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Mechanisms and function of mitophagy in adaptation to heat stress during development of *C. elegans*

Yanfang Chen

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Yanfang Chen. Mechanisms and function of mitophagy in adaptation to heat stress during development of *C. elegans*. Subcellular Processes [q-bio.SC]. Université Paris Saclay (COMUE), 2019. English. NNT : 2019SACLS217 . tel-02927784

HAL Id: tel-02927784

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Mechanisms and function of mitophagy in adaptation to heat stress during development of *C. elegans*

Thèse de doctorat de l'Université Paris-Saclay
Préparée à l'Université Paris-Sud

École doctorale n°577 : Structure et dynamique des systèmes vivants
(SDSV)
Spécialité de doctorat: Sciences de la vie et de la santé

Thèse présentée et soutenue à Gif-sur-Yvette, le 23 juillet 2019, par

Yanfang CHEN

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Acknowledge

At the beginning, I would like to thank the jury. These reporters: Nadine CAMOUGRAND and Kathrin GIESELER, who have read my thesis and provided me suggestions for better construct my thesis. These examiners: Audrey ESCLATINE and Fabrizia STAVRU, who come to attend my defense. Thank you all for your time!

The profound gratitude should go firstly to my directors: Renaud LEGOUIS and Agnès DELAHODDE. Four years ago, it was Renaud and Agnès who accepted my PhD application and offered me this opportunity to come here to start my PhD career. During the four years study in Renaud's lab, Renaud offered me instructive advices and useful suggestions for my project. My sincere gratitude to Renaud also for his constant encourage. It is not easy for me at the beginning of my PhD, I met many difficulties and always doubt myself. During this harsh time, Renaud never blamed me; on the contrary, he supported me and taught me to positively solve the problems. MERCI beaucoup, Renaud! It is a pity that I did not spend lots time with my another supervisor, Agnès DELAHODDE. But Agnès always cares about my work and spent lots of time to guide my project, correct my thesis. Each time I asked Agnès for help, she assisted me with her best.

Secondly, I want to express my heartfelt thanks to Emmanuel CULETTO. When I came to the lab, I knew nothing about *C. elegans*. It was Emmanuel who taught me hand by hand to deal with worms. Emmanuel is my teacher not only on experiments, but also on English. Although he was quite busy as a lecture in Paris-Sud, he helped me to correct my English writing for my each committee, international meeting and this thesis. I want to tell Emmanuel that I am not always a good student, but you are really an excellent teacher!

I would also thanks to all our "autophagy and development team": Céline LARGEAU, Siham ZENTOUT (M2 student), Vincent SCARCELLI, Romane LEBOUTET, Christophe LEFEBVRE, Céline JENZER. Especially Céline LARGEAU and Siham ZENTOUT, who have participate in my project and contributed to the figures in my manuscript. Without your excellent work, the manuscript would not be complete. I would like to thank Céline JENZER and Christophe LEFEBVRE for their help with some experiments. I would also thank Vincent and Romane, for your generous help in experiments as well as in life. Thank you for your patient translation and help me to call the doctor, the CAF, the bank, the FREE and so on. All your kindness makes me feel not so lonely in France which is far from my hometown. You mean friends and even families to me in France. I will miss you all.....

I also owe a specific gratitude to the people in I2BC, SDSV and Gif. I am so sorry that finally I am not able to learn French, and my English is not good, neither. The communications between us is not so smooth, but I can feel your kindness to me.

I am so deeply indebted to China Scholarship Council (CSC), for their financial support during my four years PhD study.

Last but not least, my thanks would go to my beloved families and my friends who have always been helping me out of difficulties and supporting without a word of complaint.

Thank you all, I have spent a nice period in France. I will always keep this beautiful memories in my mind.

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ABBREVIATIONS

Aβ: β-amyloid peptide

AC: actinonin

acl7: acyltransferase-like

AD: Alzheimer's disease

ads1:

Alkyldihydroxyacetonephosphate
synthase

AIM: Atg8-family interacting motif

AHA-1: Aryl hydrocarbon receptor
nuclear translocator homolog

aHS: acute heat stress

ALLO-1: Allophagy receptor -1

ALR: autophagic lysosome
reformation

ALS: amyotrophic lateral sclerosis

Ambra1: autophagy and beclin 1
regulator 1

Ams1p: α-mannosidase 1

Apf: after puparium formation

APG6: autophagy protein 6

ARF1: ADP-ribosylation factor 1

ARIH1: Ariadne RBR E3 Ubiquitin
Protein Ligase 1

ACS4: acyl-CoA synthetase long
chain 4

ATG: autophagy related gene

Atg1: autophagy related gene 1

Atg2: autophagy related gene 2

Atg3: autophagy related gene 3

Atg4: autophagy related gene 4

Atg5: autophagy related gene 5

Atg7: autophagy related gene 7

Atg8: autophagy related gene 8

Atg9: autophagy related gene 9

Atg12: autophagy related gene 12

Atg13: autophagy related gene 13

Atg14L: Autophagy-Related Protein
14-Like Protein

Atg16L: autophagy related 16-like 1

Atg18: autophagy related gene 18

Atg19: autophagy related gene 19

Atg30: autophagy related gene 30

Atg32: autophagy related gene 32

Atg36: autophagy related gene 36

Atg101: Autophagy Related 101

ATP: Adenosine triphosphate

Aup1p: autophagy-related protein phosphatase

BAG1: Bcl2-associated athanogene 1 protein

BAK1: Bcl-2 Homologous Antagonist/Killer

BAX: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma 2

BCL2L1: BCL2-like 1

Bcl2L13: Bcl-2-like protein 13

BECN1: Beclin 1

BNIP3: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3

BNIP3L: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like

BP: 2,2'-dipyridyl

BWM: body-wall muscle cell

CAF4: CCR4-associated factor 4

CAMK-II: Ca²⁺-calmoduline dependent kinase II

CCCP:
2[2(3Chlorophenyl)hydrazinylydene
]propanedinitrile

CDK-1: Cyclin-dependent kinase 1

CED-3/4: Cell death protein 3/4

CerS1: ceramide synthase 1

CK2: Casein kinase 2

CLS: chronological life span

CMA: Chaperon-mediated autophagy

CNX1: Calnexin

COPII: coat protein complex II

CORVET: class C core vacuole/endosome tethering

CPS6: Ced-3 protease suppressor 6

CRISPR: clustered regularly interspaced short palindromic repeats

Cvt: cytoplasm-to-vacuole targeting

DAF-2/16: Abnormal dauer formation protein 2/16

DCT-1: Daf-16/FOXO controlled germline tumor affecting-1

DFCP-1: Double FYVE-Containing Protein 1

DFP: deferiprone

DHAP: Dihydroxyacetone phosphate

DIC: Differential interference contrast

DMA: Dimethyl acetals

DNM1L: Dynamin-1-like protein

Dnm2: Dynamin-2

DNA: deoxyribonucleic acid

DNC: dorsal nerve cord

Drp1: dynamin-related protein 1

Dyn2: dynamin 2

ECM: extracellular matrix

EM : electron microscopy

eMi: endosomal microautophagy

Endo G: endonuclease G

EPG-2/3/4/5/8 : Ectopic P granules protein 2/3/4/5/8

ER: endoplasmic reticulum

ERMES: ER-mitochondria encounter structure

ESCRT: endosomal Sorting Complexes required for transport

FACL4: Fatty-Acid-Coenzyme A Ligase, Long-Chain 4

***fard-1*:** Fatty acyl-CoA reductase

FCCP: Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone

FIP200: focal adhesion kinase (FAK) family interacting protein of 200 kD

Fis1: fission 1

FOXO: Forkhead box O

FRDA: Friedreich's ataxia

frh1: Frataxin

FUNDC1: FUN14 Domain Containing 1

FYCO1: FYVE and coiled-coil domain-containing protein 1

GABA: gamma-amino butyric acid

GABARAP: Gamma-aminobutyric acid receptor-associated protein

GAP: GTPase-activating protein

GBF1: Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1

GDAP1: Ganglioside-induced differentiation associated protein 1

GDP: guanosine di-phosphate

GED: GTPase effector domain

GFP: green fluorescent protein

gld-1: germ line developmen-1

GLP1: Glucagon-like peptide-1

Gpb2: Guanine nucleotide-binding protein subunit beta 2

GSK3 β : Glycogen synthase kinase-3 beta

GTP: guanosine tri-phosphate

HD: Huntington's disease

HIF: hypoxia-inducible factors

Hip: Hsp70- interacting protein

HLH30: Helix Loop Helix

Hog1: High osmolarity glycerol response protein 1

Hop: Hsp70-Hsp90 organizing protein

HOPS: homotypic fusion and vacuole protein sorting

HRP: horseradish peroxidase

HSC70: heat stress cognate protein of 70 kDa

HSE: heat shock element

HSF1: Heat shock factor 1

Hsp40/70/90: heat shock protein 40/70/90

HSPA8: heat shock protein family A member 8

Htt: huntingtin

IGF1: Insulin-like growth factor 1

IKKE-1: Inhibitor of nuclear factor kappa-B kinase epsilon subunit homolog 1

ILV: internal luminal vesicles

IM: isolation membrane

IMM: inner mitochondrial membrane

IMS: intermembrane space

INF2: Inverted Formin-2

ISC: iron-sulfur-cluster

KO: knock out

LAMP2: lysosomal-associated membrane protein 2

LAMP2A: lysosomal-associated membrane protein type2A

LAP: LC3-associated phagocytosis

LD: lipid droplet

LIPL4: Lipase-Like Abhydrolase Domain-Containing Protein 4

LIR: LC3-interacting region

LC3: microtubule-associated protein light chain 3

LLPS: liquid-liquid phase separation

Irk1: Leucine-rich repeat serine/threonine protein kinase 1

LRRK2: Leucine-rich repeat kinase 2

LRS: LC3 recognition sequence

MAM: mitochondria-associated membranes

MAPK: Mitogen-activated protein kinase

MAPKK: Mitogen-activated protein kinase kinase

MARCH5: membrane-associated ring finger (C3HC4) 5

MCU: mitochondrial calcium uniporter

Mdm10/12/34: Mitochondrial distribution and morphology protein 10/12/34

MDV1/2: Mitochondrial division protein 1/2

MEF: mouse embryonic fibroblasts

Mff: mitochondrial fission factor

Mfn1/2: mitofusin 1/2

Mgm1 : Mitochondrial genome maintenance protein 1

Mi: microautophagy

MiD49/51: Mitochondrial Dynamics Protein Of 49 kDa/51 kDa

MIRO1: Mitochondrial Rho GTPase 1

Mmm1: Maintenance of mitochondrial morphology protein 1

MMP: mitochondrial membrane potential

MOs: membranous organelles

MPP+ : 1-methyl-4-phenylpyridinium

MPTP: 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine

mtDNA: mitochondrial DNA

mTOR: mechanistic target of rapamycin

MUL1 : Mitochondrial E3 Ubiquitin Protein Ligase 1

MVB: multivesicular body

MXL3: MaX-Like

Nbr1: neighbor of BRAC1 gene

NDP52: nuclear dot protein 52 kDa

NGM: Nematode growth media

NIX: NIP-3-Like Protein X

NMN: nicotinamide mononucleotide

NO: nitric oxide

NRF2/NFE2L2: NF-E2-Related Factor 2 / Nuclear Factor Erythroid 2-Related Factor 2

NS: no significance

NVT: Nbr1-mediated vacuolar targeting

OCR: oxygen consumption rate

OMM: outer mitochondrial membrane

OMMAD: outer mitochondrial membrane associated degradation

OPA1: Optic Atrophy 1

OPTN: Optineurin

OXPHOS: Oxidative phosphorylation

p62/SQST1: Sequestosome 1

PACS2: Phosphofurin Acidic Cluster Sorting Protein 2

PARL: Presenilins-associated rhomboid-like protein

PAS: phagophore assembly site

PB1: Phox and Bem1

Pbs2: Polymyxin B resistance protein 2

PD: Parkinson's disease

PE: phosphatidylethanolamine

PGAM5: phosphoglycerate mutase family member 5

PGL-1/3: P granule abnormality protein 1/3

PH: pleckstrin homology

PHB2: Prohibitin-2

PI3K: phosphatidylinositol 3 kinase

PI3KC3C1: phosphatidylinositol 3-kinase catalytic subunit type 3 complex I

PINK1: PTEN-induced kinase 1

PKA: Protein kinase A

PKC: Protein kinase C

PKM1: pyruvate kinase M1

PLEKHM1: pleckstrin homology and RUN domain containing M1

PME: paternal mitochondrial elimination

polyQ: Polyglutamine

PP1: Protein phosphatase 1

PP2A: Protein phosphatase 2

PS: phosphatidylserine

PtdIns(3)P: phosphatidylinositol 3-phosphate

PTPIP51: Protein tyrosine phosphatase interacting protein 51

Rab5: Ras-related protein Rab-5A

Rab7: Ras-related protein Rab-7A

Rab9: Ras-related protein Rab-9A

RET-1: Reticulon-like protein

RILP: Rab-interacting lysosomal protein

RNA: Ribonucleic acid

RNS: reactive nitrogen species

ROS: reactive oxygen species

SEPA-1: suppressor of ectopic P granules in autophagy

SIAH1: Seven In Absentia Homolog 1

SKN-1: Protein skinhead-1

Slt2: Suppressor of the LyTic phenotype

SM: Sec1/Munc18-like protein

SMURF1: Smad Ubiquitination Regulatory Factor 1

SNAP-29: Synaptosomal-associated protein 29

SNARE: Soluble N-ethylmaleimide-sensitive factor activating protein receptor

SNX18: Sorting nexin-18

STX17: syntaxin 17

SV: synaptic vesicles

TBC1D15: TBC1 Domain Family Member 15

TBK1: TANK-Binding Kinase 1

TEM: transmission electron microscopy

TFEB: Transcription factor EB

TIM: Translocase of the inner membrane

TMD: trans-membrane domain

TMRM: tetramethylrhodamine

TOM: translocase of the outer membrane

UA: Urolithin A

Ub: ubiquitin

UBA: ubiquitin-associated

Uba1: Ubiquitin-like modifier activating enzyme 1

Ubl: ubiquitin-like domain

ULK1/2: unc-51-like kinase 1/2

UPR: unfolded protein response

UTH1: Youth protein 1

VAMP7/8: Vesicle-associated membrane protein 7/8

VAPB: VAMP Associated Protein B

VDAC1: Voltage-dependent anion-selective channel 1

VMP1: Vacuole membrane protein 1

VNC: ventral nerve cord

Vps

3/8/11/15/16/18/30/33/34/39/41: Vacuolar protein sorting-associated protein

3/8/11/15/16/18/30/33/34/39/41

Whi2: Whiskey 2

WIPI2: WD repeat domain
phosphoinositide-interacting
protein 2

WT: wild type

YFP: Yellow fluorescent protein

Yme1: Yeast mitochondrial escape
protein 1

Zip1: Zrt- and Irt-like protein 1

I INTRODUCTION

1. Autophagy
2. *C. elegans*, a model animal for autophagy study
3. Mitophagy
4. Mitochondrial dynamics and mitophagy

1. Autophagy

Autophagy (from the Greek words auto (meaning self) and phagein (meaning to eat) is a degradation process of intracellular components through lysosome. It was first termed by De Duve in 1963(*De Duve 1963*) to describe the cell degradation process taking place in the double membraned organelle containing parts of cytosol and bits of organelle, seen under Electron Microscope. In the following years, studies in the field of autophagy were mostly descriptive: (1) showing that glucagon, insulin, amino acid deprivation and pharmacological agents such as methyl adenine and worthmannine could regulate autophagy and (2) describing autophagosome morphology and dynamic during its formation, fusion with either lysosome or endosome and their degradation. Molecular understanding of the autophagy process started when Yoshinori Ohsumi lab identified a series of genes directly involved in the autophagy process. These genes were initially named apg for AutoPhaGy related genes then further renamed atg for “AuTophagy related” gene or protein. Atg genes were first cloned from the yeast *Saccharomyces cerevisiae* after many *S.cerevisiae* autophagy defective mutants were obtained by genetic screening for mutants that survive in rich medium but died when subjected to prolonged period of nitrogen starvation (*Tsukada and Ohsumi 1993*). Both autophagy process and ATGs are highly conserved from yeast to mammalian system. This degradative process has been widely shown to play important roles in many physiological and pathological processes, such as development, growth, aging, neurodegeneration, cancer. Ohsumi was awarded the Nobel Prize in physiology or medicine in 2016 for his key contributions to the research field of autophagy.

1.1. Three types of autophagy

Based on the mechanisms of delivery of cytoplasmic components to the lysosome, autophagy has been classified in three types: microautophagy, chaperone-mediated autophagy and macroautophagy (*Boya, Reggiori et al. 2013*). Basic features characterizing these three types of autophagy are presented on [Figure1](#).

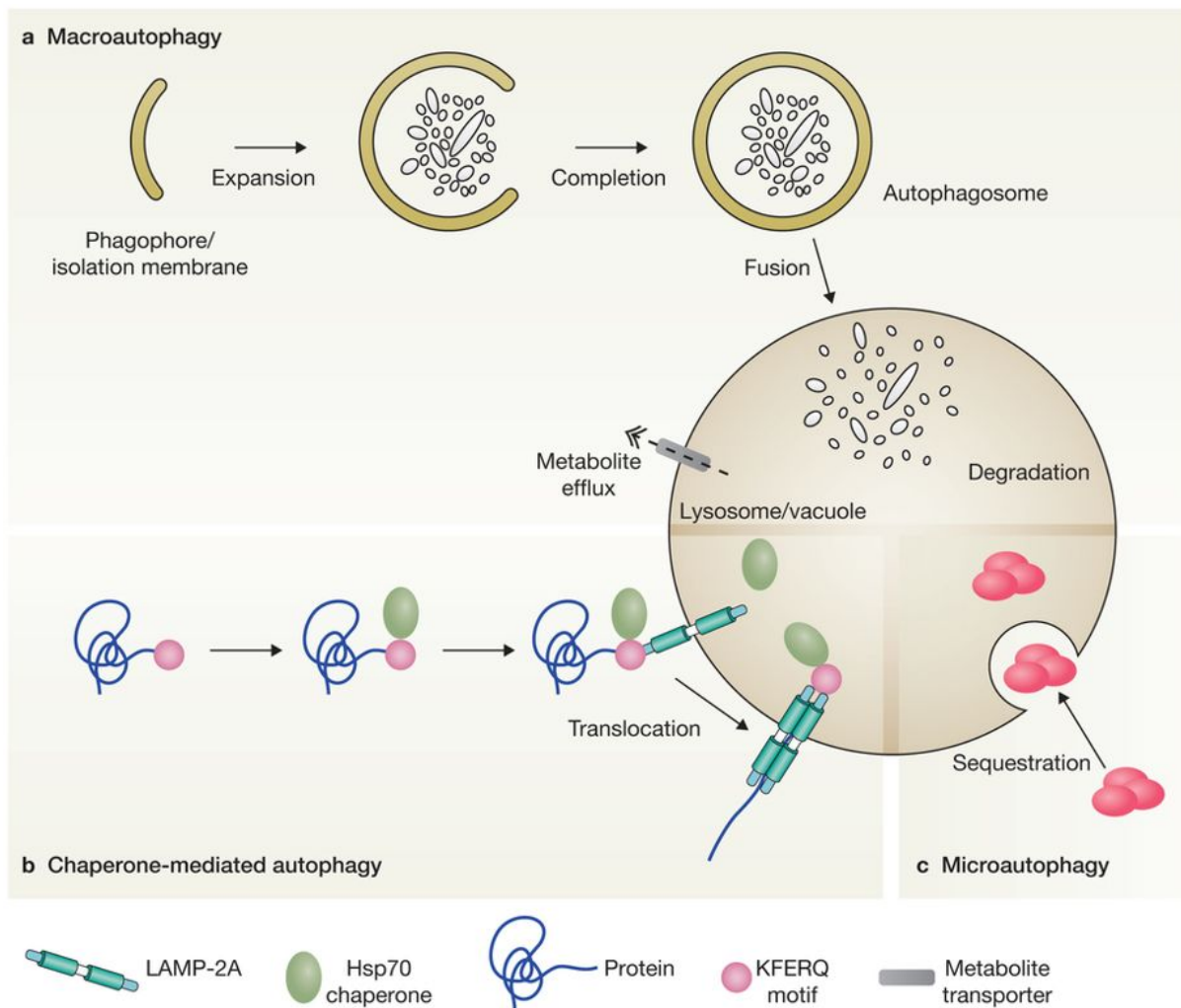


Figure 1. Three types of autophagy coexist within the eukaryotic cells (a) Macroautophagy is characterized by the cargo sequestration within a double membrane structure, which is named autophagosome. (b) Chaperone-mediated autophagy degrades proteins that containing KFERQ motif, which is recognized by chaperone Hsp70. During chaperone-mediated autophagy, LAMP2 mediates the fusion of Hsp70-protein aggregates with lysosome. Microautophagy refers to the direct sequestration of cargoes into lysosome for degradation. (c) Microautophagy directly engulfs cytoplasmic components by lysosomes. (Boya, Reggiori et al. 2013)

1.1.1. Microautophagy and endosomal microautophagy

Microautophagy (Mi) is defined as the engulfment of cytosolic cargoes directly via invagination of the lysosomal/vacuolar membrane. It is the least studied form of autophagy. It can degrade soluble proteins or even bigger cargoes such as portion of the nucleus, lipid droplets, mitochondria and endosomes (Veenhuis, Douma et al. 1983, Takahashi, Murayama et al. 1993, Tuttle and Dunn 1995, Kissova, Salin et al. 2007) . This microautophagy depends on the activity of the Endosomal Sorting Complexes required for transport (ESCRT) machinery. ESCRT components are present on the vacuolar membrane

and induced membrane dynamics in a similar way to that observed on endosome during intraluminal vesicles formation.

