\_C domain also participates in binding to other organelles (**Figure 29E**). Recent AlphaFold2 complex predictions suggest that the ATG2\_C domain could interact with the hydrophobic transmembrane surface of X-Linked Kx Blood Group (XK) scramblase which could stabilize the HsVPS13A-XK interaction at the plasma membrane (Guillén-Samander et al., 2022). In human ATG2A, the C-terminal region containing two amphipathic helices (1776-1820) is sufficient to localize to ER-mitochondria contact sites (Z. Tang et al., 2019). The same study also showed by co-immunoprecipitation that these 45 residues were sufficient to pull down TOM40. Three conserved arginines in the first helix seem to mediate TOM40 interaction since their mutation disrupts TOM40 pull down.

The ATG2\_C domain in ATG2 proteins is also important for the binding of autophagosomal membranes. In yeast, disruption of the C-terminal domain (aa residues 1347-1592) of ScATG2 which contains the ATG2\_C domain compromised its localization to the autophagosome (Kotani et al., 2018). Similarly, the deletion of the two C-terminal amphipathic helices of human ATG2A (1723-1829) and ATG2B (1863-1982) caused severe autophagy defects (Velikkakath et al., 2012).

The mechanism by which the ATG2\_C contributes to specific membrane binding is not yet fully understood. Molecular dynamics simulations show that the ATG2\_C domains from all human VPS13 proteins are deeply embedded into the membrane bilayer by disturbing the membrane with their amphipathic helices (Dall'Armellina et al., 2023). It is possible that ATG2\_C sometimes contributes to binding specific targets. However, the data presented here rather suggests that the ATG2\_C domain non-specifically associates to membranes with its hydrophobic face formed by the amphipathic helices to stabilize specific interactions engaged by other localization determinants that might otherwise be transient (**Figure 29E**).

1.5.5 The VPS13 pleckstrin homology-like domains have multiple lipid- and proteinbinding modes

Almost all VPS13 proteins harbor a PH-like domain at their C-terminus. *Arabidopsis thaliana* AtVPS13M1 and M2 both have a second PH-like domain in their N-terminal region. One exception is AtVPS13X that is devoid of PH domain at its C-terminus according to predictions realized with HHpred (Levine, 2022). Pleckstrin homology (PH) domains are present in a huge variety of proteins (Blomberg et al., 1999; Le Huray et al., 2022). PH domains fold into a seven-stranded  $\beta$ -sandwich capped at one end by a C-terminal  $\alpha$ -helix (Blomberg et al., 1999; Yoon et al., 1994) (**Figure 29F**). The PH domain fold developed hundreds of variations in structure and most of all in sequence to adapt to the functional

requirements (Le Huray et al., 2022; Scheffzek & Welti, 2012). The PH-like domains still remain recognizable by their structural arrangement.

The first function discovered for PH domains is phosphoinositide binding. PH domains are known to bind to a huge diversity of phosphoinositides through specific protein-lipid interactions taking place in a dedicated site classically described as a positively charged pocket situated in the middle of the  $\beta$ -sheets (Blomberg et al., 1999; Ferguson et al., 1995; Yoon et al., 1994). PH domains also exhibit a general membrane binding function by other means than the canonical binding pocket (Le Huray et al., 2022; Singh et al., 2021; Yamamoto et al., 2020). *In vitro* data show that a peptide containing a part of the ATG2\_C and the PH-like domain of yeast ScVPS13 (2905-3144) strongly binds to liposomes enriched in PI(4,5)P<sub>2</sub> but not in other PIP species (De et al., 2017). Similarly, the PH-like domains from ScVPS13 and HsVPS13A both bind to PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>2</sub> and efficiently bind liposomes enriched in PI(4,5)P<sub>2</sub> (Kolakowski et al., 2021), showing that VPS13 PH-like domains are indeed able to bind PIPs.

Beside their role as PIPs binding modules, PH domains have been shown to mediate protein-protein interactions (Lenoir et al., 2015; Scheffzek & Welti, 2012). The ScVPS13 and HsVPS13A PH-like domain interact with the small GTPase Arf1 (ADP ribosylation factor) according to coimmunoprecipitation assays (Kolakowski et al., 2021) (Figure 29A). Further in vivo localization assays revealed that the PH-like domain of ScVPS13 is required to interact with Arf1 at the Golgi complex (Kolakowski et al., 2021). Arf1 also plays important roles in mitochondrial morphology and division especially in association with ER-mitochondria contact sites (Ackema et al., 2014; Tábara et al., 2021). Interestingly, a fragment containing the PH-like domain of HsVPS13A localized to mitochondria (Guillén-Samander et al., 2022; Kumar et al., 2018; Yeshaw et al., 2019). The exact structural features of the HsVPS13A PH-like domain responsible for the association with mitochondria could also be precisely determined: the C-terminal  $\alpha$ -helix and the seventh  $\beta$ -strand are necessary for binding to mitochondria (Guillén-Samander et al., 2022) (Figure 29F). Whether these structural features directly interact with the mitochondrial membrane or with particular proteins remains to be determined. Human HsVPS13A directly interacts with XK, a lipid scramblase active at the PM (Park & Neiman, 2020; Ryoden et al., 2022). This interaction is mediated by the PH domain of HsVPS13A that is required to direct the protein to the PM (Guillén-Samander et al., 2022). During the HsVPS13A-XK interaction, the seventh  $\beta$ -strand of the PH-like domain apposes to a  $\beta$ -hairpin in the second cytosolic loop of XK (Guillén-Samander et al., 2022) (Figure 29F). It is worth mentioning that the seventh beta-strand of the HsVPS13A PH-like domain is necessary for both mitochondria and XK binding.

The C-terminal PH-like domain of VPS13 proteins seem to be suited for both PIP and protein binding (Kaminska, 2022). The regulation allowing the commitment to one or the other mode of binding, however, remains to be clarified.

#### 1.5.6 The APT1 region presents affinity for phosphoinositides

Often, the domain annotation APT1 (ABERRANT POLLEN TRANSMISSION 1) is found towards the C-terminal part of the hydrophobic tunnel when standard domain prediction tools are used (HHpred, HMMER). This annotation reflects a high degree of homology with the APT1 protein from *Zea mays* that forms a similar hydrophobic tunnel (Neuman, Levine, et al., 2022; Z. Xu & Dooner, 2006). This is not surprising given that APT1 is a Hobbit-like protein homologous to SABRE and KIP proteins (see **section 1.4.2**). The APT1 region is composed of seven  $\beta$ -strands that form the C-terminal part of the hydrophobic tunnel and of an  $\alpha$ -helix perpendicular to the axis of the rod (**Figure 29G**).

Despite being part of the hydrophobic tunnel, the APT1 regions from yeast ScVPS13 and human HsVPS13A have been reported to bind phosphoinositides (PI(3)P for ScVPS13 and PI(3)P and PI(5)P for HsVPS13A) (Kolakowski et al., 2020; Rzepnikowska et al., 2017) (**Figure 29G**). Similarly, the APT1 region of yeast ATG2 also binds PI(3)P (Kaminska et al., 2016). Interestingly, the I2771R mutation in HsVPS13A, situated in the second last  $\beta$ -strand at the C-terminal end of the hydrophobic tunnel, drastically reduces the ability of the APT1 region to bind PI(3)P and the structurally equivalent mutation in ScVPS13 had a similar effect (Rzepnikowska et al., 2017). These observations suggest that the C-terminal end of the hydrophobic tunnel might specifically bind to phosphoinositides to regulate its localization. Why this part of the protein would bind specifically to phosphoinositides, however, remains to be clarified. In fact, the APT1 region is structurally very similar to any part of the hydrophobic tunnel which could have the exact same binding properties. Lipid binding assays performed with N-terminal parts of the tunnel have shown an enrichment in PE and PC, but not in phosphoinositides



**Figure 30** Domain organization and RoseTTAFold structure prediction of AtVPS13M1 protein. The light grey square represent single RBG domains. Sections of 1200 amino acid residues were submitted to the RoseTTAFold server. The other overlapping fragments are not shown. It is noteworthy that all the additional canonical and non-canonical functional domains protrude from the hydrophobic tunnel, presumably to avoid disturbing the lipid transport function and to facilitate interaction with partners. PH: pleckstrin homology domain, FFAT: two phenylalanines in an acidic tract motif, VAB: VPS13 adaptor binding domain, ATG2\_C: Autophagy-related protein 2 C-terminus homology domain.

(Kumar et al., 2018). Thus, different parts of the hydrophobic tunnel might have different lipid binding affinities.

#### 1.5.7 Non-canonical functional domains

#### 1.5.7.1 Ubiquitin-associated and carbohydrate-binding domains

Many non-canonical VPS13 domains seem to be associated to ubiquitination, carbohydrate binding, or both (Levine, 2022) (Figure 27). The ubiquitin-associated (UBA) domain found in HsVPS13D is a poly-ubiquitin-binding domain implicated in the recognition of degradation targets (Wilkinson et al., 2001). The WWE motif-containing domain (hereafter WWE domain) is found in HsVPS13A and HsVPS13C. In HsVPS13C, the WWE domain is inserted between  $\beta$ -strands of the APT1 region by forming an outpocketing (Cai et al., 2022). The WWE domain is known to bind to poly-ADP-ribosylated (PARsylated) proteins and is often found in proteins implicated in the ubiquitination of PARsylated proteins (N. Li & Chen, 2014). The domain folding into a  $\beta$ -helix is exclusively found in *Arabidopsis* AtVPS13M1 and M2 proteins (Figure 27 & Figure 30). It is present in carbohydrate-binding and processing proteins, notably in the plant-specific pectate lyases (Ciccarelli et al., 2002; Zheng et al., 2021). Another domain that could be associated with carbohydrate binding is the  $\beta$ -sandwich (or  $\beta$ jellyroll) fold in HsVPS13B (Guillén-Samander & De Camilli, 2022; Levine, 2022). It has been proposed to have a carbohydrate binding function because of its similarity with carbohydrate-binding domains from the  $\alpha$ -N-acetylglucosaminidase family, even though such a fold could also have other functions given its similarity with discoidin domains implicated in collagen binding (Levine, 2022). A ricin-type lectin domain has also been identified in both HsVPS13D and AtVPS13X, which presumably folds into a  $\beta$ -trefoil. Ricin-type lectins bind to surface glycans (Cummings & L. Schnaar, 2015).

Altogether, this first collection of non-canonical VPS13 domains suggests that VPS13 proteins have acquired novel localization/interaction determinants allowing them to diversify their cellular functions. Notably, some VPS13 proteins seem to be associated with ubiquitination, which is consistent with the putative role of HsVPS13D in mitophagy (Anding et al., 2018).

#### 1.5.7.2 Additional membrane binding domains are found in plant VPS13 proteins

Other non-canonical domains are expected to associate to membranes. A C2 domain has been identified in AtVPS13M1 and M2 (**Figure 30**). C2 domains target membranes with a low lipid selectivity in a Ca<sup>2+</sup> dependent manner (Corbalan-Garcia & Gómez-Fernández, 2014). Interestingly, AtVPS13M2 lacking the C2 domain loses its localization at pollen tube tips and leads to decreased pollen germination and growth rates, two processes that heavily depend on Ca<sup>2+</sup> signaling (Tangpranomkorn et al., 2022). This suggests that the plant VPS13 C2 domains are Ca<sup>2+</sup>-dependent membrane localization determinants. Another domain folding into a  $\beta$ -tripod is also present in AtVPS13M1 and M2. This domain is usually annotated as VPS62 in standard domain prediction tools. The  $\beta$ -tripod fold is also found in human performs and plant insecticidal proteins that interact with target membranes to allow pore formation (Zaitseva et al., 2019). The presence of three and two  $\beta$ -tripod domains in respectively AtVPS13M1 and M2 suggests that they might be important localization determinants, even though their targeting mechanism is unknown.

#### 1.5.8 Reading the complexity of VPS13 localization determinants

The huge diversity of localization determinants coexisting in VPS13 proteins makes it difficult to anticipate its localization just by looking at its domain arrangement. The fact that VPS13 proteins are localized at almost all organelles and contact sites indicates that the regulation of their localization is highly complex and precise.

It seems that many localization determinants respond to signals transiently appearing in the cell depending on the physiological conditions, like the binding of the VAB domain to its adaptors or the binding of the PH-like domain to either proteins or PIPs. Others seem to bind to membranes in a more passive way like the ATG2\_C amphipathic helices, maybe to stabilize specific interactions that would otherwise be transient. We hypothesize that all the localization determinants of VPS13 proteins compete with each other at all times and that the observed localization in particular conditions is the result of the integration of all the localization signals received by the protein at a given time. The current hypothesis is corroborated by the fact that all the known localizations of yeast and human VPS13 proteins have not all been discovered at once but rather successively and often in different growth conditions or cell types.

### 1.6 VPS13 lipid transport mechanism and directionality

Even though the general structure of VPS13 proteins has already been well investigated, the exact molecular mechanisms by which lipids are extracted from the membrane, loaded and transported through the hydrophobic tunnel is still unknown.

Given the absence of full-length experimental structure, it is hard to determine the mode of lipid binding by VPS13 proteins. However, a crystal structure of the residues 21-240 (corresponding to a slightly extended Chorein\_N domain) of *Saccharomyces pombe* ATG2 bound to PE has been obtained (Osawa et al., 2019). The fatty acyl chain in sn-1 position forms hydrophobic interactions with the hydrophobic lining of the  $\beta$ -sheets composing the bottom of the cavity. The fatty acyl chain in sn-2 position, however, only engages into weak interactions with the hydrophobic surface, maybe resulting from the structural incompleteness of the protein fragment. Interestingly, the phosphoethanolamine moiety does not form any specific contacts with the protein except for the Arg224 that forms a salt bridge with the phosphate group, thus suggesting a molecular basis for nonspecific lipid transport by Chorein\_N containing RGB proteins. Given the high degree of conservation between the N-terminal domains of VPS13 and ATG2 proteins, it is probable that both engage similar modes of binding with phosphoglycerolipids. How the lipids interact inside of the tunnel in the rest of the protein remains unknown given the lack of structural data. But since RBG proteins are composed of a repetition of a structural unit resembling the crystallized

domain by (Osawa et al., 2019), it is tempting to speculate that a similar mode of binding is used all along the hydrophobic tunnel.

The desorption of a lipid molecule from a lipid bilayer is the limiting step of non-vesicular lipid transport as it is thermodynamically highly unfavorable (Somerharju, 2015). VPS13 proteins do not possess any apparent lipid selection or loading domain. It is therefore probable that they associate with other proteins to achieve this task. ATG2 has been shown to associate with ATG9, TMEM41B and VMP1 lipid scramblases (Ghanbarpour et al., 2021; Matoba et al., 2020). Recently, it has been shown that ScVPS13 and HsVPS13A respectively associate with Mcp1p and XK, two lipid scramblases, and that mutation of XK results in neurodegenerative disorders similar to HsVPS13A (Adlakha et al., 2022; Guillén-Samander et al., 2022; John Peter et al., 2017; Park et al., 2022; Park & Neiman, 2020). Furthermore, ATG9, TMEM41B and VMP1 are required for autophagy and localize to sites of autophagosome biogenesis (Y. E. Li et al., 2021; Maeda et al., 2020; Matoba et al., 2020; Melia & Reinisch, 2022). Scramblases are transmembrane proteins that flip lipids from one membrane leaflet to another in a passive way hence leading to the equilibration of lipids across a membrane bilayer. On the one hand, scramblases might favor the passive lipid transport activity of VPS13 and ATG2 proteins by dissipating the huge amount of lipids arriving at the cytosolic layer to the luminal layer (Leonzino et al., 2021; Melia & Reinisch, 2022). On the other hand, VPS13 and ATG2 proteins might take advantage of this mechanism during which the lipid is desorbed from the lipid bilayer to directly load or unload the lipid in or from their hydrophobic cavity. This hypothesis is supported by the recent imaging of the ATG2A-ATG9A complex by cryo-EM and the discovery of both interaction sites by hydrogen-deuterium exchange MS (HDX-MS) (van Vliet et al., 2022). This study suggests the existence of a direct path allowing the transfer of lipids from the cavity of ATG2A to a hydrophobic path on the ATG9A trimer to finally be inserted in the autophagosome membrane. A similar mechanism might function at the donor membrane to load lipids into the protein. A molecular dynamics study revealed an alternative model in which both ends of human VPS13 proteins directly insert into the membranes (Dall'Armellina et al., 2023). At their C-terminal ends, the amphipathic helices of the ATG2\_C domain are deeply inserted into the membrane and disturb the cytosolic lipid layer which could promote lipid insertion.

Most processes in which VPS13 proteins are implied require a directional flow of lipids. However, neither VPS13 proteins nor their associated lipid scramblases actively transport lipids rising the question of how lipid transport directionality is achieved in RBG proteins. Mathematical modelling showed that a difference in membrane tension between donor and acceptor membranes is theoretically sufficient to generate a biologically relevant bulk lipid flow through bridge-like LTPs (Y. Zhang et al., 2022). Since high membrane tension generates a pulling force and low membrane tension is indicative of an excess of lipids, lipid flow occurs from low to high membrane tension areas. One way to decrease the tension in a membrane is by synthesizing lipids. Interestingly, the lipid synthesis machinery is enriched at ER contact sites (Kannan et al., 2017; Schütter, 2020) and PC newly synthesized in the ER is preferentially

transported to the autophagosomal membranes (Orii et al., 2021). This suggests that bulk lipid transport by ATG2 might be coupled to the lipid synthesis machinery in the ER (Lees & Reinisch, 2020; Noda, 2021). Another way to create a tension gradient is by increasing membrane tension in the acceptor membrane. Morphological changes and constraints induced by cytoskeleton movements are known to induce variations in membrane tension (Fogelson & Mogilner, 2014). The cytoskeleton plays important roles in almost all the stages of autophagosome biogenesis (Kast & Dominguez, 2017; Kruppa et al., 2016). Thus, it is possible that cytoskeletal components generate tension in the nascent autophagosome by pulling or inducing curvature in the autophagosomal membranes, creating a tension gradient between donor and acceptor membranes (Y. Zhang et al., 2022).

## 1.7 VPS13 proteins in plants

Almost nothing is known about VPS13 plant homologs. In *Arabidopsis thaliana*, three putative VPS13 homologs have already been previously identified (Michaud et al., 2017; Velayos-Baeza et al., 2004). In fact, the three unknown proteins (At4g17140, At1g48090 and At5g24740) harbor the four canonical VPS13 accessory domains. In a previous publication, we proposed to rename At4g17140, At1g48090 and At5g24740 respectively AtVPS13A, B and C (Leterme & Michaud, 2022). The fourth *Arabidopsis* VPS13 protein (At3g50380) was only recently identified and the functional domains of all four plant VPS13 proteins were thoroughly described (Levine, 2022). At4g17140 and At1g48090 harbor many non-canonical accessory domains that are found neither in yeast nor in humans, are almost identical in terms of domain organization and were therefore renamed AtVPS13M1 and AtVPS13M2 ("M" for multiple), respectively. At5g24740, which was initially identified as SHRUBBY (Koizumi & Gallagher, 2013), harbors a canonical domain organization and was renamed AtVPS13S ("S" for SHRUBBY). At3g50380 lacks the C-terminal PH domain which is replaced by a bundle of amphipathic helices and has an atypical RBG domain arrangement suggesting an early evolutionary divergence from the other plant VPS13 proteins (Levine, 2022). At3g50380 was therefore renamed AtVPS13X.

The conservation of the canonical domains involved in VPS13 protein targeting in yeast and mammals suggests that plant VPS13 proteins are also associated to multiple cell compartments and MCSs. However, it is noteworthy that *Arabidopsis* VPS13 proteins present differences in their domain organization and harbor non-canonical domains that are found neither in yeast, nor in humans. This suggests a higher complexity in the plant VPS13 protein localization pattern, functions and regulation that might be specifically required for plant cell physiology.

AtVPS13M1/At4g17140 was identified in the mitochondrial outer membrane proteome of *Arabidopsis thaliana* (Duncan et al., 2011). Even though the GFP fusions of the 200 N-terminal and C-terminal amino acid residues failed to give a consistent localization, this study gives a first hint for the potential mitochondrial localization of AtVPS13M1. AtVPS13M1 was also found in the *Arabidopsis thaliana* mitochondrial complexome and migrated at a molecular weight of 650 kDa on BN-PAGE (Senkler et

al., 2017). An FFAT motif localized in the N-terminal region of AtVPS13A was also identified, suggesting an association with the ER membrane (Slee & Levine, 2019). Furthermore, AtVPS13M1 was identified in our lab in a co-immunoprecipitation with AtTOM40-HA linking this protein to mitochondria and to the MTL complex. Here, we characterize AtVPS13M1, a VPS13 homolog in *Arabidopsis thaliana*. Our objectives are 1) to describe the structural and domain evolution of plant and algae VPS13 proteins, 2) to identify the different AtVPS13M1 splice variants and their abundance, 3) to analyze its subcellular localization, 4) to investigate AtVPS13M1 lipid binding and transport properties and 5) to characterize its role in lipid homeostasis, plant growth and development using T-DNA insertion lines.

# 2 Results

# 2.1 Co-immunoprecipitation using Tom40-HA as bait identifies At4g17140/AtVPS13M1 as a possible interactant

The MTL complex plays a role in intramitochondrial trafficking and possibly projects to other membranes to establish a direct lipid import and/or export route to and/or from mitochondria (Michaud et al., 2016). Before I started my PhD, Morgane Michaud and Catherine Albrieux took a co-IP approach to identify possible MTL complex interactants localized in extramitochondrial compartments (unpublished results). To this end, AtTOM40, one of the core components of the MTL complex localized on the OMM, was C-terminally fused to an HA epitope and co-IPs were performed using anti-HA beads on DSP-crosslinked mitochondria with Col0 lines as a control. Proteomic analysis was used to determine the proteins significantly enriched in the AtTOM40-HA co-IP elution fractions compared to Col0.

As expected, the most enriched proteins were other well known components of the TOM complex (TOM20, TOM5, TOM7,...). Interestingly, one of the identified proteins was At4g17140, which is annotated as a pleckstrin homology (PH) domain-containing protein in the TAIR database and as containing VPS13 domains in InterPro. At the time of this discovery, the role of VPS13 proteins in lipid transport was still unsure but highly suspected. This protein had also been found in the outer mitochondrial membrane proteome (Duncan et al., 2011) and complexome (Senkler et al., 2017). Thus, At4g17140 seemed to be an interesting candidate as a lipid transporter at the mitochondrial membrane.

# 2.2 Long read sequencing reveals AtVPS13M1 real splice variant composition and abundance

The At4g17140/AtVPS13M1 gene has three predicted splice variants in the TAIR10 *Arabidopsis* genome database. These three variants differ from each other in four regions (**Figure 31A**). Interestingly, two of these regions are located in presumably important domains for AtVPS13M1 localization, namely the N-terminal PH domain and the VAB domain. This suggests that AtVPS13M1 localization could be influenced by the expression of different splice variants.

AtVPS13M1 is encoded by a huge nucleic acid sequence: 23 kb in genomic DNA and 13 kb in coding DNA. During the AtVPS13M1 cloning process, we found that the sequences we obtained were not entirely matching the sequence of any of the predicted splice variants. The biggest difference was observed in the fourth variable region situated in the VAB domain. Furthermore, other variable regions were arranged chimerically even though they were in agreement with the predicted splice variant sequences.



Figure 31 (A) Position of the four predicted variable regions of At4g17140/AtVPS13M1 mRNA according to TAIR10 reference genome. The protein sequence alignments of the variable regions are shown. The range of the PCR performed for long read sequencing and spanning over 7600 bp is indicated above. Note that two of the predicted variable regions are located in domains presumably important for VPS13 protein localization. (B) RNA extraction profiles from Arabidopsis calli. Four biological replicates (Repl. 1-4) of calli grown in presence and absence of phosphate (Pi) were subjected to RNA extraction. Electrophoresis on 2% agarose gels reveals that the RNAs are of excellent quality: the 28S band is twice as intense as the 18S band, no degradation is visible and small amounts of gDNA co-purified with the RNAs (see band above 28S). The contaminating gDNA was removed by DNAse treatment prior to cDNA generation. (C) Long range PCR optimization on cDNAs generated from the extracted RNAs. Several annealing temperatures (Tm) and PCR cycle numbers were tested to finally obtain a specific amplification of the 7600 bp fragment of interest. (D) Representation of the five splice variants identified by long read sequencing. Grey squares indicate AtVPS13M1 exon distribution. The map of the corresponding protein domains is drawn on top. Red squares indicate the absence of intron splicing. Orange squares represent the newly annotated exons 49 and 50 with the correct exon borders. The red arrow represents the position of a missing T nucleotide in the TAIR10 genome. The abundance of the different identified isoforms is indicated on the right.

To get more insight in the real arrangement of the variable regions, in the relative quantity of the different splice variants and in a potential differential splice variant expression in Pi deplete and replete conditions, we decided to take a long read sequencing approach, which addresses all of these questions simultaneously. We collaborated with the IGFL (Institut de Génomique Fonctionnelle de Lyon) sequencing platform to perform long read sequencing using the Nanopore technology. This approach is

particularly well suited for our biological questions because of its high processivity enabling the uninterrupted reading of very long sequences and because of its quantitative nature. However, high processivity comes with the drawback of higher error rates. Therefore, a greater depth of sequencing is necessary to obtain high accuracy, which can be achieved by amplifying the transcript of interest by PCR.

Four biological replicates of *Arabidopsis* calli were grown in presence or absence of Pi and the RNAs from calli in each condition were extracted. The presence of clear 28S and 18S bands and the absence of degradation show that the RNAs are of excellent quality (**Figure 31B**). cDNAs were then generated for each sample. Since until now we were not successful in amplifying the full-length cDNA, a PCR covering 7600 bp including all the regions predicted to be variable was run on each cDNA sample (**Figure 31A**). This PCR necessitated extensive troubleshooting as the fragment of interest was accompanied by contaminant fragments. These were probably due to off target amplification events as their appearance were dependent on the annealing temperature and number of PCR cycles (**Figure 31C**). Such difficulties have also been encountered previously for the amplification of the HsVPS13B gene during a splice variant analysis (Velayos-Baeza et al., 2004). The PCR protocol was further optimized by our collaborators who designed additional primers and bead-purified the fragment of interest. Given the encountered technical difficulties, long-read sequencing was performed on two Pi replete and two Pi deplete samples, which gave the highest quantity of fragment of interest. Nanopore sequencing and raw data analysis were entirely realized by our collaborators.

To identify biologically relevant splice variants, only the reads containing the used PCR primers at each end and having a length ranging from 5 kb to 9 kb were selected for analysis. Furthermore, only the splice variants representing more than 1% of the total reads were retained. This method enabled to identify 11 new splice variants for AtVPS13M1. Interestingly, all the isoforms were equally distributed between +Pi and –Pi conditions, suggesting that AtVPS13M1 is not differentially spliced upon phosphate starvation.

After an independent verification based on a BLAST method, five isoforms were identified as probable artefacts. Hence, six splice variants were retained and named isoforms A, D, E, G, I and J, respectively (**Figure 31D**). The isoform A is the most abundant representing alone 91.8% of the total reads and was therefore taken as a reference for the description of the other isoforms. Consistently, the sequence of isoform A corresponds to the sequence we cloned for further downstream applications. Each of the other isoforms differs from the isoform A by an unspliced intron either leading to the addition of aa residues in frame or to a frameshift. Strikingly, out of the five other transcript isoforms, only one encodes a full-length protein (isoform G) while the others encode premature STOP codons (**Figure 31D**). Interestingly, the isoform J contains a STOP codon that is in frame with the rest of the sequence. Surprisingly, the sequencing results revealed that the borders of the exons 49 and 50 situated in the fourth variable region



**Figure 32** Redefined borders of exons 49 and 50 of At4g17140/AtVPS13M1 gene. The original reference genome exon annotation is represented by grey boxes. The reannotated exons, here 49' and 50', are depicted in orange. The sequence alignments represent the right border of exon 49 and the left border of exon 50. All sequences have been aligned on the reference gDNA. At4g17140.1, .2 and .3 are the three predicted splice variants cDNA sequences. Zhang A' and Zhang B' are the isoforms as described by Zhang et al. 2020. Note the missing T base in the reference genome and in the two previously identified Zhang A' and Zhang B' sequences.

are misannotated in the reference genome (**Figure 31D** and **Figure 32**). In addition, our new sequences show that AtVPS13M1 reference coding sequence is lacking a T base at position 17 508 of the gDNA starting at the START codon. Both the misannotated exon borders and the additional T base were observed in all the newly identified splice variants (**Figure 31D** and **Figure 32**).

A previous study already identified two new isoforms for the At4g17140/AtVPS13M1 gene, which were named isoforms A and B and will be referred to as isoforms A' and B' hereafter to avoid confusion with our naming system (S. Zhang et al., 2020). The sequences of both isoforms A' and B' confirm our new exon borer annotations for the exons 49 and 50 (**Figure 32**). However, the missing T base in the reference genome discovered by our study is also missing in both A' and B' isoforms, inevitably leading to a frameshift and the appearance of early STOP codons. Apart from the missing T, the isoform A'



**Figure 33** Diagnostic PCRs to confirm the existence of the AtVPS13M1 isoforms identified by long-read sequencing. The control PCR (Ctrl) corresponds to PCR2 with a right border primer annealing in an intron predicted to be spliced in all the isoforms.

corresponds to our isoform A, thus confirming our results. The isoform B' likely corresponds to our isoform D. However, the isoform B' diverges from the reference genome and from our isoforms in two ways: 1) a 4 nt insertion just after exon 13 creates a frameshift and generates early STOP codons and 2) an 8 nt deletion at the end of exon 48, which also leads to a frameshift. Neither difference is discussed by the authors. These differences could be due to intrinsic sequencing errors as Zhang et al. based their splice variant identification on direct RNA sequencing using the Oxford Nanopore technology and reported a total of three and one reads for the isoforms A' and B', respectively.