Additionally, it has been recently proposed to formerly distinguish a second type of microautophagy which relies on lysosomal protrusion and not its invagination (*Oku and Sakai 2018*). This type of microautophagy is observed in the yeast *Pichia pastoris* when lysosome sequesters peroxisome. This mechanism seems to be Atg18 dependent.

Another type of microautophagy, named endosomal microautophagy (eMi), shares very similar features to that of microautophagy described above but involves endosome (*Sahu, Kaushik et al. 2011, Uytterhoeven, Lauwers et al. 2015, Mukherjee, Patel et al. 2016*). Notably, endosomal microautophagy could selectively degrade proteins containing KFERQ motif via binding to heat shock protein family A member 8 (HSPA8), also known as heat stress cognate protein of 70 kDa (HSC70), which directs the cargo to the endosomal membrane for internalization into multivesicular body (MVB) in an ESCRT-I and -III dependent mechanism (*Sahu, Kaushik et al. 2011, Uytterhoeven, Lauwers et al. 2015, Mukherjee, Patel et al. 2016*). Interestingly, two additional microautophagy like processes have been recently discovered. One has been identified in mouse embryonic fibroblasts (MEF) and shows that organelle such as mitochondria could be guided to the Rab5 positive early endosome in a ESCRT-0, -I and -II dependent mechanism (*Hammerling, Shires et al. 2017*). Additionally, in the fission yeast *Schizosaccharomyces pombe*, two hydrolytic enzymes and the autophagic adaptor Nbr1, are delivered into ILV inside the MVB in an ubiquitination and ESCRT-dependent mechanism. The authors named this pathway NVT (Nbr1-mediated vacuolar targeting) (*Liu and Du 2015*).

1.1.2. Chaperon-mediated autophagy (CMA)

Chaperon-mediated autophagy (CMA) is a type of autophagy that directly imports substrates to lysosome for degradation (*Majeski and Dice 2004, Massey, Kiffin et al. 2004*). In a similar way to eMi, CMA degrades proteins which interact with HSPA8 through KFERQ like motif. However, different from endosomal microautophagy, CMA is independent of ESCRT but relies on lysosomal-associated membrane protein type2A (LAMP2A) for the cargo translocation into lysosome (*Cuervo and Dice 1996*).

The substrate-chaperone complex binds to LAMP2A at its cytosolic side, triggering the translocation of the complex into lysosomal lumen (*Cuervo and Dice 2000*). As soon as the

translocation is finished, the degradation of the complex is rapid (Kon and Cuervo 2010). Although, HSPA8 is the main chaperone for CMA substrates, HSPA8-cargo interaction can also be regulated by other chaperons and co-chaperones including Hsp90, Hsp40, the Hsp70-Hsp90 organizing protein (Hop), the Hsp70-interacting protein (Hip), and the Bcl2-associated athanogene 1 protein (BAG-1) (Agarraberes and Dice 2001).

CMA is an important way for cellular protein degradation besides the ubiquitin system. Analysis of the cytosolic proteome predicted that nearly 30% of its component could be degraded via CMA (Dice 1990). This degradative mechanism probably acts in a coordinated way with other types of autophagy, to adapt cells to starvation and many other stress conditions (Kon and Cuervo 2010).

1.1.3. Macroautophagy

Unlike Mi and CMA, macroautophagy (hereafter referred to as autophagy) is characterized by the formation of double-membrane vesicle named autophagosome, which is dedicated to cytoplasmic materials sequestration and delivery either directly or after fusion with endosome, to lysosome for degradation. The cellular process and molecular mechanism of autophagy underlying autophagosome formation are conserved from yeast, *C. elegans*, *Drosophila* to mammalian cells. I decided to present a general overview of the molecular machinery of autophagy mainly based on data coming from studies in mammalian and yeast systems.

The process of autophagy is continuous and dynamic, but for the convenience of this presentation, I followed Klionsky *et al* (Yang and Klionsky 2010) proposition to separate the autophagosome formation mechanism into five distinct and consecutive steps: the initiation of autophagosome genesis through forming an isolation membrane (IM) also named phagophore, elongation and expansion of the phagophore, closure and completion of a double-membrane autophagosome, autophagosome maturation and fusion with lysosome, breakdown and degradation of the cargoes (Figure 2). Genes whose products are involved in autophagy process are termed Atg (Autophagy-related) (Klionsky 2012). The first ATG gene, ATG1, was identified in 1997 by Oshumi lab in yeast (Matsuura, Tsukada *et al.* 1997). Now, more than 40 ATGs have been revealed in yeast, many of them have homologues in mammalian and other model systems. Additionally, several metazoan specific Atg genes have been isolated by Zhang Hong (Tian, Li *et al.* 2010).

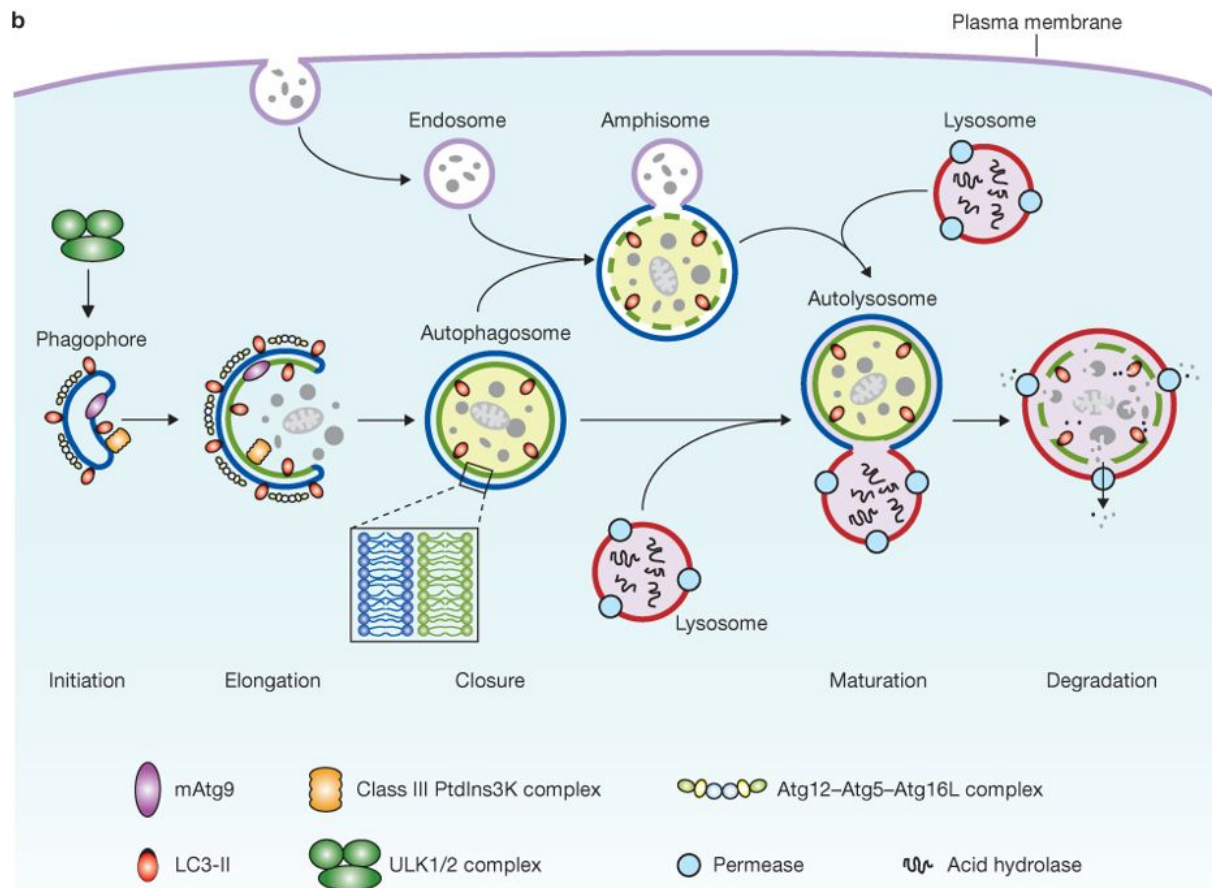


Figure 2. Autophagy process and its core machinery in mammalian cells Mammalian autophagy starts with phagophore formation, and then the double membrane structure elongates until its closure. Then, the sealed autophagosome fused with lysosome to form autolysosome. Alternatively, autophagosome can fuse with endosome to form amphisome, followed by its final fusion with lysosome. Consequently, autophagy cargoes are degraded within autolysosome due to the lysosomal acid enzymes. The core autophagy machinery which consists in four different complexes is also depicted. ULK1/2 and the PI3KC3-C1 complexes are required for phagophore initiation, while two ubiquitin-like conjugation systems Atg12-Atg5-Atg16L and Phosphatidylethanolamine Atg8/LC3 (Atg8/LC3/GABARAP-Atg4-Atg7) as well as Atg9, function downstream and are involved in the phagophore elongation. (Yang and Klionsky 2010)

1.1.4. Core machinery for autophagosome formation

Atg proteins that are part of the core autophagy machinery, assemble and interact with each other to make four major complexes. Atg proteins contained in the core autophagy machinery are critical for autophagosome formation and are conserved from yeast to mammalian. These four complexes are: the Atg1/Ulk1 complex (Atg1/Ulk1/2, Atg13, FIP200 and Atg101); the class III phosphatidylinositol 3 kinase (PI3K) complex I (PI3KC3-C1) which is composed of Beclin 1, Atg14L, Vps34, Vps15 and Ambra1; two ubiquitin-like conjugation systems: Atg12-Atg5-Atg16L and Phosphatidylethanolamine Atg8/LC3 (Atg8/LC3/GABARAP-

Atg4-Atg7) (Yang and Klionsky 2010). These complexes are displayed along with the autophagosome formation and maturation processes in **Figure 2**.

The Phagophore (or isolation membrane, IM) identified as crescent-shaped double membrane (Suzuki, Kirisako et al. 2001), is the pre-structure of autophagosome. Autophagosome nucleate at a single or several pre-autophagosomal sites named phagophore assembly site (PAS) and omegasome in yeast and higher eukaryotic organisms, respectively. In yeast, the PAS is located next to the vacuole whereas omegasomes were initially identified as a specific zone on the endoplasmic reticulum membrane which could serve as a cradle for phagophore initiation. In both locations, Phagophore formation requires the Atg1/Atg13 and PI3K complexes for its induction and nucleation. Pre-autophagosomal structure received lipids input from various vesicles including vesicles containing the unique autophagy transmembrane protein Atg9 (Noda, Kim et al. 2000), coming from the Golgi apparatus. Additionally, recycling endosomes labelled with Atg16L and probably other sources such as vesicles coming from COPII dependent vesicles from the ERGIC are recruited to the phagophore and participate to its extension (Carlsson and Simonsen 2015). The retrieval of Atg9 is dependent on Atg1/13 complex and other Atg proteins (Reggiori, Tucker et al. 2004).

Atg8/LC3/GABARAP is another important protein probably involved in phagophore expansion, and its role in autophagy process mainly relies on the modification of its C-terminal glycine by phosphatidylethanolamine (PE). This post-translational mechanism is dependent on key ubiquitin-like conjugation systems. This process starts with the Atg4 protease which cleaves the C-terminal part of the Atg8 precursor, to expose its C-terminal glycine residue. Then, this precursor form is activated by an E1-like enzyme Atg7, and then conjugated to the phagophore membrane phosphatidylethanolamine (PE) by E2-like enzyme Atg3. This conjugation activity requires Atg12-Atg5 system, which is also dependent on Atg7 (Mizushima, Noda et al. 1998, Tanida, Tanida-Miyake et al. 2002). Atg12-Atg5 further forms complex with Atg16L, and then is recruited to phagophore via the interaction with WIPI2 (Dooley, Razi et al. 2014), where Atg12-Atg5 cooperates with Atg7-Atg3 for phagophore expansion and autophagosome formation.

Atg8/LC3/GABARAP has a key role during phagophore expansion. Additionally, this protein, which is the only one to be constantly associated to autophagosome, is also involved in the

recognition and recruitment of autophagy substrates. The latter role is further discussed in [chapter 1.2](#).

Although, it is considered that the core machinery described above is essential for general autophagy process, some specific autophagy events do take place without some of these Atg genes. For instance, a type of Atg5/Atg7-independent macroautophagy takes place in mouse embryonic fibroblast (MEF). In these cells, after etoposide exposure or starvation induction, autophagosomes were still found in *Atg5^{-/-}* or *Atg7^{-/-}* genetic backgrounds. Cells which lack the lipidation of LC3 (homologue of Atg8 in mammalian cells) make autophagosome by a Rab9-dependent fusion between isolation membrane with vesicles from late endosome and trans-Golgi (*Nishida, Arakawa et al. 2009*). Another study shows that the autophagy mediated cell size reduction of *the Drosophila* intestine, requires neither Atg7 nor Atg3 but a novel E1 enzyme Uba1 (*Chang, Shrivage et al. 2013*). These works enlighten the complexity and variety of autophagy pathways.

1.1.5. Fusion of autophagosome with lysosome

Once the autophagosome is sealed, it can fuse either directly with lysosome for degradation or with endosome to form an intermediate organelle termed amphisome before its fusion with lysosome takes place (*Tooze, Hollinshead et al. 1990, Mullock, Bright et al. 1998*). Since endocytosis and autophagy deliver macromolecules to the lysosome, both pathways share many proteins involved in the regulation of fusion with lysosome. There are four important proteins/families mediating the fusion step: the small GTPase of the Rab family Rab7, the membrane containing rab7 tethering complex HOPS that may help to recruit SNARE proteins and ESCRT. The details are as followings:

Rab7 is not required for the initiation or formation of late autophagic vacuoles (*Gutierrez, Munafo et al. 2004, Jager, Bucci et al. 2004*). Gutierrez et al. (2004) studied the role of Rab7 in CHO cells, by overexpressing the dominant negative form Rab7T22N, which can not exchange GDP for GTP. They analyzed the autophagosome maturation, using either monodansylcadaverine or indirect immunolocalisation of c-myc-LC3, and fusion events with endosome and/or lysosome, marked by internalized rhodamine dextran and lysosomal enzyme cathepsine D antibody, respectively. They showed that Rab7 is recruited to autophagosomes directly from the cytoplasm but not from the fusion with vesicles containing Rab7. The authors clearly showed that the final fusion of autophagosome with

lysosome is largely impeded by the expression of Rab7T22N and results in an accumulation of larger autophagosomes and a defect in degradation of long-lived protein (*Gutierrez, Munafo et al. 2004*).

These results were confirmed and developed by Ganley et al (2011). The authors have shown in various mammalian cells that thapsigargin, an inhibitor of the Endoplasmic Reticulum Calcium ATPase pump, also blocks the autophagic flux by inhibiting the fusion of autophagosomes with endocytic compartments. Using both confocal analysis and EM coupled to immunogold detection of GFP-LC3, the authors showed that there is indeed an accumulation of autophagosomes in cells that have been starved and then treated with thapsigargin. Thapsigargin blocks the autophagic flux and led to the accumulation of autophagosomes that could not fused anymore with endosomes to give amphisomes, nor directly with lysosomes (*Ganley, Wong et al. 2011*). Interestingly, under thapsigargin treatment Rab7 is no more localized to autophagosome membrane, meanwhile it is still recruited to endosomes. Because endocytosis was not affected by thapsigargin, the authors suggested that a thapsigargin sensitive factor could be involved in Rab7 recruitment at the membrane of autophagosomes to promote their final maturation.

However, the small GTPase Rab7 controls the transport of late endosomes and lysosomes (*Guerra and Bucci 2016*). One of the key roles played by Rab7 is to cluster the autophagic structure next to lysosome for subsequent fusion. Rab7 interacts with RILP and FYCO1 to control autophagosome dynamic along the microtubule cytoskeleton, which is essential for the later degradation within lysosomes.

Additionally, Rab7 interacts with a number of key proteins required for the autophagosome-lysosome fusion: epg-5, PLEKHM1 which participates to recruit HOPS and also interacts with Atg8/LC3. Moreover, Rab7 has an additional role in Parkin-mediated autophagy compared with starvation-induced autophagy. It has been shown that the GAP protein TBC1D15 acts as an inhibitor for Rab7, governing autophagosome biogenesis in the process of mitophagy (*Yamano, Fogel et al. 2014*).

Most importantly, several data have indicated that Rab7 could recruit tethering complexes. The initial interactions, between vesicles before they fused, are mediated by dedicated protein complexes that act as tethering factors. Those proteins are capable to establish a physical link between membranes (*Epp, Rethmeier et al. 2011*). Briefly, tethering factors could be divided in two main groups, the dimeric proteins which are mainly involved in the

secretory pathway and the multimeric complexes, mainly implicated in Golgi vesicles-endosome and endosome-lysosome interactions. Specifically, two multimeric complexes, the CORVET and HOPS (Homotypic vacuolar fusion and Protein Sorting), are involved in regulating the machinery of fusion of early and late endosomes respectively. These complexes are made of a number of common subunits, Vps11, Vps16, Vps18 and Vps33, and the specific subunits Vps39, Vps41, and Vps3, Vps8 for HOPS and CORVET, respectively. Rab7 is more specifically involved in tethering by recruiting the HOPS complex.

Subunits of the HOPS complex have also a critical role to mediate membrane fusion by interacting with SNARE proteins. Homotypic fusion and vacuole protein sorting (HOPS) can recruit SNAREs to the late endosomes (*Balderhaar and Ungermann 2013*). Soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) is a protein family involved in endosome-lysosome fusion. Most of intracellular membrane fusion events (excluding mitochondria fusion) required two groups of proteins: the SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor) and the SM (Sec1/Munc18-like) proteins (*Sudhof and Rothman 2009*). The Q/t- and R/v- SNARE proteins are localized respectively on two different vesicles and interact progressively to form a quadruple helix called the trans-SNARE. The trans-SNARE complex forms a zipper that progressively closes and creates a pulling force on both membranes allowing them to fuse. The SM proteins, among which the Vps33 subunit, have been found to interact with both Q/t- and R/v-SNAREs but also binds the trans-SNARE complex. The hairpin-type tail-anchored SNARE syntaxin 17 (STX17) has been identified to be present on enclosed autophagosome and facilitate its fusion with endosome or lysosome, via the interaction with SNAP-29 (*Itakura, Kishi-Itakura et al. 2012*). Moreover, STX17–SNAP29 binary t-SNARE complex further binds to VMP8 to mediate an Atg14-dependent fusion (*Diao, Liu et al. 2015*). STX17 is recruited once the autophagosome formation is achieved and therefore could constitute a checkpoint to avoid any fusion of the lysosome with unclosed phagophores. Interestingly, STX17 could be used as a new autophagosome marker.

Homotypic fusion and vacuole protein sorting (HOPS) can recruit SNAREs to the late endosomes (*Balderhaar and Ungermann 2013*) and Vps39, a subunit of HOPS, additionally functions as a co-factor for Rab7, increases the active Rab7 pools at the endosomal membrane (*Wurmser, Sato et al. 2000*). LC3 probably also participates in the fusion step. Interestingly, in *C. elegans*, LGG-2 (LC3 homologue), but not LGG-1 (GABARAP homologue),

directly interacts with VPS-39 HOPS complex subunit and controls autophagosome-lysosome fusion (*Manil-Segalen, Lefebvre et al. 2014*).

The endosomal sorting complex required for transport (ESCRT) is a key factor for the fusion of autophagosomes with lysosomes. ESCRT complexes are needed to mature endosome into multivesicular bodies. Subsequently, MVB fuse with autophagosome to generate amphisomes. Probably, this fusion brings to autophagosomes key membrane molecules necessary for the subsequent fusion with lysosomes. Interestingly, ESCRT proteins could be involved in non-canonical functions such as vesicle tethering. A review has described the details of the roles of ESCRT in autophagy (*Lefebvre, Legouis et al. 2018*). Other proteins are also shown to be involved in the autophagosome fusion process, for instance, the ectopic P-granules autophagy protein 5 (EPG-5) in *C. elegans* (*Tian, Li et al. 2010*). More information about EPG-5 protein is discussed in [chapter 2.2.2. aggrephagy](#).

1.1.6. Autophagic lysosome reformation, ALR

Once autophagosome fused with lysosome, the autophagic cargoes are degraded by lysosomal acidic hydrolases, and the degraded materials are transported out to the cytosol via specific catabolite transporters for recycling. However, these transports do not completely end the autophagy process. In some stress conditions such as starvation, a mechanism named autophagic lysosome reformation (ALR) is triggered, immediately after the degradation step, to restore exhausted lysosomes during the autophagy process. In starved condition, mTOR is inhibited at autophagy initiation step. However it could be activated back by catabolites released from autolysosome after a prolonged starvation period, to trigger the ALR via an unknown mechanism. Moreover, the mTOR reactivation inhibits autophagy initiation and provides a negative feedback for avoiding excess autophagy (*Yu, McPhee et al. 2010*). Once ALR is triggered, tubules extruded from autolysosomes, named proto-lysosomes, and buds away from the lysosome to finally form tubules which will eventually mature into independent functional lysosomes.

1.2. Non-selective and selective autophagy

Initially, autophagy was considered to be a non-selective degradation pathway. This term means that autophagosome isolates and degrades bulk part of the cytoplasm without any specificity. Probably, it is because research activities focused on starvation-induced

autophagy for which autophagosome engulfed indistinctly bulk part of the cytoplasm containing proteins and organelles. However, a growing number of experimental results demonstrated that cells could trigger selective autophagy, i.e. an autophagy-dependent elimination (selective sequestration) of distinct cargoes which uses series of specific autophagy receptors. Autophagy receptors can specifically recognize the cargoes that need to be degraded and bridge them with autophagosome by interacting with Atg8/LC3 (*Komatsu and Ichimura 2010*). Based on the types of cargo engulfed for degradation, selective autophagy can be further classified into mitophagy (mitochondria), pexophagy (peroxisome), nucleophagy (nucleus), ER-phagy (ER), lipophagy (lipid droplets), aggrephagy (protein aggregates), xenophagy (pathogens) (*Galluzzi, Baehrecke et al. 2017*). Different cargoes for selective autophagic degradation are shown in figure3. Among them, the selective elimination of mitochondria by autophagy, i.e. mitophagy, is the most studied one. I will present more information on molecular mechanisms underlying receptor-mediated mitophagy in **chapter 3 mitophagy**. I would like to emphasize the fact that one should be careful to distinguish the autophagy receptors from the autophagy adaptors. Although autophagy adaptors participate in the substrates recognition during autophagy, they are not degraded; even they can interact with Atg8/LC3 as well as autophagy receptors. Autophagy receptors participate to the substrates recognition and interact with Atg8/LC3 during autophagy, and could be degraded, whereas autophagy adaptors serve as anchor points for the autophagy machinery (see **Figure3**).(*Galluzzi, Baehrecke et al. 2017*) (*Stolz, Ernst et al. 2014*). The different involvement of the receptors and adaptors are shown in **Figure3**.

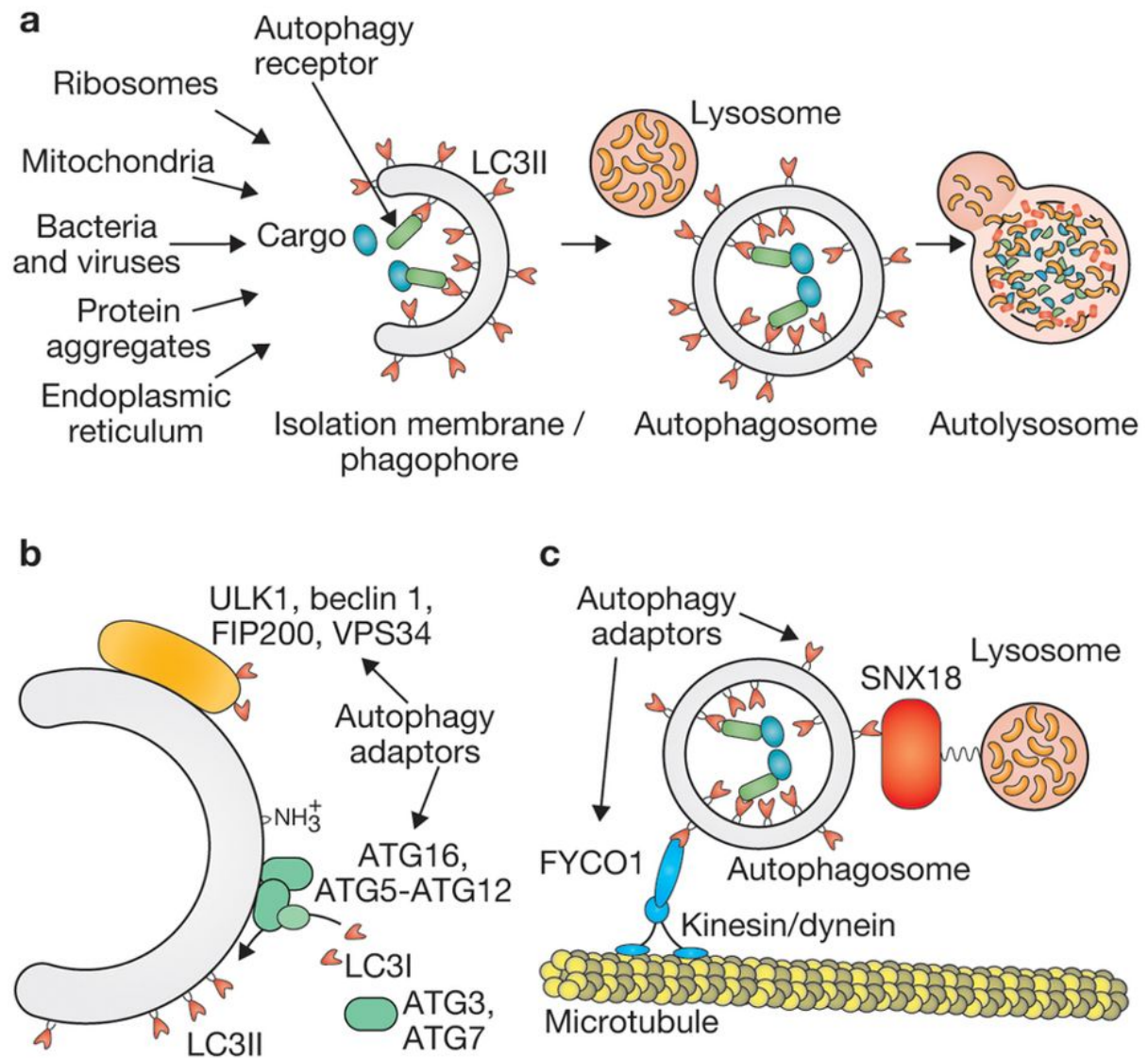


Figure 3. Functions of autophagy receptors and adaptors in selective autophagy (a) Aggregated proteins, cellular organelles such as ER, mitochondria, and ribosome, or pathogens (bacteria and viruses) can be selectively recognized by autophagy through the interaction with distinct autophagy receptors that are recognized by LC3/Atg8 via their LIR motif. (b,c) Autophagy adaptors role during autophagosome formation(b) and trafficking(c). Autophagy adaptors are involved in membrane elongation (ULK1, Beclin1, FIP200 and VPS34), LC3 lipidation (ATG16, ATG5-ATG12), autophagosome trafficking (FYCO1) and the autophagosome-lysosome fusion (SNX18). Autophagy receptors as well as cargos are engulfed by autophagosomes for degradation whereas, autophagy adaptors are only present on autophagosome/phagophore surface.(Stolz, Ernst et al. 2014)

Most autophagy receptors are characterized by the LC3-interacting region (LIR) in mammals or Atg8-family interacting motif (AIM) in yeast. LIR (or AIM) consists of a hydrophobic amino acid motif WxxL, which is necessary for the interaction with two hydrophobic pockets (W and L pockets) present in LC3 (Ichimura, Kumanomidou et al. 2008), and is conserved from yeast to mammalian (Noda, Ohsumi et al. 2010). This applies to the

identified autophagy receptors: Atg19 (Cvt pathway), Atg36 and Atg30 (pexophagy) and Atg32 (mitophagy) in yeast (Meijer, van der Klei et al. 2007, Stolz, Ernst et al. 2014); SEPA-1 in *C. elegans* ; p62, BNIP3L, BNIP3 NDP52 in mammalian. Several of the LC3 binding proteins which containing the WxxL motif are listed in **Table1**

Table 1. The Sequence alignment and binding partner of the reported autophagy receptors
(Noda, Ohsumi et al. 2010)

	AIM sequence	Binding partner
Atg19	ALTWEEL	Atg8
Atg32	SGSWQAIQP	Atg8
Atg3	VGDWEDLQD	Atg8
Atg4B	TLTYDTLRF	LC3
p62	DDDWTHLSS	LC3
NBR1	SEDYIIILP	LC3
CRT	EDDWDFLPP	GABARAP
CHC	TPDWIFLLR	GABARAP
Nix	NSSWVELPM	GABARAP
(PSSM)	DDDWVFVPM	GABARAP
SEPA-1	(TYGYQELDD)	LGG-1

A specific type of selective autophagy in *S. cerevisiae* is the cytoplasm-to-vacuole targeting (Cvt) pathway. The Cvt pathway selectively transports vacuolar hydrolases (Ape1, Ams1, etc...) into the vacuole in budding yeast cells. Although Cvt pathway shares the similar core machinery with autophagy (Scott, Hefner-Gravink et al. 1996, Xie and Klionsky 2007), Cvt vesicles (150nm) are much smaller than autophagosome (300nm-1µm). Atg19 functions as the Ape1 receptor during Cvt pathway. It binds to both CVT-Ams1p-complex and Atg8, results in the preference formation of double membrane vesicles around Cvt complex (Komatsu and Ichimura 2010). Cvt can not be considered as a type of autophagy since this pathway is biosynthetic (it helps vacuole to be efficient), rather than degradative.

1.3. Physiological roles of autophagy

Autophagy is an important way for the degradation of cellular proteins and organelles to maintain the homeostasis. It clears the damaged or redundant components as well as recycles materials in stress conditions. Autophagy plays multiple roles at the cell level which when integrated at the level of an organism are responsible for key physiological functions. Autophagy is involved in many physiological processes, such as cell death, infection,

adaptive immune response and aging. Moreover, autophagy dysfunction is tightly linked with many human diseases, like cancer, cardiomyopathies, Paget's disease, neurodegenerative diseases (Choi, Ryter et al. 2013). For example, in mammals, autophagy stimulation is critical at birth because it supplies newborn cells with nutrients and energy while maternal nutrients supply has ceased (Kuma, Hatano et al. 2004). Considering about the pages limitation, I hereafter focus on the physiological roles of autophagy that have been studied in *C. elegans* rather than going into a detailed description of the many physiological roles of autophagy. Therefore, I present how autophagy controls or participates to: (1) developmental cell death; (2) aging and (3) cancer.

1.3.1. Developmental cell death and autophagy

In some instances, cell dies with accumulating autophagosome and autolysosome within their cytoplasm. These dying cells are therefore very different from the ones that die from the type I cell death/apoptosis or necrosis and therefore this cell death mechanism has been named type II cell death or autophagic cell death (Schweichel and Merker 1973, Clarke 1990).

The first evidence of autophagic cell death was observed in *Drosophila*. As early as 1960s, researchers had observed the booming of autophagosome/autolysosomes in salivary glands and midgut during *Drosophila* metamorphosis (Schin and Clever 1965, Nopanitaya and Misch 1974). Further studies confirmed that autophagy mediated developmental cell death is required for both salivary glands and midgut degradation after puparium formation (apf), although the mechanisms may vary from tissue to tissue (Berry and Baehrecke 2007, Denton, Chang et al. 2012).

Besides its role in type II cell death, autophagy and/or key genes involved in the general autophagy machinery are involved in apoptosis. In mammalian system the autophagy protein LC3 could facilitate dead cell corpse phagocytosis for lysosomal degradation. This mechanism has been named LC3-associated phagocytosis (LAP) (Sanjuan, Dillon et al. 2007, Florey, Kim et al. 2011, Martinez, Almendinger et al. 2011). Recently, Jenzer et al showed that autophagy is required in the cell destined to apoptosis to produce the energy required to induced their phagocytosis by neighbouring cells (Jenzer, Simionato et al. 2019). This work extended in *C. elegans* the observations that apoptotic cells clearance is autophagy dependent that have also been observed during either the mouse or bird development (Qu, Zou et al. 2007, Mellen, de la Rosa et al. 2008). Additionally, this work indicates that LGG-1

and LGG-2 are not involved in LAP but are rather required differently during an autophagy process used to mature phagosome.

1.3.2. Aging and autophagy

One common character in aging cells is the accumulation of misfolded proteins and damaged organelles. Autophagy is one of the cellular pathways involved in eliminating these harmful components and therefore it is not surprising that autophagy dysregulation may influence aging. Many data indicate that autophagy induction leads to a lifespan extension in many organisms. Whereas it has been revealed that autophagy, the major pathway for intracellular degradation of proteins and organelles, is also down-regulated in aging cells (Donati, Cavallini et al. 2001, Del Roso, Vittorini et al. 2003, Nakamura, Oba et al. 2019). This may indicate the anti-aging function of autophagy. Indeed, the autophagy-deficient mutant, *Atg7^{-/-} Drosophila* is short-lived; while by contrast, the lifespan is extended by promoting autophagy function (Juhasz, Erdi et al. 2007, Simonsen, Cumming et al. 2008). In *Saccharomyces cerevisiae*, rapamycin extends yeast chronological life span (CLS) by inducing autophagy (Alvers, Wood et al. 2009).

Specifically, in *C. elegans*, autophagy activity is required for life-span extension in *daf-2* (the insulin-like receptor) mutant, as well as for feeding defect induced longevity (Melendez, Tallozy et al. 2003, Jia and Levine 2007, Hansen, Chandra et al. 2008). Moreover, *atg-7* RNAi and most other *Atg* gene down-regulation or mutant shortens lifespan in wild type worms (Hars, Qi et al. 2007).

The roles of autophagy in slowing aging and extending life span have been strongly established, whereas the mechanisms are not clear yet. Insulin-like growth factor (Melendez, Tallozy et al. 2003) and TOR (Jia and Levine 2007, Hansen, Chandra et al. 2008, Ramos, Kaeberlein et al. 2013) pathways are the main signalling pathways involved in this process. The clearance of damaged mitochondria via autophagy is also thought to contribute to reduce aging, since many genes for mitochondrial function affect longevity (Lee, Lee et al. 2003, Oh, Mukhopadhyay et al. 2006). The extraordinary high ROS level in dysfunctional mitochondria that are not cleared by autophagy is considered as one of the triggers for aging. Whereas another study demonstrates that mitochondrial permeability uncouples elevates autophagy and extend *C. elegans* lifespan (Zhou, Kreuzer et al. 2019). Spermidine induced lifespan extension is also autophagy-dependent; it may motivate the clinic

exploration of anti-aging drugs (*Eisenberg, Knauer et al. 2009*). Spermidine is a natural polyamine that can induce a modification of the chromatine structure by inhibiting histone acetyltransferases. This altered status of the chromatine leads to Atg genes transcription increase which triggers autophagy in yeast, flies, worms and human cell (*Morselli, Marino et al. 2011*).

1.3.3. Cancer and autophagy

Cancer is one of the main threatens for human health. The molecular mechanisms in cancer are extremely complicated and infinite, and the role of autophagy in cancer is debating. Here I will only give a brief introduction about the autophagy studies in cancer.

Many evidences demonstrate autophagy as tumour suppressive machinery. Autophagy has been widely recognized as a main biological event involved in both the regulation of cancer cell proliferation and in the response of several anticancer drugs. An early study found Beclin1 is mono-allelically deleted in 40–75% breast cancers and ovarian cancers (*Aita, Liang et al. 1999*). In addition, the deficiency of other ATGs also exhibits an increased tumour growth (*Marino, Salvador-Montoliu et al. 2007, Takamura, Komatsu et al. 2011*). The mutant of p53, a well-known tumour suppressor, facilitates tumorigenesis by inhibiting autophagy activity (*Tasdemir, Chiara Maiuri et al. 2008*).

There are also evidences suggesting that autophagy offers survival advantages for tumour growth, especially in metabolic stress conditions. Cancer cells are always exposed in hypoxia condition due to the insufficient vascularization, leading to an increased autophagy through glycolytic enzyme pyruvate kinase M1 (PKM1). PKM1-Atg7 knockout mice exhibit a decreased tumour growth, indicating autophagy helps cancer cells to resist metabolic stress (*Morita, Sato et al. 2018*). Autophagy knockdown also contributes to the inhibition of tumorigenesis during tumour therapy (*White 2012*).

Taken together, autophagy is a tool to suppress tumour growth; on the other hand, autophagy may be used by cancer cells to resist harsh stress.

2. *C. elegans*, a model animal for autophagy studies

2.1. General knowledge of *C. elegans*

Caenorhabditis elegans (*C. elegans*), is a nematode found on all continents (though rarely in Asia) as well as on isolated islands such as Hawaii. It does not have a specific natural habitat but seems to be present in humid temperate region. *C. elegans* is mostly found on rooting fruit and stalk, on substrate containing many microbes decomposing vegetal material and barely in soil sample (Schulenburg and Felix 2017).

Historically, there were a number of studies made in the early time of the twentieth century that reported the use of *C. elegans* to address basic biological questions. These studies were mainly done or headed by a few researchers; most of them were French people: Emile Maupas (France), Hikokura Honda (USA), Victor Nigon (France), Ellsworth Dougherty (USA) and Jean-Louis Brun (France) (Nigon and Felix 2017). Later on, in the late 1960s, Sydney Brenner who participated in the discovery of mRNA and to the elucidation of the genetic code, was looking for a model organism with which he will address questions of developmental biology and neurobiology. He used to present biochemistry as “an insurmountable barrier to the study of metazoans” and genetics as “a tunnel through this barrier”. In his mind this model organism should be a genetically tractable organism with a simple anatomy that could be reconstructed from electron micrographs. It should be easily culture in the lab having a small size and a rapid life cycle. Finally, he has chosen *C. elegans* for all these reasons. *C. elegans* became model organism as important as the yeast and *Drosophila* models. In the past 50 years, *C. elegans* has been used by many labs to understand a large variety of molecular, cellular, developmental, physiological, and behavioral biology questions and related molecular mechanism, as well as to modelize human diseases (Miguel-Aliaga, Culetto et al. 1999). Indeed, the worm has plenty of advantages making it a powerful model animal, for instance, tiny size, rapid life cycle, self-fertilizing, easy rising, transparency and so on.

2.1.1. *C. elegans* maintenance and life cycle

C. elegans has a rapid life cycle of around 3 and half days at 20°C. Its optimized growth temperature is 12°C-25°C. Although lower or higher temperature leads to sterility, lifespan modulation or even death of animals, *C. elegans* is capable of cold acclimatation. Moreover, temperature acts differently on lifespan depending on the time window it is applied. Its life cycle consists of 3 main steps: embryogenesis, larval development (L1-L4), maturation to adulthood and adulthood. It is noteworthy, *C. elegans* is capable to go to an alternative

larval development stage called dauer larvae when it experiences harsh condition such as starvation, high population density or elevated temperature during a time window encompassing L1 and L2 stages (Figure 4).

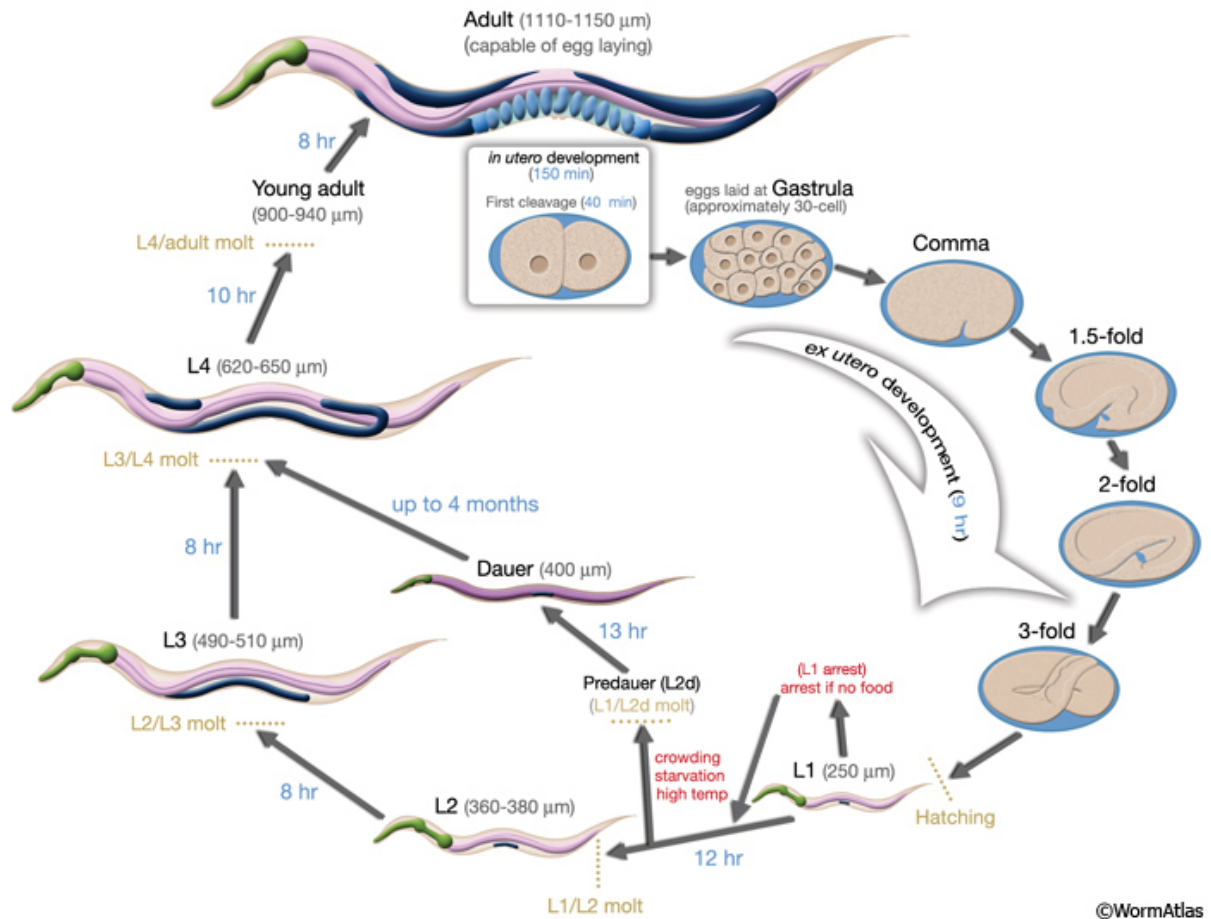


Figure 4. *C. elegans* life cycle at 22°C A life cycle for *C. elegans* consists of embryogenesis, larva development (L1-L4), adult maturation (Young Adult to adult) and sometimes an additional dauer stage. After fertilization, embryos firstly develop within the mother for 150 minutes until eggs are laid out. Blue words indicate the time it takes for the development of each stage. (from WormAtlas)

Following fertilization, the embryogenesis can be subdivided into stages as: fertilization, proliferation, gastrulation, morphogenesis, mlongation, quickening and hatching. *C. elegans* is an ectotherm/poikilotherms animal and therefore its time development is dependent of the temperature; it takes longer for worm to develop from embryo to adult at lower temperature, as shown in Table2.