To verify the biological existence of the identified isoforms, we performed diagnostic PCRs on the fragment amplified for long-read sequencing. For each isoform, two different PCRs were performed (**Figure 33**): 1) a PCR using primers annealing in two consecutive exons framing the possibly unspliced intron (PCR1) and 2) a PCR using one primer that anneals in the preceding exon and one that anneals inside the possibly unspliced intron (PCR2). PCR1 confirmed the existence of the isoform A exon arrangement as the predicted PCR fragments were observed at their expected size. PCR2 in turn confirmed the existence of the exon arrangement suspected in the five other isoforms. The control PCR (Ctrl) which corresponds to PCR2 with a right border primer annealing in an intron predicted to be spliced in all the isoforms confirmed that the method is valid as no amplification was observed. Thus, we confirm that the isoforms identified by long-read sequencing correspond to biologically existing transcripts and did not emerge through sequencing artefacts.

Altogether, our results provide a robust identification of six AtVPS13M1 splice variants. We show that isoform A is the major splice variant in calli that codes for a 4202 aa residues long protein, we reannotate the borders of exons 49 and 50 and identify a missing T in the reference genome. Given its abundance and ability to code for a full-length protein, the isoform A is likely biologically relevant. Whether the other identified splice variants also have a biological role or result from intron splicing defects is unknown and will have to be investigated.

### 2.3 AtVPS13M1 subcellular localization

Determining the localization of VPS13 proteins is essential to understand their function. Since the fulllength AtVPS13M1 gene remains recalcitrant to cloning, determining its subcellular localization



**Figure 34** Representation of the different AtVPS13M1 regions and domains (fragments) cloned for BiFC and localization studies. The exact coordinates of the cloned fragments (in amino acids from the beginning of the protein) are indicated on the right.

becomes challenging. Two different approaches were tested: 1) transient expression of different subparts (also called fragments) of AtVPS13M1 fused to GFP in a heterologous system and 2) production of a polyclonal antibody directed against AtVPS13M1 immunogenic peptides.

#### 2.3.1 Different regions of AtVPS13M1 protein localize to the endoplasmic reticulum

VPS13 proteins harbor a vast set of localization determinants distributed over the whole length of the protein that perceive and integrate various localization signals to direct the full-length protein to its final subcellular localization. The huge size of VPS13 proteins and the high number of domains potentially able to associate to organelle membranes poses complex problems in the dissection of the localization signal integration. Given the absence of full-length AtVPS13M1 clone, we set out to understand the role of different regions and domains of the protein in its localization. To do so, 16 different AtVPS13M1 fragments have been C-terminally fused to GFP (**Figure 34**), transiently expressed in *Nicotiana benthamiana* and visualized by fluorescence confocal microscopy in *Nicotiana benthamiana* leaves (**Figure 35** and **Figure 36**). Some longer fragments cover large areas of the protein to get as close as possible to the native localization. Other smaller fragments cover narrow regions or individual domains to better understand their specific role in AtVPS13M1 localization.

All the AtVPS13M1 fragments were transiently expressed under the control of the CaMV 35S promoter in Nicotiana benthamiana using the agroinfiltration technique (see materials and methods). To identify their expected association with the ER and/or mitochondria, they were co-expressed with an ER marker (ER-RFP) and/or a mitochondrial marker (mito-YFP) (Figure 35 and Figure 36). The three biggest fragments, AtVPS13M1 Nt+mid, mid and Ct, all co-localized with the ER marker. This was not completely unexpected as the Nt+mid and mid fragments carry a FFAT motif known to interact with ER-bound VAPs. Most surprisingly, all the other fragments also clearly localized to the ER upon expression. An ER localization was expected for some of the fragments like the FFAT or the fragments containing the Chorein N domain as it has previously been shown to associate with the ER in yeast (T. S. Nakamura et al., 2021), but the fact that all fragments are ER-localized raises questions about the reliability of our expression system. To verify that the linker constituted of five aa between the fragment of interest and GFP has no influence on the observed localizations, we expressed GFP alone and GFP with the linker in the same conditions. Both constructs had a cytosolic localization, suggesting that the ER localization is due to the fragments alone (Figure 37B). The PH\_Nt and  $\beta$ -tripod-containing fragments also exhibited localizations additional to the ER (Figure 37A). PH\_Nt also localized inside the nucleus and all the fragments containing a  $\beta$ -tripod domain had a partial cytosolic localization and accumulated in the nucleus. The second central  $\beta$ -tripod ( $\beta$ -tripod.2) and the two linked central  $\beta$ -tripods  $(\beta$ -tripod.1+2) additionally accumulated in what seems to be the nucleolus. Whether these nuclear localizations are specific or due to the diffusion of the overexpressed constructs is not known.



**Figure 35** Localization of AtVPS13M1 fragments transiently expressed in *Nicotiana benthamiana*. Different regions and domains of AtVPS13M1 were C-terminally fused to GFP and transiently expressed in *Nicotiana benthamiana* leaves under the control of 35S promoter. AtVPS13M1 fragments are depicted as cartoons on the left under their given name (see **Figure 34**). AtVPS13M1 fragments were transiently co-expressed with an ER marker (ER-RFP) and/or with mitochondrial marker (Mito-xFP). The mitochondrial marker is either mito-YFP or mito-RFP only for AtVPS13M1 Nt and FFAT. N/A signifies that the subcellular marker was not co-expressed. Scale bars 5 µm. See **Figure 36** for the other fragments.



**Figure 36** (Figure 35 continued) Localization of AtVPS13M1 fragments transiently expressed in *Nicotiana benthamiana*. Different regions and domains of AtVPS13M1 were C-terminally fused to GFP and transiently expressed in *Nicotiana benthamiana* leaves under the control of 35S promoter. AtVPS13M1 fragments are depicted as cartoons on the left under their given name (see Figure 34). AtVPS13M1 fragments were transiently co-expressed with an ER marker (ER-RFP) and/or with mitochondrial marker (Mito-xFP). The mitochondrial marker is mito-YFP or mito-RFP only for AtVPS13M1 Nt and FFAT. N/A signifies that the subcellular marker was not co-expressed. Scale bars 5 µm. See Figure 35 for the other fragments.



**Figure 37** (**A**) AtVPS13M1 fragments C-terminally fused to GFP and transiently expressed in *Nicotiana benthamiana* that have a localization other that the ER. AtVPS13M1 fragments are depicted as cartoons on the left under their given name (see **Figure 34**). AtVPS13M1 fragments were transiently co-expressed with an ER marker (ER-RFP) and with mitochondrial marker (Mito-YFP). N/A signifies that the subcellular marker was not co-expressed. (**B**) Controls for the localization experiment. GFP alone and GFP fused to the linker (5 amino acids) present between the AtVPS13M1 fragments and GFP were transiently expressed. Both reveal a cytoplasmic localization. (**C**) Localization control of ER-and mitochondrial markers without AtVPS13M1 fragments. Scale bars 5 μm.
In some cases, the ER marker accumulates at the vicinity of mitochondria, which led us to hypothesize that the overexpression of some fragments may lead to a tighter ER-mitochondria association (**Figure 36**; e.g. ATG2\_C). However, when we co-expressed the ER and mitochondrial markers without AtVPS13M1 fragment, these accumulations could also be observed (**Figure 37C**). In fact, this is a general phenomenon presumably due to the harsh transient overexpression system that affects the structural integrity of the ER.

The observation of such a broad ER localization is puzzling. Since AtVPS13M1 harbors many hydrophobic surfaces, it could be that most fragments specifically associate to membranes, in this case the ER. An alternative hypothesis could be the absence or insufficiency of protein partners for *Arabidopsis* AtVPS13M1 in *Nicotiana benthamiana* leading to its accumulation at the ER. Further investigation in stable *Arabidopsis* lines will be needed to decipher the native localization pattern of AtVPS13M1.

#### 2.3.2 Anti-AtVPS13M1 polyclonal antibody production

In the hope to get insight on the native localization of AtVPS13M1 by immunostaining and immunogold EM, we set out to generate specific antibodies against our protein of interest. Rabbits were inoculated with two immunogenic peptides recommended by the company in charge of the production for their high antigenicity prediction (**Figure 38A**).

The purified antibodies were applied on Col0 protein extracts to test their specificity and efficiency. Unfortunately, no signal could be detected in standard Western blot conditions (see materials and methods). Given the huge size (470 kDa) and hydrophobic nature of AtVPS13M1, we hypothesized that either the protein cannot be efficiently extracted and solubilized in the total protein fraction, or that the protein is not correctly transferred on the nitrocellulose membrane. To test this, proteins were extracted using different harsh denaturing and reducing conditions, run on an acetate gel for 3 hours to increase resolution and protein penetration, transferred for 2h on a nitrocellulose membrane and revealed with an increased antibody concentration after BSA saturation to increase the signal (**Figure 38B**). A faint band appeared at very high molecular sizes which could correspond to AtVPS13M1. However, this signal is minor compared to the bands appearing around 37 kDa. Multiple faint bands can also be observed at intermediate molecular sizes, further suggesting that the antibody has high off-target activity.

Since the antibody tests on total protein extracts were not sufficient to determine the identity of a potential AtVPS13M1 recognition, we set out to identify their efficiency by expressing two AtVPS13M1 fragments (fragment 1 and fragment 2) in a bacterial cell free system. Each fragment contains one of the two immunogenic peptides and both are fused to a His<sub>6</sub> tag (**Figure 38A**). The anti-AtVPS13M1 antibodies recognized several bands of different sizes for both fragments (**Figure 38C**). We used the His<sub>6</sub> tags to specifically recognize our constructs. The identities of both fragments are



**Figure 38** Anti-AtVPS13M1 antibody production. (A) Position of the two selected immunogenic peptides for the anti-AtVPS13M1 antibody production. Both immunogenic peptides were inoculated into rabbits once a week for 42 days. The antibodies were affinity-purified against the peptides after serum collection. Two AtVPS13M1 fragments containing the immunogenic peptides were obtained to verify the specificity and efficiency of the antibodies. (B) Antibody specificity and efficiency tests on Arabidopsis calli total protein extracts. Different protein extraction buffers were tested to optimize AtVPS13M1 solubilization and transfer: buffer B1 (Tris-HCl pH8 50 mM, EGTA 2 mM, NaCl 100 mM, DTT 1 mM, glycerol 5% (v/v), PMSF 1 mM, protease inhibitors), buffer B1 + Triton 1% (w/v), buffer B1 + n-dodecyl  $\beta$ -D-maltopyranoside (DDM) 1.5% (w/v) and buffer B2 (8M urea, Tris-HCl 50 mM pH6.8, EDTA 1mM, PMSF 1 mM, SDS 0.1% (v/v), caproic acid 5 mM, benzamidine 1 mM, DTT 1 mM). Anti-AtVPS13M1 fragments that contain the immunogenic peptides. Both fragments were produced using a bacterial *in vitro* translation system. Immunoblots were revealed with anti-AtVPS13M1 (0.2% (v/v) dilution) and anti-His antibodies. Red stars indicate the band identified as AtVPS13M1 fragment.

highlighted by the anti-His immunoblot, which allows their recognition on the anti-AtVPS13M1 immunoblot (**Figure 38C**). Hence, the anti-AtVPS13M1 antibodies are indeed able to recognize the selected epitopes but exhibit high off target activity in the tested conditions. Thus, it is probable that the specificity and efficiency is too low to perform immunostaining or immunogold EM experiments.

## 2.4 Analysis of AtVPS13M1 interactions with other proteins of interest using rBiFC and classic BiFC

AtVPS13M1 was initially identified because it co-precipitated with AtTOM40-HA, suggesting that AtVPS13M1 interacts with the mitochondrial outer membrane and probably with the TOM complex. We decided to confirm this interaction by ratiometric bimolecular fluorescence complementation (rBiFC) which is adapted from the standard BiFC technique (Grefen & Blatt, 2012). rBiFC allows to express both the bait and the prey on the same plasmid as well as a cytosolic RFP that allows the standardization of the rBiFC signal between different cells and different constructs. Since TOM40 is deeply embedded in the outer mitochondrial membrane, we chose to use one of the receptors of the TOM complex, TOM20.2, as bait in our experiment because of its large soluble domain protruding in

the cytosol. The constructs were transiently expressed in *Nicotiana benthamiana* leaves using the standard agroinfiltration technique (see materials and methods).

TOM20.3, another receptor subunit of the TOM complex, was used as positive control and its coexpression with TOM20.2 generated a YFP signal that localized to mitochondria, as expected (**Figure 39A & B**). TOM20.2 co-expression with CBR1, an outer mitochondrial membrane protein that is not part of the TOM complex and thus used as negative control, gave a significantly lower YFP signal just above detection limit. Different AtVPS13M1 fragments (AtVPS13M1 Nt, mid and Ct in **Figure 34**) fused to split YFP in both orientations were tested for interaction with Tom20.2. All the tested constructs exhibited cytosolic RFP expression, showing that the constructs were indeed present in the imaged cells (**Figure 39A**). Unfortunately, none of the AtVPS13M1 constructs gave YFP signals significantly different from the negative control, suggesting that none of them interacts with the TOM complex (**Figure 39A & B**). AtVPS13M1 Nt N-terminally fused to split YFP (nYFP-M1 Nt) gave a slightly higher YFP signal than the other constructs but was still not significantly different from the negative control (**Figure 39B**).

To verify the level of expression at the protein level, we performed Western blot analysis on the agroinfiltrated leaves (**Figure 39C**). The results revealed that 1) the level of RFP expression is not constant, 2) there is no correlation between RFP expression and AtVPS13M1 fragment expression and 3) AtVPS13M1 fragment expression correlates with the YFP signal intensity (see nYFP-AtVPS13M1 Nt YFP signal and the corresponding protein expression in **Figure 39B & C**). This was particularly worrisome as no correlation between the observed YFP fluorescence and the actual level of expression can be made solely by looking at the RFP signal. The reasons for the absence of protein detection for AtVPS13M1Ct constructs remain obscure. One reason for the absence of AtVPS13M1 Ct immunoblot signal could be its high molecular mass (214 kDa), which is difficult to transfer by Western blot. CBR1, however, is small enough to be classically transferred. Since the expression of the bait, prey and RFP are all driven by a CaMV 35S promoter, it is possible that the relative expression levels are decorrelated due to a topological interaction or steric hindrance.

To test whether the absence of signal is due to the rBiFC vector, we performed a classic BiFC experiment in which the bait and prey are co-expressed from different plasmids (**Figure 40A**). While the positive control gave a clear mitochondrial YFP signal, the negative control CBR1 as well as AtVPS13M1 mid and Ct C-terminally fused to split YFP exhibited a non-detectable YFP signal. This further suggesting that AtVPS13M1 might not directly interact with the TOM complex.



**Figure 39** Analysis of the interaction between the TOM complex and AtVPS13M1 by ratiometric bimolecular fluorescence complementation (rBiFC) results. (**A**) Schematic representation of the rBiFC vector used in our experiments. The prey and bait can be either N-terminally or C-terminally fused to split YFP, giving four different possible combinations. (**B**) Different AtVPS13M1 fragments (see figure 3) N- or C-terminally fused with the N-terminal part of the split YFP were transiently co-expressed in *Nicotiana benthamiana* leaves with TOM20.2 N-terminally fused with the C-terminal part of the split YFP. The RFP reporter indicates the presence of the rBiFC construct in the observed cells. TOM20.3 and CBR1 were used as positive and negative controls, respectively. Scale bars: 20  $\mu$ m. (**C**) Corrected and normalized YFP fluorescence expressed as a percentage of the measured RFP fluorescence. For each construct, images were taken randomly from 2 different leaves from different plants agroinfiltrated on at least two different days. Number of analyzed images is indicated above each column. (**D**) Western blot analysis on protein extracts from agroinfiltrated leaves. Western blot membranes were incubated with anti-Myc antibodies (cYFP-Myc-TOM20.2 detection), anti-HA antibodies (AtVPS13A fragments-HA-nYFP or nYFP-HA-AtVPS13A fragment detection) and anti-RFP antibodies. Red asterisks indicate the detection of expected bands.



**Figure 40** (**A**) Analysis of the interaction between the TOM complex and AtVPS13M1 by classic bimolecular fluorescence complementation (BiFC). Bait and prey are transiently expressed in *Nicotiana benthamiana* leaves from different plasmids .As for rBiFC, TOM20.3 and CBR1 were used as positive and negative control, respectively. (**B**) rBiFC constructs transiently expressed in *Arabidopsis thaliana* leaves by a method adapted from Y. Zhang et al. 2020. (**C**) Analysis of the interaction between AtVPS13M1 and MIRO1/2 by rBiFC. The constructs were transiently expressed in *Arabidopsis thaliana* leaves. Scale bars 20 µm.

The rBiFC experiments were carried out in *Nicotiana benthamiana*, which might have different VPS13 localization signals than *Arabidopsis* thus leading to AtVPS13M1 mistargeting. To verify this, we adapted a protocol enabling transient expression directly in *Arabidopsis* leaves (Y. Zhang et al., 2020). While the positive control gave a clear YFP signal, none of the AtVPS13M1 fragments tested exhibited detectable signals (**Figure 40B**).

Altogether, these results suggest that AtVPS13M1 is not directly interacting with TOM20.2 in the TOM complex. Given the huge diversity of localization determinants present on AtVPS13M1, its association to mitochondria could be mediated by another mitochondrial protein. Since MIRO1/2 has been shown to direct HsVPS13D to mitochondria in humans (Guillén-Samander et al., 2021) and that AtMIRO2 co-

migrates with AtVPS13M1 in the *Arabidopsis* mitochondrial complexome (Senkler et al., 2017), we decided to investigate the interaction between AtMIRO1/2 and AtVPS13M1 fragments. We used the rBiFC method to co-express either AtMIRO1 or AtMIRO2 with AtVPS13M1 mid, Ct or VAB. We focused on the C-terminal region of AtVPS13M1 because it was shown that the VAB domain is required for HsVPS13D recruitment by MIRO1/2 (Guillén-Samander et al., 2021). Unfortunately, no rBiFC signal could be detected for any of the tested MIRO1/2-AtVPS13M1 combinations upon transient expression in *Arabidopsis* leaves (**Figure 40C**).

#### 2.5 Investigation of AtVPS13M1 in vitro activity

#### 2.5.1 AtVPS13M1 binds glycerolipids in vitro

VPS13 proteins are now widely recognized as lipid transport proteins. The N-terminal domain of yeast ScVPS13 and human ATG2A have been shown to bind lipids *in vitro* (Kumar et al., 2018; Valverde et al., 2019). Given the high degree of conservation between the AtVPS13M1 and ScVPS13 N-termini, it is reasonable to hypothesize that AtVPS13M1 is able to bind lipids as well. To address this question, we performed an *in vitro* lipid binding assay using a purified AtVPS13M1 protein fragment and fluorescently labeled lipids.

#### 2.5.1.1 Purification of AtVPS13M1\_1-335

The *in vitro* binding experiments required to obtain a purified protein. Since we did not succeed in cloning the full-length protein, we concentrated our efforts on purifying the first 335 aa residues of AtVPS13M1 (hereafter AtVPS13M1\_1-335) and the first 800 aa residues (hereafter AtVPS13M1\_1-800) (**Figure 41A**). AtVPS13\_1-335 is homologous to the crystallized ScVPS13 fragment used for X-ray diffraction and both AtVPS13M1\_1-335 and 1-800 are homologous to a bigger ScVPS13 fragment known to bind glycerolipids *in vitro* in yeast (Kumar et al., 2018).

We first tried to overexpress AtVPS13M1\_1-800 and AtVPS13M1\_1-335 in yeast to assess its solubility. Since AtVPS13M1\_1-335 was poorly expressed in yeast, we concentrated on AtVPS13M1\_1-800. After total yeast protein extraction, the samples were centrifuged and subjected to immunoblotting. AtVPS13M1\_1-800 was clearly visible in the total fraction and in the pellet, but not in the supernatant indicating that it is not soluble (**Figure 41B**). Since our goal was to observe a lipid binding activity, we could not add detergents to increase the protein fragment solubility.

Given the difficulties encountered with our yeast expression system and with the bigger fragment, we turned to a bacterial *in vitro* translation system to express AtVPS13M1\_1-335. Small-scale preliminary tests performed by Lionel Imbert (ISBG, Grenoble) determined that AtVPS13M1\_1-335 is mostly insoluble. However, approximately 5% of our protein was found in the soluble fraction. Hence, we decided to scale-up the *in vitro* protein production and to purify the soluble part of the protein for further downstream applications. The protein of interest was affinity-purified using a standard nickel affinity column (**Figure 41C**). As expected, a huge part of AtVPS13M1\_1-335 protein precipitated and was



**Figure 41** Purification of AtVPS13M1 fragments for downstream in vitro binding experiments. (**A**) The first 335 and 800 amino acid residues of AtVPS13M1 (respectively AtVPS13M1\_1-335 and AtVPS13M1\_1-800) were C-terminally fused to a His6 tag and cloned in yeast or bacterial protein expression vectors. (**B**) Purification tests on AtVPS13M1\_1-800 expressed from two different yeast clones. The anti-His immunoblot reveals that AtVPS13M1\_1-800 is insoluble in these protein extraction conditions. The red arrow indicates the hypothetical protein size. (**C**) Purification of the protein is soluble and found in the final elution fraction. The red arrow indicates the hypothetical protein size.

found in the pellet (band at 37 kDa). A small subset of AtVPS13M1\_1-335 was found in the elution fraction but it co-eluted with a broad array of contaminants of unknown nature (**Figure 41C**). These contaminants are not truncated versions of the protein since the anti-His immunoblot recognized only one single band in the elution fraction. Since the contaminants are unlikely to have a structure and hence lipid affinity similar to AtVPS13M1\_1-335, we decided to use the eluted fraction for downstream experiments.

#### 2.5.1.2 AtVPS13M1 binds phosphoglycerolipids in vitro

To test the binding capacity of AtVPS13M1\_1-335 to isolated glycerolipids, we incubated the elution fraction with isolated PC, PE, PS and PA conjugated to a nitrobenzoxadiazole (NBD) fluorophore (**Figure 42A**). After incubation, the protein-lipid samples were run on CN-PAGE and NBD fluorescence was read directly from the gel. While for Tom20.3, used as a negative control, no signal is detected for any of the NBD-lipids at the corresponding protein size, increasing quantities of AtVPS13M1\_1-335 lead to a concomitant increase in NBD fluorescence, indicating that AtVPS13M1\_1-335 interacts with



**Figure 42** *In vitro* lipid binding assay. (**A**) The first 335 amino acid residues of AtVPS13M1 (AtVPS13M1\_1-335) were C-terminally fused to a His<sub>6</sub> tag and inserted in a bacterial protein expression vector. After purification, the AtVPS13M1\_1-335 is incubated with a fluorescently (nitrobenzoxadiazole, NBD) marked lipid to determine the lipid binding activity of the protein fragment. (**B**) AtVPS13M1\_1-335 was incubated with isolated PC, PE, PS and PA coupled to NBD and samples were run on CN-PAGE. For each NBD-coupled lipid tested, NBD fluorescence was measured (left panel) and proteins detected by Coomassie staining (right panel). Red stars on the NBD fluorescence panel represent the protein size reported from the Coomassie staining. (**C**) Western blot analysis of AtVPS13M1\_1-335 incubated with NBD-PC. Proteins were detected with No-Stain<sup>TM</sup> protein labeling reagent (left panel). NBD-fluorescence was measured directly on gel (middle panel). Anti-His immunoblot reveals that the AtVPS13M1\_1-335 coincides with the NBD-fluorescence signal (right panel).

PC, PE, PS and PA *in vitro* (**Figure 42B**). In presence of AtVPS13M1\_1-335, the NBD fluorescence is distributed over a broad range of molecular forming a smeary band. This might be due to a particular migration pattern of AtVPS13M1\_1-335 protein on CN-PAGE. This could also reflect the dynamic binding of a variable number of lipids to AtVPS13M1\_1-335, thus generating multiple types of protein-

lipid complexes that have different migration properties. The CN-PAGE anti-His immunoblot highlights a band that superposes with the NBD fluorescence band (**Figure 42C**). This strongly suggests that the observed lipid binding activity can be attributed to AtVPS13M1 alone, even though it cannot be excluded that some contaminants migrate at the same apparent molecular size as AtVPS13M1\_1-335 on CN-PAGE.

#### 2.6 Identification of four AtVPS13M1 T-DNA insertion knock out mutants

To investigate the physiological role of AtVPS13M1, we required AtVPS13M1 KO mutants. We therefore interrogated the SALK T-DNA insertion databank to find *Arabidopsis* lines with T-DNA insertions in our gene of interest (Alonso et al., 2003). The size of the T-DNA used by Alonso et al. has a considerable length and is therefore very likely to disrupt the gene it is inserted in. We identified four potential interesting lines with T-DNA insertion sites towards the beginning of the gene to maximize our chances to obtain KO mutants (**Table 17**). The four insertion lines were named *atvps13m1.1-4* (**Table 17**). For all the mutant lines, the exact insertion sites were mapped by PCR sequencing (**Table 17**; **Figure 43A**) and homozygoty has been confirmed by genotyping.



**Figure 43** (A) Position of the T-DNA insertion sites in AtVPS13M1. The T-DNAs are inserted at the beginning of the gene and three out of four are in exons. (B) Confirmation that atvps13m1.1-4 are AtVPS13M1 KO mutants by proteomic analysis. The peptides searched for in atvps13m1.1/3 and atvps13m1.2/4 mutants are distributed over the whole length of the protein. The proteins taken as reference are AT2G31390 / fructokinase and AT5G09900 / RPN5A. The protein abundances have been quantified using the IBAQ method and values are given in  $10^6$  arbitrary units. Not detected (n.d.) means that none of the indicated peptides could be detected in these experimental conditions.

**Table 17** Review of the T-DNA insertion lines identified as AtVPS13M1 KO lines. The SALK code is indicated in the left column. The T-DNA experimentally determined insertion site is indicated in the right column for each SALK line.

SALK line	Given name	gDNA insertion site
SALK_128148	atvps13m1.1	Exon 7; between nt 2317-2318
SALK_019531	atvps13m1.2	Intron 14; between nt 4199-4200
SALK_021158	atvps13m1.3	Exon 17; between nt 4985-4990
SALK_096315	atvps13m1.4	Exon 25; between nt 7761-7768

To make sure that our mutants do not express AtVPS13M1 at the protein level, we performed proteomic analysis for each mutant in triplicates since we were lacking a reliable anti-AtVPS13M1 antibody (**Figure 43**). Total proteins were extracted from callus samples and subjected to targeted proteomic analysis. In Col0, several AtVPS13M1-specific peptides distributed over the whole length of the protein could be detected with a substantial amount of counts. In the four T-DNA insertion lines, on the opposite, no specific peptide could be detected. Thus, the four T-DNA insertion lines *atvps13m1.1-4* are KO for AtVPS13M1 at the protein level and can be used for phenotypic analysis.

#### 2.7 AtVPS13M1 participates in *Arabidopsis* lipid homeostasis

The widely recognized role of VPS13 proteins in non-vesicular lipid transport made us wonder if AtVPS13M1 also plays a role in plant lipid homeostasis. To investigate the implication of AtVPS13M1 in intracellular lipid trafficking, we used our Pi starvation system to induce membrane lipid remodeling in plant cells requiring extensive lipid transport between compartments.

To achieve this, Col0 and the AtVPS13M1 KO mutants *atvps13m1.1* and *atvps13m1.3* calli were cultured in presence and absence of Pi and their total glycerolipid content was analyzed. The lipidomic analysis results show that Col0 calli accumulate galactolipids and have decreased amounts of phospholipids during Pi starvation compared to Pi replete conditions, as expected (**Figure 44A**). A similar lipid remodeling is observed in both *atvps13m1* mutants, showing that remodeling is not impaired by the absence of AtVPS13M1. However, the *atvps13m1* mutants both have a higher galactolipid and phospholipid content in both +Pi and –Pi conditions. These differences can be observed for almost all glycerolipids, but are statistically significant for DGDG, PE and PC amounts (**Figure 44A** and **Figure 44B**). For DGDG, only *atvps13m1.3* exhibits a significant accumulation compared to Col0 in -Pi conditions (**Figure 44B**). For PE, atvps13m1.1 significantly increases in +Pi while both mutants have a significant accumulation in –Pi (**Figure 44B**). For PC, both mutants show significant increases in both +Pi and –Pi conditions (**Figure 44B**).

To determine if *atvps13m1.1* and *atvps13m1.3* have an altered lipid remodeling efficiency upon Pi starvation, we compared the fold change for each lipid amount in -Pi versus +Pi conditions (**Figure 44C**). The results show that DGDG tends to accumulate in *atvps13m1.3* -Pi conditions compared to Col0. Interestingly, both *atvps13m1* mutants have smaller fold changes for PE and PC compared to

Col0, suggesting that these lipids likely accumulate in the mutants upon Pi starvation due to a partial degradation defect. Both effects could be explained by a reduction in global lipid transport capacity due to the absence of AtVPS13M1. DGDG might be less transported to its target organelles leading to its overproduction in the chloroplast. Similarly, PE and PC might be less transported to their degradation sites thus leading to their accumulation. Neither of these hypotheses can be proven with this dataset and further investigation is needed to identify the cause of this effect.



**Figure 44** Lipidomic analysis of *Arabidopsis thaliana* whole calli. In all histograms, the represented values correspond to the median of three biological replicates and the error bars represent the range between minimum and maximum values. Galactolipids are indicated in green, phospholipids in red and neutral lipids in black. (A) Lipid quantities in phosphate replete (+Pi) and deplete (-Pi) conditions for glycerolipids of *Arabidopsis* whole calli. Quantities are expressed in nmol of lipids per mg of callus. Statistical significance between conditions is indicated by stars (p value < 0,05). (B) Zoom on the data of DGDG, PE and PC lipid quantities in phosphate replete (+Pi). Quantities are expressed in nmol of lipids per mg of callus. Statistical significance between conditions is indicated by stars (p value < 0,05). (C) Log<sub>2</sub> of fold change in lipid quantities between phosphate deplete and replete conditions for DGDG, PE and PC of *Arabidopsis* whole calli. Statistical significance between conditions is indicated by stars (p value < 0,05). (C) Log<sub>2</sub> of fold change in lipid quantities between phosphate deplete and replete conditions for DGDG, PE and PC of *Arabidopsis* whole calli. Statistical significance between conditions is indicated by stars (p value < 0,05).