Table2. Developmental timing of *C. elegans* at different growth temperatures. (from *wormatlas*)

	"16°C" (16.0 ± 0.3°C)	"20°C" (19.5 ± 0.5°C)	"25°C" (25.0 ± 0.2°C)
Egg laid	0 hr	0 hr	0 hr
Egg hatches	16-18 hr	10-12 hr	8-9 hr
First-molt lethargus	36.5 hr	26 hr	18.0 hr
Second-molt lethargus	48 hr	34.5 hr	25.5 hr
Third-molt lethargus	60.0 hr	43.5 hr	31 hr
Fourth-molt lethargus	75 hr	56 hr	39 hr
Egg-laying begins	~90 hr	~65 hr	~47 hr
Egg-laying maximal	~140 hr	~96 hr	~62 hr
Egg-laying ends	~180 hr	~128 hr	~88 hr
Length at first molt	360 µm	370 µm	380 µm
Length at second molt	490 µm	480 µm	510 µm
Length at third molt	650 µm	640 µm	620 µm
Length at fourth molt	900 µm	850 µm	940 µm
Length at egg- laying onset	1150 µm	1060 µm	1110 µm
Maximal egg- laying rate	5.4/hr	9.1/hr	8.1/hr
Total eggs laid	275	280	170

Figure 4 exhibits an example of life cycle at 22°C, embryogenesis takes 9 hours to enter first larva stage (L1) since laid eggs. The periods for L1-L4, lasts 12h, 8h, 8h and 10h separately, and each stage ends up with the molting of an old cuticle. After the fourth molting, worm reaches adult stage, but progenies are produced until several hours later. The majority of *C. elegans* is hermaphrodite and self-fertilizing, with a small ratio (<0.2%) being males. Per adult hermaphrodite is able to produce up to 300 self-progenies due to the limit of sperms, and then the spare oocytes are laid out without fertilization that are unable to produce offspring. However, once crossed with males, hermaphrodite can continue fertilization and produce 700 more progenies utilizing the sperms from male. The egg-laying

of hermaphrodite lasts 3 days at 20°C and the maximal rate is 9.1/hr, occurring 30h later since the laying starts. The newly formed embryos are retained within the mother's body until they reach the 24-cell stage. Notably, in food deprivation or other conditions, mother is no longer able to lay embryos, and the retained embryos hatch out leading to mother's death. The hermaphrodite lives 2-3 weeks in optimal conditions after the termination of egg lay. The body length varies from 0.25 (L1 larva) to 1 millimetre (adult) during lifespan, which allows the observation and picking under dissecting stereomicroscope.

L1 larvae enter to dauer stage due to unfavourable environments, and this alternative L2&L3 stage can last for months, followed by L4 stage again in food sufficient condition (*Golden and Riddle 1984, Hu 2007*). The dauer larvae have strong cuticles to protect against the harsh environments, and plug mouth pumps to avoid eating.

In the laboratory, *C. elegans* is always cultured on agar plates (or in liquid), which are seeded with bacteria *Escherichia coli* as food supply. The agar plates used for worm culture are NGM plates. And the *E. coli* strain applied on these plates is OP50, whose growth is limited to a lawn on NGM plates. This limited bacteria lawn provides convenience for plates keeping and worm observation. And an extreme limited OP50 lawn is applied in the cross of males with a hermaphrodite to promote their mating (*book: C. elegans, a practical approach, part of maintenance of C. elegans, by Theresa Stiernagle*). Worms should be transferred regularly to ensure the food supply, and the detailed frequency depends on the worm number and growth rate.

2.1.2. *C. elegans* tissues

One advantage for applying *C. elegans* as a model animal is its well characterized tissues (Please find the worm anatomy in [Figure5](#)). The main tissues: epidermis, muscles, digestive system, reproductive tissue and nervous system are introduced as following:

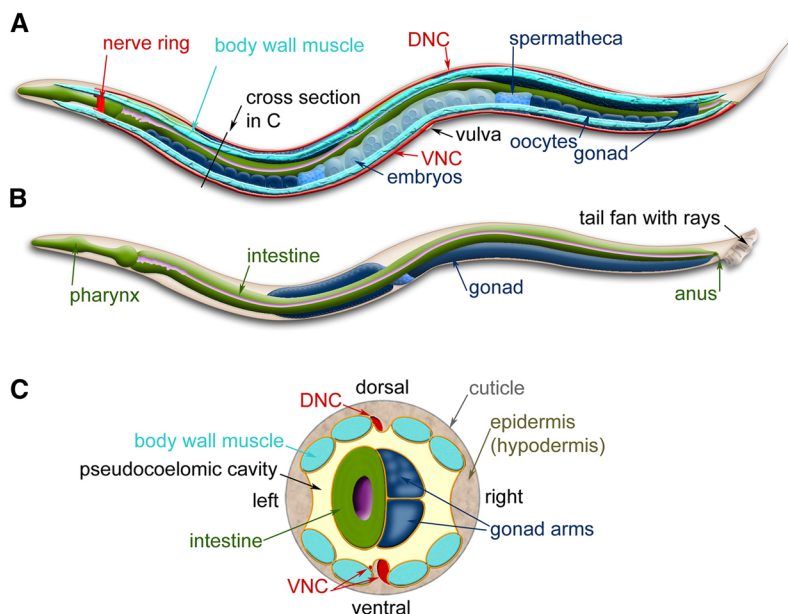


Figure 5. *C. elegans* anatomy (a,b) The lateral views of major tissues within a hermaphrodite (a) and male (b). (a) Both sperms and oocytes are presented in hermaphrodite; (b) while a male just produce sperms via a J-shaped germline. The tail fan with rays controls the mating behaviour with the hermaphrodite. For the convenience of observation, the nervous system and muscles are omitted in this view. (c) the vertical section of the indicated position in (a). The worm is coated by the cuticle, under which then lie sequentially epidermis and body wall muscle cells, forming the body cavity. The intestine and germline are within the body cavity. The dorsal nerve cord (DNC) and ventral nerve cord (VNC) are located at the dorsal and ventral separately. (from wormbook)

2.1.2.1. Epidermis

Epidermis, that also termed as hypodermis and regarded as the skin for *C. elegans*, is the outer epithelial layer formed by cell fusions to form large multinucleate epidermal cells. This layer provides a protective cuticle of specialized extracellular matrix (ECM) by secreting collagen, lipids, glycoproteins and other materials (*Chisholm and Hardin 2005, Page and Johnstone 2007*). Epidermis also functions as the scaffold for worm shaping by connecting to muscles. During larva development, each molting represents the renewal of epidermis to accommodate worm growth. The worm epidermis is a suitable system for the study of cell movements, cell fusion, wound healing (*Xu, Hsiao et al. 2012*) and innate immune respond (*Chisholm and Hardin 2005, Podbilewicz 2006*).

Seam cells are a special cell type in hypodermis, and are required for the formation of stage-specific cuticles through synthesis of various collagen proteins (*Thein, McCormack et al. 2003*). There are ten seam cells on each side of the L1 larva. All seam cells, except for H0, divide in a stem-cell-like pattern before each molting. Until the final molting, seam cells

differentiate terminally and all fuse into one longitudinal syncytium (*Sulston and Horvitz 1977*). Seam cells are used in exploring the roles of autophagy in dauer formation (*Melendez, Talloczy et al. 2003*) and could also respond to HS-induced autophagy (*Kumsta, Chang et al. 2017*), indicating seam cells or epidermis is a good tool for the study of autophagy in *C. elegans*.

2.1.2.2. Muscles

There are different categories of muscle tissue in *C. elegans*. One type is responsible for the worm locomotion and cells belonging to this tissue are named body-wall muscle cells (BWMs). These cells are located just beneath the hypodermis, in region where the epidermis is particularly thin. Body wall muscle cells which are spindle-shaped, line the general worm cavity alongside the length of the animal. The 95 BWM cells are arranged in four quadrants: left and right dorsal and ventral quadrants. Therefore, 4 BWM cells can be generally seen on *C. elegans* cross-sections (**Figure 5C**). Although the *C. elegans* epidermis is a syncytium, muscle cells are mononucleated and do not fuse with each other, but they work cooperatively since these cells are electrically coupled through a number of gap junctions. The BWM cells contractile filament are quite similar to the one described in vertebrate skeletal striated muscle cells. One main difference is for worm movement which is controlled by the arms connected to ventral and dorsal cord (*White, Southgate et al. 1976*). A number of reviews dealing with BWM could be read to access to much detail characterization (*Moerman and Fire 1997*)(*Waterston, 1988*). Besides BWM cells, there are other types of muscle cells, including muscles in the pharyngeal area and around the vulva, and the enteric muscles.

Per body wall muscle cell is consisted of three parts: the contractile filament lattice, a noncontractile body containing the nucleus and the cytoplasm with mitochondria, and the muscle arms. The mitochondria within the body wall muscle cells are striated along with the filament and their network morphology is easily observed with a mitochondria-targeted fluorescence marker, or staining dye. For this reason, *C. elegans* muscle is viewed as a good tissue for mitochondria-related studies (*Palikaras and Tavernarakis 2017*).

2.1.2.3. The digestive system

The *C. elegans* digestive system (or alimentary system) has a simple structure composed of only 3 regions: pharynx, intestine and rectum (hindgut). The major roles of intestine are: to take up food, to break food down into nutrient, to absorb nutrients and minerals. A tube-like neuromuscular pump called the pharynx takes in bacteria. Its structure is much more complicated. It is made of 58 cells including epithelial, muscle and nerve cells. Its rhythmic contraction which is generated by an inner kind of pace maker made of one neuron M2 creates within the lumen a kind of depression that allows the animal to swallow bacteria to the buccal cavity. Broken bacteria are then directed to the intestine through the pharyngo-intestinal valves. The pump behaviour is a major character to distinguish live animals from the dead ones, and the decreased pump activity is always linked with aging (Fang, Waltz et al. 2017).

The intestine system is a simple tube distributed as two lines along the worm length and includes 20 polyploid epithelial cells, enterocytes, lines a lumen where food flux. *C. elegans* intestine has been applied to the study of understanding pathogens infection and immune responds (Darby 2005, Balla and Troemel 2013, Diogo and Bratanich 2014). Additionally, *C. elegans* intestine plays a role in detoxification and storage of nutrients. It is also involved in integrating multiple signals coming from diverse anatomical structure to subsequently provide information to the all body to adapt. The intestine also synthesizes vitellogenine which is subsequently transferred to the developing oocytes where it is used as nutrient supply during worm embryogenesis.

2.1.2.4. Reproductive tissue

The anatomical appearances are different from males to hermaphrodites in *C. elegans*. The most obvious difference is the smaller body size in adult males. The gonad in hermaphrodites is symmetric with two U-shaped germline parts, including the formation of oocytes and sperms, as well as the fertilized embryos. Comparatively, the male gonad has only one part, and produce sperms without oocytes or even embryos. Alternatively, the male developed a fan-shaped tail whose movement is mediated by neurons and the supporting cells, for the mating with a hermaphrodite (Emmons 2005, Herman 2006). In general, the major study of *C. elegans* is in hermaphrodites but not males.

The maturation of oocytes within the germline includes both mitosis and meiosis processes, provides a model for studying cell mitosis/meiosis or germline proliferation in stem cell

biology (Kimble and Crittenden 2005). And the mutant of *gld* (for germ line development)-1 which controls the mitosis leads to an abundant of differentiated cells within the gonad, that characterized as a tumour model in *C. elegans* (Francis, Barton et al. 1995, Ciosk, DePalma et al. 2006).

2.1.2.5. The nervous system

C. elegans has emerged as a good model animal for the study in neurons for several decades. 302 and 383 neurons are found in adult hermaphrodite or male, respectively (Sulston and Horvitz 1977, White, Southgate et al. 1986). Neurons are distributed in head, ventral and dorsal cord, and tail. Different from human neurons, the neurons in *C. elegans* make synapses and connections through *en passant*, a side by side way for neurites pass. However, *C. elegans* shares the same chemicals for signalling as in vertebrates, including acetylcholine, dopamine, glutamate, gamma-amino butyric acid (GABA) (Hobert 2013), that is a power advantage for studying neuro-related diseases. For instance, a recent study in *C. elegans* revealed that the neurotoxic huntingtin (Htt) protein with a polyQ trace could be concentrated as exophers and then extruded out of neurons, and the exophers increased with aging. This is similar to the human neurodegenerative diseases pattern, which is also related to mitochondrial function (Melentijevic, Toth et al. 2017).

2.2. *C. elegans* is an ideal model animal for autophagy study

As I described above, *C. elegans* has many advantages as a model animal, and indeed, it has been applied to the studies in many fields. In this part, I will concentrate on introducing the study of autophagy in *C. elegans*. It has been more than 15 years since *C. elegans* has been chosen as an animal model for autophagy study. Using both genomic approaches to look for *C. elegans* homologous gene to Atg in its genome and brilliant genetic screening assay, a series of Atg homologues have been identified in worms (Tian, Li et al. 2010), that greatly facilitated the exploration of autophagy within *C. elegans*. The identified autophagy genes in *C. elegans* and their homologues in both yeast and mammalian are listed in **Table3** (Chen, Scarcelli et al. 2017), with few genes lacking the corresponding homologue in other systems. Notably, **Table3** indicates that the autophagy-related genes are conserved from yeast, worm, to mammal. I have described these autophagy genes in one published review (see the review in the **annexes**), so I will not write again here. In this review, we also share

the approaches and tools for studying autophagy in *C. elegans*. Another advantage to use *C. elegans* as the model animal study is the involvement of autophagy in many physiological processes during worm lifespan (shown in **Figure 6**), including embryogenesis (allophagy, aggrephagy, apoptotic cell death), larva development (stress resistance) and aging (lipophagy, pathologies neurodegeneration) (Jenzer, Simionato et al. 2015). In this chapter, these different types of autophagy will be discussed separately according to their physiological roles.

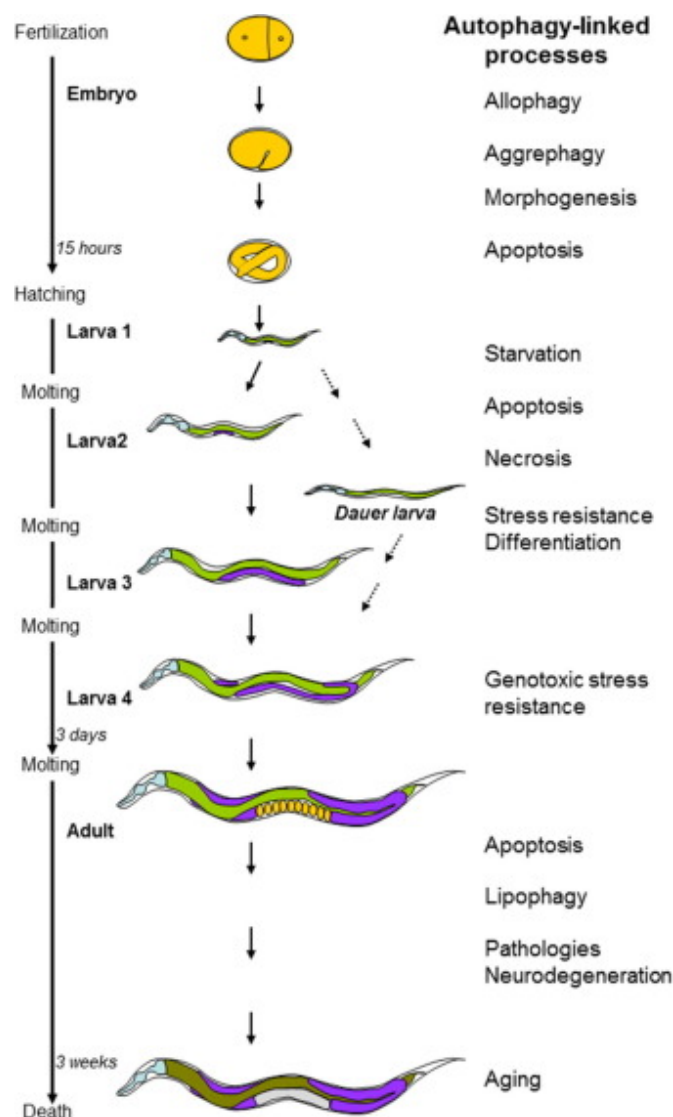


Figure 6. The autophagy is essential for various physiological processes during whole life (Jenzer, Simionato et al. 2015)

Table 3 Autophagy genes in *C. elegans*, and homologs

<i>C. elegans</i> Genes	Mutant Alleles or RNAi	Mammalian Homologs	Yeast Genes
<i>atg-2</i>	<i>bp576</i>	ATG2	ATG2
<i>atg-3</i>	<i>bp412/RNAi</i>	ATG3	ATG3
<i>atg-4.1</i>	<i>bp410</i>	ATG4A, ATG4B	ATG4
<i>atg-4.2</i>	<i>tm3948</i>	ATG4C, ATG4D	ATG4
<i>atg-5</i>	<i>bp546/RNAi</i>	ATG5	ATG5
<i>atg-7</i>	<i>bp422/RNAi</i>	ATG7	ATG7
<i>atg-9</i>	<i>bp564/RNAi</i>	ATG9	ATG9
<i>atg-10</i>	<i>bp421, bp588/RNAi</i>	ATG10	ATG10
<i>atg-16.1</i>	<i>gk668615d</i>	ATG16L1	ATG16
<i>atg-16.2</i>	<i>bp636, ok3224</i>	ATG16L2	
<i>atg-18</i>	<i>gk378/RNAi</i>	WIPI1, WIPI2	ATG18
<i>bec-1</i>	<i>ok691, bp613, ok700/RNAi</i>	BECN1	VPS30/ATG6
<i>epg-1</i>	<i>bp414</i>	KIAA0652	ATG13
<i>epg-2</i>	<i>bp444/RNAi</i>	?	VPS34
<i>epg-3</i>	<i>bp405/RNAi</i>	VPM1	VPS34
<i>epg-4</i>	<i>bp425/RNAi</i>	EI24	VPS34
<i>epg-5</i>	<i>tm3425/RNAi</i>	EPG5	VPS34
<i>epg-6</i>	<i>bp242</i>	WIPI3, WIPI4	
<i>epg-7</i>	<i>tm2508</i>	FIP200	VPS34
<i>epg-8</i>	<i>bp251</i>	ATG14	ATG14
<i>epg-9</i>	<i>bp320</i>	ATG101	VPS34
<i>let-363</i>	<i>h98/RNAi</i>	TOR	
<i>lgg-1</i>	<i>tm348/RNAi</i>	GABARAP	ATG8
<i>lgg-2</i>	<i>tm5755/RNAi</i>	LC3	ATG8
<i>lgg-3</i>	<i>RNAi</i>	ATG12	ATG12
<i>pgl-3</i>	<i>bp439</i>	?	
<i>rab-7</i>	<i>ok511/RNAi</i>	RAB7	
<i>sepa-1</i>	<i>bp456</i>	?	
<i>sqst-1</i>	<i>ok2892</i>	SQSTM1/p62	
<i>unc-51</i>	<i>e369/RNAi</i>	ULK1	ATG1
<i>vps-34</i>	<i>h741</i>	VPS34	VPS34
<i>vps-39</i>	<i>tm2253</i>	VPS39	
<i>vps-41</i>	<i>ep402</i>	VPS4	

2.2.1. Allophagy

Allophagy term designs allogeneic (non-self) organelle autophagy. This specific autophagy mechanism takes place in *C. elegans* after fertilization during which paternal mitochondria and membranous organelles goes into the oocyte. One great feature of mitochondria which differs from other organelles is that it carries its own DNA within the matrix. In most animals, mitochondrial DNA (mtDNA) is maternally inherited (*Birky 2001*). Although, in humans the

central dogma of maternal inheritance of mtDNA remains valid; there are some exceptional pathological cases in which paternal mtDNA could be passed to the offspring (Luo, Valencia et al. 2018). In general, paternal mtDNA is removed in early embryogenesis, before or just after fertilization, by diverse mechanisms in different model animals. Both in mice and *Drosophila*, paternal mtDNA is mainly eliminated before fertilization, whereas the removal of sperm mitochondria and their mtDNA in *C. elegans* is by a selective autophagy (allophagy) process after fertilization. Three published papers in 2011 demonstrated this process and termed it as allophagy (Al Rawi, Louvet-Vallee et al. 2011, Sato and Sato 2011). The process of allophagy is shown in Figure7 and described as following.