**Figure 45** Lipidomic analysis of *Arabidopsis thaliana* isolated mitochondria. In all histograms, the represented values correspond to the median of three biological replicates and the error bars represent the range between minimum and maximum values. Galactolipids are indicated in green, phospholipids in red and neutral lipids in black. (A) Lipid quantities in phosphate replete (+Pi) and deplete (-Pi) conditions for glycerolipids of *Arabidopsis* mitochondria. Quantities are expressed in nmol of lipids per  $\mu$ g of mitochondrial proteins. No statistical significance between conditions could be determined (p value < 0,05). (B) Log<sub>2</sub> of fold change in lipid quantities between phosphate deplete and replete conditions for DGDG, PE and PC in *Arabidopsis* mitochondria. No statistical significance between conditions could be determined (p value < 0,05).

Since we suspect that AtVPS13M1 is associated to mitochondria, we wondered whether its absence could trigger lipid remodeling defects in mitochondria in standard conditions and upon Pi starvation. To address this question, mitochondria from Col0, *atvps13m1.1* and *atvps13m1.3* were isolated by cell fractionation and their glycerolipidome was determined. The quantification of mitochondrial glycerolipid contents revealed a high variability in the data (**Figure 45A**). Consequently, no statistical significance could be determined for mitochondrial glycerolipids. Why such a huge variability appeared in our lipidomics data is not clear. The mitochondria isolation is a long, multistep process including



**Figure 46** Glycerolipid distribution in *Arabidopsis thaliana* isolated mitochondria in phosphate replete (+Pi) and deplete (-Pi) conditions. The represented values correspond to the median of three biological replicates and the error bars represent the range between minimum and maximum values. Galactolipids are indicated in green, phospholipids in red and neutral lipids in black. Quantities are expressed in percentage of total lipids. No statistical significance between conditions could be determined (p value < 0,05).

several washes. Since the absolute lipid quantity is normalized by the mitochondrial protein content, variations in protein quantities due to callus growth conditions might induce the observed variability in normalized lipid content. This hypothesis is corroborated by the fact that less variability is observed when lipid class quantities are expressed as a percentage of total lipid content (**Figure 46**). Nonetheless, the expected lipid remodeling occurring in –Pi is clearly visible in Col0, *atvps13m1.1* and *atvps13m1.3* mitochondria with accumulation of DGDG and degradation of phospholipids, mainly of PE and PC (**Figure 45A**). However, no significant difference in lipid remodeling efficiency could be observed between Col0 and the *atvps13m1* mutants (**Figure 45B**), suggesting that the absence of AtVPS13M1 does not affect mitochondrial lipid homeostasis. Interestingly, *atvps13m1.1* and *atvps13m1.3* mitochondria both seem to contain less diphosphatidylglycerol (DPG, cardiolipin) compared to Col0, which might indicate mitochondrial destabilization, but data variability is too high to conclude (**Figure 45A**).

Altogether, our data suggest that AtVPS13M1 contributes to lipid homeostasis of *Arabidopsis* whole calli but does not seem to play a role in mitochondrial lipid homeostasis as the perturbation might be compensated by other pathways. How AtVPS13M1 affects lipid distribution upon Pi starvation and which lipids are transported between which compartments remains to be discovered.

#### 2.8 AtVPS13M1 mutant phenotype analysis

#### 2.8.1 AtVPS13M1 plants growth exhibit no growth phenotype

To determine if AtVPS13M1 plays a role in *Arabidopsis* growth, *atvps13m1.1-4* mutants were sown on soil and grown with a 8h-16h light-dark photoperiod at 22°C. *atvps13m1.2-4* had a slightly more vigorous aspect in the beginning of their development (**Figure 47A**), probably due to the slightly faster germination dynamics. However, this vigorous aspect rapidly vanished to become undiscernible after 16 days of growth (**Figure 47B**). No particular phenotype could be detected in these conditions.

#### 2.8.2 Cold stress

In yeast, Csf1/Tweek, a member of the RBG protein family, was identified as being important in cold stress response (John Peter, Cheung, et al., 2022). Similarly, the *Caenorhabditis elegans* protein LPD-3/Tweek is important for cold stress resistance (C. Wang et al., 2022). To test if AtVPS13M1 plays a role in cold stress acclimation in *Arabidopsis thaliana*, *atvps13m1.1-4* mutants were sown, grown for 2 weeks at 22°C with a 8h-16h light-dark photoperiod and eventually grown at 4°C. After 16 days, atvps13m1.2-4 grew more vigorously, as previously observed (**Figure 48A**). After 69 days of growth at 4°C, *atvps13m1.1* grew substantially slower than Col0 while *atvps13m1.2-4* grew like Col0 (**Figure 48B**). The fact that *atvps13m1.1* grew differently than the three other mutants suggests that it might have an additional T-DNA insertion making it different from the others. To conclude, since *atvps13m1.2-4* do not show any cold sensitivity, it is unlikely that AtVPS13M1 plays a major role in cold acclimation.



**Figure 47** Growth of *Arabidopsis* Col0 and *atvps13m1* mutants on soil. Plants were grown with a 8h-16h lightdark photoperiod at 22°C and a light intensity of 100  $\mu$ E for 9 days (**A**) and 16 days (**B**).



**Figure 48** Growth of *Arabidopsis* Col0 and *atvps13m1* mutants on soil in cold stress. Plants were grown with a 8h-16h light-dark photoperiod at 4°C and a light intensity of 100  $\mu$ E for 16 days (**A**) and 69 days (**B**).

#### 2.8.3 Pollen tube germination

Pollen tubes are one of the fastest elongating structures in plants. Thus, during pollen tube growth, huge quantities of membranes have to be generated in a very short time and transported to the pollen tube tip, implying efficient transport mechanisms. Pollen tube growth-related membrane generation is thought to heavily rely on vesicular transport (Adhikari et al., 2020). However, it has previously been shown that the proteins KINKY POLLEN (KIP; At5g49680) in *Arabidopsis thaliana* as well as APT1 in *Zea mays* are required for pollen tube growth (Procissi et al., 2003; Z. Xu & Dooner, 2006). KIP and APT1 are SABRE-like proteins (from SABRE; SAB; At1g58250) identified to have a general effect on tip growth. SABRE, KIP and APT1 have recently been identified as members of the RBG protein family which are supposed to function as lipid transport proteins since they harbor a long hydrophobic groove and are located at membrane contact sites (Neuman, Levine, et al., 2022). Thus, it is tempting to speculate that SABRE, KIP and APT1 might provide lipids for membrane biogenesis during pollen tube growth in plants. To test if AtVPS13M1 also plays a role during tip growth, we investigated pollen germination of *atvps13m1* mutants.

Pollen from Col0 and *atvps13m1.1-4* plants were incubated in pollen germination medium and the rate of germination was determined after 30min, 6h and 24h (**Figure 49**). As expected, the pollen germination rate increases over time for all plant lines. At each time point, no difference in germination rate between Col0 and the atvps13m1 mutants was observed. Only two biological replicates were done

for this experiment. Hence, no statistical test could be performed. However, it seems unlikely that any of the mutants would show a different behavior in a third biological replicate. Furthermore, a recent preprint analyzed the germination rate of AtVPS13M1 and AtVPS13M2. While they found that AtVPS13M2 had significantly lower germination rates than Col0, no difference was observed for AtVPS13M1 (Tangpranomkorn et al., 2022), which is consistent with our finding.



**Figure 49** Pollen germination rate determination for atvps13m1 mutants. *Arabidopsis* Col0 and atvps13m1.1-4 mutant pollen grains were harvested and the germination rate was determined at 30 min, 6h and 24h after the harvest. For each condition, six images containing at least 30 pollen grains were analyzed. Two biological replicates were performed (n=2). Values represent the median and error bars represent minimum and maximum values.

#### 3 Discussion and perspectives

VPS13 proteins have gained a lot of attention in the last decade due to the high number of cellular processes they seem to influence and because they are a founding member of a novel and peculiar family of lipid transport proteins: the repeating  $\beta$ -groove or bridge-like lipid transport proteins (Braschi et al., 2022; Levine, 2022; Neuman, Levine, et al., 2022; Toulmay et al., 2022). While their study in yeast and humans has already taken a huge leap forward, VPS13 proteins have remained almost unstudied for their function as bulk lipid transporters in plants. Here, we conducted the first systematic analysis of a VPS13 protein in *Arabidopsis thaliana* in the context of non-vesicular lipid transport and open many new paths for their investigation.

#### 3.1 AtVPS13M1 gene holds an unexpected diversity of splice variants

We took a long-read sequencing approach using Nanopore technology to unveil the splice variant diversity of the At4g17140/AtVPS13M1 gene and to identify a possible splice variant expression bias in phosphate deplete versus replete conditions. We identified six novel splice variants not described in TAIR (isoforms A, D, E, G, I, J). These splice variants differ from the predicted sequences by unspliced introns and exon border annotations. We found that phosphate starvation had no impact on the abundance of the different isoforms. Among all the identified isoforms, isoform A was found to be highly abundant making up almost 92% of the reads and corresponding to the sequence we cloned for further experiments. Surprisingly, only two of the novel splice variants encode full-length proteins (isoforms A and G) while the unspliced introns of the other four lead to frameshifts and early STOP codons. The existence of all the newly defined splice variants has been confirmed by PCR. Altogether, we provide the robust characterization of the At4g17140/AtVPS13M1 transcriptional landscape.

To attain a satisfactory depth of sequencing, a fragment containing all the predicted variable regions of AtVPS13M1 gene was amplified by PCR from coding DNA prior to Nanopore long-read sequencing. While this reinforces the confidence in our sequencing results, an additional amplification step might introduce a quantitative bias. In fact, the respective isoforms may have been amplified with different efficiencies, thus interfering with the relative abundances of the identified isoforms. We found that isoform A represented almost 92% of the reads while the other isoforms represented only 1% or 2% each. While it is possible that the PCR amplification slightly modified the respective isoform A abundances compared to the real mRNA distribution, it seems unlikely that the dominant isoform A abundance is solely due to PCR artefacts. The differences in abundance observed between the minor isoforms, however, should be handled with care.

The unbalanced distribution of AtVPS13M1 isoform abundances raises the question of the biological relevance of each of the isoforms. Since isoform A is overly abundant and encodes a full-length protein, it is highly probable that this exon arrangement is biologically relevant and performs most, if not all, AtVPS13M1 biochemical functions in *A. thaliana* calli. Among the five other identified isoforms, only

isoform G encodes a full-length protein while the other exon configurations introduce early STOP codons. Given the low abundance of these isoforms, it is possible that they emerged through defaults in the splicing process, which is particularly plausible for a gene of this size. It is however unlikely that they emerged through sequencing artefacts because their existence was verified by PCR. Even though it is improbable that truncated AtVPS13M1 could be functional, it cannot be ruled out that such AtVPS13M1 fragments could play a role in cells. In humans, the transcriptional landscape of VPS13 genes has already been investigated (Kolehmainen et al., 2003; Rampoldi et al., 2001; Velayos-Baeza et al., 2004). It is interesting to note that the human VPS13 genes all have at least two major splice variants but also present multiple minor isoforms. None of these isoforms have been functionally investigated apart from two HsVPS13A isoforms (Rampoldi et al., 2001). Interestingly, Zhang et al. also identified the isoform A in their long-read RNA sequencing study with minor differences addressed earlier (S. Zhang et al., 2020), further supporting the hypothesis that this isoform is biologically relevant. The same study identified what seems to be our isoform D with the described differences (see results), but does not identify any other isoform. Thus, the biological relevance of the isoforms D, E, G, I and J remains to be investigated.

The tissue-specific distribution of the AtVPS13M1 splice variants is an aspect we have not addressed in our study. According to the BAR (Bio-Analytic Resource for Plant Biology) tissue expression database, At4g17140/AtVPS13M1 is not preferentially expressed in any *A. thaliana* tissue but rather presents a ubiquitous expression pattern (Nakabayashi et al., 2005; Schmid et al., 2005). This suggests that the AtVPS13M1 splice variant expression pattern observed in calli might be the same in whole plants. Zhang et al. used 14 days old seedlings and floral buds in their direct RNA sequencing approach in which they identified the equivalents of the isoforms A and D (S. Zhang et al., 2020). This indicates that the most expressed isoform in calli is also found in whole plant tissues. The splice variant abundance distribution of human VPS13 proteins do not show any tissue specificity either and are ubiquitously expressed (Kolehmainen et al., 2003; Rampoldi et al., 2001; Velayos-Baeza et al., 2004). Future studies should focus on determining whether plant tissue-specific AtVPS13M1 splice variants exist and investigate their biological relevance in light of the function of the tissue.

#### 3.2 AtVPS13M1 subcellular localization remains elusive

Determining the localization of AtVPS13M1 is essential to understand its cellular functions. Since we were not successful in cloning the full-length AtVPS13M1 protein, we took advantage of its multidomain organization and the assumption that several individual domains each contribute to the final localization of VPS13 proteins. We generated a large set of AtVPS13M1 parts (fragments) C-terminally fused to GFP to dissect its localization determination scheme. Surprisingly, all the generated fragments co-localized with an ER-marker after transient expression in *Nicotiana benthamiana*. Only the Nterminal PH domain and the  $\beta$ -tripod fragments displayed a secondary localization in the nucleus and/or the cytoplasm. The recurrent ER localization raised doubts on the veracity of the observed localizations. Our attempt to determine AtVPS13M1 native localization by immunostaining and immunogold EM was cut short by the low efficiency of the anti-AtVPS13M1 antibodies. We further investigated the possible association of AtVPS13M1 with mitochondria by BiFC and rBiFC. Using AtTOM20.2, AtMIRO1 or AtMIRO2 as bait for different AtVPS13M1 fragments transiently expressed in both *Nicotiana benthamiana* and *Arabidopsis thaliana* leaves did not reveal any interaction signal. Altogether, our results constitute a huge work for the determination of AtVPS13M1 localization. Despite our efforts, localization and interaction data remain inconclusive and need further investigation.

The observation that all the tested AtVPS13M1 fragments localized to the ER was unexpected and puzzling (Figure 35 & Figure 36). We used a heterologous overexpression system to determine the localizations of the GFP-fused fragments. Since we supposed that AtVPS13M1 localization, like yeast and human VPS13 proteins, is in part determined by protein partners located in the target membranes, we suspected that the right protein partners were not present in N. benthamiana to allow a correct localization. Furthermore, the high amount of expressed AtVPS13M1 fragments might saturate the protein partners. In fact, we suspect that VPS13 proteins are expressed ubiquitously but at very low levels in plants as exemplified by the low number of reads (3 in total) obtained for At4g17140/AtVPS13M1 in direct RNA sequencing (S. Zhang et al., 2020). In addition, the overexpressed AtVPS13M1 fragments might compete with the N. benthamiana VPS13 proteins. Why the fragments associate precisely with the ER is unclear. The hydrophobic nature of the AtVPS13M1 fragments might induce the association with intracellular membranes and the most extended membrane system in plant cells (outside the chloroplast) happens to be the ER. However, not all fragments harbored extensive hydrophobic surfaces and still associated with the ER. We suspected that the AtVPS13M1 fragments and/or the linker separating the fragment from the GFP contained a motif that induces the association with the ER. We found no known ER-association motif in any of the AtVPS13M1 fragments and the linker alone fused to GFP had a cytosolic localization (Figure 37B). Some fragments also had a cytosolic and/or nuclear localization in addition to their ER localization (Figure 37A). No fragment harbored a nuclear localization signal. It is commonly accepted that proteins with a molecular mass smaller than about 30 kDa can diffuse freely from the cytoplasm to the nucleus but free diffusion of proteins up to 90 kDa has been observed in HeLa cells (R. Wang & Brattain, 2007). eGFP has a molecular mass of 27 kDa and our smallest fusion construct (PH\_Nt-GFP) had a molecular mass of 40 kDa. The biggest fusion protein observed inside the nucleus ( $\beta$ -tripod.1+2-GFP) had a molecular mass of 73 kDa. It is therefore plausible that these nuclear localizations are due to mere diffusion.

The contribution of the respective AtVPS13M1 localization determinants to the resulting protein targeting remains unresolved. Interestingly, a recent pre-print focused on the role of AtVPS13M2 in pollen germination and pollen tube elongation (Tangpranomkorn et al., 2022). Notably, they show that AtVPS13M2 marks the site of pollen tube elongation and that it localizes at the tip of the growing pollen tube in an actin-dependent manner. The authors further determined that AtVPS13M2 associates with

membrane structures implicated in exocytosis like the exocyst, which helped to conclude that AtVPS13M2 regulates vesicular trafficking to the tip of the pollen tube. It is therefore tempting to speculate that AtVPS13M2 localizes to the plasma membrane and to exocytic vesicles in pollen tubes. However, this remains to be confirmed. The only differences in domain arrangement between AtVPS13M1 and AtVPS13M2 are an additional C-terminal  $\beta$ -tripod domain and an additional FFAT motif for AtVPS13M1 (**Figure 27**). Despite their structural resemblance, AtVPS13M1 has no impact on pollen germination time or rate in contrast to AtVPS13M2 (Tangpranomkorn et al., 2022) (**Figure 49**). This suggests the possibility that AtVPS13M1 is targeted to different membranes than AtVPS13M2 due to the additional FFAT and  $\beta$ -tripod domains.

The presence of a FFAT motif in AtVPS13M1 strongly suggests that it associates to the ER by binding to VAPs (Slee & Levine, 2019). Furthermore, AtVPS13M1 was found in the *A. thaliana* mitochondrial proteome (Duncan et al., 2011), mitochondrial complexome (Senkler et al., 2017) and it was identified as a potential AtTOM40-HA interactor in a co-IP experiment (Michaud & Albrieux, unpublished). It is therefore tempting to speculate that AtVPS13M1 localizes at ER-mitochondria contact sites. Multiple VPS13 proteins in yeast and humans have already been identified at ER-mitochondria contact sites or associated to mitochondria (see introduction) (Dziurdzik & Conibear, 2021). Up to now, only a restricted number of VPS13 domains have been found to promote association with mitochondria. In yeast, the ScVPS13 VAB domain interacts with the mitochondria-localized Mcp1 protein through a PxP motif (John Peter et al., 2017). The human ATG2A ATG2\_C domain was found to associate to mitochondria, probably by interacting with TOM40 (Z. Tang et al., 2019). The C-terminal PH domain of human HsVPS13A was also shown to localize to mitochondria (Guillén-Samander et al., 2022; Kumar et al., 2018; Yeshaw et al., 2019). All these domains are also present in AtVPS13M1, suggesting that it might have the capacity to associate to mitochondria. However, there is currently no indication that the same domains in different VPS13 protein homologs promote the same localization.

We investigated the possible association of AtVPS13M1 with mitochondria by BiFC and rBiFC. Using AtTOM20.2, AtMIRO1 or AtMIRO2 as bait for different AtVPS13M1 fragments transiently expressed in both *Nicotiana benthamiana* and *Arabidopsis thaliana* leaves yielded no interaction signal. The most straightforward conclusion is that the lack of interaction signal reflects the absence of direct interaction between bait and prey. AtVPS13M1 was found in co-IP performed with AtTOM40-HA, but since the co-IP was realized on DSP-crosslinked mitochondria, the suspected interaction might be indirect. Hence, AtVPS13M1 might not be in close proximity of AtTOM20.2. Furthermore, TOM20 is known to associate to the TOM complex in a transient manner as it presumably serves different mitochondrial functions (Pitt & Buchanan, 2021). Similarly, we suspected AtMIRO1/2 to be able to interact with AtVPS13M1 because AtMIRO2 co-migrated with AtVPS13M1 on BN-PAGE (Senkler et al., 2017) and because human HsVPS13D interacts with MIRO1/2 (Guillén-Samander et al., 2021). However, none of these observations constitutes a proof of direct interaction between AtVPS13M1 and AtMIRO1/2 in *A*.

*thaliana*, and our results suggest they are not directly interacting. Another reason for the lack of interaction could be the titration of the AtVPS13M1 fragments by the ER membrane. In fact, we used the same fragments for BiFC/rBiFC experiments than for the GFP fusions. Since all the fragments seem to associate to the ER, it is possible that not enough AtVPS13M1 fragments are left to interact with the bait and give a measurable YFP signal. The BiFC method can be criticized in many aspects including the tendency of the split fluorescent protein to self-assemble, the association with unrelated proteins and many more (Horstman et al., 2014). One less addressed issue is the maturation time of the fluorophore which can reach up to 50 min for split YFP (Rose et al., 2010). Thus, dynamic protein-protein interactions like the one we suspect for AtVPS13M1 may not allow the timely maturation of the split fluorophore.

Taken together, our data constitute the first systematic study of AtVPS13M1 sub-cellular localization. It is clear that many questions and uncertainties stand out from our heterologous transient expression localization and interaction experiments. To further characterize AtVPS13M1 localization and interactors, it will be important to express the full-length protein in stable lines and in quantities that match the native expression levels. To achieve this, we took advantage of the A. thaliana JAtY library that inserted the whole A. thaliana genomic DNA in TACs (transformation ready bacterial artificial chromosomes). We used a dedicated recombineering technique (Brumos et al., 2020) on the relevant A. thaliana genome section to generate full-length AtVPS13M1 fused to mCherry, to a triple tandem of YFP-derived YPet fluorescent protein and to the  $\beta$ -glucuronidase (GUS) reporter system. To maintain recombinant AtVPS13M1 functional, we inserted the desired reporter proteins at a site known to maintain VPS13 protein function (Lang et al., 2015). These constructs have been transformed in the atvps13m1.3 KO mutant plant line to avoid competition with the endogenous AtVPS13M1. If the transformations are effective, this opens up a whole array of experiments for future studies. First, this approach gives access to the native AtVPS13M1 localization either by standard fluorescence confocal microscopy or by immunogold EM. Second, the improved YPet fluorophore allows its use in FRET experiments to further investigate the proximity between AtVPS13M1 and potential interactors. These lines will also allow co-IP experiments to determine AtVPS13M1 interactors that could recruit the protein to its target site. Finally, the GUS reporter system will allow to assess the tissue-specific AtVPS13M1 expression levels in planta, and particularly in response to Pi starvation. All these perspectives are already quite ambitious and address numerous key questions. However, the way AtVPS13M1 and VPS13 proteins in general are targeted to specific locations remains to be solved.

## 3.3 AtVPS13M1 binds multiple phosphoglycerolipids *in vitro* and participates in lipid homeostasis *in vivo*

VPS13 proteins are lipid transport proteins known to transport lipids between membranes. While the lipid binding properties of VPS13 and VPS13-like proteins have already been demonstrated *in vitro* (Kumar et al., 2018; Valverde et al., 2019), their impact on the global lipidome *in vivo* has never been

shown. To assess AtVPS13M1 lipid binding capacity, we realized *in vitro* lipid binding assays using the 335 first AtVPS13M1 amino acids and fluorescently labelled phosphoglycerolipids. We show that AtVPS13M1\_1-335 efficiently binds several phosphoglycerolipids. To ask whether AtVPS13M1 is important for *A. thaliana* cells and mitochondria lipid homeostasis *in vivo*, we analyzed the lipidomes of *atvps13m1* T-DNA insertion KO mutants in whole cells and isolated mitochondria both in phosphate deplete and replete conditions. Our results show that the *atvps13m1* mutant lipidomes are perturbed in whole cells but not in mitochondria. Notably, *atvps13m1* mutant cells accumulate DGDG in –Pi conditions and degrade less PE and PC in both +Pi and –Pi conditions. We propose that AtVPS13M1 participates in the transport of DGDG to its target organelles during phosphate deplete and replete conditions.

The N-terminal part of VPS13 proteins is highly conserved in eukaryotes. This part contains the Chorein N domain and the beginning of the hydrophobic tunnel that characterizes RBG. In vitro binding assays determined that the N-terminal parts of yeast VPS13 and human ATG2A are able to bind phospholipids (Kumar et al., 2018; Valverde et al., 2019). Notably, the first 1350 aa residues of yeast ScVPS13 efficiently bind phosphoglycerolipids even after harsh washing steps. Even if PC was found in slightly higher quantities, no binding specificity was determined as various phosphoglycerolipids but no other lipid types were bound to the protein fragment. A similar experiment carried out with human ATG2A showed that it is able to bind tens of phosphoglycerolipids. Our experiments clearly show that the first 335 aa residues of AtVPS13M1 efficiently bind several fluorescently labeled phosphoglycerolipids, namely PC, PA, PS and PA (Figure 42). Given the high degree of conservation of this segment across eukaryotes, this result was expected and confirms the functional homology between VPS13 proteins. While we were able to determine AtVPS13M1 lipid binding capacity through our experiments, we provide no binding specificity or preference data. Future experiments should address the question of a possible lipid binding bias and whether AtVPS13M1 is also able to bind plantspecific lipids like DGDG. To do so, we already started to optimize the protein fragment purification protocol in order to increase the quantity and purity of AtVPS13M1. This should allow to realize binding assays directly with total A. thaliana lipid extracts containing all the natively found glycerolipids species whose identities and quantities will be determined by mass spectrometry. We are also planning an *in* vitro lipid transfer assay as previously described (Hanna et al., 2022; Kumar et al., 2018; Valverde et al., 2019) allowing to determine the lipid transfer capacity of AtVPS13M1 (Figure 50).



**Figure 50** FRET-based *in vitro* assay to detect lipid transport activity. A donor liposome is composed of 1palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), soy-PE, 18:1 DGS-NTA(Ni) and two fluorescently labeled lipids; nitrobenzoxadiazole (NBD)-PS and rhodamine (Rh)-PE. In donor liposomes, NBD-PS fluorescence is quenched by FRET with Rh-PE. Acceptor liposomes are composed of POPC, soy-PE and brain phosphatidylinositol 4,5-bisphosphate (PI(4,5)P<sub>2</sub>). To test if a particular protein or protein part is able to transport lipids from one liposome to another, the peptide of interest is fused at its C-terminus with the PH domain of rat PLC $\Delta$  and a His<sub>6</sub> tag separated by a flexible linker from human E-Syt2. The PH domain binds to the PI(4,5)P<sub>2</sub> of the acceptor liposome while the His<sub>6</sub> tag binds to the DGS-NTA (Ni) of the donor liposome thus creating a tether between donor and acceptor liposomes. If the peptide of interest is able to transfer lipids between both types of liposomes, the fluorescently labeled lipids will be diluted resulting in the alleviation of the NBD-PS quenching leading to an increase in fluorescence intensity over time.

VPS13 proteins drive bulk lipid transport between organelles (Leonzino et al., 2021; Melia & Reinisch, 2022; Reinisch & Prinz, 2021). Lipid transport by VPS13 proteins *per se* is thought to be non-specific as exemplified by *in vitro* binding and transfer experiments (Kumar et al., 2018). Whether and how other RBG protein-associated proteins determine the specificity and/or directionality of lipid transport is currently under debate (Ghanbarpour et al., 2021; Melia & Reinisch, 2022). Nevertheless, a huge volume of lipids is presumably transported through VPS13 proteins and more generally through RBG proteins. Given the high number of membrane contact sites at which VPS13 proteins have been localized, the depletion of one or more VPS13 proteins might result in severe lipidome perturbation. While many cellular and physiological effects have been observed in absence of VPS13 proteins (Dziurdzik & Conibear, 2021; Guillén-Samander & De Camilli, 2022), changes in total lipidomes have rarely or only partially been reported to date.

We show that callus depleted in AtVPS13M1 have a perturbed lipidome on the whole cell level, especially upon phosphate starvation (**Figure 44**). Notably, we observed that DGDG accumulated in *atvps13m1.3* upon phosphate limitation. DGDG is exclusively synthetized in the chloroplast and is massively exported to extra-chloroplastic membranes to compensate for the degradation of phosphoglycerolipids upon Pi starvation (Andersson et al., 2005; Hartel et al., 2001; Jouhet et al., 2003;

Moellering & Benning, 2011; Tjellström et al., 2008). Why DGDG accumulates in absence of AtVPS13M1 is not clear. We speculated that AtVPS13M1 might be implicated in the delivery of DGDG to its target organelles (vacuole, plasma membrane, mitochondria). This possibility does not imply that AtVPS13M1 is localized at chloroplast membrane contact sites as DGDG might transit through other compartments prior to its final delivery. Failing to deliver DGDG to their target site might stimulate a feedback loop leading to the overproduction of DGDG to try to compensate for the lack of DGDG in one or more extra-chloroplastic compartments. This possible explanation is highly speculative and needs to be investigated. We also showed that *atvps13m1.1* and *atvps13m1.3* accumulate PC and PE in both Pi replete and deplete conditions. We further show that their accumulation during Pi starvation is due to a lack of degradation rather than a mere accumulation. Some PC and PE degradation enzymes are known to function at particular sites upon Pi starvation. NPC4 functions at the plasma membrane (Y. Nakamura et al., 2005) while PLDZ2 degrades PC to PA at the tonoplast and localizes at sites of close proximity between vacuole-mitochondria and vacuole-chloroplast in Pi limited conditions (Shimamura et al., 2022; Yamaryo et al., 2008). NPC5 has also been proposed to be recruited at particular sites when required (Gaude et al., 2008). These observations lead to the hypothesis that phospholipids are processed at specialized degradation sites in proximity of multiple membranes, which might favor the transport to and from these sites. We propose that AtVPS13M1 might be implicated in the transport of PC and PE to their degradation sites upon phosphate limitation. According to this hypothesis, the absence of AtVPS13M1 would lead to the accumulation of PC and PE caused by a lack of degradation. Interestingly, an accumulation of PC and PE in the *atvps13m1* mutants was also observed in Pi replete conditions, though in a less pronounced way. In A. thaliana, it is estimated that as many genes are implied in lipid turnover than in lipid biosynthesis (Li-Beisson et al., 2013). These encode (phospho)lipases, hydrolases and enzymes involved in  $\beta$ -oxidation. Despite their importance in lipid metabolism, very little is known about their mechanism of action and localization. The same enzymes involved in phospholipid degradation during Pi starvation can be implied in lipid turnover. Notably, this is the case for the phosphatidic acid phosphatases AtPAH1 and AtPAH2 in absence of which the eukaryotic pathway is compromised (Y. Nakamura et al., 2009). Phospholipid turnover requires the transport of lipids to the processing enzymes. The accumulation of PC and PE in absence of AtVPS13M1 might reflect the decreased lipid transport capacity toward the phospholipid remodeling/turnover sites.

As we suspected that AtVPS13M1 is associated to mitochondria, we hypothesized that the mitochondrial lipidome would be affected in absence of AtVPS13M1. The absence of lipidome perturbation in isolated mitochondria from *atvps13m1.1* and *atvps13m1.3* was unfortunate, but not unexpected (**Figure 45** & **Figure 46**). Non-vesicular glycerolipid transport to mitochondria is carried out by a huge diversity of MCS effectors, especially localized at ER-mitochondria contact sites (see general introduction). Notably, multiple LTPs are transporting the same lipids to mitochondria, leading to a high level of

redundancy in the lipid transport routes. In yeast, the deletion of the ERMES complex residing at ERmitochondria contact sites causes growth and mitochondrial defects that can be rescued by dominant point mutations in ScVPS13 (Lang et al., 2015; Park et al., 2016) or by the forced recruitment of ScVPS13 to mitochondria (John Peter et al., 2017). Furthermore, deletion of ScVPS13 had no effect on yeast growth or mitochondrial function (John Peter, Petrungaro, et al., 2022). Surprisingly, even the deletion of all five RBG proteins in yeast only resulted in a 40% decrease in DPG production in mitochondria without affecting other phosphoglycerolipids and did not impair growth or mitochondrial function (Toulmay et al., 2022). Finally, it was shown that ScVPS13 has a rather low contribution to lipid transport to yeast mitochondria in vivo (John Peter, Petrungaro, et al., 2022). These examples illustrate that VPS13 proteins can easily be bypassed as far as their function in lipid transport to mitochondria is concerned. Even though the knowledge gained in yeast cannot simply be applied to plants, it is conceivable that AtVPS13M1 can be bypassed in a similar way. AtVPS13M2 has an almost identical domain organization and structure as AtVPS13M1 suggesting that AtVPS13M2 could compensate for the loss of AtVPS13M1. Similarly, the other A. thaliana VPS13 protein paralogs AtVPS13S and AtVPS13X could be redundant with AtVPS13M1. Alternatively, AtVPS13M1 might also be redundant with other not yet identified LTPs. Finally, it could simply be that AtVPS13M1 is not implied in lipid transport to mitochondria either because it is located elsewhere or because it carries out functions at the mitochondrial membrane other than lipid transport. It is also worth noticing that a high variability between biological replicates was observed in our mitochondrial lipidome results for unexplained reasons. Therefore, a slight effect of the absence of AtVPS13M1 on the mitochondrial lipidome might have gone unnoticed.

The absence of localization data for AtVPS13M1 makes it hard to determine which organelles could be affected by the depletion of AtVPS13M1. Future studies should concentrate on determining the precise localization of AtVPS13M1 to analyze the lipidome of organelles of interest accordingly. Redundancy in the non-vesicular lipid transport routes is another difficulty that needs to be addressed. One way to circumvent redundancy is to find specific conditions in which a LTP becomes critical for cell physiology and/or lipid homeostasis. In yeast, the RBG protein Csf1 was shown to be important for the transport of lipids associated to membrane homeoviscous adaptation to cold (John Peter, Cheung, et al., 2022; John Peter, Schie, et al., 2022; Tokai et al., 2000) while the depletion of Csf1 had no impact at normal growth temperatures. Phosphate starvation in plants is a convenient inducible stress condition that we use to trigger extensive membrane remodeling necessitating non-vesicular lipid transport. Interestingly, AtVPS13M1 seems to be important for DGDG, PC and PE homeostasis during Pi limitation. It would be interesting to identify other conditions or biological processes in which AtVPS13M1 is even more influential and combine these with localization data to alleviate experimental ambiguities. Finally, generating *atvps13m1 atvps13m2* double mutants may help to remove some redundancy as both proteins are very similar.

Altogether, our data suggest that AtVPS13M1 is indeed a lipid transport protein with lipid binding properties *in vitro* and with an effect on membrane lipid homeostasis *in vivo*. Its exact role and mechanism of action in plant cells, however, remains to be determined.

#### 3.4 *atvps13m1* mutant plants phenotype analysis

As stated above, finding the conditions or biological processes in which the absence of a lipid transport protein will have a visible impact can be an arduous task. To identify some of these conditions and/or processes, we observed the *atvps13m1* mutant plant phenotypes. The plants grown in normal conditions presented no obvious growth phenotype. Plants subjected to cold stress revealed that *atvps13m1.2-4* were not different from Col0 while *atvps13m1.1* had a growth delay, suggesting that it might be different from the other T-DNA insertion mutants. Finally, we found that pollen germination was not impaired or delayed in the *atvps13m1* mutants. Overall, our data did not identify phenotypic differences between Col0 and *atvps13m1* plants in the tested conditions.

Few studies conducted phenotypic analysis on VPS13 mutant plants. The only A. thaliana VPS13 protein that has been extensively phenotyped is At5g24740 / AtVPS13S / SHRUBBY (Koizumi & Gallagher, 2013). Briefly, *atvps13s* mutants had a shrubby appearance, had shorter roots, produced much smaller plants than wild type, had curled leaves and presented short-petaled flowers. Furthermore, atvps13s plants were both male and female sterile, produced no visible pollen and produced no seeds. Other studies also identified a VPS13S homolog in Taraxacum officinale (Dandelion) as a determinant of diplosporic apomixis (Cornaro et al., 2023; Van Dijk et al., 2020). This dramatic *atvps13s* phenotype stands in contrast with our observations and raises questions on the reasons for such a difference. Like for lipid transport, AtVPS13M2 might partially compensate for the loss of AtVPS13M1 given their high degree of similarity. Therefore, we suspect that a *atvps13m1 atvps13m2* double KO mutant would have a visible phenotype, which should be the aim of future studies. Tangpranomkorn et al. reported that AtVPS13M2 presents a decrease in pollen germination rate and defects in pollen tube growth while neither phenotype was observed for AtVPS13M1 (Tangpranomkorn et al., 2022). Our results corroborate these observations. It is interesting to note that both *atvps13s* and *atvps13m2* mutants display defects linked to reproduction, even if no obvious phenotypes in seed production quantity and quality was observed in our mutant lines. It would therefore be interesting to focus on the more precise observation of reproductive parts in our *atvps13m1* mutants. Interestingly, *atvps13s* mutant roots had an abnormal cell layer organization and were significantly shorter. Related observations have also been reported for the Hobbit-like KINKY POLLEN protein mutants that also had defects in root tip growth (Procissi et al., 2003). Conducting an in depth analysis of root growth and root tip structure might therefore be an interesting prospect. We are currently analyzing *atvps13m1* mutant seedling root lengths on agar.

#### 3.5 Concluding remarks and future prospects

Investigating a protein the size of AtVPS13M1 was a challenging undertaking. We managed to identify the real sequence of AtVPS13M1 and implemented it on the general protein domain organization. We show that AtVPS13M1 is able to bind multiple phosphoglycerolipids *in vitro* and participates in lipid homeostasis in whole cells but likely not in mitochondria. AtVPS13M1 was particularly important for the maintenance of lipid homeostasis upon phosphate limitation, suggesting an important role in plant adaptation to phosphate starvation. Our AtVPS13M1 localization studies remain inconclusive but pave the way for further investigations including the use of the full-length protein. The still elusive localization of AtVPS13M1 makes it difficult to establish an elaborate working model. Nevertheless, we propose that AtVPS13M1 localizes at membrane contact sites between two organelles where it is implied in bulk lipid transport both in phosphate replete and deplete conditions (**Figure 51**).

Most importantly, the systematic investigation of AtVPS13M1 opened many new paths for future research. Investigating the role of the different VPS13 domains in its localization and protein function is an important prospect in the field. VPS13 proteins perform a huge variety of cellular functions that sometimes have no obvious link with their presumed biochemical activity. Understanding the link between VPS13 localization, regulation and function would allow to reconcile its various seemingly moonlight activities with its primary biochemical function. AtVPS13M1 has more annotated functional domains than its yeast and human counterparts, making it an interesting and challenging candidate to



**Figure 51** Model for AtVPS13M1 mode of action in *Arabidopsis thaliana* cells. AtVPS13M1 localizes to one or more membrane contact sites between not yet identified organelles where it mediates bulk lipid transport both in phosphate replete and deplete conditions. The protein model presented on the right is the RoseTTAFold prediction of *Chaetomium thermophilum* CtVPS13 protein.

study its presumably multifaceted regulation of localization and function. What lipids are transported by AtVPS13M1 and between which compartments is another outstanding question. Answering these fundamental questions will pave the way for new investigations on non-vesicular lipid transport to different plant organelles. While the phosphate starvation-induced membrane remodeling is an already well investigated phenomenon, the non-vesicular transport routes taken by lipids during this process are still widely unknown. By detecting a possible role of AtVPS13M1 in lipid homeostasis during phosphate limitation, we identified a new potential lipid transport route important for plant adaptation to phosphate starvation. Finally, the high degree of structural similarity between VPS13 proteins raises questions about the evolutionary origin of VPS13 proteins. To get more insight on the origin and diversity of VPS13 proteins in plants and algae, we are currently performing an advanced phylogenetic analysis.

# General conclusion and perspectives

The organization of the intracellular space into biochemically different compartments is an important paradigm in cell biology. This compartmentalization of the cell's biochemical activities has long held the view that organelles play their role in a rather isolated way and exchange metabolites with the cellular environment to sustain their function and to deliver their products. The discovery of membrane contact sites between virtually all organelles added an additional layer of complexity to the organellar functions. Compartments dynamically associate and dissociate from one another to perform specific functions depending on the physiological requirements of the cell. One particularly important function of membrane contact sites is non-vesicular lipid transport. The fact that organelles can directly exchange lipids by contacting each other adds a much more dynamic aspect to the regulation of membrane composition and organelle biogenesis. The field of non-vesicular lipid transport has known an enormous leap forward in the last two decades with the discovery of multiple types of lipid transport proteins and lipid transport mechanisms (Reinisch & Prinz, 2021; Wong et al., 2019). While much knowledge has already been accumulated in yeast and mammals, the fields of membrane contact sites and non-vesicular lipid transport are still in their infancy in plants (Leterme & Michaud, 2022). Our study contributes to fill this still considerable gap of knowledge.

Apart from the need to gain fundamental knowledge, studying membrane contact sites and non-vesicular lipid transport in plants is important for several other reasons. As stated before, the lipid transport mechanisms are conserved throughout evolution rather than the proteins themselves. Studying similar processes in different organisms helps to understand lipid transport mechanisms from different perspectives. Furthermore, new mechanisms discovered in plants could enrich the general knowledge and lead to new discoveries in other organisms. In plants, the formation of membrane contact sites and the transport of lipids is often associated with stress responses (Leterme & Michaud, 2022; Michaud & Jouhet, 2019). Since plants are sedentary organisms, these mechanisms are crucial for their adaptation to environmental stresses. Thus, better understanding these processes might pave the way for developing plant management strategies. An abiotic stress commonly encountered by plants in nature and in agriculture is phosphate limitation (Dissanayaka et al., 2021; Plaxton & Tran, 2011). Phosphate starvation response implies dramatic membrane lipid remodeling and necessitates extensive lipid transport between organelles. While phosphate starvation-induced membrane lipid remodeling has already been well characterized from a lipidomic point of view, how lipids are transported between organelles remains widely unknown.

The MTL complex is a huge mitochondrial complex spanning through both mitochondrial membranes. One of its key components, AtMIC60, plays an important role in intramitochondrial lipid trafficking, probably by mediating OM-IM tethering and by locally stabilizing membranes (Michaud et al., 2016). We took a biochemical approach to further characterize the composition and structure of the MTL complex. Our study proposes that the MTL complex is composed of not yet identified core components that dynamically interact with other mitochondrial and/or extramitochondrial complexes/components depending on the physiological needs. The lipid enrichment likely plays an important role in stabilizing the interactions between the different sub-complexes. Future work should allow to better define and understand the identity and functions of the MTL complex. A particularly important perspective is the investigation of the MTL complex interaction with the chloroplast. In this context, our laboratory is currently investigating OMP24, a chloroplast outer membrane protein found in the initial MTL complex proteome. The first results suggest that OMP24 overexpression leads to a mitochondrial lipidome perturbation in phosphate-limited conditions and to an alteration of mitochondrial morphology. This suggests that the MTL complex may not only be implied in intramitochondrial lipid transport, but that it might be able to contact other membranes to create a direct lipid exchange route between organelles. OMP24 is therefore an interesting candidate for mediating lipid exchange between chloroplasts and mitochondria, especially during phosphate starvation response. Furthermore, this potential mitochondria-chloroplast structure would be the first description of an ERMIONE-like network in plants. In addition, we have currently investigated the role of a small subset of MTL complex components in lipid transport, but others could also play a key role in this process. It could be the case for VDAC proteins that are in the MTL complex and were recently shown to have a scramblase activity (Jahn et al., 2022). Thus, this complex remains an important platform to identify key candidates involved in mitochondrial lipid transport and contact sites formation in plants.

While the MTL complex definitely plays a role in non-vesicular lipid transport to mitochondria during phosphate starvation, its role is probably indirect. Until now, no lipid transport protein has ever been described as directly playing a role in lipid transport associated to phosphate starvation-induced membrane remodeling. AtVPS13M1, a VPS13 homolog in Arabidopsis thaliana, is therefore the first LTP to participate in lipid homeostasis in phosphate-starved plants. This role opens multiple important perspectives for the study of membrane contact sites and non-vesicular lipid transport in plants. Defining AtVPS13M1 localization will allow to identify membrane contact sites that are important during phosphate starvation. Furthermore, identifying AtVPS13M1 partners might reveal other proteins and pathways important for lipid transport. This will also bring insight in the VPS13 lipid transport mechanism that still remains elusive. The current models state that bridge-like LTPs like VPS13 and ATG2 associate to membrane scramblases and the lipid synthesis machinery to promote lipid desorption, unloading and transport directionality (Ghanbarpour et al., 2021; Leonzino et al., 2021; Melia & Reinisch, 2022; Reinisch & Prinz, 2021; van Vliet et al., 2022). The identification of AtVPS13M1 partners might help to confirm this model. Molecular dynamics studies might also contribute to understand these basic principles (Dall'Armellina et al., 2023; Y. Zhang et al., 2022) and to design experiments allowing to experimentally confirm them. The study of AtVPS13M1 also raises questions on the way lipid transport protein activity is regulated at membrane contact sites. LTPs can be regulated at the expression level. Since LTPs function at membrane contact sites, the extent of organelle contact is a convenient way to regulate the extent of non-vesicular lipid transport. The activity of LTPs can

directly be influenced by post-translational modifications or binding of regulatory molecules. Finally, the recruitment of LTPs to particular sites is an important regulatory element. The regulation of VPS13 proteins is just beginning to be understood. While not much is known about the control of VPS13 expression, their recruitment to sites where lipid transport is needed has been documented (Bean et al., 2018). The current model for VPS13 protein recruitment to membrane contact sites states that the multiple VPS13 functional domains each sense localization signals and that the final observed protein localization is the result of the integration of these various signals. Since AtVPS13M1 harbors the canonical VPS13 functional domains as well as several non-canonical domains, some of which are plant specific, AtVPS13M1 constitutes an interesting platform to study VPS13 activity regulation by differential recruitment.

The field of non-vesicular lipid transport is full of challenging questions we have not directly addressed in our study. To what extent non-vesicular lipid transport contributes to cellular lipid transport is a major issue as it is difficult to directly measure. The answer is assuredly different depending on the considered organelle and organism. Endosymbiotic organelles probably get a bigger contribution from nonvesicular lipid transport than endomembranous organelles. A related question is the rate at which lipids are transported by LTPs between compartment and the relevant timescales. New techniques including new lipid tags might help to assess these values (John Peter, Petrungaro, et al., 2022; Liang et al., 2023). Redundancy between the lipid transport routes is a difficulty inherent to the study of LTPs. The existence of redundancy in non-vesicular lipid transport raises questions on the utility of having transport systems that have the same function. Why would an organism conserve functionally identical systems if keeping all of them costs precious energy? The most probable answer is that no lipid transport route is fully redundant with another, but rather that we are not (yet) able to detect the subtle differences in contribution of the individual transport routes (inspired by Benoît Kornmann's seminar at the EMBO Workshop, Luca, Italy, October 2022). Thus, future studies should focus on removing these layers of apparent redundancy either by finding physiological conditions in which a route becomes important (John Peter, Cheung, et al., 2022; Tokai et al., 2000), by artificially creating/finding genetic conditions enabling to isolate a route of interest (John Peter, Schie, et al., 2022; Michel & Kornmann, 2022) or by combining both. We used phosphate starvation as a condition to trigger membrane lipid remodeling that presumably requires non-vesicular lipid transport to create stringent conditions. Both the MTL complex (Michaud et al., 2016) and AtVPS13M1 (this study) have an effect on the efficiency of phosphate starvation-induced lipid remodeling. Finding new ways to identify LTPs is an obvious goal for future studies, especially in plants. Applying a transposon screening approach similar to SATAY (Saturated transposon analysis in yeast) would be an interesting way (Michel & Kornmann, 2022). Such approaches already exist, for example the T-DNA insertion mapping of the A. thaliana genome (Alonso et al., 2003) or using random mutagenesis. However, it has to be pointed out that these large-scale screening methods are much more tedious to realize in plants.

In conclusion, we investigated the MTL complex and AtVPS13M1, two actors in non-vesicular glycerolipid transport contributing to the important lipid remodeling taking place during plant adaptation to phosphate starvation. We propose a model for the MTL complex organization and composition and a mechanism of action for AtVPS13M1. Most importantly, we open several new paths for future studies aiming to discover new non-vesicular lipid transport routes in plant cells.

### References
Aaltonen, M. J., Friedman, J. R., Osman, C., Salin, B., di Rago, J., Nunnari, J., Langer, T., & Tatsuta, T. (2016). *MICOS and phospholipid transfer by Ups2–Mdm35 organize membrane lipid synthesis in mitochondria*. 10.

Abrisch, R. G., Gumbin, S. C., Wisniewski, B. T., Lackner, L. L., & Voeltz, G. K. (2020). Fission and fusion machineries converge at ER contact sites to regulate mitochondrial morphology. *Journal of Cell Biology*, *219*(4), e201911122. https://doi.org/10.1083/jcb.201911122

Ackema, K. B., Hench, J., Böckler, S., Wang, S. C., Sauder, U., Mergentaler, H., Westermann, B., Bard, F., Frank, S., & Spang, A. (2014). The small GTP ase Arf1 modulates mitochondrial morphology and function. *The EMBO Journal*, *33*(22), 2659-2675. https://doi.org/10.15252/embj.201489039

Adhikari, P. B., Liu, X., & Kasahara, R. D. (2020). Mechanics of Pollen Tube Elongation: A Perspective. *Frontiers in Plant Science*, *11*, 589712. https://doi.org/10.3389/fpls.2020.589712

Adlakha, J., Hong, Z., Li, P., & Reinisch, K. M. (2022). Structural and biochemical insights into lipid transport by VPS13 proteins. *Journal of Cell Biology*, 221(5), e202202030. https://doi.org/10.1083/jcb.202202030

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., ... Ecker, J. R. (2003). *Genome-Wide Insertional Mutagenesis of.* 301, 8.

Andersson, M. X., Larsson, K. E., Tjellström, H., Liljenberg, C., & Sandelius, A. S. (2005). Phosphatelimited Oat. *Journal of Biological Chemistry*, 280(30), 27578-27586. https://doi.org/10.1074/jbc.M503273200

Anding, A. L., Wang, C., Chang, T.-K., Sliter, D. A., Powers, C. M., Hofmann, K., Youle, R. J., & Baehrecke, E. H. (2018). Vps13D Encodes a Ubiquitin-Binding Protein that Is Required for the Regulation of Mitochondrial Size and Clearance. *Current Biology*, 28(2), 287-295.e6. https://doi.org/10.1016/j.cub.2017.11.064

Awai, K., Marechal, E., Block, M. A., Brun, D., Masuda, T., Shimada, H., Takamiya, K. -i., Ohta, H., & Joyard, J. (2001). Two types of MGDG synthase genes, found widely in both 16:3 and 18:3 plants, differentially mediate galactolipid syntheses in photosynthetic and nonphotosynthetic tissues in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, *98*(19), 10960-10965. https://doi.org/10.1073/pnas.181331498

#### B

Babiychuk, E., Müller, F., Eubel, H., Braun, H.-P., Frentzen, M., & Kushnir, S. (2003). *Arabidopsis* phosphatidylglycerophosphate synthase 1 is essential for chloroplast differentiation, but is dispensable for mitochondrial function : Arabidopsis *phosphatidylglycerophosphate synthase 1. The Plant Journal*, *33*(5), 899-909. https://doi.org/10.1046/j.1365-313X.2003.01680.x

Baek, M., DiMaio, F., Anishchenko, I., Dauparas, J., Ovchinnikov, S., Lee, G. R., Wang, J., Cong, Q., Kinch, L. N., Schaeffer, R. D., Millán, C., Park, H., Adams, C., Glassman, C. R., DeGiovanni, A., Pereira, J. H., Rodrigues, A. V., van Dijk, A. A., Ebrecht, A. C., ... Baker, D. (2021). Accurate prediction of protein structures and interactions using a three-track neural network. *Science*, *373*(6557), 871-876. https://doi.org/10.1126/science.abj8754

Baldwin, H. A., Wang, C., Kanfer, G., Shah, H. V., Velayos-Baeza, A., Dulovic-Mahlow, M., Brüggemann, N., Anding, A., Baehrecke, E. H., Maric, D., Prinz, W. A., & Youle, R. J. (2021). VPS13D promotes peroxisome biogenesis. *Journal of Cell Biology*, 220(5), e202001188. https://doi.org/10.1083/jcb.202001188

Balla, T. (2013). Phosphoinositides : Tiny Lipids With Giant Impact on Cell Regulation. *Physiological Reviews*, 93(3), 1019-1137. https://doi.org/10.1152/physrev.00028.2012

Bankaitis, V. A., Johnson, L. M., & Emr, S. D. (1986). Isolation of yeast mutants defective in protein targeting to the vacuole. *Proceedings of the National Academy of Sciences*, 83(23), 9075-9079. https://doi.org/10.1073/pnas.83.23.9075

Baslam, M., Oikawa, K., Kitajima-Koga, A., Kaneko, K., & Mitsui, T. (2016). Golgi-to-plastid trafficking of proteins through secretory pathway: Insights into vesicle-mediated import toward the plastids. *Plant Signaling & Behavior*, *11*(9), e1221558. https://doi.org/10.1080/15592324.2016.1221558

Basso, V., Marchesan, E., & Ziviani, E. (2020). A trio has turned into a quartet : DJ-1 interacts with the IP3R-Grp75-VDAC complex to control ER-mitochondria interaction. *Cell Calcium*, 87, 102186. https://doi.org/10.1016/j.ceca.2020.102186

Baum, D. A., & Baum, B. (2014). An inside-out origin for the eukaryotic cell. *BMC Biology*, *12*(1), 76. https://doi.org/10.1186/s12915-014-0076-2

Bean, B. D. M., Dziurdzik, S. K., Kolehmainen, K. L., Fowler, C. M. S., Kwong, W. K., Grad, L. I., Davey, M., Schluter, C., & Conibear, E. (2018). Competitive organelle-specific adaptors recruit Vps13 to membrane contact sites. *Journal of Cell Biology*, *217*(10), 3593-3607. https://doi.org/10.1083/jcb.201804111

Bernhard, W., & Rouiller, C. (1956). Close topographical relationship between mitochondria and ergastoplasm of liver cells in a definite phase of cellular activity. *The Journal of Biophysical and Biochemical Cytology*, 2(4), 73-78. https://doi.org/10.1083/jcb.2.4.73

Beutner, G., Rück, A., Riede, B., & Brdiczka, D. (1998). Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. *Biochimica et Biophysica Acta* (*BBA*) - *Biomembranes*, *1368*(1), 7-18. https://doi.org/10.1016/S0005-2736(97)00175-2

Beutner, G., Rück, A., Riede, B., Welte, W., & Brdiczka, D. (1996). Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. *FEBS Letters*, *396*(2-3), 189-195. https://doi.org/10.1016/0014-5793(96)01092-7

Birnboim, H. C., & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research*, 7(6), 1513-1523. https://doi.org/10.1093/nar/7.6.1513

Blomberg, N., Baraldi, E., Nilges, M., & Saraste, M. (1999). The PH superfold : A structural scaffold for multiple functions. *Trends in Biochemical Sciences*, 24(11), 441-445. https://doi.org/10.1016/S0968-0004(99)01472-3

Böckler, S., & Westermann, B. (2014). Mitochondrial ER Contacts Are Crucial for Mitophagy in Yeast. *Developmental Cell*, 28(4), 450-458. https://doi.org/10.1016/j.devcel.2014.01.012

Bohnert, M., Wenz, L.-S., Zerbes, R. M., Horvath, S. E., Stroud, D. A., von der Malsburg, K., Müller, J. M., Oeljeklaus, S., Perschil, I., Warscheid, B., Chacinska, A., Veenhuis, M., van der Klei, I. J., Daum, G., Wiedemann, N., Becker, T., Pfanner, N., & van der Laan, M. (2012). Role of mitochondrial inner

membrane organizing system in protein biogenesis of the mitochondrial outer membrane. *Molecular Biology of the Cell*, 23(20), 3948-3956. https://doi.org/10.1091/mbc.e12-04-0295

Botos, I., Majdalani, N., Mayclin, S. J., McCarthy, J. G., Lundquist, K., Wojtowicz, D., Barnard, T. J., Gumbart, J. C., & Buchanan, S. K. (2016). Structural and Functional Characterization of the LPS Transporter LptDE from Gram-Negative Pathogens. *Structure*, 24(6), 965-976. https://doi.org/10.1016/j.str.2016.03.026

Boudière, L., Michaud, M., Petroutsos, D., Rébeillé, F., Falconet, D., Bastien, O., Roy, S., Finazzi, G., Rolland, N., Jouhet, J., Block, M. A., & Maréchal, E. (2014). Glycerolipids in photosynthesis : Composition, synthesis and trafficking. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1837*(4), 470-480. https://doi.org/10.1016/j.bbabio.2013.09.007

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Braschi, B., Bruford, E. A., Cavanagh, A. T., Neuman, S. D., & Bashirullah, A. (2022). The bridge-like lipid transfer protein (BLTP) gene group : Introducing new nomenclature based on structural homology indicating shared function. *Human Genomics*, *16*(1), 66. https://doi.org/10.1186/s40246-022-00439-3

Brickner, J. H., & Fuller, R. S. (1997). SOI1 Encodes a Novel, Conserved Protein That Promotes TGN– Endosomal Cycling of Kex2p and Other Membrane Proteins by Modulating the Function of Two TGN Localization Signals. *Journal of Cell Biology*, *139*(1), 23-36. https://doi.org/10.1083/jcb.139.1.23

Brumos, J., Zhao, C., Gong, Y., Soriano, D., Patel, A. P., Perez-Amador, M. A., Stepanova, A. N., & Alonso, J. M. (2020). An Improved Recombineering Toolset for Plants. *The Plant Cell*, *32*(1), 100-122. https://doi.org/10.1105/tpc.19.00431

Buseman, C. M., Tamura, P., Sparks, A. A., Baughman, E. J., Maatta, S., Zhao, J., Roth, M. R., Esch, S. W., Shah, J., Williams, T. D., & Welti, R. (2006). Wounding Stimulates the Accumulation of Glycerolipids Containing Oxophytodienoic Acid and Dinor-Oxophytodienoic Acid in Arabidopsis Leaves. *Plant Physiology*, *142*(1), 28-39. https://doi.org/10.1104/pp.106.082115

#### С

Cai, S., Wu, Y., Guillén-Samander, A., Hancock-Cerutti, W., Liu, J., & De Camilli, P. (2022). In situ architecture of the lipid transport protein VPS13C at ER–lysosome membrane contacts. *Proceedings of the National Academy of Sciences*, *119*(29), e2203769119. https://doi.org/10.1073/pnas.2203769119

Carmichael, R. E., & Schrader, M. (2022). Determinants of Peroxisome Membrane Dynamics. *Frontiers in Physiology*, *13*, 834411. https://doi.org/10.3389/fphys.2022.834411

Casares, D., Escribá, P. V., & Rosselló, C. A. (2019). Membrane Lipid Composition : Effect on Membrane and Organelle Structure, Function and Compartmentalization and Therapeutic Avenues. *International Journal of Molecular Sciences*, 20(9), 2167. https://doi.org/10.3390/ijms20092167

Castro, I. G., Shortill, S. P., Dziurdzik, S. K., Cadou, A., Ganesan, S., Valenti, R., David, Y., Davey, M., Mattes, C., Thomas, F. B., Avraham, R. E., Meyer, H., Fadel, A., Fenech, E. J., Ernst, R., Zaremberg, V., Levine, T. P., Stefan, C., Conibear, E., & Schuldiner, M. (2022). Systematic analysis of membrane contact sites in Saccharomyces cerevisiae uncovers modulators of cellular lipid distribution. *ELife*, *11*, e74602. https://doi.org/10.7554/eLife.74602

Chacinska, A., Lind, M., Frazier, A. E., Dudek, J., Meisinger, C., Geissler, A., Sickmann, A., Meyer, H. E., Truscott, K. N., Guiard, B., Pfanner, N., & Rehling, P. (2005). Mitochondrial Presequence

Translocase : Switching between TOM Tethering and Motor Recruitment Involves Tim21 and Tim17. *Cell*, *120*(6), 817-829. https://doi.org/10.1016/j.cell.2005.01.011

Chen, Y.-L., Chen, L.-J., Chu, C.-C., Huang, P.-K., Wen, J.-R., & Li, H. (2018). TIC236 links the outer and inner membrane translocons of the chloroplast. *Nature*, *564*(7734), 125-129. https://doi.org/10.1038/s41586-018-0713-y

Cheng, X., & Bezanilla, M. (2021). SABRE populates ER domains essential for cell plate maturation and cell expansion influencing cell and tissue patterning. *ELife*, *10*, e65166. https://doi.org/10.7554/eLife.65166

Chitwood, P. J., & Hegde, R. S. (2019). The Role of EMC during Membrane Protein Biogenesis. *Trends in Cell Biology*, 29(5), 371-384. https://doi.org/10.1016/j.tcb.2019.01.007