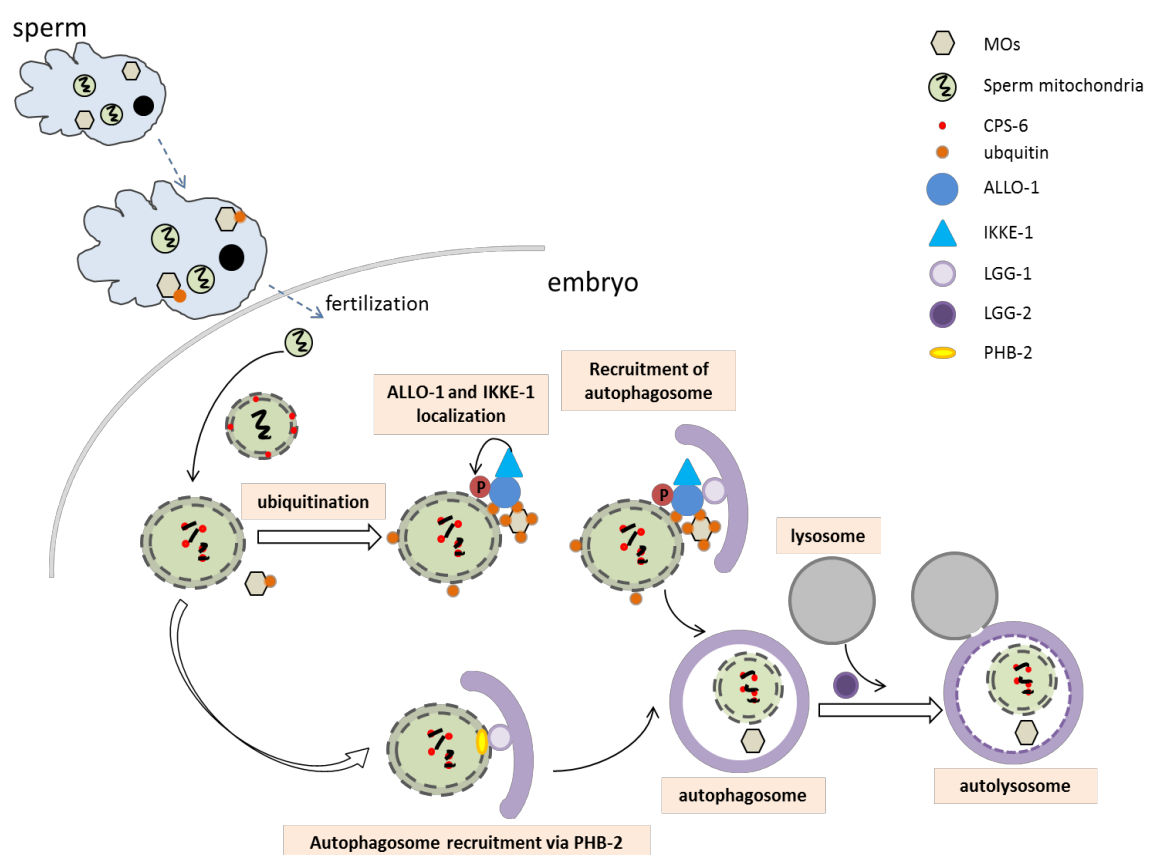


Figure7. The process of allophagy Firstly, membranous organelles (MOs), a specific spermatozoid organelle, are already ubiquitinated within sperm. As soon as fertilization occurs, MOs and paternal mitochondria are further ubiquitinated, allowing recognition and binding by the autophagy receptor, ALLO-1. ALLO-1 interacts with the IKKE-1 kinase which phosphorylates ALLO-1 and probably other substrates that could be present on mitochondria. Then ALLO-1 interacts with LGG-1 through its LIR domain which drives the formation of autophagosome around MOs and paternal mitochondria. Paternal mitochondria are also selected via a specific mitophagy receptor, the OMM protein PHB-2. The fusion of autophagosome with lysosome is mediated by LGG-2.

Before entering oocyte, sperm membranous organelles (MOs) are ubiquitinated, contributing to targeting them for autophagosome later (*Al Rawi, Louvet-Vallee et al. 2011, Sato and Sato 2011*).

Upon fertilization, different microscopy analysis shows that paternal mitochondria are depolarized and damaged internally rapidly. The alteration of the sperm originated mitochondria triggers a translocation of mitochondrial endonuclease G, CPS-6, from the intermembrane space to the matrix. In addition, it happens before the assembly of autophagosome. The matrix CPS-6 mediates the breakdown of mitochondrial inner membranes and further mtDNA degradation from sperm. In *Drosophila*, the Endo G mediated mtDNA degradation occurs before fertilization, which is different from that in *C. elegans*. The autophagosome engulfment for paternal mitochondria during allophagy is slowed down in *cps-6* mutant, suggesting CPS-6 induced mitochondrial breakdown may be a sensor of autophagy during paternal mitochondria degradation in zygote *C. elegans* (*Zhou, Li et al. 2016*).

Next, how paternal mitochondria and MOs are selected during allophagy? Paternal organelles are tagged by ubiquitylation before autophagosome formation. However, the mutant of common autophagy receptor p62, which could recognize ubiquitin signal, has no effect on the degradation of neither MOs nor sperm-specific mitochondria. The mutant of *pink-1* or *pdr-1* also has effect on allophagy. Alternatively, ALLO-1 has been identified as an autophagy receptor for allophagy. ALLO-1 accumulates on the ubiquitinated paternal organelles to promote their degradation through the direct interaction of the LIR motif with LGG-1. The activity of ALLO-1 is regulated by its kinase IKKE-1 (*Sato, Sato et al. 2018*).

Beth Levine lab has revealed prohibitin 2 (PHB-2) as another receptor regulating allophagy. Different from ALLO-1, PHB-2 is a mitophagy specific receptor. Previously identified mitophagy receptors are mainly located on the outer mitochondrial membrane (OMM), that is logical for its interaction with cytosol-distributed LC3 families (*Liu, Feng et al. 2012, Pickrell and Youle 2015, Bingol and Sheng 2016, Yamaguchi, Murakawa et al. 2016*). Nonetheless, PHB-2 is an inner mitochondrial membrane (IMM) protein, the study of PHB-2 in mammalian cells have explained that the proteasome-dependent OMM rupture in PINK1/Parkin-mediated mitophagy wipes out the obstacle for PHB-2 and LC3 direct interaction. And the exposure of PHB-2 in cytosol is required for PINK1/Parkin-mediated mitophagy (*Wei, Chiang et al. 2017*). In *C. elegans*, paternal *phb-2* RNAi results in the

impaired degradation of paternal mitochondria and its mtDNA in early embryo, this study suggests that PHB-2 is required for the eliminating of transmission of paternal organelles to offspring in *C. elegans* (Wei, Chiang et al. 2017). But what mediates the exposure of PHB-2 in sperm mitochondria of zygote needs further study, and the detectable permeabilization of paternal mitochondria may be a clue for it. In parallel, sperm originated mitochondria have fuzzy membrane with cracks, and electron dense spots in the matrix (Zhou, Li et al. 2016). In conclusion, the cooperation of ALLO-1, PHB-2, and ubiquitin for allophagy is not completely clear yet.

Autophagy is the main pathway to degrade paternal mitochondria mtDNA, and MOs. LGG-1 and LGG-2, the homologues of mammalian GABARAP and LC3 respectively, both appear in big clusters and colocalize specifically with paternal mitochondria and MOs after fertilization. Using genetic approaches and *lgg-1* and *lgg-2* mutant to block autophagy activity, MOs degradation is impaired and it is still detectable until 100-200-cell stage, while the degradation in WT is completed no later than 8-cell stage (Al Rawi, Louvet-Vallee et al. 2011, Sato and Sato 2011). Further studies revealed LGG-1 and LGG-2 specific roles in allophagy. Allophagic clusters are completely abolished in *lgg-1* null mutant but present for longer time in *lgg-2*, and the long-lasting autophagic clusters are identified as non-lysosome fused autophagosomes. These datas illustrate that *lgg-1* is essential for autophagosome formation, whereas *lgg-2* has a role in autophagosome fusion with lysosome. Moreover, LGG-2 interacts with VPS-39, an HOPS complex subunit to mediate the tethering of autophagosome with lysosome (Manil-Segalen, Lefebvre et al. 2014). Another study also proved that LGG-2 is important for autophagosome acidification, but instead of directly promoting its fusion with lysosome, LGG-2 mainly contributes downstream of Rab5-Rab7 to autophagosome locating to pericentrosomal area where lysosome are localized and therefore improve acidification (Djeddi, Al Rawi et al. 2015).

2.2.2. Aggrephagy

2.2.2.1. Degradation of germline P granule

Depending on the animal, two different mechanisms control the germline cells specification: inductive and determinative. The inductive mechanism is present in mammals and avians where the germ line fate is triggered by an inductive signalling

pathways and cell-cell communications. Cells of the embryo are specified to become germline cells independently of pre-existing maternal information. In other organisms, germline lineage is established through asymmetric segregation during embryonic cell divisions of maternal intrinsic factors. These maternal pre-existing factors specify the germline fate to the cell which inherited these maternal cytoplasmic determinants (Lehmann 2012). In fact, *C. elegans* germline fate has been thought to be determined by the asymmetric repartition of maternal determinants called P granules. Recent data indicate that P granules are not required to distinguish soma from germline (Gallo, Wang et al. 2010) but are probably required to preserve the totipotency of germline cells by preventing the expression of somatic development fate (Updike, Knutson et al. 2014).

P granules are a type of ribonucleoprotein aggregates made of maternal mRNA and proteins such as PGL-1, which are synthesised in germline and passed to offspring through oocytes. Large amount of P granules are present during embryogenesis due to their function on the cell differentiation, which are classified into two categories based on their location. The P granules in germline cells exist throughout worm development and are restricted to germline precursor cells. Some P granules that have not been segregated into germline precursor and partitioned into future somatic cells are degraded. More specifically, PGL-1 and PGL-3 are selectively eliminated by autophagy (Hird, Paulsen et al. 1996, Zhang, Yan et al. 2009). In autophagy mutant, these proteins aggregate and form large aggregate named PGL granules. A genetic screening showed *sepa-1* (suppressor of ectopic P granule in autophagy mutants) gene is essential for these PGL granules elimination. This gene encodes a coiled-coil domain-containing protein SEPA-1 zygotically (Lu, Wu et al. 2013). SEPA-1 bridges PGL granules with autophagy via its direct interaction with both PGL-3 and LGG-1, and facilitates the aggregation and further recognition by autophagy. SEPA-1 itself also aggregates and can be degraded through autophagy, but independent of PGL-1 and PGL-3 (Zhang, Yan et al. 2009), therefore SEPA-1 is an autophagic receptor. The *C. elegans* genome contains several *sepa-1* homologues that could constitute additional autophagy receptor in other type of selective autophagy.

Additional genetic screen has been performed to identify genes involved in the somatic elimination of PGL granules during *C. elegans* embryogenesis. Several previously uncharacterized atg genes, identified in this screen including *epg-3,-4* and *-5*, are required for both *C. elegans* PGL granules and p62 homologue SQST-1 degradation (Tian, Li et al.

2010). Although, PGL granules and SQST-1 eliminations shares similar autophagy degradation machinery during embryogenesis, EPG-2, is specifically involved in PGL granule component elimination. EPG-2 is a scaffold protein which interacts with both SEPA-1 and LGG-1, it is a nematode-specific autophagy related gene, since no homologue has been found in other model organism.

Further studies have found that autophagy-dependent degradation of PGL granules requires their correct assembling to the certain size and biophysical properties, which is formed by liquid-liquid phase separation (LLPS) mechanism (*Brangwynne, Eckmann et al. 2009, Zhang, Wang et al. 2018*). PGL-1 fails to form granules and has a diffused distribution in *pgl-3* mutant embryo, and SEPA-1 promotes LLPS for both PGL-1 and PGL-3. EPG-2 is another protein involved in LLPS process for two roles: on one hand, it promotes LLPS and drives PGL-1, PGL-3 and SEPA-1 into the less dynamic gel-like structure; on the other hand, EPG-2 coats PGL-1, PGL-3 and SEPA-1 droplets and controls their sizes by avoiding overgrowth. Through these functions, EPG-2 makes their biophysical properties amenable for autophagic degradation (*Zhang, Wang et al. 2018*).

2.2.2.2. Degradation of SQST-1-positive aggrephagy during embryogenesis

p62, also called sequestosome 1 (SQSTM1), is one of the most studied autophagy receptor in mammals. p62 contains three important domains: Phox and Bem1 (PB1) domain at N-terminal, LRS (LC3 recognition sequence) or LIR motif and ubiquitin-associated (UBA) domain at C-terminal. p62 is polymerized through its PB1 domain (*Lamark, Perander et al. 2003*), and this oligomerization is not necessary but stimulates its autophagic degradation (*Ichimura, Kumanomidou et al. 2008*). LIR motif mediates its direct interaction with LC3 and engulfment by autophagosome (*Pankiv, Clausen et al. 2007, Ichimura, Kumanomidou et al. 2008*), and the ubiquitination is tagged to UBA domain for the formation of ubiquitinated protein aggregates (*Cavey, Ralston et al. 2005*), which serves as a label for autophagy recognition. The aggregates of p62 accumulate in several neurodegenerative diseases.

During embryogenesis, the *C. elegans* p62 homologue, SQST-1 is also removed continuously via autophagy and kept in diffused location. However, when autophagy is inhibited by *epg-3,-4* or *-5* mutants, p62 forms numerous aggregates. Besides, there are also other EPG proteins functional in aggrephagy during embryogenesis. During the process of autophagy, these EPG proteins have diverse roles which are indicated in **Figure8**.

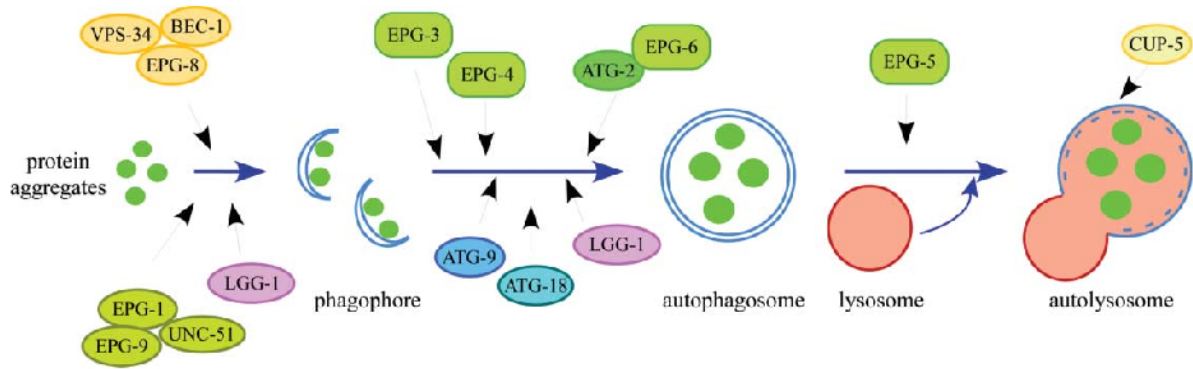


Figure 8. The hierarchical order of autophagy genes in the aggrephagy pathway EPG-8 is predicted to be the Atg14 homologue and functions in the initiation step of aggrephagy. Similar to EPG-8, EPG-1 and EPG-9 are also involved in the initiation step. During phagophore elongation, EPG-6, a WIPI homologue, may interact with ATG-2, while EPG-3 and -4 are ER proteins mediating phagophore growing. ATG-9, ATG-18 and LGG-1 all function for autophagosome formation. EPG-5 is related with the fusion between autophagosome and lysosome (Zhang, Chang et al. 2015).

EPG-3 (homologue of VMP1 in mammals) is located on endoplasmic reticulum (ER), and regulates autophagosome formation at the ER-isolation membrane (ER-IM) contact sites. EPG-3 negatively regulates the contacts of ER-IM, and the loss of EPG-3 leads to an enhanced tethering which suppresses the release of IM for autophagosome closing (Tian, Li et al. 2010, Zhao, Chen et al. 2017). EPG-4 is another ER protein, and the similar phenotype between *epg-4* and *epg-3* mutants suggests that EPG-4 is also involved in the autophagosome formation step. But the detailed mechanism has not been explored yet (Tian, Li et al. 2010).

EPG-5 binds to Rab7 and VAMP7/8 for the recruitment of late endosome or lysosome. Meanwhile, EPG-5 contains a LIR motif for the interaction with LGG-1 on the autophagosome, stabilizing and facilitating the *trans*-SNARE complex for autophagosome fusion. The *epg-5* mutant causes fusion defect between autophagosome and other endocytic vesicles, resulting in the accelerated hybrids of endosomes /lysosomes. In conclusion, EPG-5 functions at the fusion step during autophagy (Wang, Miao et al. 2016).

EPG-6 is a WD40 repeat-containing protein with a phosphatidylinositol 3-phosphate (PtdIns(3)P)-binding activity, and it shares the homologous function with human WIPI4. EPG-6 directly binds with ATG-2 to mediate the process of autophagosome formation from omegasome, and the mutation of either *epg-6* or *atg-2* generates increased early autophagic structures during embryo development. In addition, ATG-18, the homologue of

WIP1/2, is another WD40 repeat PtdIns(3)P effector and recruited to PAS by PtdIns(3)P in yeast, which also contributes to the early autophagy step. But *atg-18* mutated embryo shows much smaller SQST-1 aggregates comparing with *epg-6* mutant, suggesting a distinct role of ATG-18 for autophagosome biogenesis (Lu, Yang et al. 2011).

EPG-8 is a potential Atg-14 homologue in *C. elegans* based both on their conserved structural properties and similar functions during autophagy (Yang and Zhang 2011). As well as in other *epg* mutant, *epg-8* mutated embryo has increased SQST-1 aggregates, suggesting the defect of autophagy. Nevertheless, *epg-8* mutant dramatically decreases LGG-1 puncta, while LGG-1-I and LGG-1-II proteins levels are elevated, indicating that *epg-8* is required for the recruitment of LGG-1-II to autophagic structures (Yang and Zhang 2011). Notably, the functions of *epg-8* does not highly correspond with that of Atg14, for instance, the knockdown of mammalian Atg14L impairs both LC-3-II protein level and its puncta (Sun, Fan et al. 2008, Matsunaga, Saitoh et al. 2009).

There're many other proteins involved in the autophagic degradation of SQST-1 aggregates and PGL granules, like the well-known Atg8 families: LGG-1 and LGG-2, which is not further introduced here. By applying genetic screening for the genes involved in autophagy during *C. elegans* embryogenesis, series autophagy related genes have been identified (Tian, Li et al. 2010), and the other genes and corresponding homologues are shown in my review (Chen, Scarcelli et al. 2017).

2.2.3. Autophagy in stress conditions

Harsh environments impair the worm survival, development and ageing, while autophagy has been proved to play roles in worm resistance to stress conditions. Here, three stresses that applied on *C. elegans* are described: (1) starvation; (2) high temperatures; (3) Dauer formation.

2.2.3.1. Autophagy in starvation condition

Starvation is a common induction for autophagy in yeast and mammals, as well as in *C. elegans* (Lum, Bauer et al. 2005, Yorimitsu and Klionsky 2005, Kang, You et al. 2007). Dual roles, however, have been explored in starvation condition in *C. elegans*. The physiological autophagy level is beneficial for long-live survival whereas the insufficient or excessive autophagy level is toxic (Kang, You et al. 2007). Autophagy is elevated in starved worms, and

the depletion of autophagy functions significantly decreases the worm survival, which could be rescued by the food addition. As it has been revealed that the excess autophagy may be toxic in cellular level (*Kroemer and Jaattela 2005, Levine and Yuan 2005, Kang, You et al. 2007*), overactivating autophagy by *gpb-2* mutant, which mediates muscarinic acetylcholine signalling pathway, leads to the defect in pharyngeal muscles and contributes to worm death. Moreover, the *gpb-2* mutant has no apoptotic changes, indicating it does not function through an autophagy-apoptotic pattern. It is not clear how autophagy promotes the survivals in starved worms, one possibility is that autophagy enables to recycle materials in nutrient deprivation condition. A recent study highlights a link between autophagy and mitochondria in starvation condition. Starvation induced the mitochondrial fragmentation, damaged respiratory functions, and decreased mitochondrial contents via nonselective autophagy. These data indicate that autophagy mediated mitochondrial physiological changes is key for worm survival during starvation (*Hibshman, Leuthner et al. 2018*).