Ciccarelli, F. D., Copley, R. R., Doerks, T., Russell, R. B., & Bork, P. (2002). CASH – a β-helix domain widespread among carbohydrate-binding proteins. *Trends in Biochemical Sciences*, 27(2), 59-62. https://doi.org/10.1016/S0968-0004(01)02046-1

Connerth, M., Tatsuta, T., Haag, M., Klecker, T., Westermann, B., & Langer, T. (2012). Intramitochondrial Transport of Phosphatidic Acid in Yeast by a Lipid Transfer Protein. *Science*, 338(6108), 815-818. https://doi.org/10.1126/science.1225625

Copeland, D. E., & Dalton, A. J. (1959). An Association between Mitochondria and the Endoplasmic Reticulum in Cells of the Pseudobranch Gland of a Teleost. *The Journal of Biophysical and Biochemical Cytology*, *5*(3), 393-396. https://doi.org/10.1083/jcb.5.3.393

Corbalan-Garcia, S., & Gómez-Fernández, J. C. (2014). Signaling through C2 domains : More than one lipid target. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1838(6), 1536-1547. https://doi.org/10.1016/j.bbamem.2014.01.008

Cornaro, L., Banfi, C., Cucinotta, M., Colombo, L., & van Dijk, P. J. (2023). Asexual reproduction through seeds : The complex case of diplosporous apomixis. *Journal of Experimental Botany*, erad054. https://doi.org/10.1093/jxb/erad054

Cruz-Ramírez, A., Oropeza-Aburto, A., Razo-Hernández, F., Ramírez-Chávez, E., & Herrera-Estrella, L. (2006). Phospholipase DZ2 plays an important role in extraplastidic galactolipid biosynthesis and phosphate recycling in *Arabidopsis* roots. *Proceedings of the National Academy of Sciences*, 103(17), 6765-6770. https://doi.org/10.1073/pnas.0600863103

Cummings, R. D., & L. Schnaar, R. (2015). R-Type Lectins. In A. Varki, R. D. Cummings, J. D. Esko, P. Stanley, G. W. Hart, M. Aebi, A. G. Darvill, T. Kinoshita, N. H. Packer, J. H. Prestegard, R. L. Schnaar, & P. H. Seeberger (Éds.), *Essentials of Glycobiology* (3rd éd.). Cold Spring Harbor Laboratory Press. http://www.ncbi.nlm.nih.gov/books/NBK453065/

#### D

Da Costa, R., Bordessoules, M., Guilleman, M., Carmignac, V., Lhussiez, V., Courot, H., Bataille, A., Chlémaire, A., Bruno, C., Fauque, P., Thauvin, C., Faivre, L., & Duplomb, L. (2020). Vps13b is required for acrosome biogenesis through functions in Golgi dynamic and membrane trafficking. *Cellular and Molecular Life Sciences*, 77(3), 511-529. https://doi.org/10.1007/s00018-019-03192-4

Dabrowski, R., Tulli, S., & Graef, M. (2022). Parallel phospholipid transfer by Vps13 and Atg2 determines autophagosome biogenesis dynamics [Preprint]. Cell Biology. https://doi.org/10.1101/2022.11.10.516013

Dall'Armellina, F., Stagi, M., & Swan, L. E. (2023). In silico modeling human VPS13 proteins associated with donor and target membranes suggests lipid transfer mechanisms. *Proteins: Structure, Function, and Bioinformatics*, 91(4), 439-455. https://doi.org/10.1002/prot.26446

de Brito, O. M., & Scorrano, L. (2008). Mitofusin 2 tethers endoplasmic reticulum to mitochondria. *Nature*, 456(7222), 605-610. https://doi.org/10.1038/nature07534

De, M., Oleskie, A. N., Ayyash, M., Dutta, S., Mancour, L., Abazeed, M. E., Brace, E. J., Skiniotis, G., & Fuller, R. S. (2017). The Vps13p–Cdc31p complex is directly required for TGN late endosome transport and TGN homotypic fusion. *Journal of Cell Biology*, *216*(2), 425-439. https://doi.org/10.1083/jcb.201606078

De Vos, K. J., Mórotz, G. M., Stoica, R., Tudor, E. L., Lau, K.-F., Ackerley, S., Warley, A., Shaw, C. E., & Miller, C. C. J. (2012). VAPB interacts with the mitochondrial protein PTPIP51 to regulate calcium homeostasis. *Human Molecular Genetics*, 21(6), 1299-1311. https://doi.org/10.1093/hmg/ddr559

Dellaporta, S. L., Wood, J., & Hicks, J. B. (1983). A plant DNA minipreparation : Version II. *Plant Molecular Biology Reporter*, 1(4), 19-21. https://doi.org/10.1007/BF02712670

Demé, B., Cataye, C., Block, M. A., Maréchal, E., & Jouhet, J. (2014). Contribution of galactoglycerolipids to the 3-dimensional architecture of thylakoids. *The FASEB Journal*, 28(8), 3373-3383. https://doi.org/10.1096/fj.13-247395

Di Mattia, T., Martinet, A., Ikhlef, S., McEwen, A. G., Nominé, Y., Wendling, C., Poussin-Courmontagne, P., Voilquin, L., Eberling, P., Ruffenach, F., Cavarelli, J., Slee, J., Levine, T. P., Drin, G., Tomasetto, C., & Alpy, F. (2020). FFAT motif phosphorylation controls formation and lipid transfer function of inter-organelle contacts. *The EMBO Journal*, *39*(23). https://doi.org/10.15252/embj.2019104369

Di Mattia, T., Wilhelm, L. P., Ikhlef, S., Wendling, C., Spehner, D., Nominé, Y., Giordano, F., Mathelin, C., Drin, G., Tomasetto, C., & Alpy, F. (2018). Identification of MOSPD2, a novel scaffold for endoplasmic reticulum membrane contact sites. *EMBO Reports*, 19(7). https://doi.org/10.15252/embr.201745453

Dissanayaka, D. M. S. B., Ghahremani, M., Siebers, M., Wasaki, J., & Plaxton, W. C. (2021). Recent insights into the metabolic adaptations of phosphorus-deprived plants. *Journal of Experimental Botany*, 72(2), 199-223. https://doi.org/10.1093/jxb/eraa482

Dubochet, J., Adrian, M., Chang, J.-J., Homo, J.-C., Lepault, J., McDowall, A. W., & Schultz, P. (1988). Cryo-electron microscopy of vitrified specimens. *Quarterly Reviews of Biophysics*, 21(2), 129-228. https://doi.org/10.1017/S0033583500004297

Dumont, M., Cataye, C., Lehner, A., Maréchal, E., Lerouge, P., Falconet, D., & Mollet, J.-C. (2015). A Simple Protocol for the Immunolabelling of Arabidopsis Pollen Tube Membranes and Cell Wall Polymers. *BIO-PROTOCOL*, *5*(12). https://doi.org/10.21769/BioProtoc.1502

Duncan, O., Taylor, N. L., Carrie, C., Eubel, H., Kubiszewski-Jakubiak, S., Zhang, B., Narsai, R., Millar, A. H., & Whelan, J. (2011). Multiple Lines of Evidence Localize Signaling, Morphology, and Lipid Biosynthesis Machinery to the Mitochondrial Outer Membrane of Arabidopsis. *Plant Physiology*, *157*(3), 1093-1113. https://doi.org/10.1104/pp.111.183160

Dziurdzik, S. K., Bean, B. D. M., Davey, M., & Conibear, E. (2020). A VPS13D spastic ataxia mutation disrupts the conserved adaptor-binding site in yeast Vps13. *Human Molecular Genetics*, 29(4), 635-648. https://doi.org/10.1093/hmg/ddz318 Dziurdzik, S. K., & Conibear, E. (2021). The Vps13 Family of Lipid Transporters and Its Role at Membrane Contact Sites. *Int. J. Mol. Sci.*, 16.

#### Ε

Eisenberg-Bord, M., Shai, N., Schuldiner, M., & Bohnert, M. (2016). A Tether Is a Tether Is a Tether : Tethering at Membrane Contact Sites. *Developmental Cell*, *39*(4), 395-409. https://doi.org/10.1016/j.devcel.2016.10.022

Elbaz-Alon, Y., Eisenberg-Bord, M., Shinder, V., Stiller, S. B., Shimoni, E., Wiedemann, N., Geiger, T., & Schuldiner, M. (2015). Lam6 Regulates the Extent of Contacts between Organelles. *Cell Reports*, *12*(1), 7-14. https://doi.org/10.1016/j.celrep.2015.06.022

Elbaz-Alon, Y., Guo, Y., Segev, N., Harel, M., Quinnell, D. E., Geiger, T., Avinoam, O., Li, D., & Nunnari, J. (2020). PDZD8 interacts with Protrudin and Rab7 at ER-late endosome membrane contact sites associated with mitochondria. *Nature Communications*, *11*(1), 3645. https://doi.org/10.1038/s41467-020-17451-7

Elbaz-Alon, Y., Rosenfeld-Gur, E., Shinder, V., Futerman, A. H., Geiger, T., & Schuldiner, M. (2014). A Dynamic Interface between Vacuoles and Mitochondria in Yeast. *Developmental Cell*, *30*(1), 95-102. https://doi.org/10.1016/j.devcel.2014.06.007

Engström, P., Zambryski, P., Van Montagu, M., & Stachel, S. (1987). Characterization of Agrobacterium tumefaciens virulence proteins induced by the plant factor acetosyringone. *Journal of Molecular Biology*, *197*(4), 635-645. https://doi.org/10.1016/0022-2836(87)90470-0

Enyenihi, A. H., & Saunders, W. S. (2003). Large-Scale Functional Genomic Analysis of Sporulation and Meiosis in *Saccharomyces cerevisiae*. *Genetics*, *163*(1), 47-54. https://doi.org/10.1093/genetics/163.1.47

Epand, R. F., Schlattner, U., Wallimann, T., Lacombe, M.-L., & Epand, R. M. (2007). Novel Lipid Transfer Property of Two Mitochondrial Proteins that Bridge the Inner and Outer Membranes. *Biophysical Journal*, 92(1), 126-137. https://doi.org/10.1529/biophysj.106.092353

Essigmann, B., Güler, S., Narang, R. A., Linke, D., & Benning, C. (1998). Phosphate availability affects the thylakoid lipid composition and the expression of *SQD1*, a gene required for sulfolipid biosynthesis in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, 95(4), 1950-1955. https://doi.org/10.1073/pnas.95.4.1950

#### $\mathbf{F}$

Faber, A. I. E., van der Zwaag, M., Schepers, H., Eggens-Meijer, E., Kanon, B., IJsebaart, C., Kuipers, J., Giepmans, B. N. G., Freire, R., Grzeschik, N. A., Rabouille, C., & Sibon, O. C. M. (2020). Vps13 is required for timely removal of nurse cell corpses. *Development*, dev.191759. https://doi.org/10.1242/dev.191759

Fasimoye, R., Dong, W., Nirujogi, R. S., Rawat, E. S., Iguchi, M., Nyame, K., Phung, T. K., Bagnoli, E., Prescott, A. R., Alessi, D. R., & Abu-remaileh, M. (2022). *Golgi-IP, a novel tool for multimodal analysis of Golgi molecular content* [Preprint]. Cell Biology. https://doi.org/10.1101/2022.11.22.517583

Ferguson, K. M., Lemmon, M. A., Schlessinger, J., & Sigler, P. B. (1995). Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell*, *83*(6), 1037-1046. https://doi.org/10.1016/0092-8674(95)90219-8

Fernandez-Martinez, J., LaCava, J., & Rout, M. P. (2016). Density Gradient Ultracentrifugation to Isolate Endogenous Protein Complexes after Affinity Capture. *Cold Spring Harbor Protocols*, 2016(7), pdb.prot087957. https://doi.org/10.1101/pdb.prot087957

Fogelson, B., & Mogilner, A. (2014). Computational Estimates of Membrane Flow and Tension Gradient in Motile Cells. *PLoS ONE*, *9*(1), e84524. https://doi.org/10.1371/journal.pone.0084524

Folch, J., Lees, M., & Stanley, G. H. S. (1957). A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES. *Journal of Biological Chemistry*, 226(1), 497-509. https://doi.org/10.1016/S0021-9258(18)64849-5

Föller, M., Hermann, A., Gu, S., Alesutan, I., Qadri, S. M., Borst, O., Schmidt, E., Schiele, F., Hagen, J. M. vom, Saft, C., Schöls, L., Lerche, H., Stournaras, C., Storch, A., & Lang, F. (2012). Choreinsensitive polymerization of cortical actin and suicidal cell death in chorea-acanthocytosis. *The FASEB Journal*, *26*(4), 1526-1534. https://doi.org/10.1096/fj.11-198317

Freyre, C. A. C., Rauher, P. C., Ejsing, C. S., & Klemm, R. W. (2019). MIGA2 Links Mitochondria, the ER, and Lipid Droplets and Promotes De Novo Lipogenesis in Adipocytes. *Molecular Cell*, *76*(5), 811-825.e14. https://doi.org/10.1016/j.molcel.2019.09.011

Friedman, J. R., Kannan, M., Toulmay, A., Jan, C. H., Weissman, J. S., Prinz, W. A., & Nunnari, J. (2018). Lipid Homeostasis Is Maintained by Dual Targeting of the Mitochondrial PE Biosynthesis Enzyme to the ER. *Developmental Cell*, 44(2), 261-270.e6. https://doi.org/10.1016/j.devcel.2017.11.023

Friedman, J. R., Lackner, L. L., West, M., DiBenedetto, J. R., Nunnari, J., & Voeltz, G. K. (2011). ER Tubules Mark Sites of Mitochondrial Division. *Science*, *334*(6054), 358-362. https://doi.org/10.1126/science.1207385

Furuita, K., Jee, J., Fukada, H., Mishima, M., & Kojima, C. (2010). Electrostatic Interaction between Oxysterol-binding Protein and VAMP-associated Protein A Revealed by NMR and Mutagenesis Studies. *Journal of Biological Chemistry*, 285(17), 12961-12970. https://doi.org/10.1074/jbc.M109.082602

#### G

Galmes, R., Houcine, A., Vliet, A. R., Agostinis, P., Jackson, C. L., & Giordano, F. (2016). ORP5/ORP8 localize to endoplasmic reticulum-mitochondria contacts and are involved in mitochondrial function. *EMBO Reports*, *17*(6), 800-810. https://doi.org/10.15252/embr.201541108

Gao, H., Metz, J., Teanby, N. A., Ward, A. D., Botchway, S. W., Coles, B., Pollard, M. R., & Sparkes, I. (2016). In Vivo Quantification of Peroxisome Tethering to Chloroplasts in Tobacco Epidermal Cells Using Optical Tweezers. *Plant Physiology*, *170*(1), 263-272. https://doi.org/10.1104/pp.15.01529

Gao, Y., Xiong, J., Chu, Q.-Z., & Ji, W.-K. (2022). PDZD8-mediated lipid transfer at contacts between the ER and late endosomes/lysosomes is required for neurite outgrowth. *Journal of Cell Science*, *135*(5), jcs255026. https://doi.org/10.1242/jcs.255026

Gatta, A. T., & Levine, T. P. (2017). Piecing Together the Patchwork of Contact Sites. *Trends in Cell Biology*, 27(3), 214-229. https://doi.org/10.1016/j.tcb.2016.08.010

Gaude, N., Nakamura, Y., Scheible, W.-R., Ohta, H., & Dörmann, P. (2008). Phospholipase C5 (NPC5) is involved in galactolipid accumulation during phosphate limitation in leaves of Arabidopsis. *The Plant Journal*, *56*(1), 28-39. https://doi.org/10.1111/j.1365-313X.2008.03582.x

Gauthier, J., Meijer, I. A., Lessel, D., Mencacci, N. E., Krainc, D., Hempel, M., Tsiakas, K., Prokisch, H., Rossignol, E., Helm, M. H., Rodan, L. H., Karamchandani, J., Carecchio, M., Lubbe, S. J., Telegrafi, A., Henderson, L. B., Lorenzo, K., Wallace, S. E., Glass, I. A., ... Campeau, P. M. (2018). Recessive mutations in *VPS13D* cause childhood onset movement disorders. *Annals of Neurology*, *83*(6), 1089-1095. https://doi.org/10.1002/ana.25204

Ghanbarpour, A., Valverde, D. P., Melia, T. J., & Reinisch, K. M. (2021). A model for a partnership of lipid transfer proteins and scramblases in membrane expansion and organelle biogenesis. *Proceedings of the National Academy of Sciences*, *118*(16), e2101562118. https://doi.org/10.1073/pnas.2101562118

Ghosh, A. K., Chauhan, N., Rajakumari, S., Daum, G., & Rajasekharan, R. (2009). At4g24160, a Soluble Acyl-Coenzyme A-Dependent Lysophosphatidic Acid Acyltransferase. *Plant Physiology*, *151*(2), 869-881. https://doi.org/10.1104/pp.109.144261

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, 6(5), 343-345. https://doi.org/10.1038/nmeth.1318

Giménez-Andrés, M., Čopič, A., & Antonny, B. (2018). The Many Faces of Amphipathic Helices. *Biomolecules*, 8(3), 45. https://doi.org/10.3390/biom8030045

González Montoro, A., Auffarth, K., Hönscher, C., Bohnert, M., Becker, T., Warscheid, B., Reggiori, F., van der Laan, M., Fröhlich, F., & Ungermann, C. (2018). Vps39 Interacts with Tom40 to Establish One of Two Functionally Distinct Vacuole-Mitochondria Contact Sites. *Developmental Cell*, 45(5), 621-636.e7. https://doi.org/10.1016/j.devcel.2018.05.011

Grefen, C., & Blatt, M. R. (2012). A 2in1 cloning system enables ratiometric bimolecular fluorescence complementation (rBiFC). *BioTechniques*, 53(5), 311-314. https://doi.org/10.2144/000113941

Grippa, A., Buxó, L., Mora, G., Funaya, C., Idrissi, F.-Z., Mancuso, F., Gomez, R., Muntanyà, J., Sabidó, E., & Carvalho, P. (2015). The seipin complex Fld1/Ldb16 stabilizes ER–lipid droplet contact sites. *Journal of Cell Biology*, *211*(4), 829-844. https://doi.org/10.1083/jcb.201502070

Guan, S., Zhao, L., & Peng, R. (2022). Mitochondrial Respiratory Chain Supercomplexes : From Structure to Function. *International Journal of Molecular Sciences*, 23(22), 13880. https://doi.org/10.3390/ijms232213880

Guillén-Samander, A., & De Camilli, P. (2022). Endoplasmic Reticulum Membrane Contact Sites, Lipid Transport, and Neurodegeneration. *Cold Spring Harbor Perspectives in Biology*, a041257. https://doi.org/10.1101/cshperspect.a041257

Guillén-Samander, A., Leonzino, M., Hanna, M. G., Tang, N., Shen, H., & De Camilli, P. (2021). VPS13D bridges the ER to mitochondria and peroxisomes via Miro. *The Journal of Cell Biology*, 220(5). https://doi.org/10.1083/jcb.202010004

Guillén-Samander, A., Wu, Y., Pineda, S. S., García, F. J., Eisen, J. N., Leonzino, M., Ugur, B., Kellis, M., Heiman, M., & De Camilli, P. (2022). A partnership between the lipid scramblase XK and the lipid transfer protein VPS13A at the plasma membrane. *Proceedings of the National Academy of Sciences*, *119*(35), e2205425119. https://doi.org/10.1073/pnas.2205425119

#### Η

Hanna, M. G., Suen, P. H., Wu, Y., Reinisch, K. M., & De Camilli, P. (2022). SHIP164 is a chorein motif lipid transfer protein that controls endosome–Golgi membrane traffic. *Journal of Cell Biology*, 221(6), e202111018. https://doi.org/10.1083/jcb.202111018

Harner, M., Körner, C., Walther, D., Mokranjac, D., Kaesmacher, J., Welsch, U., Griffith, J., Mann, M., Reggiori, F., & Neupert, W. (2011). The mitochondrial contact site complex, a determinant of mitochondrial architecture : Molecular architecture of mitochondria. *The EMBO Journal*, 30(21), 4356-4370. https://doi.org/10.1038/emboj.2011.379

Harper, C. S., White, A. J., & Lackner, L. L. (2020). The multifunctional nature of mitochondrial contact site proteins. *Current Opinion in Cell Biology*, 65, 58-65. https://doi.org/10.1016/j.ceb.2020.02.010

Hartel, H., Dormann, P., & Benning, C. (2001). *Galactolipids not associated with the photosynthetic apparatus in phosphate-deprived plants*. 6.

Hayashi, T., Kishida, M., Nishizawa, Y., Iijima, M., Koriyama, C., Nakamura, M., Sano, A., & Kishida, S. (2012). Subcellular localization and putative role of VPS13A/chorein in dopaminergic neuronal cells. *Biochemical and Biophysical Research Communications*, 419(3), 511-516. https://doi.org/10.1016/j.bbrc.2012.02.047

Hermanson, G. T. (2013). Homobifunctional Crosslinkers. In *Bioconjugate Techniques* (p. 275-298). Elsevier. https://doi.org/10.1016/B978-0-12-382239-0.00005-4

Hernández-Alvarez, M. I., Sebastián, D., Vives, S., Ivanova, S., Bartoccioni, P., Kakimoto, P., Plana, N., Veiga, S. R., Hernández, V., Vasconcelos, N., Peddinti, G., Adrover, A., Jové, M., Pamplona, R., Gordaliza-Alaguero, I., Calvo, E., Cabré, N., Castro, R., Kuzmanic, A., ... Zorzano, A. (2019). Deficient Endoplasmic Reticulum-Mitochondrial Phosphatidylserine Transfer Causes Liver Disease. *Cell*, *177*(4), 881-895.e17. https://doi.org/10.1016/j.cell.2019.04.010

Hicks, G., & Jia, Z. (2018). Structural Basis for the Lipopolysaccharide Export Activity of the Bacterial Lipopolysaccharide Transport System. *International Journal of Molecular Sciences*, *19*(9), 2680. https://doi.org/10.3390/ijms19092680

Hirabayashi, Y., Kwon, S.-K., Paek, H., Pernice, W. M., Paul, M. A., Lee, J., Erfani, P., Raczkowski, A., Petrey, D. S., Pon, L. A., & Polleux, F. (2017). ER-mitochondria tethering by PDZD8 regulates Ca <sup>2+</sup> dynamics in mammalian neurons. *Science*, *358*(6363), 623-630. https://doi.org/10.1126/science.aan6009

Hochholdinger, F., Yu, P., & Marcon, C. (2018). Genetic Control of Root System Development in Maize. *Trends in Plant Science*, 23(1), 79-88. https://doi.org/10.1016/j.tplants.2017.10.004

Homblé, F., Krammer, E.-M., & Prévost, M. (2012). Plant VDAC : Facts and speculations. *Biochimica et Biophysica Acta* (*BBA*) - *Biomembranes*, *1818*(6), 1486-1501. https://doi.org/10.1016/j.bbamem.2011.11.028

Hönscher, C., Mari, M., Auffarth, K., Bohnert, M., Griffith, J., Geerts, W., van der Laan, M., Cabrera, M., Reggiori, F., & Ungermann, C. (2014). Cellular Metabolism Regulates Contact Sites between Vacuoles and Mitochondria. *Developmental Cell*, 30(1), 86-94. https://doi.org/10.1016/j.devcel.2014.06.006

Hoppins, S., Collins, S. R., Cassidy-Stone, A., Hummel, E., DeVay, R. M., Lackner, L. L., Westermann, B., Schuldiner, M., Weissman, J. S., & Nunnari, J. (2011). A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. *Journal of Cell Biology*, *195*(2), 323-340. https://doi.org/10.1083/jcb.201107053

Horibata, Y., Ando, H., Zhang, P., Vergnes, L., Aoyama, C., Itoh, M., Reue, K., & Sugimoto, H. (2016). StarD7 Protein Deficiency Adversely Affects the Phosphatidylcholine Composition, Respiratory Activity, and Cristae Structure of Mitochondria. *Journal of Biological Chemistry*, 291(48), 24880-24891. https://doi.org/10.1074/jbc.M116.736793

Horstman, A., Tonaco, I., Boutilier, K., & Immink, R. (2014). A Cautionary Note on the Use of Split-YFP/BiFC in Plant Protein-Protein Interaction Studies. *International Journal of Molecular Sciences*, 15(6), 9628-9643. https://doi.org/10.3390/ijms15069628

Horvath, S. E., & Daum, G. (2013). Lipids of mitochondria. *Progress in Lipid Research*, 52(4), 590-614. https://doi.org/10.1016/j.plipres.2013.07.002

Hsueh, Y.-C., Ehmann, C., Flinner, N., Ladig, R., & Schleiff, E. (2017). The plastid outer membrane localized LPTD1 is important for glycerolipid remodelling under phosphate starvation: LptD orthologues in *A. thaliana. Plant, Cell & Environment, 40*(8), 1643-1657. https://doi.org/10.1111/pce.12973

Huh, W.-K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., & O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature*, 425(6959), 686-691. https://doi.org/10.1038/nature02026

Hung, C.-H., Kobayashi, K., Wada, H., & Nakamura, Y. (2015). Isolation and characterization of a phosphatidylglycerophosphate phosphatase1, PGPP1, in Chlamydomonas reinhardtii. *Plant Physiology and Biochemistry*, *92*, 56-61. https://doi.org/10.1016/j.plaphy.2015.04.002

Huynen, M. A., Mühlmeister, M., Gotthardt, K., Guerrero-Castillo, S., & Brandt, U. (2016). Evolution and structural organization of the mitochondrial contact site (MICOS) complex and the mitochondrial intermembrane space bridging (MIB) complex. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1863*(1), 91-101. https://doi.org/10.1016/j.bbamcr.2015.10.009

#### Ι

Ibarguren, M., López, D. J., & Escribá, P. V. (2014). The effect of natural and synthetic fatty acids on membrane structure, microdomain organization, cellular functions and human health. *Biochimica et Biophysica* Acta (BBA) - Biomembranes, 1838(6), 1518-1528. https://doi.org/10.1016/j.bbamem.2013.12.021

Imbert, L., Lenoir-Capello, R., Crublet, E., Vallet, A., Awad, R., Ayala, I., Juillan-Binard, C., Mayerhofer, H., Kerfah, R., Gans, P., Miclet, E., & Boisbouvier, J. (2021). In Vitro Production of Perdeuterated Proteins in H2O for Biomolecular NMR Studies. In Y. W. Chen & C.-P. B. Yiu (Éds.), *Structural Genomics* (Vol. 2199, p. 127-149). Springer US. https://doi.org/10.1007/978-1-0716-0892-0\_8

Insolera, R., Lőrincz, P., Wishnie, A. J., Juhász, G., & Collins, C. A. (2021). Mitochondrial fission, integrity and completion of mitophagy require separable functions of Vps13D in Drosophila neurons. *PLOS Genetics*, *17*(8), e1009731. https://doi.org/10.1371/journal.pgen.1009731

Itoh, T., Toh-e, A., & Matsui, Y. (2004). Mmr1p is a mitochondrial factor for Myo2p-dependent inheritance of mitochondria in the budding yeast. *The EMBO Journal*, 23(13), 2520-2530. https://doi.org/10.1038/sj.emboj.7600271

Iwasawa, R., Mahul-Mellier, A.-L., Datler, C., Pazarentzos, E., & Grimm, S. (2011). Fis1 and Bap31 bridge the mitochondria-ER interface to establish a platform for apoptosis induction : Fis1 induces apoptosis via Bap31. *The EMBO Journal*, *30*(3), 556-568. https://doi.org/10.1038/emboj.2010.346

#### J

Jacobs, M. E., DeSouza, L. V., Samaranayake, H., Pearlman, R. E., Siu, K. W. M., & Klobutcher, L. A. (2006). The *Tetrahymena thermophila* Phagosome Proteome. *Eukaryotic Cell*, *5*(12), 1990-2000. https://doi.org/10.1128/EC.00195-06 Jahn, H., Bartoš, L., Holthuis, J. C. M., Vácha, R., & Menon, A. K. (2022). *Mitochondrial phospholipid import mediated by VDAC, a dimeric beta barrel scramblase* [Preprint]. Biochemistry. https://doi.org/10.1101/2022.10.17.512472

Janer, A., Morris, J. L., Krols, M., Antonicka, H., Aaltonen, M. J., Lin, Z.-Y., Gingras, A.-C., Prudent, J., & Shoubridge, E. A. (2022). *ESYT1 tethers the endoplasmic reticulum to mitochondria and is required for mitochondrial lipid and calcium homeostasis* [Preprint]. Cell Biology. https://doi.org/10.1101/2022.11.14.516495

Jeong, H., Park, J., Jun, Y., & Lee, C. (2017). Crystal structures of Mmm1 and Mdm12–Mmm1 reveal mechanistic insight into phospholipid trafficking at ER-mitochondria contact sites. *Proceedings of the National Academy of Sciences*, *114*(45). https://doi.org/10.1073/pnas.1715592114

Jeong, H., Park, J., & Lee, C. (2016). Crystal structure of Mdm12 reveals the architecture and dynamic organization of the ERMES complex. *EMBO Reports*, *17*(12), 1857-1871. https://doi.org/10.15252/embr.201642706

Jeyasimman, D., Ercan, B., Dharmawan, D., Naito, T., Sun, J., & Saheki, Y. (2021). PDZD-8 and TEX-2 regulate endosomal PI(4,5)P2 homeostasis via lipid transport to promote embryogenesis in C. elegans. *Nature Communications*, *12*(1), 6065. https://doi.org/10.1038/s41467-021-26177-z

Jin, K., Musso, G., Vlasblom, J., Jessulat, M., Deineko, V., Negroni, J., Mosca, R., Malty, R., Nguyen-Tran, D.-H., Aoki, H., Minic, Z., Freywald, T., Phanse, S., Xiang, Q., Freywald, A., Aloy, P., Zhang, Z., & Babu, M. (2015). Yeast Mitochondrial Protein–Protein Interactions Reveal Diverse Complexes and Disease-Relevant Functional Relationships. *Journal of Proteome Research*, *14*(2), 1220-1237. https://doi.org/10.1021/pr501148q