2.2.3.2. Autophagy in heat stress condition

The optimal temperature for *C. elegans* maintaining is between 12-25°C, higher temperature is regarded as a heat stress which may induce protein aggregation, Ca²⁺ leakage and be toxic for worms (*Kampinga 1993, Kourtis, Nikolettou et al. 2012, Lanner, Georgiou et al. 2012*). Thermotolerance of *C. elegans* is linked with insulin-like signalling pathway, as the knockout of *daf-2* (insulin/IGF receptor homolog) induces DAF-16 translocation to nucleus and further promotes worm survival in heat stress condition (*Lithgow, White et al. 1995, Lin, Hsin et al. 2001, McColl, Rogers et al. 2010*). Moreover, the thrashing movement in heat stress (35°C, 4h) treated worms could recover in 20°C but fails in *daf-16* mutant worms (*Furuhashi and Sakamoto 2014*). Since autophagy is related to lifespan extension in *daf-2* mutant, autophagy may also be involved in thermotolerance in *C. elegans*. There are three more studies exploring the role of autophagy in heat stress condition in *C. elegans*.

Tavernarakis group has applied a severe heat stress treatment (37°C, 7h) that differs from Tsubasa's study, to trigger selective autophagy (*Palikaras, Lionaki et al. 2015*). They identified a novel mitophagy receptor DCT-1 in *C. elegans*, which is controlled by DAF-16 or low insulin/IGF-1 signalling. This study would be further discussed in mitophagy part.

Hansen group has applied a hormetic (36°C, 1h) rather than a severe heat stress to adult *C. elegans* and observed an increase of autophagosomes in multi tissues, including seam cells in epidermis, muscle, nerve ring and intestine, with tissue-specific manners. Not only the autophagic structures, but also the transcription levels of autophagy-related genes are elevated by heat stress, suggesting that autophagy responses are regulated at transcriptional levels. These autophagic markers increase is also observed in HSF-1 overexpression worms, and further amplified by BafA, the inhibitor for the fusion of autophagosome with lysosome. HSF-1 is a transcription factor which binds to the heat shock elements (HSEs) in heat stress condition and mediates genes expression. Interestingly, many autophagy-related genes contain putative HSEs in their promoter regions, suggesting that autophagy responding upon hormetic heat stress are induced possibly via the transcriptional regulator HSF-1, which however remains to be determined. As it has been reported, HSF-1 overexpression promotes worm longevity and resistance to stress (*Hsu, Murphy et al. 2003, Morley and Morimoto 2004*), and hormetic heat stress also increases longevity in an autophagy-dependent manner. Lastly, the hormetic heat stress improves proteostasis by reducing PolyQ aggregates in intestine and neuron tissues, which could extend lifespan in *C. elegans*. The beneficial autophagy responses in hormetic heat stress condition rely on worm age, and the maximum effect is in one-day adults, whereas the effect declines in old adults (*Kumsta, Chang et al. 2017*).

Heat stress also leads to the alteration of autophagy during embryogenesis. Embryos laid in heat stress condition (26°C) causes the PGL granules accumulation. The accumulation of PGL granules due to the accelerated LLPS and the formation of large droplets, are resistant to autophagic degradation. And the persisted PGL granules confer embryonic viability in heat stress condition. Heat stress increases mTORC1 mediated phosphorylation of PGL proteins which changes their biophysical properties and could count for the phase separation. Although the basal autophagy activity is not affected in this condition, it provides another role of autophagy in heat stress adaptation (*Zhang, Wang et al. 2018*).

2.2.3.3. Autophagy and dauer formation

As mentioned previously in [chapter 2.1.1](#), *C. elegans* larvae arrest to an alternative L3 stage named dauer in stress conditions. Dauer formation requires numerous changes in energy metabolism and body morphologies, including intestinal fat storage, pharyngeal

constriction and cuticle sealing. *daf-2(e1370)* is a temperature-sensitive strain with inappropriate constitutive dauer entry at 25°C, while extends lifespan at lower temperature. Growing at 25°C, *daf-2(e1370)* animals enter dauer stage, accompanied with an increased autophagy in seam cells, which is essential for dauer formation. Depleting autophagy by RNAi against *bec-1*, the homologue of APG6/VPS30 (in yeast) and *beclin1* (in mammalian), *daf-2 (e1370)* worms fail to form dauer with an abnormal morphology, and die quickly at 25°C (Melendez, Tallozy et al. 2003). However, the mechanisms by which autophagy mediates dauer formation are not clear yet.

2.2.4. Autophagy and longevity

Autophagy activity is also required for lifespan extension as well as for dauer formation. Although the detailed mechanisms are still on debate, it is for sure that autophagy is involved in several pathways that affect worm lifespan. First, both HSF-1 and DAF-16 function downstream of DAF-2 to activate expression of targeted genes, including some genes encoding small heat shock proteins (sHSPs), which make a contribution to longevity. These sHSPs are considered to prevent protein aggregation by forming large oligomers to bind to the unfolded proteins (Van Montfort, Slingsby et al. 2001, Giese and Vierling 2002, Melendez, Tallozy et al. 2003), which may be one explanation for the lifespan extension in *daf-2* mutant worms. HSF-1 knockdown alone induces a rapid-aging phenotype and a short lifespan (Garigan, Hsu et al. 2002).

Besides insulin-like signal pathway, the dietary restriction is another key for longevity, which has been proved in *C. elegans* (Kaeberlein, Smith et al. 2006, Lee, Wilson et al. 2006), as well as in yeast, *Drosophila* and mammal. Dietary deprivation in adult *C. elegans* significantly extends lifespan and enhances resistance to oxidative or thermal stress, via a mechanism that is distinct from insulin like pathway and the Sir2-family deacetylase, SIR-2.1. However, dietary deprivation could not further increase the lifespan of *eat-2(ad465)* mutant worms, indicating they share the same pathway in controlling longevity (Kaeberlein, Smith et al. 2006, Lee, Wilson et al. 2006). The feeding-defect, *eat-2* mutant, shows an extended lifespan, which depends on autophagic genes, for instance, *bec-1*, *vps-34* and *atg-7*.

The autophagy mediated lipid metabolism may be a key factor for lifespan extension in *C. elegans*. On one hand, lipophagy may be activated in starved condition for the energy supplement. On the other hand, autophagy defect not only leads to the less lipid storage in

intestine in WT but also impair the increasing of lipid contents in long-lived worms, as *daf-2* mutant and *glp-1* mutant. Moreover, the reduced LDs are not related to the reduced pharyngeal pumping, nutrient absorption or defecation (Lapierre, Silvestrini et al. 2013), but autophagy seems to function directly in the lipid metabolism. It is interesting that the triacylglycerol lipase, LIPL-4, is also required for lifespan extension in *glp-1* and *daf-2* mutants, and the overexpression of LIPL-4 is sufficient to confer a long lifespan that relies on DAF-16 and autophagy activity (Lapierre, Gelino et al. 2011).

In addition, I have mentioned in [chapter 2.2.3.2](#) that hormetic heat stress could also increase longevity in an autophagy-dependent manner.

However, autophagy may not always be beneficial for longevity. A recently published study demonstrated that HLH-30/TFEB regulated autophagy plays dual roles during high-glucose diet condition in *C. elegans* (Franco-Juarez, Mejia-Martinez et al. 2018). High glucose diet has been proved to be deleterious for health and could shorten lifespan via down-regulating DAF-16/FOXO in *C. elegans* (Lee, Murphy et al. 2009). And autophagy is activated in an HLH-30-dependent manner. Unexpectedly, the blockage of HLH-30 by protein phosphatases PP2A and PP1 which inhibits autophagy flux extends worm lifespan in high-glucose diet exposure. To sum up, autophagy is a two-edge sword that can either prolong or shorten lifespan in different conditions (Franco-Juarez, Mejia-Martinez et al. 2018).(Franco-Juarez, Mejia-Martinez et al. 2018).

2.2.5. Autophagy and neurodegenerative diseases

One of the main features for neurodegenerative diseases, e.g., Huntington's disease, Parkinson's disease and Alzheimer's disease, is the accumulation of protein aggregates. And autophagy as a major cellular pathway for degradation, is tightly associated with these neurodegenerative diseases (Cataldo, Hamilton et al. 1996, Anglade, Vyas et al. 1997, Sapp, Schwarz et al. 1997, Nixon, Wegiel et al. 2005).

Using transgenic worms expressing human β -amyloid peptide in muscle cells could mimic the pathogenesis of Alzheimer's disease and help to understand its mechanisms (Sapp, Schwarz et al. 1997). Christopher D. Link group has used this *C. elegans* model and found that the A β overexpression is toxic to muscle cells and impairs the fusion of autophagosome with lysosome. Whereas the accumulated autophagosomes are promoted to form autolysosomes in *daf-2* mutant, in which the insulin-receptor signal is decreased. Moreover,

the autophagy activity is required for the suppression of A β -toxicity mediated by reduced DAF-2 signaling (Florez-McClure, Hohsfield et al. 2007).

Different from Alzheimer's disease, the pathogenic feature in Huntington's disease is polyglutamine (PolyQ) aggregates, which is toxic and may lead to neurodegenerative disease. The polyglutamine repeats are prone to form aggregates due to the overlong glutamine residues, and autophagy is the major way for polyQ aggregates degradation (Ravikumar, Duden et al. 2002, Goldberg 2003). In *C. elegans* muscle cells, overexpressed polyQ fragments containing 40 repeats (Q40-YFP) form into aggregates, and the autophagy defect could further accelerate these aggregates and muscle disorder. Moreover, autophagy inactivation by *bec-1*, *atg-7* or *atg-18* RNAi also increases the human huntingtin fragment (Htn-Q150) aggregation in *C. elegans* ASH sensory neurons, associated with an increased neuronal degeneration (Jia, Hart et al. 2007). The autophagy function to decrease polyglutamine toxicity has also been confirmed in *C. elegans* intestine (Khan, Yamanaka et al. 2008). More importantly, polyQ aggregates are mediated by distinct states and mechanisms. The *C. elegans* intestine specifically expressing Q44-GFP is a model for studying osmotic stress condition. Q44-GFP aggregates irreversibly in hyperosmotic condition (500mM NaCl) comparing with control condition (50mM NaCl), which is neither induced in heat shock (37°C) nor in oxidative stress (200um juglone). *daf-2* mutant, which inhibits the aggregates in aging worms, fails to prevent the osmotic stress induced aggregates (Moronetti Mazzeo, Dersh et al. 2012).

It may exist other pathways which cooperate with autophagy to clear aggregations within neurons. A recent paper demonstrated a novel way for protein aggregates removal in *C. elegans* neurons. It is reported that neurons in adult *C. elegans* could extrude protein aggregates and organelles that are engulfed by membrane-surrounded vesicles named exophers. Exopher production occurs throughout worm life, while increases in neurotoxic condition, achieved by expressing Htt-Q128 (containing human huntingtin protein fragment) or amyloid- β protein (Alzheimer's disease fragment). The contents of exopher are protein aggregates and even large organelles including lysosomes and mitochondria, and the exophers prefer to exclude the damaged mitochondrial with high oxidative matrix than the healthy ones. A defect of autophagy (*lgg-1* RNAi, *atg-7* RNAi) or mitophagy (*pink-1* RNAi, *dct-1* RNAi) increases the occurrence of exophers, but whether autophagy or mitophagy is involved in exophers exclusion is not further discussed. It's not clear for the fate of exophers

after jettison, but part of the apoptotic genes are involved, indicating it is distinct from the classical removal of apoptotic corpses. In addition, some exopher contents are transferred to hypodermis for releasing into pseudocoelomic fluid and later taken up by coelomocytes (*Melentijevic, Toth et al. 2017*).

2.2.6. Other examples of autophagy associated process

2.2.6.1. Lipophagy

It has been reported that in mice hepatocytes, lipid droplets (LDs) are engulfed by autophagosome and then degraded in starvation condition. This process is named lipophagy. There're few studies on lipophagy in *C. elegans*. In a paper in 2013, LIPL-1 and -3 were identified as the lysosomal lipases that are required for lipid breakdown during lipophagy in starved *C. elegans* (*O'Rourke and Ruvkun 2013*). This process is mediated via the cooperation of MXL-3 and HLH-30 (homologue of TFEB, a well-known transcription factor ED which regulates autophagy genes). In favourable food condition, MXL-3 acts as an inhibitor for the expression of lysosomal lipase genes, while during fasting, MXL-3 is removed from nucleus and alternatively, HLH-30 translocates to nucleus for upregulating autophagy genes and acid lipase genes, resulting in the activated lipophagy (*Lapierre, De Magalhaes Filho et al. 2013*).

2.2.6.2. Autophagy mediated apoptotic cell death

During *C. elegans* embryogenesis, 131 somatic cells undergo programmed cell death (*Sulston, Schierenberg et al. 1983*), and are eliminated through phagocytosis. Cell corpses accumulate in autophagy null function mutants, including *bec-1* (*Cheng, Wu et al. 2013, Huang, Jia et al. 2013*). In addition, LGG-1 and LGG-2, the homologues of GABARAP and LC3 respectively, have different roles in the degradation of apoptotic cell during *C. elegans* embryogenesis. LGG-1 is enriched in the apoptotic cells for the exposure of PS, which facilitates the recognition for these cells; by contrast, the LGG-2 labelled autophagosomes in phagocytic cells help to promote the fusion between phagosome and lysosome for efficient clearance. Notably, the functions of both LGG-1 and LGG-2 depend on the canonical autophagy machinery (*Jenzer, Simionato et al. 2019*).

To sum up, *C. elegans* has emerged into a mature model animal for the understanding of autophagy and expanded the insights in this field, and the study of selective autophagy has gained more and more attentions, for instance, one of the most important -mitophagy.

3. Mitophagy

Mitochondrial homeostasis (turn-over) is mainly based on two opposed mechanisms: mechanism that promotes mitochondria biogenesis by addition and import into mitochondria of new lipids and proteins respectively and mechanisms that participate to the clearance of dysfunctional mitochondria. Both mechanisms are probably modulated by developmental (intrinsic) and environmental contexts. Mitochondrial clearance is mainly based on a selective autophagy mechanism called mitophagy. Besides, mitochondria or piece of damaged mitochondria could be removed via endosomal microautophagy (see [chapter 1.1.1](#)), outer mitochondrial membrane associated degradation (OMMAD), exospheres excretion (see [chapter 2.2.5](#)) and so on (*Pickles, Vigié et al. 2018*). In this chapter, I will mainly focus on mitophagy.

3.1. Mitophagy concept

Mitophagy is termed for the process that selectively engulfs mitochondria into autophagosomes, followed by fusion with lysosomes for degradation. [The first evidence of mitophagy was observed by Christian de Duve in 1966, and the term mitophagy was first coined by Sidney Scott and Daniel Klionsky in 1998 \(*Scott and Klionsky 1998*\). Whereas the term of mitophagy was popularized by JJ Lemaster based on the work performed in yeast by Nadine Camougrand and her team \(*Kissova, Deffieu et al. 2004*\).](#) Bulk autophagy shares with mitophagy its classical molecular machinery. Specifically, mitophagy is characterized by a series of specific receptors for the selective recognition of mitochondria that should be degraded. It is possible to distinguish three types of mitophagy, basal mitophagy, stress induced mitophagy and developmentally programmed mitophagy. Until now, the mitophagy mediators that have been identified are Atg32, Parkin/PINK1, NIX (BNIP3L), FUNDC1, PHB-2, DCT-1. I choose to describe some of the results that paved the way to the discovery of mitophagy mechanism in yeast. Then, I will present the molecular pathways regulating mitophagy in mammals. Finally, I will present and discuss the *C. elegans* mitophagy

litterature. A brief sketch illustrates three mitophagy pathways in yeast and mammals in **Figure9**.

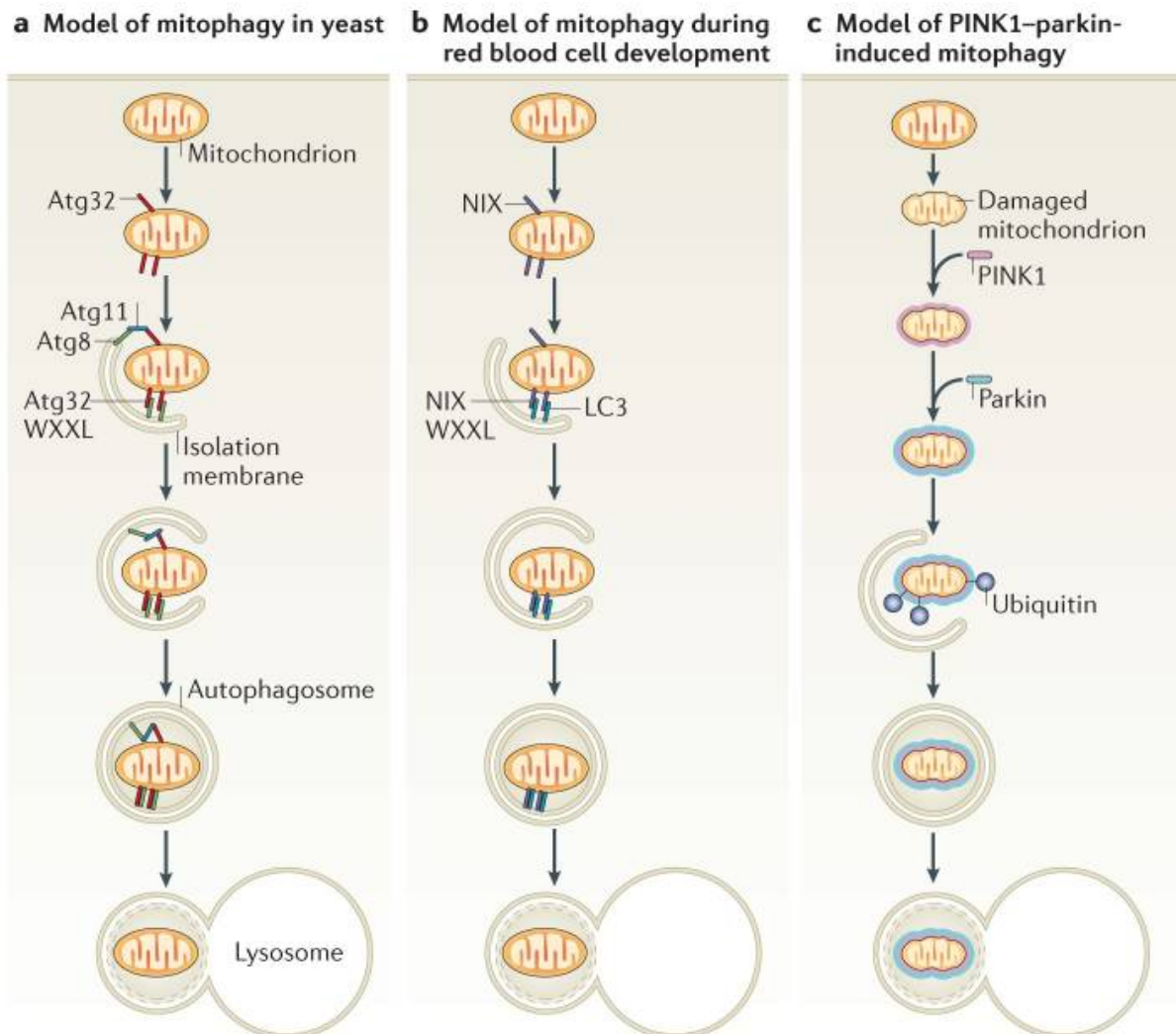


Figure 9. Different types of mitophagy in yeast and mammals In yeast, an outer mitochondrial membrane protein Atg32 can act as an autophagy receptor which possesses an Atg8 interacting motif WXXL, leading to the specific recognition of mitochondria by autophagy. Additionally, Atg32 can also interact with Atg11, which is an Atg8 binding protein. During red blood cell maturation in most mammals, mitochondria are removed by a mechanism involving mitophagy. Similar to yeast, NIP3-like protein X (NIX; also known as BNIP3L), an outer mitochondrial protein, also has a LC3 interacting region (LIR) WXXL, which can recruit isolation membranes to mitochondria and promote mitophagy. In addition to these mechanisms, PTEN-induced putative kinase protein 1 (PINK1) and Parkin pathway is also involved in mitochondrial removal. When mitochondria are damaged and loss membrane potential, PINK1 is stabilized on mitochondrial membrane and thus recruits the E3 ubiquitin ligase Parkin. Parkin then ubiquitylates mitochondrial proteins, activating mitophagy to remove mitochondria. (Youle and Narendra 2011)

3.2. Mitophagy in yeast

In yeast, it has been shown that mitophagy is induced when: (1) cells are cultured in a nonfermentable medium (lactate medium (YPL) or glycerol medium) and then shifted to nitrogen starvation medium or treated with rapamycin which inhibits Tor (Kissova, Deffieu et al. 2004); (2) cells are in the stationary phase and cultured in a nonfermentable medium (lactate medium (YPL) or glycerol medium) (Kanki and Klionsky 2008); (3) cells are pre-cultured in a fermentable medium and then exposed to nitrogen starvation (Bockler and Westermann 2014). UTH1 is the first identified gene to be involved in yeast mitophagy. (Kissova, Deffieu et al. 2004). *uth-1* mutant blocks mitochondria elimination during this process, without affecting general autophagy activity. The *Uth1p* product, Uth1p, is an outer mitochondrial membrane protein containing a SUN domain, may be specifically involved in mitophagy process (Kissova, Deffieu et al. 2004). Another mitophagy receptor identified in yeast is Aup1p (autophagy-related protein phosphatase), a highly conserved phosphatase protein that localizes at the mitochondrial intermembrane space (Tal, Winter et al. 2007). Aup1p is required for efficient mitophagy in stationary phase in *Saccharomyces cerevisiae*, and *aup1Δ* mutant impairs cell survival in long-term stationary phase cultures condition. The function of Aup1p is similar as UTH-1, and may be involved in mitochondria recognition for sequestration into autophagosomes (Kissova, Deffieu et al. 2004).