John Peter, A. T., Cheung, N. J., & Kornmann, B. (2022). Csf1 : A Putative Lipid Transport Protein Required for Homeoviscous Adaptation of the Lipidome. *Contact*, *5*, 251525642211019. https://doi.org/10.1177/25152564221101974

John Peter, A. T., Herrmann, B., Antunes, D., Rapaport, D., Dimmer, K. S., & Kornmann, B. (2017). Vps13-Mcp1 interact at vacuole–mitochondria interfaces and bypass ER–mitochondria contact sites. *Journal of Cell Biology*, *216*(10), 3219-3229. https://doi.org/10.1083/jcb.201610055

John Peter, A. T., Petrungaro, C., Peter, M., & Kornmann, B. (2022). METALIC reveals interorganelle lipid flux in live cells by enzymatic mass tagging. *Nature Cell Biology*, 24(6), 996-1004. https://doi.org/10.1038/s41556-022-00917-9

John Peter, A. T., Schie, S. N. S., Cheung, N. J., Michel, A. H., Peter, M., & Kornmann, B. (2022). Rewiring phospholipid biosynthesis reveals resilience to membrane perturbations and uncovers regulators of lipid homeostasis. *The EMBO Journal*, *41*(7). https://doi.org/10.15252/embj.2021109998

Jones, E. W. (1977). PROTEINASE MUTANTS OF SACCHAROMYCES CEREVISIAE. Genetics, 85(1), 23-33. https://doi.org/10.1093/genetics/85.1.23

Josts, I., Stubenrauch, C. J., Vadlamani, G., Mosbahi, K., Walker, D., Lithgow, T., & Grinter, R. (2017). The Structure of a Conserved Domain of TamB Reveals a Hydrophobic  $\beta$  Taco Fold. *Structure (London, England: 1993)*, 25(12), 1898-1906.e5. https://doi.org/10.1016/j.str.2017.10.002

Jouhet, J., Lupette, J., Clerc, O., Magneschi, L., Bedhomme, M., Collin, S., Roy, S., Maréchal, E., & Rébeillé, F. (2017). LC-MS/MS versus TLC plus GC methods : Consistency of glycerolipid and fatty acid profiles in microalgae and higher plant cells and effect of a nitrogen starvation. *PLOS ONE*, *12*(8), e0182423. https://doi.org/10.1371/journal.pone.0182423

Jouhet, J., Maréchal, E., Baldan, B., Bligny, R., Joyard, J., & Block, M. A. (2004). Phosphate deprivation induces transfer of DGDG galactolipid from chloroplast to mitochondria. *Journal of Cell Biology*, *167*(5), 863-874. https://doi.org/10.1083/jcb.200407022

Jouhet, J., Maréchal, E., Bligny, R., Joyard, J., & Block, M. A. (2003). Transient increase of phosphatidylcholine in plant cells in response to phosphate deprivation. *FEBS Letters*, *544*(1-3), 63-68. https://doi.org/10.1016/S0014-5793(03)00477-0

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S. A. A., Ballard, A. J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., ... Hassabis, D. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature*, *596*(7873), 583-589. https://doi.org/10.1038/s41586-021-03819-2

#### Κ

Kaminska, J. (2022). The PH-like Domain of VPS13 Proteins—A Determinant of Localization to the Golgi Apparatus or to the Plasma Membrane. *Contact*, *5*, 251525642211060. https://doi.org/10.1177/25152564221106024

Kaminska, J., Rzepnikowska, W., Polak, A., Flis, K., Soczewka, P., Bala, K., Sienko, M., Grynberg, M., Kaliszewski, P., Urbanek, A., Ayscough, K., & Zoladek, T. (2016). Phosphatidylinositol-3-phosphate regulates response of cells to proteotoxic stress. *The International Journal of Biochemistry & Cell Biology*, *79*, 494-504. https://doi.org/10.1016/j.biocel.2016.08.007

Kannan, M., Lahiri, S., Liu, L.-K., Choudhary, V., & Prinz, W. A. (2017). Phosphatidylserine synthesis at membrane contact sites promotes its transport out of the ER. *Journal of Lipid Research*, 58(3), 553-562. https://doi.org/10.1194/jlr.M072959

Kast, D. J., & Dominguez, R. (2017). The Cytoskeleton–Autophagy Connection. *Current Biology*, 27(8), R318-R326. https://doi.org/10.1016/j.cub.2017.02.061

Kawai, S., Hashimoto, W., & Murata, K. (2010). Transformation of *Saccharomyces cerevisiae* and other fungi : Methods and possible underlying mechanism. *Bioengineered Bugs*, 1(6), 395-403. https://doi.org/10.4161/bbug.1.6.13257

Kawano, S., Tamura, Y., Kojima, R., Bala, S., Asai, E., Michel, A. H., Kornmann, B., Riezman, I., Riezman, H., Sakae, Y., Okamoto, Y., & Endo, T. (2018). Structure–function insights into direct lipid transfer between membranes by Mmm1–Mdm12 of ERMES. *Journal of Cell Biology*, *217*(3), 959-974. https://doi.org/10.1083/jcb.201704119

Kelly, A. A., & Dörmann, P. (2002). DGD2, an Arabidopsis Gene Encoding a UDP-Galactosedependent Digalactosyldiacylglycerol Synthase Is Expressed during Growth under Phosphate-limiting Conditions. *Journal of Biological Chemistry*, 277(2), 1166-1173. https://doi.org/10.1074/jbc.M110066200

Khawar, M. B., Gao, H., & Li, W. (2019). Mechanism of Acrosome Biogenesis in Mammals. *Frontiers in Cell and Developmental Biology*, 7, 195. https://doi.org/10.3389/fcell.2019.00195

Kim, H., Lee, S., Jun, Y., & Lee, C. (2022). Structural basis for mitoguardin-2 mediated lipid transport at ER-mitochondrial membrane contact sites. *Nature Communications*, *13*(1), 3702. https://doi.org/10.1038/s41467-022-31462-6

Klodmann, J., Senkler, M., Rode, C., & Braun, H.-P. (2011). Defining the *Protein Complex Proteome* of Plant Mitochondria. *Plant Physiology*, *157*(2), 587-598. https://doi.org/10.1104/pp.111.182352

Kobayashi, K., Awai, K., Nakamura, M., Nagatani, A., Masuda, T., & Ohta, H. (2009). Type-B monogalactosyldiacylglycerol synthases are involved in phosphate starvation-induced lipid remodeling, and are crucial for low-phosphate adaptation. *The Plant Journal*, *57*(2), 322-331. https://doi.org/10.1111/j.1365-313X.2008.03692.x

Koike, S., & Jahn, R. (2019). SNAREs define targeting specificity of trafficking vesicles by combinatorial interaction with tethering factors. *Nature Communications*, *10*(1), 1608. https://doi.org/10.1038/s41467-019-09617-9

Koizumi, K., & Gallagher, K. L. (2013). Identification of SHRUBBY, a SHORT-ROOT and SCARECROW interacting protein that controls root growth and radial patterning. *Development*, *140*(6), 1292-1300. https://doi.org/10.1242/dev.090761

Kojima, R., Endo, T., & Tamura, Y. (2016). A phospholipid transfer function of ER-mitochondria encounter structure revealed in vitro. *Scientific Reports*, 6(1), 30777. https://doi.org/10.1038/srep30777

Kolakowski, D., Kaminska, J., & Zoladek, T. (2020). The binding of the APT1 domains to phosphoinositides is regulated by metal ions in vitro. *Biochimica et Biophysica Acta (BBA)* - *Biomembranes*, *1862*(9), 183349. https://doi.org/10.1016/j.bbamem.2020.183349

Kolakowski, D., Rzepnikowska, W., Kaniak-Golik, A., Zoladek, T., & Kaminska, J. (2021). The GTPase Arf1 Is a Determinant of Yeast Vps13 Localization to the Golgi Apparatus. *International Journal of Molecular Sciences*, 22(22), 12274. https://doi.org/10.3390/ijms222212274

Kolehmainen, J., Black, G. C. M., Saarinen, A., Chandler, K., Clayton-Smith, J., Träskelin, A.-L., Perveen, R., Kivitie-Kallio, S., Norio, R., Warburg, M., Fryns, J.-P., Chapelle, A. de la, & Lehesjoki, A.-E. (2003). Cohen Syndrome Is Caused by Mutations in a Novel Gene, COH1, Encoding a Transmembrane Protein with a Presumed Role in Vesicle-Mediated Sorting and Intracellular Protein Transport. *The American Journal of Human Genetics*, 72(6), 1359-1369. https://doi.org/10.1086/375454

Körner, C., Barrera, M., Dukanovic, J., Eydt, K., Harner, M., Rabl, R., Vogel, F., Rapaport, D., Neupert, W., & Reichert, A. S. (2012). The C-terminal domain of Fcj1 is required for formation of crista junctions and interacts with the TOB/SAM complex in mitochondria. *Molecular Biology of the Cell*, 23(11), 2143-2155. https://doi.org/10.1091/mbc.e11-10-0831

Kornmann, B. (2020). The endoplasmic reticulum-mitochondria encounter structure : Coordinating lipid metabolism across membranes. *Biological Chemistry*, 401(6-7), 811-820. https://doi.org/10.1515/hsz-2020-0102

Kornmann, B., Currie, E., Collins, S. R., Schuldinger, M., Nunnari, J., Weissman, J., & Walter, P. (2009). An ER-Mitochondria Tethering Complex Revealed by a Synthetic Biology Screen. *Science*, *325*(5939), 477-481. https://doi.org/10.1126/science.1174447

Kornmann, B., Osman, C., & Walter, P. (2011). The conserved GTPase Gem1 regulates endoplasmic reticulum-mitochondria connections. *Proceedings of the National Academy of Sciences*, *108*(34), 14151-14156. https://doi.org/10.1073/pnas.1111314108

Kors, S., Kurian, S. M., Costello, J. L., & Schrader, M. (2022). Controlling contacts—Molecular mechanisms to regulate organelle membrane tethering. *BioEssays*, 2200151. https://doi.org/10.1002/bies.202200151

Kotani, T., Kirisako, H., Koizumi, M., Ohsumi, Y., & Nakatogawa, H. (2018). The Atg2-Atg18 complex tethers pre-autophagosomal membranes to the endoplasmic reticulum for autophagosome formation.

*Proceedings of the National Academy of Sciences*, *115*(41), 10363-10368. https://doi.org/10.1073/pnas.1806727115

Kruppa, A. J., Kendrick-Jones, J., & Buss, F. (2016). Myosins, Actin and Autophagy. *Traffic*, 17(8), 878-890. https://doi.org/10.1111/tra.12410

Kumar, N., Leonzino, M., Hancock-Cerutti, W., Horenkamp, F. A., Li, P., Lees, J. A., Wheeler, H., Reinisch, K. M., & De Camilli, P. (2018). VPS13A and VPS13C are lipid transport proteins differentially localized at ER contact sites. *Journal of Cell Biology*, *217*(10), 3625-3639. https://doi.org/10.1083/jcb.201807019

Kundu, D., & Pasrija, R. (2020). The ERMES (Endoplasmic Reticulum and Mitochondria Encounter Structures) mediated functions in fungi. *Mitochondrion*, 52, 89-99. https://doi.org/10.1016/j.mito.2020.02.010

Kwok, E. Y., & Hanson, M. R. (2004). Plastids and stromules interact with the nucleus and cell membrane in vascular plants. *Plant Cell Reports*, 23(4), 188-195. https://doi.org/10.1007/s00299-004-0824-9

#### L

Lackner, L. L. (2019). The Expanding and Unexpected Functions of Mitochondria Contact Sites. *Trends in Cell Biology*, 29(7), 580-590. https://doi.org/10.1016/j.tcb.2019.02.009

Lahiri, S., Chao, J. T., Tavassoli, S., Wong, A. K. O., Choudhary, V., Young, B. P., Loewen, C. J. R., & Prinz, W. A. (2014). A Conserved Endoplasmic Reticulum Membrane Protein Complex (EMC) Facilitates Phospholipid Transfer from the ER to Mitochondria. *PLOS Biology*, *12*(10), 18.

Lambers, H. (2022). Phosphorus Acquisition and Utilization in Plants. *Annual Review of Plant Biology*, 73(1), 17-42. https://doi.org/10.1146/annurev-arplant-102720-125738

Lang, A. B., Peter, A. T. J., Walter, P., & Kornmann, B. (2015). ER-mitochondrial junctions can be bypassed by dominant mutations in the endosomal protein Vps13. *Journal of Cell Biology*, 210(6), 883-890. https://doi.org/10.1083/jcb.201502105

Le Huray, K. I. P., Wang, H., Sobott, F., & Kalli, A. C. (2022). Systematic simulation of the interactions of pleckstrin homology domains with membranes. *Science Advances*, 8(27), eabn6992. https://doi.org/10.1126/sciadv.abn6992

Lees, J. A., & Reinisch, K. M. (2020). Inter-organelle lipid transfer : A channel model for Vps13 and chorein-N motif proteins. *Current Opinion in Cell Biology*, 65, 66-71. https://doi.org/10.1016/j.ceb.2020.02.008

Lei, Y., Wen, X., & Klionsky, D. J. (2022). Vps13 is Required for Efficient Autophagy in *Saccharomyces cerevisiae*. *Contact*, *5*, 251525642211363. https://doi.org/10.1177/25152564221136388

Leiba, J., Sabra, A., Bodinier, R., Marchetti, A., Lima, W. C., Melotti, A., Perrin, J., Burdet, F., Pagni, M., Soldati, T., Lelong, E., & Cosson, P. (2017). Vps13F links bacterial recognition and intracellular killing in *Dictyostelium*. *Cellular Microbiology*, *19*(7), e12722. https://doi.org/10.1111/cmi.12722

Lenoir, M., Kufareva, I., Abagyan, R., & Overduin, M. (2015). Membrane and Protein Interactions of the Pleckstrin Homology Domain Superfamily. *Membranes*, 5(4), 646-663. https://doi.org/10.3390/membranes5040646 Leonzino, M., Reinisch, K. M., & De Camilli, P. (2021). Insights into VPS13 properties and function reveal a new mechanism of eukaryotic lipid transport. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1866*(10), 159003. https://doi.org/10.1016/j.bbalip.2021.159003

Lesage, S., Drouet, V., Majounie, E., Deramecourt, V., Jacoupy, M., Nicolas, A., Cormier-Dequaire, F., Hassoun, S. M., Pujol, C., Ciura, S., Erpapazoglou, Z., Usenko, T., Maurage, C.-A., Sahbatou, M., Liebau, S., Ding, J., Bilgic, B., Emre, M., Erginel-Unaltuna, N., ... Brice, A. (2016). Loss of VPS13C Function in Autosomal-Recessive Parkinsonism Causes Mitochondrial Dysfunction and Increases PINK1/Parkin-Dependent Mitophagy. *The American Journal of Human Genetics*, *98*(3), 500-513. https://doi.org/10.1016/j.ajhg.2016.01.014

Leterme, S., & Michaud, M. (2022). Non-vesicular glycerolipids transport in plant cells. In *Advances in Botanical Research* (Vol. 101, p. 121-189). Elsevier. https://doi.org/10.1016/bs.abr.2021.07.001

Leterme, S., Michaud, M., & Jouhet, J. (2021). Isolation of Mitochondria for Lipid Analysis. In D. Bartels & P. Dörmann (Éds.), *Plant Lipids* (Vol. 2295, p. 337-349). Springer US. https://doi.org/10.1007/978-1-0716-1362-7\_18

Levine, T. P. (2019). Remote homology searches identify bacterial homologues of eukaryotic lipid transfer proteins, including Chorein-N domains in TamB and AsmA and Mdm31p. *BMC Molecular and Cell Biology*, 20(1), 43. https://doi.org/10.1186/s12860-019-0226-z

Levine, T. P. (2022). Sequence Analysis and Structural Predictions of Lipid Transfer Bridges in the Repeating Beta Groove (RBG) Superfamily Reveal Past and Present Domain Variations Affecting Form, Function and Interactions of VPS13, ATG2, SHIP164, Hobbit and Tweek. *Contact*, *5*, 251525642211343. https://doi.org/10.1177/25152564221134328

Li, C., Duckney, P., Zhang, T., Fu, Y., Li, X., Kroon, J., De Jaeger, G., Cheng, Y., Hussey, P. J., & Wang, P. (2022). TraB family proteins are components of ER-mitochondrial contact sites and regulate ER-mitochondrial interactions and mitophagy. *Nature Communications*, *13*(1), 5658. https://doi.org/10.1038/s41467-022-33402-w

Li, C., Guan, Z., Liu, D., & Raetz, C. R. H. (2011). Pathway for lipid A biosynthesis in *Arabidopsis thaliana* resembling that of *Escherichia coli*. *Proceedings of the National Academy of Sciences*, 108(28), 11387-11392. https://doi.org/10.1073/pnas.1108840108

Li, L., Lavell, A., Meng, X., Berkowitz, O., Selinski, J., van de Meene, A., Carrie, C., Benning, C., Whelan, J., De Clercq, I., & Wang, Y. (2019). Arabidopsis DGD1 SUPPRESSOR1 Is a Subunit of the Mitochondrial Contact Site and Cristae Organizing System and Affects Mitochondrial Biogenesis. *The Plant Cell*, *31*(8), 1856-1878. https://doi.org/10.1105/tpc.18.00885

Li, M., Qin, C., Welti, R., & Wang, X. (2006). Double Knockouts of Phospholipases D  $\zeta$  1 and D  $\zeta$  2 in Arabidopsis Affect Root Elongation during Phosphate-Limited Growth But Do Not Affect Root Hair Patterning. *Plant Physiology*, 140(2), 761-770. https://doi.org/10.1104/pp.105.070995

Li, M., Welti, R., & Wang, X. (2006). Quantitative Profiling of Arabidopsis Polar Glycerolipids in Response to Phosphorus Starvation. Roles of Phospholipases D  $\zeta 1$  and D  $\zeta 2$  in Phosphatidylcholine Hydrolysis and Digalactosyldiacylglycerol Accumulation in Phosphorus-Starved Plants. *Plant Physiology*, 142(2), 750-761. https://doi.org/10.1104/pp.106.085647

Li, N., & Chen, J. (2014). ADP-Ribosylation : Activation, Recognition, and Removal. *Molecules and Cells*, *37*(1), 9-16. https://doi.org/10.14348/molcells.2014.2245

Li, P., Lees, J. A., Lusk, C. P., & Reinisch, K. M. (2020). Cryo-EM reconstruction of a VPS13 fragment reveals a long groove to channel lipids between membranes. *Journal of Cell Biology*, 219(5), e202001161. https://doi.org/10.1083/jcb.202001161

Li, S. C., & Kane, P. M. (2009). The yeast lysosome-like vacuole : Endpoint and crossroads. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1793*(4), 650-663. https://doi.org/10.1016/j.bbamcr.2008.08.003

Li, Y. E., Wang, Y., Du, X., Zhang, T., Mak, H. Y., Hancock, S. E., McEwen, H., Pandzic, E., Whan, R. M., Aw, Y. C., Lukmantara, I. E., Yuan, Y., Dong, X., Don, A., Turner, N., Qi, S., & Yang, H. (2021). TMEM41B and VMP1 are scramblases and regulate the distribution of cholesterol and phosphatidylserine. *Journal of Cell Biology*, 220(6), e202103105. https://doi.org/10.1083/jcb.202103105

Liang, D., Luan, L., & Baskin, J. M. (2023). *Imaging interorganelle phospholipid transport by extended synaptotagmins using bioorthogonally tagged lipids* [Preprint]. Cell Biology. https://doi.org/10.1101/2023.02.01.526230

Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M. X., Arondel, V., Bates, P. D., Baud, S., Bird, D., DeBono, A., Durrett, T. P., Franke, R. B., Graham, I. A., Katayama, K., Kelly, A. A., Larson, T., Markham, J. E., Miquel, M., Molina, I., Nishida, I., ... Ohlrogge, J. (2013). Acyl-Lipid Metabolism. *The Arabidopsis Book*, *11*, e0161. https://doi.org/10.1199/tab.0161

Lin, Y.-C., Kobayashi, K., Wada, H., & Nakamura, Y. (2018). Phosphatidylglycerophosphate phosphatase is required for root growth in Arabidopsis. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1863*(6), 563-575. https://doi.org/10.1016/j.bbalip.2018.02.007

Liu, Y., Ma, X., Fujioka, H., Liu, J., Chen, S., & Zhu, X. (2019). DJ-1 regulates the integrity and function of ER-mitochondria association through interaction with IP3R3-Grp75-VDAC1. *Proceedings of the National Academy of Sciences*, *116*(50), 25322-25328. https://doi.org/10.1073/pnas.1906565116

Liu, Y.-T., Senkler, J., Herrfurth, C., Braun, H.-P., & Feussner, I. (2023). Defining the lipidome of Arabidopsis leaf mitochondria : Specific lipid complement and biosynthesis capacity. *Plant Physiology*, kiad035. https://doi.org/10.1093/plphys/kiad035

Lopez-Fabuel, I., Le Douce, J., Logan, A., James, A. M., Bonvento, G., Murphy, M. P., Almeida, A., & Bolaños, J. P. (2016). Complex I assembly into supercomplexes determines differential mitochondrial ROS production in neurons and astrocytes. *Proceedings of the National Academy of Sciences*, *113*(46), 13063-13068. https://doi.org/10.1073/pnas.1613701113

Luo, Q., Yang, X., Yu, S., Shi, H., Wang, K., Xiao, L., Zhu, G., Sun, C., Li, T., Li, D., Zhang, X., Zhou, M., & Huang, Y. (2017). Structural basis for lipopolysaccharide extraction by ABC transporter LptB2FG. *Nature Structural & Molecular Biology*, *24*(5), 469-474. https://doi.org/10.1038/nsmb.3399

#### Μ

Maeda, S., Yamamoto, H., Kinch, L. N., Garza, C. M., Takahashi, S., Otomo, C., Grishin, N. V., Forli, S., Mizushima, N., & Otomo, T. (2020). Structure, lipid scrambling activity and role in autophagosome formation of ATG9A. *Nature Structural & Molecular Biology*, 27(12), 1194-1201. https://doi.org/10.1038/s41594-020-00520-2

Maréchal, E. (2018). Primary Endosymbiosis: Emergence of the Primary Chloroplast and the Chromatophore, Two Independent Events. In E. Maréchal (Éd.), *Plastids* (Vol. 1829, p. 3-16). Springer US. https://doi.org/10.1007/978-1-4939-8654-5\_1

Maréchal, E., Azzouz, N., Santos de Macedo, C., Block, M. A., Feagin, J. E., Schwarz, R. T., & Joyard, J. (2002). Synthesis of Chloroplast Galactolipids in Apicomplexan Parasites. *Eukaryotic Cell*, 1(4), 653-656. https://doi.org/10.1128/EC.1.4.653-656.2002

Martin, W. F., Garg, S., & Zimorski, V. (2015). Endosymbiotic theories for eukaryote origin. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*(1678), 20140330. https://doi.org/10.1098/rstb.2014.0330

Matoba, K., Kotani, T., Tsutsumi, A., Tsuji, T., Mori, T., Noshiro, D., Sugita, Y., Nomura, N., Iwata, S., Ohsumi, Y., Fujimoto, T., Nakatogawa, H., Kikkawa, M., & Noda, N. N. (2020). Atg9 is a lipid scramblase that mediates autophagosomal membrane expansion. *Nature Structural & Molecular Biology*, 27(12), 1185-1193. https://doi.org/10.1038/s41594-020-00518-w

Maulucci, G., Cohen, O., Daniel, B., Sansone, A., Petropoulou, P. I., Filou, S., Spyridonidis, A., Pani, G., De Spirito, M., Chatgilialoglu, C., Ferreri, C., Kypreos, K. E., & Sasson, S. (2016). Fatty acid-related modulations of membrane fluidity in cells: Detection and implications. *Free Radical Research*, *50*(sup1), S40-S50. https://doi.org/10.1080/10715762.2016.1231403

McLelland, G.-L., & Fon, E. A. (2018). Principles of mitochondrial vesicle transport. *Current Opinion in Physiology*, *3*, 25-33. https://doi.org/10.1016/j.cophys.2018.02.005

Melia, T. J., & Reinisch, K. M. (2022). A possible role for VPS13-family proteins in bulk lipid transfer, membrane expansion and organelle biogenesis. *Journal of Cell Science*, *135*(5), jcs259357. https://doi.org/10.1242/jcs.259357

Michaud, M. (2018). Analysis of the MTL Supercomplex at Contact Sites Between Mitochondria and Plastids. In E. Maréchal (Éd.), *Plastids* (Vol. 1829, p. 173-188). Springer US. https://doi.org/10.1007/978-1-4939-8654-5\_12

Michaud, M., & Duchêne, A.-M. (2012). Macromolecules Trafficking to Plant Mitochondria. In *Advances in Botanical Research* (Vol. 63, p. 347-421). Elsevier. https://doi.org/10.1016/B978-0-12-394279-1.00012-0

Michaud, M., Gros, V., Tardif, M., Brugière, S., Ferro, M., Prinz, W. A., Toulmay, A., Mathur, J., Wozny, M., Falconet, D., Maréchal, E., Block, M. A., & Jouhet, J. (2016). AtMic60 Is Involved in Plant Mitochondria Lipid Trafficking and Is Part of a Large Complex. *Current Biology*, *26*(5), 627-639. https://doi.org/10.1016/j.cub.2016.01.011

Michaud, M., & Jouhet, J. (2019). Lipid Trafficking at Membrane Contact Sites During Plant Development and Stress Response. *Frontiers in Plant Science*, 10, 2. https://doi.org/10.3389/fpls.2019.00002

Michaud, M., Prinz, W. A., & Jouhet, J. (2017). Glycerolipid synthesis and lipid trafficking in plant mitochondria. *The FEBS Journal*, 284(3), 376-390. https://doi.org/10.1111/febs.13812

Michel, A. H., & Kornmann, B. (2022). SAturated Transposon Analysis in Yeast (SATAY) for Deep Functional Mapping of Yeast Genomes. In F. Devaux (Éd.), *Yeast Functional Genomics* (Vol. 2477, p. 349-379). Springer US. https://doi.org/10.1007/978-1-0716-2257-5\_20

Miège, C., Maréchal, E., Shimojima, M., Awai, K., Block, M. A., Ohta, H., Takamiya, K., Douce, R., & Joyard, J. (1999). Biochemical and topological properties of type A MGDG synthase, a spinach chloroplast envelope enzyme catalyzing the synthesis of both prokaryotic and eukaryotic MGDG: MGDG synthase A of spinach chloroplast envelope membranes. *European Journal of Biochemistry*, 265(3), 990-1001. https://doi.org/10.1046/j.1432-1327.1999.00801.x

Miliara, X., Garnett, J. A., Tatsuta, T., Abid Ali, F., Baldie, H., Pérez-Dorado, I., Simpson, P., Yague, E., Langer, T., & Matthews, S. (2015). Structural insight into the TRIAP 1/ PRELI -like domain family of mitochondrial phospholipid transfer complexes. *EMBO Reports*, *16*(7), 824-835. https://doi.org/10.15252/embr.201540229

Miyata, N., Fujii, S., & Kuge, O. (2017). Cooperative function of Fmp30, Mdm31, and Mdm32 in Ups1-independent cardiolipin accumulation in the yeast Saccharomyces cerevisiae. *Scientific Reports*, 12.

Miyata, N., Goda, N., Matsuo, K., Hoketsu, T., & Kuge, O. (2018). Porin proteins have critical functions in mitochondrial phospholipid metabolism in yeast. 13.

Miyata, N., Watanabe, Y., Tamura, Y., Endo, T., & Kuge, O. (2016). Phosphatidylserine transport by Ups2–Mdm35 in respiration-active mitochondria. *Journal of Cell Biology*, 214(1), 77-88. https://doi.org/10.1083/jcb.201601082

Mizoi, J., Nakamura, M., & Nishida, I. (2007). Defects in CTP:PHOSPHORYLETHANOLAMINE CYTIDYLYLTRANSFERASE Affect Embryonic and Postembryonic Development in *Arabidopsis*. *The Plant Cell*, *18*(12), 3370-3385. https://doi.org/10.1105/tpc.106.040840

Moellering, E. R., & Benning, C. (2011). Galactoglycerolipid metabolism under stress : A time for remodeling. 16(2), 10.

Monteiro-Cardoso, V. F., Rochin, L., Arora, A., Houcine, A., Jääskeläinen, E., Kivelä, A. M., Sauvanet, C., Le Bars, R., Marien, E., Dehairs, J., Neveu, J., El Khallouki, N., Santonico, E., Swinnen, J. V., Tareste, D., Olkkonen, V. M., & Giordano, F. (2022). ORP5/8 and MIB/MICOS link ER-mitochondria and intra-mitochondrial contacts for non-vesicular transport of phosphatidylserine. *Cell Reports*, *40*(12), 111364. https://doi.org/10.1016/j.celrep.2022.111364

Mueller, S. J., Lang, D., Hoernstein, S. N. W., Lang, E. G. E., Schuessele, C., Schmidt, A., Fluck, M., Leisibach, D., Niegl, C., Zimmer, A. D., Schlosser, A., & Reski, R. (2014). Quantitative Analysis of the Mitochondrial and Plastid Proteomes of the Moss *Physcomitrella patens* Reveals Protein Macrocompartmentation and Microcompartmentation. *Plant Physiology*, *164*(4), 2081-2095. https://doi.org/10.1104/pp.114.235754

Mueller, S. J., & Reski, R. (2015). Mitochondrial Dynamics and the ER: The Plant Perspective. *Frontiers in Cell and Developmental Biology*, *3*. https://doi.org/10.3389/fcell.2015.00078

Mueller-Roeber, B., & Pical, C. (2002). Inositol Phospholipid Metabolism in Arabidopsis. Characterized and Putative Isoforms of Inositol Phospholipid Kinase and Phosphoinositide-Specific Phospholipase C. *Plant Physiology*, *130*(1), 22-46. https://doi.org/10.1104/pp.004770

Mueller-Schuessele, S. J., & Michaud, M. (2018). Plastid Transient and Stable Interactions with Other Cell Compartments. In E. Maréchal (Éd.), *Plastids* (Vol. 1829, p. 87-109). Springer US. https://doi.org/10.1007/978-1-4939-8654-5\_6

Mukherjee, I., Ghosh, M., & Meinecke, M. (2021). *MICOS and the mitochondrial inner membrane morphology - when things get out of shape*. 57.