Subsequently, to identify genes involved in the molecular machinery of the mitophagy process, two similar genetic screens have been performed in the labs of Oshumi and Klionsky. Both screens rely on monitoring mitophagy, when cells were in a nonfermentable carbon source medium, by visualizing mitochondria membrane protein tagged with a fluorophore addressed to the vacuole. Then, these authors analysed the effect of nonessential gene deletion array on mitophagy; looking for gene deletion strains that were defective in transporting mito-GFP to the vacuole in this mitophagy-triggered medium. It is noteworthy that Aup1 and Uth1, proteins that have been suggested to be required for mitophagy, were not retrieved in these genetic screens.

Both teams identified Atg32 as mitophagy receptor in yeast (Kanki, Wang et al. 2009, Okamoto, Kondo-Okamoto et al. 2009) and **Figure9**. Atg32 is a transmembrane protein locating at OMM with its N-terminal in cytosol containing a WXXI motif for immediately interacting and recruiting Atg8 for mitochondria engulfment. Overexpression of Atg32 facilitates mitophagy, while the null mutant of Atg32 does not cause any defect of mitochondria in normal condition. In addition, the mitophagy receptor Atg32 could also

interact with Atg11, an adaptor protein, to recruit the redundant/damaged mitochondria to the vacuole surface. Another function of Atg11 is to recruit fission machinery to mitochondria to promote mitochondrial fragmentation during mitophagy (*Kanki, Wang et al. 2009, Okamoto, Kondo-Okamoto et al. 2009*).

Atg32 is an essential factor for the selective elimination of mitochondria in yeast but is not involved in the bulk autophagy. How Atg32 is regulated during mitophagy? Two mechanisms are responsible for the regulation Atg32 activity. Firstly, Atg32 undergoes proteolytic process at its C-terminal in intermembrane space (IMS) upon mitophagy induction, which is mediated by the mitochondrial i-AAA protease Yme1 and essential for effective mitophagy. Depletion of *yme1* blocks Atg32 process and lowers its binding affinity with Atg11, resulting in a mitophagy defect. How the C-terminal processing affects its interaction at N-terminal remains unknown (*Wang, Jin et al. 2013*). Secondly, Atg32 activity is modulated via phosphorylation. Atg32 is phosphorylated at Ser114 and Ser119 residus when mitophagy is induced (for example in nitrogen starvation). Point mutations at both sites that blocks Atg32 phosphorylation prevent its interaction with Atg11 and mitophagy, whereas its binding ability with Atg8 is not impaired. Two kinases, Hog1 and Pbs2, which are involved in HOG signaling pathway, have been identified to be relating to Atg32 phosphorylation. Hog1 is a MAPK and Pbs2 is a MAPKK upstream of Hog1, but neither of them could directly phosphorylate Atg32. Their function is more upstream in mitophagy pathway (*Aoki, Kanki et al. 2011*). Additionally, Kai Mao *et al* found that another MAPK signalling pathway Slt2 also regulates mitophagy, as well as pexophagy (*Mao, Wang et al. 2011*). A following study identified CK2, downstream of Hog1, as the kinase directly phosphorylating Atg32 at Ser114 and Ser119, which is required for the Atg32-Atg11 interaction and mitophagy (*Kanki, Kurihara et al. 2013*).

3.3. Mitophagy in mammals

Since this initial molecular description of mitophagy in yeast, multiple *in vivo* evidences for the physiological importance of mitophagy have been put forward in other organisms. In mammals, there are more different factors and signaling pathways related to mitophagy regulation compared to the situation in yeast. Mitophagy-mediated mitochondrial quality/quantity controls are involved in multi physiological processes, including neurodegeneration diseases, programmed red blood cells maturation, apoptotic cell death,

stress-responses (drug, hypoxia, heat shock, etc...) that challenge mitochondria functioning. The many mitophagy mechanisms that coexist within the cell could be broadly separated as basal, stress-induced and developmentally programmed mitophagies. In the following chapter I summarize mitophagy studies in mammalian system and present these distinct mechanisms.

3.3.1. PINK1/Parkin mediated mitophagy

Parkinson's disease (PD) is a progressive neurodegenerative disorder which is caused by the loss of dopaminergic neurons in the substantia nigra pars compacta. Mutations in Parkin and/or PINK1 are associated with PD involving many mechanisms including compromised mitophagy (*Deas, Wood et al. 2011*).

3.3.1.1. PINK1, a sensor for mitophagy initiation and Parkin activation

PTEN-induced putative kinase 1 (PINK1) serves as a sensor for mitochondrial polarization. In healthy state, PINK1 with an N-terminal mitochondrial targeting sequence is imported into mitochondria through the TOM and TIM complexes. Translocated PINK1 is continuously clipped by the matrix processing peptidase MPP and then cleaved by the intermembrane space protease PARL (PINK1/PGAM5-associated rhomboid-like protease) (*Jin, Lazarou et al. 2010, Deas, Plun-Favreau et al. 2011, Meissner, Lorenz et al. 2011*). The resulting N-terminal cleaved Pink1 is released into the cytosol for further proteasomal degradation (*Yamano and Youle 2013*). While, in damaged mitochondria with a defective inner mitochondrial membrane potential, PINK1 cleavage is compromised, stabilizing PINK1 on the outer mitochondrial membrane (OMM), likely by forming a PINK1 dimer, which is capable of intermolecular phosphorylation, within a protein complex made of Tom subunits (*Okatsu, Uno et al. 2013*). The accumulation of activated PINK1 on OMM causes a series of changes (described as following) that promote mitophagy.

a) PINK1 phosphorylates Ser65 on residual ubiquitin attached to the mitochondria surface to give phosphorylated-ubiquitin (Phospho-Ub). This process is upstream and independent of Parkin (*Kane, Lazarou et al. 2014*). Parkin protein an E3 ubiquitin ligase, which is autoinhibited in cytosol in control condition, is recruited to mitochondria via its direct binding to phosphorylated ubiquitin (*Wauer, Simicek et al. 2015, Kumar, Chaugule et al. 2017*). Parkin binding to phospho-ubiquitin leads to a conformational change which release

the ubiquitin-like domain (Ubl) of Parkin and the phosphorylation site Ser65, whereas the pUb-bound parkin remains autoinhibited (*Gladkova, Maslen et al. 2018*).

b) Next step, PINK1 phosphorylates the E3 ligase Parkin recruited to mitochondria at Ser65 (*Shiba-Fukushima, Imai et al. 2012*), and the phospho-mutants (S65A and S65E) exhibit a delay but not a total inhibition for Parkin translocation. However, the phosphorylation at Ser65 alone is not sufficient for mitochondrial translocation of Parkin, since several well-characterised pathogenic Parkin mutants, that maintain the p-Ser65 in CCCP-induced mitophagy, are not able to complete the translocation. This indicates that other domains are related to Parkin activation. Indeed, the catalytic site of phosphor-Ubl Parkin is still obstructed, and the release needs further interaction between phosphor-Ubl and Parkin core to form a new activating element named ACT. Finally, ACT exposes the catalytic Cys of RING2 domain and enables the access for the binding of E2 ubiquitin ligase. Meanwhile, the repressor element (REP) is also released at this step, and then Parkin is fully activated (*Gladkova, Maslen et al. 2018*). The PINK1 mediated activation of Parkin is shown in **Figure10**.

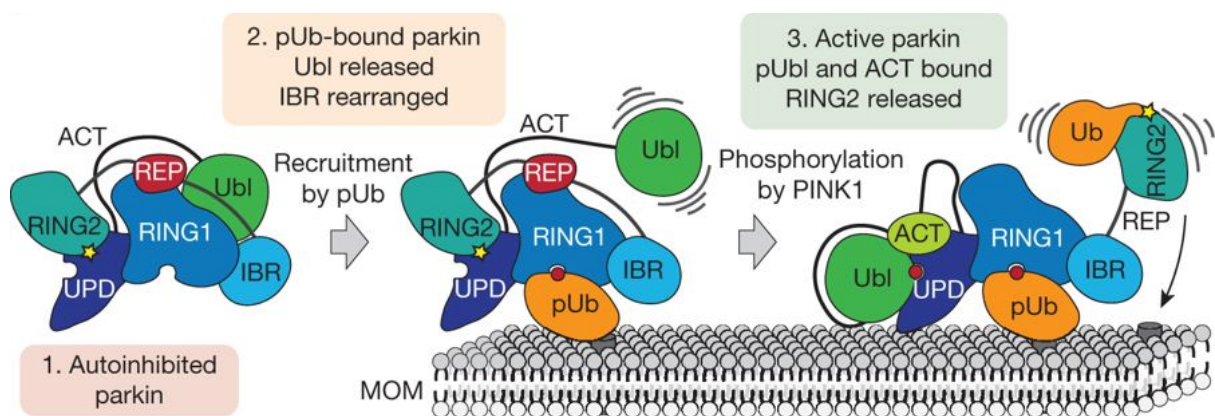


Figure10. The process of Parkin activation Normally, Parkin is autoinhibited by several mechanisms, for example, the active site of the catalytic RING2 domain is impeded by the UPD. Phospho-ubiquitin binding to Parkin release Ubl domain and reposition IBR, leading to the partial activation of Parkin. Release of Ubl enables PINK1 to phosphorylate Parkin at Ubl, resulting in the Ubl domain and ACT element to bind the UPD and thus full activation of Parkin. (*Gladkova, Maslen et al. 2018*).

These conformational changes clearly reveals how Parkin is activated by PINK1 and partly explains why the AR-JP mutants (K211N, K161N) that block the Ubl and UPD binding are

unable to release the Parkin activity by Ser65 phosphorylation (*Corti, Lesage et al. 2011, Pickrell and Youle 2015, Gladkova, Maslen et al. 2018*).

c) Guanosine triphosphatase mitofusin 2 (Mfn2), the mitochondrial fusion factor, is also a substrate of PINK1. Previous studies have suggested that Mfn2 participates in mitophagy downstream of Parkin/PINK1 and is ubiquitinated upon mitophagy induction (*Gegg, Cooper et al. 2010, Rakovic, Grunewald et al. 2011*). A later study demonstrated that PINK1 phosphorylates Mfn2 at Ser442 and Thr111, and the phosphorylated Mfn2 mediates the recruitment of Parkin to damaged mitochondria. In mouse cardiac myocytes, Mfn2 is involved in mitochondrial depolarization and the translocation of Parkin. Thus, Mfn2 functions as a mitochondrial receptor for Parkin and is required for mitophagy. Moreover, Parkin translocation to mitochondria in Mfn2 depleted embryonic fibroblasts (*Chan, Salazar et al. 2011*) indicates one or more compensatory mechanisms for Parkin recruiting. The phosphorylated form of mitofusin can interact with Parkin which probably ubiquitynyle Mfn2 which is then addressed to the proteasome for degradation. This may help to separate damaged organelle because it destabilizes the contact site between ER and mitochondria since Mfn2 is probably a key protein to stabilize the MAMs (*McLelland, Goiran et al. 2018*).

3.3.1.2. Receptors involved in Parkin-mediated mitophagy

Parkin mainly catalyses the Lys-63-linked polyubiquitylation of OMM proteins (*Geisler, Holmstrom et al. 2010*), which leads to their recognition by mitophagy receptors. These proteins could interact both with damaged mitochondria and autophagosome through the direct binding with both ubiquitin and LC3/GABARap via their LIR motif. Therefore, these proteins are able to tether damaged mitochondria, labelled by ubiquitine and polyubiquitin chains to the maturing autophagosome labelled by LC3/GABARAP (*Bjorkoy, Lamark et al. 2005, Pankiv, Clausen et al. 2007*). p62 is one of the most common receptor for selective autophagy. In Parkin-mediated mitophagy, p62 is rapidly recruited to mitochondria after parkin-directed ubiquitylation. The *p62^{-/-}* mouse embryonic fibroblasts (MEFs) exhibit no defect in Parkin translocation, suggesting that p62 acts downstream of Parkin/PINK1. Casein kinase 2 (CK2) directly phosphorylates p62 at serine 403 on its ubiquitin-associated (UBA) domain to increase its affinity to polyubiquitin chain. This phosphorylation triggers more efficiently targeting of polyubiquitinated proteins into “sequestosomes” for autophagosome entry (*Matsumoto, Wada et al. 2011*). However, the

p62 deficient mouse embryonic fibroblasts (MEFs) fail to form mitochondrial clusters at the perinuclear region, which are observed in WT and transported through microtubules. This mitochondrial clustering requires full Parkin activity, and Parkin mutants that abolish its E3 ligase activity after translocation, prevent the clustering. *p62* mediates the aggregation by polymerizing at its PB1 domain. Whereas the pathophysiological significance of the mitochondrial clustering is unclear, since the *p62*^{-/-} cell that blocks this process appears to be unimpeded for mitochondrial degradation (Narendra, Kane et al. 2010, Okatsu, Saisho et al. 2010).

Optineurin (OPTN) is another receptor acting downstream of Parkin during mitophagy process. Mutations in this gene lead to primary open-angle glaucoma (Rezaie, Child et al. 2002) and amyotrophic lateral sclerosis (ALS) (Maruyama, Morino et al. 2010), two neurodegenerative diseases in which mitochondrial defects have been observed. In CCCP-induced mitophagy, optineurin is recruited to parkin-ubiquitinated mitochondria and stabilized via Ub binding, and subsequently binds to LC3 via its LIR domain for the assembly of autophagosome around damaged mitochondria. The ALS-associated OPTN-E478G mutant, which blocks its binding ability with ubiquitin, disrupts the autophagosome formation. Parkin is also required for the recruitment and stabilization of OPTN to damaged mitochondria. Of note, in HeLa cells, where the parkin expression is undetectable, optineurin could still translocate to damaged mitochondria but in a specific and transient way (Wong and Holzbaur 2014).

The Parkin-mediated recruitment of optineurin and *p62* are independent of each other. It seems that *p62* preferentially locates to domains between adjacent mitochondria to regulate mitochondrial clustering rather than to recruit LC3 during Parkin-mediated mitophagy. By contrast, optineurin is recruited all over the damaged mitochondria and acts as a receptor in LC3 recruitment that is necessary for efficient mitochondrial degradation during CCCP-induced and Parkin-mediated mitophagy (Wong and Holzbaur 2014). This explains why LC3 can still be recruited and mitochondrial clearance is not altered in absence of *p62* during Parkin-mediated mitophagy.

Although the PINK–PARKIN pathway is the key mechanism involved in stress induced mitophagy, more specifically to help cells to deal with mitochondria uncoupler such as FCCCP (Jin, Lazarou et al. 2010), it is not related to hypoxia-induced mitophagy. Literature indicates that Parkin knockdown does not prevent mitochondrial degradation and LC3-I to II

transition in response to hypoxia (Wu, Lin et al. 2016). Instead, FUNDC1 is the key receptor in hypoxia-induced mitophagy.

3.3.2. FUNDC1 mediated mitophagy

The human FUNDC1 (FUN14 domain containing 1) protein contains three transmembrane domains that anchor this protein exclusively to the OMM. Its N-terminus faces cytosol while the carboxy terminus stretches into the mitochondrial intermembrane space. There is a LIR region at the amino terminus of this protein and FUNDC1 has been identified as mitophagy receptor (Liu, Feng et al. 2012). In hypoxia treated HeLa cells, the *FUNDC1*-knockdown markedly prevents hypoxia induced mitochondrial fragmentation and degradation, however, neither the conversion of LC3-II nor the number of GFP-LC3 puncta is impaired, suggesting FUNDC1 mediates highly selective mitochondrial clearance under hypoxic conditions. It seems that the roles of FUNDC1 in mitophagy are distinct due to the different inductions. For instance, it was further confirmed that in starvation condition, *FUNDC1* knockdown has neither effect on general autophagy nor in mitophagy. The uncoupler, carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) induced mitophagy only partially requires FUNDC1, while the FCCP-induced mitochondrial network morphology alteration is FUNDC1-independent (Liu, Feng et al. 2012).

FUNDC1 mediates its mitophagy receptor function mainly via its LIR motif (Y(18)xxL(21)). In hypoxia condition the impaired mitophagy by *FUNDC1* knockdown could be rescued by WT FUNDC1 expression but not the LC3-interaction-deficient FUNDC1 mutants. The overexpression of WT FUNDC1 is sufficient to recruit GFP-LC3 puncta and induce mitochondrial degradation via autophagy (Liu, Feng et al. 2012).

Moreover, FUNDC1 is responsible for mitochondria dynamic regulation through interaction with both the fission protein DNM1L/DRP1 and the fusion OPA1 protein. DRP-1 is recruited by FUNDC1 to the damaged mitochondria, which is essential for mitochondrial fission and subsequent degradation (Chen, Chen et al. 2016). Hypoxia condition relocates FUNDC1, which interact with the inner membrane fusion protein OPA1, to the ER mitochondria contact sites where it interacts with the ER protein Calnexin. This may help to stabilize contact sites between mitochondria and ER to promote any fission process. Moreover, FUNDC1 is capable to bind Drp1, the key mitochondrial fission protein (Wu, Lin et al. 2016). How FUNDC1 cooperates with other proteins at the ER-mitochondria contact sites for

mitochondrial dynamic regulation will be further discussed on the [chapter 4.3.1 DRP1-dependent mitophagy](#).

Taken together, FUNDC1 seems to coordinate two mechanisms: (1) mitophagy. As a mitophagy receptor, FUNDC1 could bind to LC3 and thus recruit autophagosomes; (2) mitochondrial fragmentation. FUNDC1 is accumulated at ER-mitochondria contact sites for DRP-1 dependent mitochondrial fission, which is required for mitochondrial degradation.

3.3.2.1. Regulation of FUNDC1 by phosphorylation

The phosphorylation status of FUNDC1 is critical for its activity. The activity of FUNDC1 is regulated through phosphorylation/dephosphorylation reactions at Ser13 which is near to the LIR motif. In healthy mitochondria, FUNDC1 Ser13 is kept in a phosphorylated state by kinase CK2, which could be blocked by the selective CK2 inhibitor Tetrabromobenzotriazole (TBBt) (*Zien, Duncan et al. 2005*). Upon hypoxia or FCCP treatment, the phosphoryl group at Ser13 is removed by PGAM5 (phosphoglycerate mutase family member 5), a mitochondrial-localized Ser/Thr phosphatase. The FUNDC1 Ser13 dephosphorylation promotes FUNDC1 binding affinity with LC3, while the Ser13A mutant that prevents its phosphorylation results in a greater ability to increase autophagy flux. In addition, PGAM5 knockdown or phosphatase-dead mutant (H105A) dramatically reduces the turnover of LC3-II and GFP-LC3 puncta, as well as mitochondria degradation (*Chen, Han et al. 2014*).

FUNDC1 is not only phosphorylated at Ser13 by CK2 but also at Tyr18 by Src kinase (*Liu, Feng et al. 2012*). Both phosphorylations prevent its interaction with LC3. The two sites are phosphorylated by distinct mechanisms but function cooperatively to regulate mitophagy, and only the combined inhibition of both CK2 and Src could lead to mitochondrial protein degradation and formation of mitoautophagosomes (*Chen, Han et al. 2014*). Another key amino-acid, Ser17, is also involved in FUNDC1 activity regulation. Hypoxia and mitochondrial uncouplers could elevate ULK1 and promote its translocation to damaged mitochondria, where it binds FUNDC1 and phosphorylates FUNDC1 Ser-17 to enhance its binding affinity with LC3 (*Wu, Lin et al. 2016*). Therefore, these results suggest that FUNDC1 is an ULK1 adaptor, one key component of the basic autophagy molecular machinery.

Both BCL2 and BCL2L1, are antiapoptotic proteins that sequester the proapoptotic proteins BAX and BAK1 to prevent the mitochondrial outer membrane permeabilization for cytochrome c release and subsequent apoptosis (*Cheng, Wei et al. 2001*). BCL2, BCL2L1

(which contains BH4 domain) and BAX, BAK1 (which contain 3 BH domains: BH1, BH2, and BH3) belong to BCL2-family proteins (*Adams and Cory 1998*). In addition, BCL2L1, but not BCL2, could suppress PGAM5 by direct binding, to prevent FUNDC1 dephosphorylation of at Ser13 and subsequent mitophagy under hypoxia or FCCP treatment (*Wu, Xue et al. 2014*).

The anti-apoptotic proteins BCL2 and BCL2L1/Bcl-X(L) bind and inhibit Beclin-1 and BNIP3L, both putative BH3-only proteins, to prevent starvation-induced autophagy or apoptosis (*Oberstein, Jeffrey et al. 2007*). Although Beclin1 has been proved to function in starvation-induced autophagy, it does not affect FUNDC1-induced mitophagy.