Muñoz-Braceras, S., Calvo, R., & Escalante, R. (2015). TipC and the chorea-acanthocytosis protein VPS13A regulate autophagy in *Dictyostelium* and human HeLa cells. *Autophagy*, *11*(6), 918-927. https://doi.org/10.1080/15548627.2015.1034413

Muñoz-Braceras, S., Tornero-Écija, A. R., Vincent, O., & Escalante, R. (2019). VPS13A is closely associated with mitochondria and is required for efficient lysosomal degradation. *Disease Models & Mechanisms*, *12*(2), dmm036681. https://doi.org/10.1242/dmm.036681

Murcha, M. W., Elhafez, D., Millar, A. H., & Whelan, J. (2005). The C-terminal Region of TIM17 Links the Outer and Inner Mitochondrial Membranes in Arabidopsis and Is Essential for Protein Import. *Journal of Biological Chemistry*, 280(16), 16476-16483. https://doi.org/10.1074/jbc.M413299200

Murcha, M. W., Kmiec, B., Kubiszewski-Jakubiak, S., Teixeira, P. F., Glaser, E., & Whelan, J. (2014). Protein import into plant mitochondria: Signals, machinery, processing, and regulation. *Journal of Experimental Botany*, 65(22), 6301-6335. https://doi.org/10.1093/jxb/eru399

Murcha, M. W., Wang, Y., & Whelan, J. (2012). A molecular link between mitochondrial preprotein transporters and respiratory chain complexes. *Plant Signaling & Behavior*, 7(12), 1594-1597. https://doi.org/10.4161/psb.22250

Murley, A., Sarsam, R. D., Toulmay, A., Yamada, J., Prinz, W. A., & Nunnari, J. (2015). Ltc1 is an ER-localized sterol transporter and a component of ER–mitochondria and ER–vacuole contacts. *Journal of Cell Biology*, 209(4), 539-548. https://doi.org/10.1083/jcb.201502033

Murphy, S. E., & Levine, T. P. (2016). VAP, a Versatile Access Point for the Endoplasmic Reticulum : Review and analysis of FFAT-like motifs in the VAPome. *Biochimica et Biophysica Acta (BBA)* -*Molecular and Cell Biology of Lipids*, 1861(8), 952-961. https://doi.org/10.1016/j.bbalip.2016.02.009

#### Ν

Nakabayashi, K., Okamoto, M., Koshiba, T., Kamiya, Y., & Nambara, E. (2005). Genome-wide profiling of stored mRNA in Arabidopsis thaliana seed germination : Epigenetic and genetic regulation of transcription in seed: Molecular profiling in Arabidopsis seed. *The Plant Journal*, *41*(5), 697-709. https://doi.org/10.1111/j.1365-313X.2005.02337.x

Nakamura, T. S., Suda, Y., Muneshige, K., Fujieda, Y., Okumura, Y., Inoue, I., Tanaka, T., Takahashi, T., Nakanishi, H., Gao, X.-D., Okada, Y., Neiman, A. M., & Tachikawa, H. (2021). Suppression of Vps13 adaptor protein mutants reveals a central role for PI4P in regulating prospore membrane extension. *PLOS Genetics*, *17*(8), e1009727. https://doi.org/10.1371/journal.pgen.1009727

Nakamura, Y. (2013). Phosphate starvation and membrane lipid remodeling in seed plants. *Progress in Lipid Research*, 52(1), 43-50. https://doi.org/10.1016/j.plipres.2012.07.002

Nakamura, Y., Awai, K., Masuda, T., Yoshioka, Y., Takamiya, K., & Ohta, H. (2005). A Novel Phosphatidylcholine-hydrolyzing Phospholipase C Induced by Phosphate Starvation in Arabidopsis. *Journal of Biological Chemistry*, 280(9), 7469-7476. https://doi.org/10.1074/jbc.M408799200

Nakamura, Y., Koizumi, R., Shui, G., Shimojima, M., Wenk, M. R., Ito, T., & Ohta, H. (2009). *Arabidopsis* lipins mediate eukaryotic pathway of lipid metabolism and cope critically with phosphate starvation. *Proceedings of the National Academy of Sciences*, *106*(49), 20978-20983. https://doi.org/10.1073/pnas.0907173106

Nakanishi, H., Suda, Y., & Neiman, A. M. (2007). Erv14 family cargo receptors are necessary for ER exit during sporulation in Saccharomyces cerevisiae. *Journal of Cell Science*, *120*(5), 908-916. https://doi.org/10.1242/jcs.03405

Namba, T. (2019). BAP31 regulates mitochondrial function via interaction with Tom40 within ERmitochondria contact sites. *Science Advances*, 5(6), eaaw1386. https://doi.org/10.1126/sciadv.aaw1386

Neiman, A. M. (2011). Sporulation in the Budding Yeast *Saccharomyces cerevisiae*. *Genetics*, 189(3), 737-765. https://doi.org/10.1534/genetics.111.127126

Nerlich, A., von Orlow, M., Rontein, D., Hanson, A. D., & Dörmann, P. (2007). Deficiency in Phosphatidylserine Decarboxylase Activity in the *psd1 psd2 psd3* Triple Mutant of Arabidopsis Affects Phosphatidylethanolamine Accumulation in Mitochondria. *Plant Physiology*, *144*(2), 904-914. https://doi.org/10.1104/pp.107.095414

Neuman, S. D., & Bashirullah, A. (2018). Hobbit regulates intracellular trafficking to drive insulindependent growth during *Drosophila* development. *Development*, *145*(11), dev161356. https://doi.org/10.1242/dev.161356

Neuman, S. D., Jorgensen, J. R., Cavanagh, A. T., Smyth, J. T., Selegue, J. E., Emr, S. D., & Bashirullah, A. (2022). The Hob proteins are novel and conserved lipid-binding proteins at ER–PM contact sites. *Journal of Cell Science*, *135*(5), jcs259086. https://doi.org/10.1242/jcs.259086

Neuman, S. D., Levine, T. P., & Bashirullah, A. (2022). A novel superfamily of bridge-like lipid transfer proteins. *Trends in Cell Biology*, S0962892422000836. https://doi.org/10.1016/j.tcb.2022.03.011

Noda, N. N. (2021). Atg2 and Atg9: Intermembrane and interleaflet lipid transporters driving autophagy. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1866(8), 158956. https://doi.org/10.1016/j.bbalip.2021.158956

#### 0

Oikawa, K., Matsunaga, S., Mano, S., Kondo, M., Yamada, K., Hayashi, M., Kagawa, T., Kadota, A., Sakamoto, W., Higashi, S., Watanabe, M., Mitsui, T., Shigemasa, A., Iino, T., Hosokawa, Y., & Nishimura, M. (2015). Physical interaction between peroxisomes and chloroplasts elucidated by in situ laser analysis. *Nature Plants*, 1(4), 15035. https://doi.org/10.1038/nplants.2015.35

Orii, M., Tsuji, T., Ogasawara, Y., & Fujimoto, T. (2021). Transmembrane phospholipid translocation mediated by Atg9 is involved in autophagosome formation. *Journal of Cell Biology*, 220(3), e202009194. https://doi.org/10.1083/jcb.202009194

Osawa, T., Kotani, T., Kawaoka, T., Hirata, E., Suzuki, K., Nakatogawa, H., Ohsumi, Y., & Noda, N. N. (2019). Atg2 mediates direct lipid transfer between membranes for autophagosome formation. *Nature Structural & Molecular Biology*, *26*(4), 281-288. https://doi.org/10.1038/s41594-019-0203-4

Ott, C., Ross, K., Straub, S., Thiede, B., Götz, M., Goosmann, C., Krischke, M., Mueller, M. J., Krohne, G., Rudel, T., & Kozjak-Pavlovic, V. (2012). Sam50 Functions in Mitochondrial Intermembrane Space Bridging and Biogenesis of Respiratory Complexes. *Molecular and Cellular Biology*, *32*(6), 1173-1188. https://doi.org/10.1128/MCB.06388-11

#### Р

Park, J.-S., Halegoua, S., Kishida, S., & Neiman, A. M. (2015). A Conserved Function in Phosphatidylinositol Metabolism for Mammalian Vps13 Family Proteins. *PLOS ONE*, *10*(4), e0124836. https://doi.org/10.1371/journal.pone.0124836

Park, J.-S., Hu, Y., Hollingsworth, Nancy. M., Miltenberger-Miltenyi, G., & Neiman, A. M. (2022). Interaction between VPS13A and the XK scramblase is important for VPS13A function in humans. *Journal of Cell Science*, jcs.260227. https://doi.org/10.1242/jcs.260227

Park, J.-S., & Neiman, A. M. (2012). *VPS13* regulates membrane morphogenesis during sporulation in *Saccharomyces cerevisiae*. *Journal of Cell Science*, *125*(12), 3004-3011. https://doi.org/10.1242/jcs.105114 Park, J.-S., & Neiman, A. M. (2020). XK is a partner for VPS13A : A molecular link between Chorea-Acanthocytosis and McLeod Syndrome. *Molecular Biology of the Cell*, 31(22), 2425-2436. https://doi.org/10.1091/mbc.E19-08-0439-T

Park, J.-S., Okumura, Y., Tachikawa, H., & Neiman, A. M. (2013). *SPO71* Encodes a Developmental Stage-Specific Partner for Vps13 in Saccharomyces cerevisiae. *Eukaryotic Cell*, *12*(11), 1530-1537. https://doi.org/10.1128/EC.00239-13

Park, J.-S., Thorsness, M. K., Policastro, R., McGoldrick, L. L., Hollingsworth, N. M., Thorsness, P. E., & Neiman, A. M. (2016). Yeast Vps13 promotes mitochondrial function and is localized at membrane contact sites. *Molecular Biology of the Cell*, 27(15), 2435-2449. https://doi.org/10.1091/mbc.e16-02-0112

Pérez-Sancho, J., Tilsner, J., Samuels, A. L., Botella, M. A., Bayer, E. M., & Rosado, A. (2016). Stitching Organelles : Organization and Function of Specialized Membrane Contact Sites in Plants. *Trends in Cell Biology*, *26*(9), 705-717. https://doi.org/10.1016/j.tcb.2016.05.007

Petrungaro, C., & Kornmann, B. (2019). Lipid exchange at ER-mitochondria contact sites : A puzzle falling into place with quite a few pieces missing. *Current Opinion in Cell Biology*, 57, 71-76. https://doi.org/10.1016/j.ceb.2018.11.005

Pietra, S., Lang, P., & Grebe, M. (2015). *SABRE* is required for stabilization of root hair patterning in *Arabidopsis thaliana*. *Physiologia Plantarum*, *153*(3), 440-453. https://doi.org/10.1111/ppl.12257

Pitt, A. S., & Buchanan, S. K. (2021). A Biochemical and Structural Understanding of TOM Complex Interactions and Implications for Human Health and Disease. *Cells*, *10*(5), 1164. https://doi.org/10.3390/cells10051164

Plaxton, W. C., & Tran, H. T. (2011). Metabolic Adaptations of Phosphate-Starved Plants. *Plant Physiology*, 156(3), 1006-1015. https://doi.org/10.1104/pp.111.175281

Poirier, Y., Thoma, S., Somerville, C., & Schiefelbein, J. (1991). A Mutant of Arabidopsis Deficient in Xylem Loading of Phosphate. 97, 7.

Potting, C., Tatsuta, T., König, T., Haag, M., Wai, T., Aaltonen, M. J., & Langer, T. (2013). TRIAP1/PRELI Complexes Prevent Apoptosis by Mediating Intramitochondrial Transport of Phosphatidic Acid. *Cell Metabolism*, *18*(2), 287-295. https://doi.org/10.1016/j.cmet.2013.07.008

Prinz, W. A. (2014). Bridging the gap : Membrane contact sites in signaling, metabolism, and organelle dynamics. *Journal of Cell Biology*, 205(6), 759-769. https://doi.org/10.1083/jcb.201401126

Prinz, W. A., Toulmay, A., & Balla, T. (2020). The functional universe of membrane contact sites. *Nature Reviews Molecular Cell Biology*, 21(1), 7-24. https://doi.org/10.1038/s41580-019-0180-9

Procissi, A., Guyon, A., Pierson, E. S., Giritch, A., Knuiman, B., Grandjean, O., Tonelli, C., Derksen, J., Pelletier, G., & Bonhomme, S. (2003). *KINKY POLLEN* encodes a SABRE-like protein required for tip growth in *Arabidopsis* and conserved among eukaryotes. *The Plant Journal*, *36*(6), 894-904. https://doi.org/10.1046/j.1365-313X.2003.01933.x

#### R

Rainteau, D., Humbert, L., Delage, E., Vergnolle, C., Cantrel, C., Maubert, M.-A., Lanfranchi, S., Maldiney, R., Collin, S., Wolf, C., Zachowski, A., & Ruelland, E. (2012). Acyl Chains of Phospholipase D Transphosphatidylation Products in Arabidopsis Cells : A Study Using Multiple Reaction Monitoring Mass Spectrometry. *PLoS ONE*, *7*(7), e41985. https://doi.org/10.1371/journal.pone.0041985

Rampelt, H., Zerbes, R. M., van der Laan, M., & Pfanner, N. (2017). Role of the mitochondrial contact site and cristae organizing system in membrane architecture and dynamics. *Biochimica et Biophysica Acta* (*BBA*) - *Molecular Cell Research*, *1864*(4), 737-746. https://doi.org/10.1016/j.bbamcr.2016.05.020

Rampoldi, L., Dobson-Stone, C., Rubio, J. P., Danek, A., Chalmers, R. M., Wood, N. W., Verellen, C., Ferrer, X., Malandrini, A., Fabrizi, G. M., Brown, R., Vance, J., Pericak-Vance, M., Rudolf, G., Carrè, S., Alonso, E., Manfredi, M., Németh, A. H., & Monaco, A. P. (2001). A conserved sorting-associated protein is mutant in chorea-acanthocytosis. *Nature Genetics*, *28*(2), 119-120. https://doi.org/10.1038/88821

Raychaudhuri, S., & Prinz, W. A. (2008). Nonvesicular phospholipid transfer between peroxisomes and the endoplasmic reticulum. *Proceedings of the National Academy of Sciences*, *105*(41), 15785-15790. https://doi.org/10.1073/pnas.0808321105

Raymond, C. K., Howald-Stevenson, I., Vater, C. A., & Stevens, T. H. (1992). Morphological classification of the yeast vacuolar protein sorting mutants : Evidence for a prevacuolar compartment in class E vps mutants. *Molecular Biology of the Cell*, *3*(12), 1389-1402. https://doi.org/10.1091/mbc.3.12.1389

Redding, K., Brickner, J. H., Marschall, L. G., Nichols, J. W., & Fuller, R. S. (1996). Allele-specific suppression of a defective trans-Golgi network (TGN) localization signal in Kex2p identifies three genes involved in localization of TGN transmembrane proteins. *Molecular and Cellular Biology*, *16*(11), 6208-6217. https://doi.org/10.1128/MCB.16.11.6208

Reinisch, K. M., & Prinz, W. A. (2021). Mechanisms of nonvesicular lipid transport. *Journal of Cell Biology*, 220(3), e202012058. https://doi.org/10.1083/jcb.202012058

Richardson, L. G. L., & Schnell, D. J. (2020). Origins, function, and regulation of the TOC–TIC general protein import machinery of plastids. *Journal of Experimental Botany*, 71(4), 1226-1238. https://doi.org/10.1093/jxb/erz517

Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A., & Pozzan, T. (1998). Close Contacts with the Endoplasmic Reticulum as Determinants of Mitochondrial Ca <sup>2+</sup> Responses. *Science*, *280*(5370), 1763-1766. https://doi.org/10.1126/science.280.5370.1763

Robinson, J. S., Klionsky, D. J., Banta, L. M., & Emr, S. D. (1988). Protein sorting in Saccharomyces cerevisiae : Isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. *Molecular and Cellular Biology*, 8(11), 4936-4948. https://doi.org/10.1128/mcb.8.11.4936-4948.1988

Rogers, L. D., & Foster, L. J. (2007). The dynamic phagosomal proteome and the contribution of the endoplasmic reticulum. *Proceedings of the National Academy of Sciences*, 104(47), 18520-18525. https://doi.org/10.1073/pnas.0705801104

Rolland, N., Bouchnak, I., Moyet, L., Salvi, D., & Kuntz, M. (2018). The Main Functions of Plastids. In E. Maréchal (Éd.), *Plastids* (Vol. 1829, p. 73-85). Springer US. https://doi.org/10.1007/978-1-4939-8654-5\_5

Rose, R. H., Briddon, S. J., & Holliday, N. D. (2010). Bimolecular fluorescence complementation: Lighting up seven transmembrane domain receptor signalling networks: Investigating 7TM receptor function by BiFC. *British Journal of Pharmacology*, *159*(4), 738-750. https://doi.org/10.1111/j.1476-5381.2009.00480.x

Rothman, J. H., Howald, I., & Stevens, T. H. (1989). Characterization of genes required for protein sorting and vacuolar function in the yeast Saccharomyces cerevisiae. *The EMBO Journal*, 8(7), 2057-2065. https://doi.org/10.1002/j.1460-2075.1989.tb03614.x

Rothman, J. H., & Stevens, T. H. (1986). Protein sorting in yeast: Mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway. *Cell*, 47(6), 1041-1051. https://doi.org/10.1016/0092-8674(86)90819-6

Rowland, A. A., & Voeltz, G. K. (2012). Endoplasmic reticulum-mitochondria contacts : Function of the junction. *Nature Reviews Molecular Cell Biology*, *13*(10), 607-615. https://doi.org/10.1038/nrm3440

Ryoden, Y., Segawa, K., & Nagata, S. (2022). Requirement of Xk and Vps13a for the P2X7-mediated phospholipid scrambling and cell lysis in mouse T cells. *Proceedings of the National Academy of Sciences*, *119*(7), e2119286119. https://doi.org/10.1073/pnas.2119286119

Rzepnikowska, W., Flis, K., Kaminska, J., Grynberg, M., Urbanek, A., Ayscough, K. R., & Zoladek, T. (2017). Amino acid substitution equivalent to human chorea-acanthocytosis I2771R in yeast Vps13 protein affects its binding to phosphatidylinositol 3-phosphate. *Human Molecular Genetics*, 26(8), 1497-1510. https://doi.org/10.1093/hmg/ddx054

#### S

Sagar, S., & Singh, A. (2021). Emerging role of phospholipase C mediated lipid signaling in abiotic stress tolerance and development in plants. *Plant Cell Reports*, 40(11), 2123-2133. https://doi.org/10.1007/s00299-021-02713-5

Saita, S., Tatsuta, T., Lampe, P. A., König, T., Ohba, Y., & Langer, T. (2018). PARL partitions the lipid transfer protein STARD7 between the cytosol and mitochondria. *The EMBO Journal*, *37*(4). https://doi.org/10.15252/embj.201797909

Samaranayake, H. S., Cowan, A. E., & Klobutcher, L. A. (2011). Vacuolar protein sorting protein 13A, TtVPS13A, localizes to the tetrahymena thermophila phagosome membrane and is required for efficient phagocytosis. *Eukaryotic Cell*, *10*(9), 1207-1218. https://doi.org/10.1128/EC.05089-11

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, 74(12), 5463-5467. https://doi.org/10.1073/pnas.74.12.5463

Sassano, M. L., Felipe-Abrio, B., & Agostinis, P. (2022). ER-mitochondria contact sites; a multifaceted factory for Ca2+ signaling and lipid transport. *Frontiers in Cell and Developmental Biology*, *10*, 988014. https://doi.org/10.3389/fcell.2022.988014

Sassano, M. L., van Vliet, A. R., Vervoort, E., Van Eygen, S., Van den Haute, C., Pavie, B., Roels, J., Swinnen, J. V., Spinazzi, M., Moens, L., Casteels, K., Meyts, I., Pinton, P., Marchi, S., Rochin, L., Giordano, F., Felipe-Abrio, B., & Agostinis, P. (2023). PERK recruits E-Syt1 at ER–mitochondria contacts for mitochondrial lipid transport and respiration. *Journal of Cell Biology*, 222(3), e202206008. https://doi.org/10.1083/jcb.202206008

Schattat, M., Barton, K., & Mathur, J. (2011). Correlated behavior implicates stromules in increasing the interactive surface between plastids and ER tubules. *Plant Signaling & Behavior*, 6(5), 715-718. https://doi.org/10.4161/psb.6.5.15085

Scheffzek, K., & Welti, S. (2012). Pleckstrin homology (PH) like domains—Versatile modules in protein-protein interaction platforms. *FEBS Letters*, 586(17), 2662-2673. https://doi.org/10.1016/j.febslet.2012.06.006

Schlattner, U., Tokarska-Schlattner, M., Ramirez, S., Tyurina, Y. Y., Amoscato, A. A., Mohammadyani, D., Huang, Z., Jiang, J., Yanamala, N., Seffouh, A., Boissan, M., Epand, R. F., Epand, R. M., Klein-

Seetharaman, J., Lacombe, M.-L., & Kagan, V. E. (2013). Dual Function of Mitochondrial Nm23-H4 Protein in Phosphotransfer and Intermembrane Lipid Transfer. *Journal of Biological Chemistry*, 288(1), 111-121. https://doi.org/10.1074/jbc.M112.408633

Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D., & Lohmann, J. U. (2005). A gene expression map of Arabidopsis thaliana development. *Nature Genetics*, *37*(5), 501-506. https://doi.org/10.1038/ng1543

Schütter, M. (2020). Local Fatty Acid Channeling into Phospholipid Synthesis Drives Phagophore Expansion during Autophagy. 30.

Scorrano, L., De Matteis, M. A., Emr, S., Giordano, F., Hajnóczky, G., Kornmann, B., Lackner, L. L., Levine, T. P., Pellegrini, L., Reinisch, K., Rizzuto, R., Simmen, T., Stenmark, H., Ungermann, C., & Schuldiner, M. (2019). Coming together to define membrane contact sites. *Nature Communications*, *10*(1), 1287. https://doi.org/10.1038/s41467-019-09253-3

Seifert, W., Kühnisch, J., Maritzen, T., Horn, D., Haucke, V., & Hennies, H. C. (2011). Cohen Syndrome-associated Protein, COH1, Is a Novel, Giant Golgi Matrix Protein Required for Golgi Integrity. *Journal of Biological Chemistry*, 286(43), 37665-37675. https://doi.org/10.1074/jbc.M111.267971

Seifert, W., Kühnisch, J., Maritzen, T., Lommatzsch, S., Hennies, H. C., Bachmann, S., Horn, D., & Haucke, V. (2015). Cohen Syndrome-associated Protein COH1 Physically and Functionally Interacts with the Small GTPase RAB6 at the Golgi Complex and Directs Neurite Outgrowth. *Journal of Biological Chemistry*, 290(6), 3349-3358. https://doi.org/10.1074/jbc.M114.608174

Senkler, J., Senkler, M., Eubel, H., Hildebrandt, T., Lengwenus, C., Schertl, P., Schwarzländer, M., Wagner, S., Wittig, I., & Braun, H.-P. (2017). The mitochondrial complexome of *Arabidopsis thaliana*. *The Plant Journal*, *89*(6), 1079-1092. https://doi.org/10.1111/tpj.13448

Seong, E., Insolera, R., Dulovic, M., Kamsteeg, E., Trinh, J., Brüggemann, N., Sandford, E., Li, S., Ozel, A. B., Li, J. Z., Jewett, T., Kievit, A. J. A., Münchau, A., Shakkottai, V., Klein, C., Collins, C. A., Lohmann, K., Warrenburg, B. P., & Burmeister, M. (2018). Mutations in *VPS13D* lead to a new recessive ataxia with spasticity and mitochondrial defects. *Annals of Neurology*, *83*(6), 1075-1088. https://doi.org/10.1002/ana.25220

Sesaki, H., & Jensen, R. E. (2004). Ugo1p Links the Fzo1p and Mgm1p GTPases for Mitochondrial Fusion. *Journal of Biological Chemistry*, 279(27), 28298-28303. https://doi.org/10.1074/jbc.M401363200

Sheikholeslam, S. N., & Weeks, D. P. (1987). Acetosyringone promotes high efficiency transformation of Arabidopsis thaliana explants by Agrobacterium tumefaciens. *Plant Molecular Biology*, 8(4), 291-298. https://doi.org/10.1007/BF00021308

Shen, J. L., Fortier, T. M., Zhao, Y. G., Wang, R., Burmeister, M., & Baehrecke, E. H. (2021). Vmp1, Vps13D, and Marf/Mfn2 function in a conserved pathway to regulate mitochondria and ER contact in development and disease. *Current Biology*, *31*(14), 3028-3039.e7. https://doi.org/10.1016/j.cub.2021.04.062

Shimamura, R., Ohashi, Y., Taniguchi, Y. Y., Kato, M., Tsuge, T., & Aoyama, T. (2022). Arabidopsis PLDζ1 and PLDζ2 localize to post-Golgi membrane compartments in a partially overlapping manner. *Plant Molecular Biology*, *108*(1-2), 31-49. https://doi.org/10.1007/s11103-021-01205-0

Shirane, M., Wada, M., Morita, K., Hayashi, N., Kunimatsu, R., Matsumoto, Y., Matsuzaki, F., Nakatsumi, H., Ohta, K., Tamura, Y., & Nakayama, K. I. (2020). Protrudin and PDZD8 contribute to

neuronal integrity by promoting lipid extraction required for endosome maturation. *Nature Communications*, *11*(1), 4576. https://doi.org/10.1038/s41467-020-18413-9

Shui, W., Sheu, L., Liu, J., Smart, B., Petzold, C. J., Hsieh, T., Pitcher, A., Keasling, J. D., & Bertozzi, C. R. (2008). Membrane proteomics of phagosomes suggests a connection to autophagy. *Proceedings of the National Academy of Sciences*, *105*(44), 16952-16957. https://doi.org/10.1073/pnas.0809218105

Silva-Pavez, E., Ahumada-Castro, U., Lovy, A., & Cárdenas, J. C. (2021). Ca <sup>2+</sup> transfer to mitochondria : A spark of life in unexpected conditions. *Molecular & Cellular Oncology*, 8(1), 1839341. https://doi.org/10.1080/23723556.2020.1839341

Simionato, D., Block, M. A., La Rocca, N., Jouhet, J., Maréchal, E., Finazzi, G., & Morosinotto, T. (2013). The Response of Nannochloropsis gaditana to Nitrogen Starvation Includes *De Novo* Biosynthesis of Triacylglycerols, a Decrease of Chloroplast Galactolipids, and Reorganization of the Photosynthetic Apparatus. *Eukaryotic Cell*, *12*(5), 665-676. https://doi.org/10.1128/EC.00363-12

Singh, N., Reyes-Ordoñez, A., Compagnone, M. A., Moreno, J. F., Leslie, B. J., Ha, T., & Chen, J. (2021). Redefining the specificity of phosphoinositide-binding by human PH domain-containing proteins. *Nature Communications*, *12*(1), 4339. https://doi.org/10.1038/s41467-021-24639-y

Slee, J. A., & Levine, T. P. (2019). Systematic Prediction of FFAT Motifs Across Eukaryote Proteomes Identifies Nucleolar and Eisosome Proteins With the Predicted Capacity to Form Bridges to the Endoplasmic Reticulum. *Contact*, *2*, 251525641988313. https://doi.org/10.1177/2515256419883136

Smertenko, A., Assaad, F., Baluška, F., Bezanilla, M., Buschmann, H., Drakakaki, G., Hauser, M.-T., Janson, M., Mineyuki, Y., Moore, I., Müller, S., Murata, T., Otegui, M. S., Panteris, E., Rasmussen, C., Schmit, A.-C., Šamaj, J., Samuels, L., Staehelin, L. A., ... Žárský, V. (2017). Plant Cytokinesis: Terminology for Structures and Processes. *Trends in Cell Biology*, 27(12), 885-894. https://doi.org/10.1016/j.tcb.2017.08.008

Somerharju, P. (2015). Is Spontaneous Translocation of Polar Lipids between Cellular Organelles Negligible? *Lipid Insights*, 8s1, LPI.S31616. https://doi.org/10.4137/LPI.S31616

Spinelli, J. B., & Haigis, M. C. (2018). The multifaceted contributions of mitochondria to cellular metabolism. *Nature Cell Biology*, 20(7), 745-754. https://doi.org/10.1038/s41556-018-0124-1

Stege, J. T., Laub, M. T., & Loomis, W. F. (1999). Tip genes act in parallel pathways of earlyDictyostelium development. *Developmental Genetics*, 25(1), 64-77. https://doi.org/10.1002/(SICI)1520-6408(1999)25:1<64::AID-DVG7>3.0.CO;2-1

Stoica, R., De Vos, K. J., Paillusson, S., Mueller, S., Sancho, R. M., Lau, K.-F., Vizcay-Barrena, G., Lin, W.-L., Xu, Y.-F., Lewis, J., Dickson, D. W., Petrucelli, L., Mitchell, J. C., Shaw, C. E., & Miller, C. C. J. (2014). ER–mitochondria associations are regulated by the VAPB–PTPIP51 interaction and are disrupted by ALS/FTD-associated TDP-43. *Nature Communications*, *5*(1), 3996. https://doi.org/10.1038/ncomms4996

Subra, M., Dezi, M., Bigay, J., Lacas-Gervais, S., Di Cicco, A., Araújo, A. R. D., Abélanet, S., Fleuriot, L., Debayle, D., Gautier, R., Patel, A., Roussi, F., Antonny, B., Lévy, D., & Mesmin, B. (2023). VAP-A intrinsically disordered regions enable versatile tethering at membrane contact sites. *Developmental Cell*, *58*(2), 121-138.e9. https://doi.org/10.1016/j.devcel.2022.12.010

Sutoh, K., Sanuki, N., Sakaki, T., & Imai, R. (2010). Specific induction of TaAAPT1, an ER- and Golgilocalized ECPT-type aminoalcoholphosphotransferase, results in preferential accumulation of the phosphatidylethanolamine membrane phospholipid during cold acclimation in wheat. *Plant Molecular Biology*, 72(4-5), 519-531. https://doi.org/10.1007/s11103-009-9588-5 Swayne, T. C., Zhou, C., Boldogh, I. R., Charalel, J. K., McFaline-Figueroa, J. R., Thoms, S., Yang, C., Leung, G., McInnes, J., Erdmann, R., & Pon, L. A. (2011). Role for cER and Mmr1p in Anchorage of Mitochondria at Sites of Polarized Surface Growth in Budding Yeast. *Current Biology*, 21(23), 1994-1999. https://doi.org/10.1016/j.cub.2011.10.019

Szabadkai, G., Bianchi, K., Várnai, P., De Stefani, D., Wieckowski, M. R., Cavagna, D., Nagy, A. I., Balla, T., & Rizzuto, R. (2006). Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca2+ channels. *Journal of Cell Biology*, *175*(6), 901-911. https://doi.org/10.1083/jcb.200608073

#### Т

Tábara, L.-C., Morris, J. L., & Prudent, J. (2021). The Complex Dance of Organelles duringMitochondrialDivision.TrendsinCellBiology,31(4),241-253.https://doi.org/10.1016/j.tcb.2020.12.005

Tamura, N., Nishimura, T., Sakamaki, Y., Koyama-Honda, I., Yamamoto, H., & Mizushima, N. (2017). Differential requirement for ATG2A domains for localization to autophagic membranes and lipid droplets. *FEBS Letters*, *591*(23), 3819-3830. https://doi.org/10.1002/1873-3468.12901

Tamura, Y., Endo, T., Iijima, M., & Sesaki, H. (2009). Ups1p and Ups2p antagonistically regulate cardiolipin metabolism in mitochondria. *Journal of Cell Biology*, *185*(6), 1029-1045. https://doi.org/10.1083/jcb.200812018

Tamura, Y., Kawano, S., & Endo, T. (2020). Lipid homeostasis in mitochondria. *Biological Chemistry*, 401(6-7), 821-833. https://doi.org/10.1515/hsz-2020-0121

Tan, T., Özbalci, C., Brügger, B., Rapaport, D., & Dimmer, K. S. (2013). Mcp1 and Mcp2, two novel proteins involved in mitochondrial lipid homeostasis. *Journal of Cell Science*, jcs.121244. https://doi.org/10.1242/jcs.121244

Tang, J., Zhang, K., Dong, J., Yan, C., Hu, C., Ji, H., Chen, L., Chen, S., Zhao, H., & Song, Z. (2020). Sam50–Mic19–Mic60 axis determines mitochondrial cristae architecture by mediating mitochondrial outer and inner membrane contact. *Cell Death & Differentiation*, 27(1), 146-160. https://doi.org/10.1038/s41418-019-0345-2

Tang, Z., Takahashi, Y., He, H., Hattori, T., Chen, C., Liang, X., Chen, H., Young, M. M., & Wang, H.-G. (2019). TOM40 Targets Atg2 to Mitochondria-Associated ER Membranes for Phagophore Expansion. *Cell Reports*, 28(7), 1744-1757.e5. https://doi.org/10.1016/j.celrep.2019.07.036

Tangpranomkorn, S., Igarashi, M., Ishizuna, F., Kato, Y., Suzuki, T., Fujii, S., & Takayama, S. (2022). *A land plant specific VPS13 mediates polarized vesicle trafficking in germinating pollen* [Preprint]. Plant Biology. https://doi.org/10.1101/2022.11.01.514778

Tatsuta, T., & Langer, T. (2017). Intramitochondrial phospholipid trafficking. *Biochimica et Biophysica Acta* (*BBA*) - *Molecular and Cell Biology of Lipids*, 1862(1), 81-89. https://doi.org/10.1016/j.bbalip.2016.08.006

Tatsuta, T., Scharwey, M., & Langer, T. (2014). Mitochondrial lipid trafficking. *Trends in Cell Biology*, 24(1), 44-52. https://doi.org/10.1016/j.tcb.2013.07.011

Tilsner, J., Nicolas, W., Rosado, A., & Bayer, E. M. (2016). Staying Tight : Plasmodesmal Membrane Contact Sites and the Control of Cell-to-Cell Connectivity in Plants. *Annual Review of Plant Biology*, 67(1), 337-364. https://doi.org/10.1146/annurev-arplant-043015-111840

Tjellström, H., Andersson, M. X., Larsson, K. E., & Sandelius, A. S. (2008). *Membrane phospholipids as a phosphate reserve : The dynamic nature of phospholipid-to-digalactosyl diacylglycerol exchange in higher plants*. 11.

Tokai, M., Kawasaki, H., Kikuchi, Y., & Ouchi, K. (2000). Cloning and Characterization of the *CSF1* Gene of *Saccharomyces cerevisiae*, Which Is Required for Nutrient Uptake at Low Temperature. *Journal of Bacteriology*, *182*(10), 2865-2868. https://doi.org/10.1128/JB.182.10.2865-2868.2000

Toulmay, A., Whittle, F. B., Yang, J., Bai, X., Diarra, J., Banerjee, S., Levine, T. P., Golden, A., & Prinz, W. A. (2022). Vps13-like proteins provide phosphatidylethanolamine for GPI anchor synthesis in the ER. *Journal of Cell Biology*, 221(3), e202111095. https://doi.org/10.1083/jcb.202111095

Trost, M., English, L., Lemieux, S., Courcelles, M., Desjardins, M., & Thibault, P. (2009). The Phagosomal Proteome in Interferon-γ-Activated Macrophages. *Immunity*, *30*(1), 143-154. https://doi.org/10.1016/j.immuni.2008.11.006

Trotter, P. J., & Voelker, D. R. (1995). Identification of a Non-mitochondrial Phosphatidylserine Decarboxylase Activity (PSD2) in the Yeast Saccharomyces cerevisiae. *Journal of Biological Chemistry*, 270(11), 6062-6070. https://doi.org/10.1074/jbc.270.11.6062

#### V

Valverde, D. P., Yu, S., Boggavarapu, V., Kumar, N., Lees, J. A., Walz, T., Reinisch, K. M., & Melia, T. J. (2019). *ATG2 transports lipids to promote autophagosome biogenesis*. 12.

van der Bliek, A. M., Shen, Q., & Kawajiri, S. (2013). Mechanisms of Mitochondrial Fission and Fusion. *Cold Spring Harbor Perspectives in Biology*, 5(6), a011072-a011072. https://doi.org/10.1101/cshperspect.a011072

Van Dijk, P. J., Op den Camp, R., & Schauer, S. E. (2020). Genetic Dissection of Apomixis in Dandelions Identifies a Dominant Parthenogenesis Locus and Highlights the Complexity of Autonomous Endosperm Formation. *Genes*, *11*(9), 961. https://doi.org/10.3390/genes11090961

van Vliet, A. R., Chiduza, G. N., Maslen, S. L., Pye, V. E., Joshi, D., De Tito, S., Jefferies, H. B. J., Christodoulou, E., Roustan, C., Punch, E., Hervás, J. H., O'Reilly, N., Skehel, J. M., Cherepanov, P., & Tooze, S. A. (2022). ATG9A and ATG2A form a heteromeric complex essential for autophagosome formation. *Molecular Cell*, S1097276522010176. https://doi.org/10.1016/j.molcel.2022.10.017

Vance, J. E. (1990). Phospholipid synthesis in a membrane fraction associated with mitochondria. *Journal of Biological Chemistry*, 265(13), 7248-7256. https://doi.org/10.1016/S0021-9258(19)39106-9

Velayos-Baeza, A., Vettori, A., Copley, R. R., Dobson-Stone, C., & Monaco, A. P. (2004). Analysis of the human VPS13 gene family. *Genomics*, 84(3), 536-549. https://doi.org/10.1016/j.ygeno.2004.04.012

Velikkakath, A. K. G., Nishimura, T., Oita, E., Ishihara, N., & Mizushima, N. (2012). Mammalian Atg2 proteins are essential for autophagosome formation and important for regulation of size and distribution of lipid droplets. *Molecular Biology of the Cell*, 23(5), 896-909. https://doi.org/10.1091/mbc.e11-09-0785

Vercellino, I., & Sazanov, L. A. (2022). The assembly, regulation and function of the mitochondrial respiratory chain. *Nature Reviews Molecular Cell Biology*, 23(2), 141-161. https://doi.org/10.1038/s41580-021-00415-0

Voet, D., & Voet, J. G. (2011). Biochemistry (4th éd.). John Wiley& SonsInc.

Voinnet, O., Rivas, S., Mestre, P., & Baulcombe, D. (2003). Retracted : An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus: *An enhanced transient expression system in* N. benthamiana. *The Plant Journal*, 33(5), 949-956. https://doi.org/10.1046/j.1365-313X.2003.01676.x

von der Malsburg, K., Müller, J. M., Bohnert, M., Oeljeklaus, S., Kwiatkowska, P., Becker, T., Loniewska-Lwowska, A., Wiese, S., Rao, S., Milenkovic, D., Hutu, D. P., Zerbes, R. M., Schulze-Specking, A., Meyer, H. E., Martinou, J.-C., Rospert, S., Rehling, P., Meisinger, C., Veenhuis, M., ... van der Laan, M. (2011). Dual Role of Mitofilin in Mitochondrial Membrane Organization and Protein Biogenesis. *Developmental Cell*, *21*(4), 694-707. https://doi.org/10.1016/j.devcel.2011.08.026

#### W

Wada, H., Shintani, D., & Ohlrogge, J. (1997). Why do mitochondria synthesize fatty acids? Evidence for involvement in lipoic acid production. *Proceedings of the National Academy of Sciences*, 94(4), 1591-1596. https://doi.org/10.1073/pnas.94.4.1591

Wang, C., Wang, B., Pandey, T., Long, Y., Zhang, J., Oh, F., Sima, J., Guo, R., Liu, Y., Zhang, C., Mukherjee, S., Bassik, M., Lin, W., Deng, H., Vale, G., McDonald, J. G., Shen, K., & Ma, D. K. (2022). A conserved megaprotein-based molecular bridge critical for lipid trafficking and cold resilience. *Nature Communications*, *13*(1), 6805. https://doi.org/10.1038/s41467-022-34450-y

Wang, J., Chu, Q., Chang, W., Deng, L., & Ji, W.-K. (2022). A complex containing RhoBTB3-SHIP164-Vps26B promotes the biogenesis of early endosome buds at Golgi-endosome contacts [Preprint]. Cell Biology. https://doi.org/10.1101/2022.09.30.510409

Wang, J., Fang, N., Xiong, J., Du, Y., Cao, Y., & Ji, W.-K. (2021). An ESCRT-dependent step in fatty acid transfer from lipid droplets to mitochondria through VPS13D–TSG101 interactions. *Nature Communications*, *12*(1), 1252. https://doi.org/10.1038/s41467-021-21525-5

Wang, R., & Brattain, M. G. (2007). The maximal size of protein to diffuse through the nuclear pore is larger than 60 kDa. *FEBS Letters*, *581*(17), 3164-3170. https://doi.org/10.1016/j.febslet.2007.05.082

Wang, Y., Carrie, C., Giraud, E., Elhafez, D., Narsai, R., Duncan, O., Whelan, J., & Murcha, M. W. (2012). Dual Location of the Mitochondrial Preprotein Transporters B14.7 and Tim23-2 in Complex I and the TIM17:23 Complex in *Arabidopsis* Links Mitochondrial Activity and Biogenesis. *The Plant Cell*, 24(6), 2675-2695. https://doi.org/10.1105/tpc.112.098731

Wenzel, E. M., Elfmark, L. A., Stenmark, H., & Raiborg, C. (2022). ER as master regulator of membrane trafficking and organelle function. *Journal of Cell Biology*, 221(10), e202205135. https://doi.org/10.1083/jcb.202205135

White, R. R., Lin, C., Leaves, I., Castro, I. G., Metz, J., Bateman, B. C., Botchway, S. W., Ward, A. D., Ashwin, P., & Sparkes, I. (2020). Miro2 tethers the ER to mitochondria to promote mitochondrial fusion in tobacco leaf epidermal cells. *Communications Biology*, *3*(1), 161. https://doi.org/10.1038/s42003-020-0872-x

Wideman, J. G., & Muñoz-Gómez, S. A. (2016). The evolution of ERMIONE in mitochondrial biogenesis and lipid homeostasis : An evolutionary view from comparative cell biology. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1861(8), 900-912. https://doi.org/10.1016/j.bbalip.2016.01.015

Wilkinson, C. R. M., Seeger, M., Hartmann-Petersen, R., Stone, M., Wallace, M., Semple, C., & Gordon, C. (2001). Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. *Nature Cell Biology*, *3*(10), 939-943. https://doi.org/10.1038/ncb1001-939

Wong, L. H., Gatta, A. T., & Levine, T. P. (2019). Lipid transfer proteins: The lipid commute via shuttles, bridges and tubes. *Nature Reviews Molecular Cell Biology*, 20(2), 85-101. https://doi.org/10.1038/s41580-018-0071-5

Wozny, M. R., Di Luca, A., Morado, D. R., Picco, A., Hoffmann, P. C., Miller, E. A., Vanni, S., & Kukulski, W. (2022). *Supramolecular architecture of the ER-mitochondria encounter structure in its native environment* [Preprint]. Cell Biology. https://doi.org/10.1101/2022.04.12.488000

Wu, M., Gu, J., Guo, R., Huang, Y., & Yang, M. (2016). Structure of Mammalian Respiratory Supercomplex I 1 III 2 IV 1. *Cell*, *167*(6), 1598-1609.e10. https://doi.org/10.1016/j.cell.2016.11.012

Wu, P., Ma, L., Hou, X., Wang, M., Wu, Y., Liu, F., & Deng, X. W. (2003). Phosphate Starvation Triggers Distinct Alterations of Genome Expression in Arabidopsis Roots and Leaves. *Plant Physiology*, *132*(3), 1260-1271. https://doi.org/10.1104/pp.103.021022

#### Х

Xie, J., Marusich, M. F., Souda, P., Whitelegge, J., & Capaldi, R. A. (2007). The mitochondrial inner membrane protein Mitofilin exists as a complex with SAM50, metaxins 1 and 2, coiled-coil-helix coiled-coil-helix domain-containing protein 3 and 6 and DnaJC11. *FEBS Letters*, *581*(18), 3545-3549. https://doi.org/10.1016/j.febslet.2007.06.052

Xu, C., Moellering, E. R., Muthan, B., Fan, J., & Benning, C. (2010). Lipid Transport Mediated by Arabidopsis TGD Proteins is Unidirectional from the Endoplasmic Reticulum to the Plastid. *Plant and Cell Physiology*, *51*(6), 1019-1028. https://doi.org/10.1093/pcp/pcq053

Xu, Z., & Dooner, H. K. (2006). The Maize *aberrant pollen transmission 1* Gene Is a *SABRE / KIP* Homolog Required for Pollen Tube Growth. *Genetics*, 172(2), 1251-1261. https://doi.org/10.1534/genetics.105.050237

#### Y

Yamamoto, E., Domański, J., Naughton, F. B., Best, R. B., Kalli, A. C., Stansfeld, P. J., & Sansom, M. S. P. (2020). Multiple lipid binding sites determine the affinity of PH domains for phosphoinositide-containing membranes. *Science Advances*, 6(8), eaay5736. https://doi.org/10.1126/sciadv.aay5736

Yamano, K., Tanaka-Yamano, S., & Endo, T. (2010). Tom7 Regulates Mdm10-mediated Assembly of the Mitochondrial Import Channel Protein Tom40. *Journal of Biological Chemistry*, 285(53), 41222-41231. https://doi.org/10.1074/jbc.M110.163238

Yamaryo, Y., Dubots, E., Albrieux, C., Baldan, B., & Block, M. A. (2008). Phosphate availability affects the tonoplast localization of PLDζ2, an *Arabidopsis thaliana* phospholipase D. *FEBS Letters*, 582(5), 685-690. https://doi.org/10.1016/j.febslet.2008.01.039

Yang, R.-Y., Xue, H., Yu, L., Velayos-Baeza, A., Monaco, A. P., & Liu, F.-T. (2016). Identification of VPS13C as a Galectin-12-Binding Protein That Regulates Galectin-12 Protein Stability and Adipogenesis. *PLOS ONE*, *11*(4), e0153534. https://doi.org/10.1371/journal.pone.0153534

Yeo, H. K., Park, T. H., Kim, H. Y., Jang, H., Lee, J., Hwang, G., Ryu, S. E., Park, S. H., Song, H. K., Ban, H. S., Yoon, H., & Lee, B. I. (2021). Phospholipid transfer function of PTPIP51 at mitochondria-associated ER membranes. *EMBO Reports*, 22(6). https://doi.org/10.15252/embr.202051323

Yeshaw, W. M., van der Zwaag, M., Pinto, F., Lahaye, L. L., Faber, A. I., Gómez-Sánchez, R., Dolga, A. M., Poland, C., Monaco, A. P., van IJzendoorn, S. C., Grzeschik, N. A., Velayos-Baeza, A., & Sibon,

O. C. (2019). Human VPS13A is associated with multiple organelles and influences mitochondrial morphology and lipid droplet motility. *ELife*, *8*, e43561. https://doi.org/10.7554/eLife.43561

Yoon, H. S., Hajduk, P. J., Petros, A. M., Olejniczak, E. T., Meadows, R. P., & Fesik, S. W. (1994). Solution structure of a pleckstrin-homology domain. *Nature*, *369*(6482), 672-675. https://doi.org/10.1038/369672a0

Yoshida, Y. (2018). The cellular machineries responsible for the division of endosymbiotic organelles. *Journal of Plant Research*, *131*(5), 727-734. https://doi.org/10.1007/s10265-018-1050-9

Yu, B., Xu, C., & Benning, C. (2002). *Arabidopsis* disrupted in *SQD2* encoding sulfolipid synthase is impaired in phosphate-limited growth. *Proceedings of the National Academy of Sciences*, 99(8), 5732-5737. https://doi.org/10.1073/pnas.082696499

Yuan, W., Akşit, A., de Boer, R., Krikken, A. M., & van der Klei, I. J. (2022). Yeast Vps13 is Crucial for Peroxisome Expansion in Cells With Reduced Peroxisome-ER Contact Sites. *Frontiers in Cell and Developmental Biology*, *10*, 842285. https://doi.org/10.3389/fcell.2022.842285

#### Ζ

Zaitseva, J., Vaknin, D., Krebs, C., Doroghazi, J., Milam, S. L., Balasubramanian, D., Duck, N. B., & Freigang, J. (2019). Structure–function characterization of an insecticidal protein GNIP1Aa, a member of an MACPF and  $\beta$ -tripod families. *Proceedings of the National Academy of Sciences*, *116*(8), 2897-2906. https://doi.org/10.1073/pnas.1815547116

Zhang, S., Li, R., Zhang, L., Chen, S., Xie, M., Yang, L., Xia, Y., Foyer, C. H., Zhao, Z., & Lam, H.-M. (2020). New insights into Arabidopsis transcriptome complexity revealed by direct sequencing of native RNAs. *Nucleic Acids Research*, 48(14), 7700-7711. https://doi.org/10.1093/nar/gkaa588

Zhang, Y., Chen, M., Siemiatkowska, B., Toleco, M. R., Jing, Y., Strotmann, V., Zhang, J., Stahl, Y., & Fernie, A. R. (2020). A Highly Efficient Agrobacterium-Mediated Method for Transient Gene Expression and Functional Studies in Multiple Plant Species. *Plant Communications*, *1*(5), 100028. https://doi.org/10.1016/j.xplc.2020.100028

Zhang, Y., Ge, J., Bian, X., & Kumar, A. (2022). Quantitative models of lipid transfer and membrane contact formation. *Contact*, *5*, 25152564221096024.

Zheng, L., Xu, Y., Li, Q., & Zhu, B. (2021). Pectinolytic lyases : A comprehensive review of sources, category, property, structure, and catalytic mechanism of pectate lyases and pectin lyases. *Bioresources and Bioprocessing*, 8(1), 79. https://doi.org/10.1186/s40643-021-00432-z

Zhu, S., Liang, C., Tian, J., & Xue, Y. (2022). Advances in Plant Lipid Metabolism Responses to Phosphate Scarcity. *Plants*, *11*(17), 2238. https://doi.org/10.3390/plants11172238

# Scientific publications and

## communications

## 1 Scientific publications

Nicolas, E., Oger, C. A., Nguyen, N., Lambin, M., Draime, A., **Leterme, S. C.**, ... & Hallet, B. F. (2017). Unlocking Tn3-family transposase activity in vitro unveils an asymmetric pathway for transposome assembly. *PNAS*, 114(5), E669-E678. doi.org/10.1073/pnas.1611701114

Billey, E., Magneschi, L., **Leterme, S.**, Bedhomme, M., Andres-Robin, A., Poulet, L., ... & Marechal, E. (2021). Characterization of the Bubblegum acyl-CoA synthetase of Microchloropsis gaditana. *Plant Physiology*, 185(3), 815-835. doi.org/10.1093/plphys/kiaa110

Leterme, S., Michaud, M., & Jouhet, J. (2021). Isolation of mitochondria for lipid analysis. In *Plant Lipids* (pp. 337-349). Humana, New York, NY. doi.org/10.1007/978-1-0716-1362-7\_18

Leterme, S. & Michaud, M. (2022). Non-vesicular glycerolipids transport in plant cells. In *Advances in Botanical Research*. doi.org/10.1016/bs.abr.2021.07.001

**Leterme, S.**, & Michaud, M. (2023). Mitochondrial membrane biogenesis: A new pathway for lipid transport mediated by PERK/E-Syt1 complex. *Journal of Cell Biology*, 222(3), e202301132. doi.org/ff10.1083/jcb.202301132

### 2 Scientific communications

**Leterme S.**, Albrieux C., Couté Y., Brugière S., Jouhet J., Michaud M. Structural and functional characterization of a complex involved in lipid transport to mitochondria during plant adaptation to phosphate starvation. **Poster presentation**. Bilayers at the ILL 2019, December 2019, Grenoble (France).

**Leterme S.**, Albrieux C., Couté Y., Brugière S., Jouhet J., Michaud M. Structural and functional characterization of a complex involved in lipid transport to mitochondria during plant adaptation to phosphate starvation. **Poster presentation**. ASBMB Experimental Biology 2021, April 2021, online.

**Leterme S.**, Albrieux C., Couté Y., Brugière S., Jouhet J., Michaud M. Lipid transport to mitochondria during plant adaptation to phosphate starvation. **Oral presentation**. Young Mitochondria Investigators Symposium 2021, online.

**Leterme S.**, Albrieux C., Couté Y., Brugière S., Gillet B., Hughes S., Dellinger J., Imbert L., Jouhet J., Michaud M. Identification and characterization of a VPS13 homolog in *Arabidopsis thaliana*. **Poster presentation**. Conférences Jacques Monod: Molecular basis for membrane remodeling and organization, May 2022, Roscoff (France).

**Leterme S.**, Albrieux C., Couté Y., Brugière S., Gillet B., Hughes S., Dellinger J., Imbert L., Jouhet J., Michaud M. Identification and characterization of a VPS13 homolog in *Arabidopsis thaliana*. **Oral presentation**. International Symposium of Plant Lipids (ISPL) 2022, Grenoble (France).

Leterme S., Albrieux C., Couté Y., Brugière S., Gillet B., Hughes S., Dellinger J., Imbert L., Jouhet J., Michaud M. Identification and characterization of a VPS13 homolog in *Arabidopsis thaliana*. Poster

presentation. EMBO workshop 2022: ER: The master regulator of membrane trafficking, October 2022, Lucca (Italy).

Leterme S., Albrieux C., Couté Y., Brugière S., Gillet B., Hughes S., Dellinger J., Imbert L., Jouhet J., Michaud M. Identification and characterization of a VPS13 homolog in *Arabidopsis thaliana*. Oral presentation. Gordon Conference, January 2023, Galveston (Texas, USA).

Leterme S., Albrieux C., Couté Y., Brugière S., Gillet B., Hughes S., Dellinger J., Imbert L., Jouhet J., Michaud M. Identification and characterization of a VPS13 homolog in *Arabidopsis thaliana*. Poster presentation. Gordon Conference, January 2023, Galveston (Texas, USA).



# Caractérisation du complexe MTL et de AtVPS13M1 : deux acteurs impliqués dans le remodelage des lipides membranaires induit par la carence en phosphate chez *Arabidopsis thaliana*

Les cellules eucaryotes sont divisées en plusieurs compartiments délimités par une ou plusieurs membranes. Les membranes des organites sont composées d'un arrangement spécifique de lipides et de protéines qui assurent l'identité de chaque compartiment. Comme seuls quelques compartiments cellulaires sont capables de synthétiser l'ensemble de leurs lipides, un échange massif de lipides s'opère entre les organites. Le transport de lipides peut être assuré par des voies vésiculaires et/ou par des voies non vésiculaires. Le transport non vésiculaire de lipides est assuré par des protéines de transport de lipides (LTPs) qui facilitent le transfert direct des lipides d'une membrane à l'autre au niveau des sites de contact membranaire (MCSs). Si les LTPs ont déjà été bien étudiées chez la levure et chez l'humain, seul un petit nombre d'entre elles ont été caractérisées chez les plantes. La carence en phosphate (Pi) est un stress abiotique auquel sont couramment soumis les plantes dans la nature et au cours de laquelle une dégradation partielle des phospholipides se produit afin d'augmenter la disponibilité intracellulaire du Pi. Les phospholipides dégradés sont dynamiquement remplacés par du digalactosyldiacylglycérol (DGDG), un lipide non phosphoré synthétisé exclusivement dans le plaste. Le remodelage lipidique s'opère probablement par des voies de transport lipidique non vésiculaires, ce qui souligne son importance dans le maintien de l'intégrité membranaire en cas de stress. Nous caractérisons ici deux acteurs impliqués dans le remodelage membranaire induit par la carence en phosphate : le complexe MTL (mitochondrial transmembrane lipoprotein) et AtVPS13M1.

Le complexe MTL est un énorme complexe enrichi en lipides et principalement composé de protéines situées dans les deux membranes mitochondriales. Le complexe MTL est important pour le trafic intramitochondrial de lipides pendant l'adaptation des plantes à la carence en Pi. Nous avons tenté de purifier le complexe MTL par plusieurs techniques biochimiques afin d'affiner sa composition et d'étudier son architecture et sa structure. Nos résultats mettent en évidence la nature modulaire du complexe MTL qui peut vraisemblablement se présenter sous la forme d'un arrangement variable de différents composants. AtVPS13M1 est un homologue de vacuolar protein sorting 13 (VPS13) chez *Arabidopsis thaliana*. Des données récentes montrent que les protéines VPS13 sont d'énormes LTPs impliquées dans le transport massif de lipides et qu'elles sont localisées à de multiples sites de contact membranaire entre différents organites. Nous avons identifié six nouveaux variants d'épissage d'AtVPS13M1 et montré que l'une des isoformes domine largement les autres suggérant une importance biologique. Nous montrons que AtVPS13M1 est capable de lier plusieurs phosphoglycérolipides in vitro et participe à l'homéostasie des lipides en présence et en absence de Pi. Nos études de localisation d'AtVPS13M1 restent à affiner mais suggèrent une association avec le réticulum endoplasmique.

Dans l'ensemble, nous proposons une approche originale dans laquelle nous identifions et caractérisons de nouvelles voies de transport de lipides non vésiculaires importantes lors du remodelage des lipides membranaires induit par la carence en Pi chez les plantes.

**Mots clés :** Complexe MTL, VPS13, carence de phosphate, transport non-vésiculaire de lipides, mitochondrie, sites de contact membranaires

## Characterization of the MTL complex and AtVPS13M1: two actors involved in phosphate starvation-induced membrane lipid remodeling in *Arabidopsis thaliana*

Eukaryotic cells are divided into several compartments delineated by one or more membranes. Organelle membranes are composed of a specific arrangement of lipids and proteins that ensure the identity of each compartment. As only a few cell compartments are able to synthesize their whole set of lipids, a massive exchange of lipids occurs between organelles. The transport of lipids can be mediated by vesicular pathways and/or by non-vesicular pathways. Non-vesicular lipid transport can be mediated by lipid transport proteins (LTPs) that facilitate the direct transfer of lipids from one membrane to another at membrane contact sites (MCSs). While LTPs have already been well investigated in yeast and human cells, only a small number have been characterized in plants. Phosphate (Pi) starvation is an abiotic stress commonly encountered by plants in nature during which a partial degradation of phospholipids occurs to increase the intracellular Pi availability. The degraded phospholipids are dynamically replaced by digalactosyldiacylglycerol (DGDG), a non-phosphorous plastid-synthetized lipid exclusively synthetized in the plastid. Lipid remodeling likely uses non-vesicular lipid transport routes highlighting its importance in the maintenance of membrane integrity during stress. Here, we characterize two actors involved in phosphate starvation-induced membrane remodeling: the mitochondrial transmembrane lipoprotein (MTL) complex and AtVPS13M1.

The MTL complex is a huge complex enriched in lipids and mainly composed of proteins located in both mitochondrial membranes. The MTL complex is important for intramitochondrial lipid trafficking during plant adaptation to Pi starvation. We attempt to purify the MTL complex by several biochemical techniques to allow the refinement of its composition and the study of its architecture and structure. Our results highlight the modular nature of the MTL complex that can presumably be found as a variable arrangement of different components. AtVPS13M1 is a vacuolar protein sorting 13 (VPS13) homolog in *Arabidopsis thaliana*. Recent data show that VPS13 proteins are huge LTPs implied in bulk lipid transport localized at multiple membrane contact sites between organelles. We identify six novel splice variants for AtVPS13M1 and show that the transcriptional landscape is widely dominated by only one presumably biologically relevant isoform. We show that AtVPS13M1 is able to bind multiple phosphoglycerolipids *in vitro* and participates in lipid homeostasis in Pi replete and deplete conditions. AtVPS13M1 localization remains to be further investigated but our preliminary studies suggest an association at least with the endoplasmic reticulum.

Overall, we provide an original approach in which we identify and characterize novel non-vesicular lipid transport routes important for Pi starvation-induced membrane lipid remodeling in plants.

**Keywords:** MTL complex, VPS13, phosphate starvation, non-vesicular lipid transport, mitochondria, membrane contact sites

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