3.3.2.2. The ubiquitin regulation of FUNDC1

As a mitophagy receptor, FUNDC1 is degraded along with mitochondria, however, evidences showed that the degradation of FUNDC1 could be largely prevented by proteasome inhibitor, MG132, suggesting that FUNDC1 protein level is also regulated by the proteasomal pathway. Mitochondrial E3 ligase MARCH5 (membrane-associated ring finger (C3HC4) 5) could modulate mitochondrial morphology by targeting MiD49, a mitochondrial receptor of Drp1 (*Yonashiro, Ishido et al. 2006*). March5 is also involved in the proteasomal degradation of FUNDC1. In hypoxia condition, MARCH5 mediates FUNDC1 ubiquitylation at lysine 119 site to triggers it to proteasome, before mitophagy induction. MARCH5 mediated FUNDC1 degradation fine-tunes the hypoxia-induced mitophagy. Knockdown of *MARCH5* enhances mitochondrial sensitivity against stresses and leads to an abundant mitophagy process (*Chen, Liu et al. 2017*).

Additional levels of FUNDC1 regulation have been discovered. One study indicates that the micro RNA miR-137 decreases the FUNDC1 expression and therefore the hypoxia-induced mitophagy level (*Li, Zhang et al. 2014*).

3.3.3. NIX (BNIP3L) mediated mitophagy

In most mammals, the maturation of red blood cells (erythrocyte) from enucleated immature red blood cells named reticulocytes, termed reticulocyte maturation, necessitates a complete mitochondria clearance via a mitophagy pathway (*Kundu, Lindsten et al. 2008, Mortensen, Ferguson et al. 2010*) depending on a specific mitophagy receptor: the BCL2-related protein NIX (BNIP3L). This is a very important physiological process since *Nix*^{-/-} homozygote mouse, accumulates immature erythroid cells that still contain mitochondria,

causing anemia, reticulocytosis, and erythroid-myeloid hyperplasia (*Diwan, Koesters et al. 2007, Schweers, Zhang et al. 2007*).

The commonly known function of BCL2-related proteins is regulating programmed cell death. NIX/BNIP3L contains a single BH3 domain that suppresses the function of anti-apoptotic Bcl-2 proteins (Danial NN, Korsmeyer SJ, 2004, Cell). Under death-inducing stimuli, BH3 domain on NIX/BNIP3L is spurred to further activate BAX and BAK and on the other hand, inhibit anti-apoptotic BCL2-related proteins, in turn leading to cytochrome *c* release, caspase activation, and apoptosis (*Jurgensmeier, Xie et al. 1998*).

Whereas the role of NIX/BNIP3L for mitochondrial clearance during reticulocyte maturation does not depend on proapoptotic pathway, since BAX, BAK, or other proapoptotic protein are not required (*Schweers, Zhang et al. 2007*). Instead, NIX/BNIP3L is a potential autophagy receptor, which contains a LIR motif (WXXL-like motif) at the N-terminal to directly interact with LC3 on the OMM (*Novak, Kirkin et al. 2010, Hanna, Quinsay et al. 2012, Zhu, Massen et al. 2013*). Although, NIX is not required for the induction of autophagy, and it is mandatory for mitochondrial incorporation into autophagosomes and autophagosome maturation during terminal erythroid differentiation (*Schweers, Zhang et al. 2007*). Interestingly the NIX-dependent mitophagy is Atg5 and Atg7 independent but the Rheb small GTPase could be involved in the NIX-dependent mitophagy mechanism

3.3.4. Other mitophagy mediators

I have illustrated the widely-studied mitophagy mediators in mammals: PINK1/Parkin, FUNDC1 and NIX. There are additional mitophagy receptors. The IMM protein Prohibitin2 (PHB2) is a novel mitophagy receptor, has already described in allophagy section. Meanwhile, researchers also revealed other proteins regulating mitophagy. These protein functions are described in the following chapters.

3.3.4.1. NDP52 and TBK1 cooperate during mitophagy

NDP52 (nuclear dot protein 52), which has been originally described as a myosin VI binding partner, is also a selective autophagic receptor for the recognition of cytosol invading pathogens during xenophagy, (*Mostowy, Sancho-Shimizu et al. 2011, Wong and Holzbaur 2014*). During xenophagy, NDP52 teams up with OPTN and p62 to facilitate the removal of cytosol-invading bacteria (*Mostowy, Sancho-Shimizu et al. 2011, Thurston, Wandel et al.*

2012). Moreover, NDP52 has been suggested to be also involved in PINK1- PARKIN mediated mitophagy. Heo et al. (2015) reported that depolarized mitochondria recruit poly-UB chains on OMM depending on PINK-1-PARKIN pathway, which could further promote the translocation of various mitophagy receptors including OPTN and NDP52. At this step, the kinase TBK1 (TANK-Binding Kinase 1) is also translocated to OMM and activated via the phosphorylation at Ser172, which requires OPTN and NDP52. The activated TBK1 in turn cooperates with PINK1 to promote phosphorylation of autophagy adaptors/receptors, and thus stabilizes their binding to either poly-UB chains or LC3B, which is essential for efficient degradation of depolarized mitochondria via selective autophagy (Heo, Ordureau et al. 2015).

3.3.4.2. MUL1, an OMM E3 ligase

Although, Parkin is the main E3 ubiquitin ligase involved in stress-induced mitophagy, there are other ubiquitin ligase participating in mitophagy such as Gp78 (Fu, St-Pierre et al. 2013), SMURF1, SIAH1, ARIH1 and MUL1.

Sodium selenite is an inducer of mitochondrial ROS and membrane potential loss, and low dose of sodium selenite stimulates efficient mitophagy (Huang, Nie et al. 2009) whereas high dose treatment leads to apoptosis. It is striking that the depletion of several well-established E3 ligases related to mitophagy has no effect on selenite-induced mitophagy, such as Parkin, MARCH5. Instead, siRNA against *MUL1*, which encodes an OMM E3 ubiquitin ligase, could inhibit LC3 puncta formation and mitochondrial proteins degradation. However, the involvement of MUL1 is highly specific for selenite-induced mitophagy, since it does not take part in FCCP, amino acid starvation or hypoxia-induced mitophagy/autophagy (Li, Qi et al. 2015).

Similarly to FCCP- or hypoxia-induced mitophagy, ULK1 translocates to mitochondria in selenite treated cells and acts as a substrate for MUL1 to be polyubiquitinated, which triggers its degradation by both proteasome and autolysosomes. The link between MUL1-mediated mitophagy and ULK1 degradation is not clear yet. As ULK1 is a key factor for the initiation of autophagosomes, how does it regulate mitochondria degradation during mitophagy is of great interest. It is proposed that the high level of ROS within the intermembrane space induced by selenite stimulates the ULK1 translocation to mitochondria for mitophagy initiation (Li, Qi et al. 2015). In addition, it has been reported in

mice that PARKIN and MUL1 play redundant roles in elimination of paternal mitochondria (Rojansky, Cha et al. 2016).

3.3.4.3. Bcl2L13/Bcl-Rambo

Kinya Otsu group used an UniProt database (<http://www.uniprot.org/>) screening and identified Bcl-2-like protein 13 (Bcl2-L-13) as the first Atg32 functional homologue in mammals. This homologue shares the following molecular features with the yeast Atg32: mitochondrial localization; it contains a LIR motif (WXXL/I motif); acidic amino acid clusters; and single membrane-spanning topology (Murakawa, Yamaguchi et al. 2015). Bcl2-L-13 could partially rescue the mitophagy defect in *atg32Δ* yeast at stationary phase. As a Bcl-2 family protein, Bcl2-L-13 (Bcl-Rambo) contains four BH motifs, however, it induces apoptotic signal via its C-terminal transmembrane domain rather than the BH motifs (Kataoka, Holler et al. 2001).

Bcl2-L-13 contains a functional LC3-interacting region (LIR) WXXL/I motif at the residues 273–276, on the other hand, its BH domains are important for mitochondrial fragmentation. CCCP promotes the interaction of Bcl2-L-13 with LC3 and mitochondrial fission. When mitochondrial fragmentation is induced, the Ser272 close to LIR is phosphorylated via an unknown kinase. This phosphorylation improves its binding with LC3 and subsequent autophagic machinery recruitment. Moreover, this Bcl2-L-13 mediated mitophagy does not require Parkin nor ubiquitination (Murakawa, Yamaguchi et al. 2015), but is dependent on ULK1 complex (Murakawa, Okamoto et al. 2019).

3.3.5. Lipid-mediated mitophagy

In addition to autophagy/mitophagy receptor proteins, mitophagy selectivity could also be achieved via lipid receptor: ceramide and cardiolipin (Sentelle, Senkal et al. 2012). Ceramide is a bioactive sphingolipid that mediates cell death (Ogretmen and Hannun 2004). More recently, it has been revealed that C18-pyridinium ceramide mediates lethal mitophagy and subsequent tumor suppression in human cancer cells. Through either exogenous addition or endogenous generation by ceramide synthase 1 (CerS1), C18-pyridinium ceramide, which exclusively localizes at the OMM, binds to the lipidated LC3B (LC3B-II) and recruits autophagosome to mitochondria. LC3B-II recruitment depends on Drp-1-mediated mitochondrial fission, since the knockdown of Drp-1 prevents the OMM localization of

ceramide and fails to undergo mitophagy (*Sentelle, Senkal et al. 2012*). Of note, the ceramide-mediated mitophagy is independent on apoptotic activity.

Alternatively, cardiolipin, located at the inner mitochondrial membrane (IMM), serves as an important defensive function for the elimination of damaged mitochondria in both primary and transformed neuronal cells (*Chu, Ji et al. 2013*). In healthy mitochondria, cardiolipin is restricted to IMM for supporting cristae, stabilizing respiratory chain complexes (*Beyer and Nuscher 1996*), and modulating autophagy and immunity functions (*Singh, Ornatowski et al. 2010*). In rotenone, staurosporine and 6-hydroxydopamine stimulated mitophagy, cardiolipin translocates to the OMM surface and interacts with LC3. The knockdown of cardiolipin synthase or phospholipid scramblase-3, which transports cardiolipin from IMM to the OMM, impairs the delivery of mitochondria to autophagosomes (*Chu, Ji et al. 2013*). Clearance of cardiolipin-exposed mitochondria by mitophagy probably helps to prevent cardiolipin oxidation and accumulation of pro-apoptotic signals (*Kagan, Tyurin et al. 2005, Chu, Ji et al. 2013*).

3.3.6. Mitophagy related diseases

Mitophagy is a key mechanism involved in mitochondrial quality and quantity controls and consequently its defect, is associated with many human diseases, including diabetes, cardiomyopathy, cancer and neurodegenerative diseases (*Redmann, Dodson et al. 2014*). Here I choose to describe the link between mitophagy and neurodegenerative diseases, with particular emphasize on Parkinson's disease.

Neurodegenerative diseases are a complex group of pathologies characterised by the progressive loss of neurons and/or neuronal functions, leading to severe cognitive inabilities, locomotor deficiency, and ultimately death in affected patients. The well-known neurodegenerative diseases, Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and Amyotrophic Lateral Sclerosis (ALS), share, at the cell level, some pathology features such as progressive accumulation of specific protein aggregates within the cell indicating that these pathologies could be due to proteostasis imbalance (*Rodolfo, Campello et al. 2018*).

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease with an occurrence of 1%–2% population worldwide (*de Rijk and Bijl 1998*). This disease is characterized by a progressive and selective elimination of dopamine neuron from the pars

compacta of substantia nigra. Early in 1990s, it had been reported that the autosomal recessive PD is associated with the presence of mutations in the PARK2 and PARK6 genes, coding for the Parkin and PINK1 proteins respectively (*Kitada, Asakawa et al. 1998, Lucking and Brice 2000*). Early in 1983, a medical case study found that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) administration in human lead to parkinsonism symptom. Later on additional studies indicate that MPTP is transformed by astrocytes monoamine oxidase in MPP⁺ which is then specifically taken up and concentrated in dopaminergic neurons. Afterward, different studies indicate that MPP⁺ inhibits the mitochondrial OXPHOS complex I and definitively linking Parkinson's disease and mitochondria function (*Langston 2017*).

Then, R. J. Youle and other researchers revealed that cytosolic Parkin is recruited by PINK1 to depolarized mitochondria to mediate mitophagy (*Narendra, Tanaka et al. 2008, Matsuda and Tanaka 2010, Vives-Bauza, Tocilescu et al. 2010*). Altogether these results establish a general accepted conclusion that mutations of mitophagy genes that lead to mitophagy deficiency are associated with PD. On the other hand, augmenting mitophagy by activating the PINK1/Parkin pathway is an attractive target for therapeutic intervention for PD and a variety of maternally inherited mitochondrial diseases (*Pickrell and Youle 2015*).

3.4. Mitophagy in *C. elegans*

Mitochondrial function is vital for cellular homeostasis and organism health, and dysfunctional mitochondria, which could trigger cell apoptosis, inflammation and senescence (*Fang, Waltz et al. 2017*), are considered as toxic for cells. Mitochondria homeostasis is dependent on mechanisms that on one hand to promote biogenesis i.e. import of proteins, lipids and mitDNA replication and on the other hand are capable of removing damaged mitochondria or piece of damaged mitochondria: i.e. mitophagy, endosomal microautophagy. Mitochondrial quality and quantity control via autophagy pathway is required for worm healthy aging and lifespan maintenance.

C. elegans has emerged as a model animal for the study of mitophagy within the last ten years. The first evidence of mitophagy in *C. elegans* appears during paternal mitochondrial degradation after fertilization, and this process, named allophagy has been discussed on the [section 2.2.1 Allophagy](#). Besides, mitophagy in *C. elegans* is also associated with physiological processes of stress resistance, aging and neurodegenerative diseases, which have been reported by several studies.

3.4.1. Basal or induced mitophagy affects worm lifespan

A systematic RNAi screen demonstrated that inactivation of genes encoding mitochondrial proteins affect *C. elegans* longevity. To researchers' surprise, mutants with impaired mitochondrial function show an extended lifespan, which is accompanied by a lower ATP content and decrease oxygen consumption (Lee, Lee et al. 2003). They hypothesize that these mitochondrial mutants live longer because they produce less ROS (Rapid Oxygen Species). The major ROS site productions are the mitochondrial OXPHOS Complex I and III. Consequently a reduced mitochondrial activity should lead to ROS decrease production and worm exposition to this deteriorous molecules. However, it is not able to completely count for the mechanism by which the impaired mitochondria in mutant worms promote longevity. Whereas a recent study demonstrates that mitochondrial permeability uncouples elevates autophagy and extended *C. elegans* lifespan. In addition, the mitochondrial defect associated with lifespan extension is not totally dependent on insulin-like pathway (Lee, Lee et al. 2003). In another publication, *clk-1*, which is defective in ubiquinone biosynthesis, is also long-lived (Lakowski and Hekimi 1996). These data suggest that while the severe mitochondrial dysfunction is lethal, partial loss of mitochondrial function could be beneficial (Antebi 2007).

3.4.1.1. DCT-1 mediated mitophagy in aging and stress conditions

Tavernarakis group has shown that during *C. elegans* aging, mitochondrial content gradually increases. This phenotype is further enhanced in *bec-1*, *pdr-1/Parkin* and *pink-1/Pink1* RNAi; demonstrating mitochondria are partly removed via autophagy during aging. This is probably another case of programmed mitophagy. Somehow, during life cycle mitophagy accompanied aging. And the morphology of accumulated mitochondria in mitophagy deficient worms is mainly fragmented, different from that in WT worms. More importantly, they identified DCT-1 to be the *C. elegans* NIX/BNIP3L homologue, which plays a crucial role as a mitophagy receptor, during aging. Knockdown of *dct-1* also exhibits increased mitochondrial mass and abnormal mitochondrial morphology, observed in *pdr-1* (RNAi) and *pink-1* (RNAi) mutants (Palikaras, Lionaki et al. 2015).

DCT-1 (DAF-16/FOXO Controlled, germline Tumour affecting-1) is localized at the OMM, and contains a LIR motif (WXXL) which mediates the interaction with the *C. elegans*

Atg8/LC3 homologue LGG-1 (Palikaras, Lionaki et al. 2015). DCT-1 expression is ubiquitously expressed in somatic cells throughout the development. Its expression is partially controlled by DAF-16, which is activated in low insulin/IGF-1 signalling (Oh, Mukhopadhyay et al. 2006, Pinkston-Gosse and Kenyon 2007).

The lifespan extension in *daf-2* mutant is reduced in either *dct-1*, *pdr-1* or *pink-1*, indicating that mitophagy participates to the extended lifespan of *daf-2*. In addition, longevity which is induced by moderate mitochondrial dysfunction (*clk-1* and *isp-1*) is also dependent on DCT-1 and PINK-1.

These authors hypothesize that dysfunctional mitochondria accumulation in mitophagy mutants diminishes worm resistance during aging. To confirm this hypothesis, mitophagy defective worms were challenged by a series of mitochondrial stressors: the uncoupler FCCP/CCCP, ultraviolet, paraquat, starvation and heat shock. As expected, the defective mitophagy worms with impaired mitochondrial function are more sensitive to these stress inducers (Palikaras, Lionaki et al. 2015).

To further explore the *C. elegans* mitophagy mechanism, the authors constructed genetic tools to directly visualize mitophagy events within *C. elegans* muscle cells. They took advantage of employing the transgenic worms expressing both DsRed::LGG-1 and mitoGFP in muscle cells, labelling autophagic structures and mitochondria respectively, to trace mitophagy events by mitophagy inducers. Treatment with CCCP, paraquat or heat shock triggers both mitochondrial fragmentation and LGG-1 puncta. Moreover, the majority of LGG-1 puncta are colocalized with mitochondria, which indicates mitophagy events. Muscle-specific expressed mitoRosella that consists of a pH-sensitive GFP and a pH-insensitive DsRed, could indicate mitophagy stimulation by the GFP/DsRed ratio reduction due to the quenching of GFP in acidic autolysosome condition (Rosado, Mijaljica et al. 2008). In either *pdr-1/pink-1* or *dct-1* depletion worms, the LGG-1 puncta are almost completely inhibited and mitochondrial degradation is impaired. Taken together, DCT-1, as well as PDR-1 and PINK-1, are essential genes for mitophagy events during aging and in stress conditions in *C. elegans* (Palikaras, Lionaki et al. 2015).

What are the molecular links between DCT-1, PDR-1 and PINK-1 in *C. elegans* (Figure 11)? Researchers found that ubiquitylation of the DCT-1 Lys26 increased upon mitophagy induction, and is PDR-1 and PINK1 dependent.

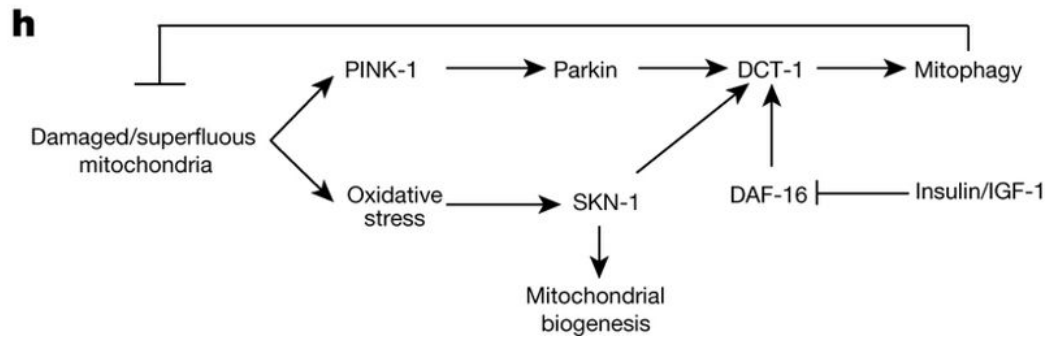


Figure 11. Cooperated pathways of mitochondrial biogenesis and mitophagy Under stress condition, PINK1/Parkin pathway mediates the activation of DCT-1, which is the mitophagy receptor required for selective elimination of damaged mitochondria. On the other hand, the oxidative stress within mitochondria triggers SKN-1 and subsequent mitochondrial biogenesis. The DCT-1 production is also elevated by the transcriptional factor SKN-1 to increase mitophagy. The insulin/IGF-1 signalling pathway negatively controls the expression of *dct-1* through DAF-16. (Palikaras, Lionaki et al. 2015).

This study also indicates that, SKN-1 regulates mitophagy process via regulating *dct-1* expression (Palikaras, Lionaki et al. 2015). SKN-1 is the mammalian NRF2/NFE2L2 homologue, which is activated under oxidative stress conditions to mediate the expression of downstream genes including mitochondrial associated genes (Zarse, Schmeisser et al. 2012, Ghose, Park et al. 2013). In *C. elegans*, SKN-1 promotes mitochondrial biogenesis via up-regulating several mitochondria genes. In normal condition, *skn-1(RNAi)* leads to a mutant phenotype characterized by modification of mitochondrial morphology, increased cytoplasmic Ca^{2+} and mitochondrial membrane depolarization, which may be due to the decreased expression of *dct-1*. SKN-1 depletion blocks DCT-1 mediated mitophagy induced in *daf-2* mutant or by various stressors (Palikaras, Lionaki et al. 2015).

In summary, when mitophagy is induced, the oxidative stress produced by damaged mitochondria activates SKN-1, which acts as a transcriptional factor mediating the expression of not only *dct-1* to induce mitophagy, but also mitochondrial genes for mitochondrial biogenesis, forming a loop for mitochondrial homeostasis and integrity (Palikaras, Lionaki et al. 2015). **Figure 11** indicates how mitophagy and mitochondrial biogenesis are coupled by SKN-1.

3.4.1.2. Tomatidine induced mitophagy

A recent study revealed the beneficial effect of tomatidine in *C. elegans* lifespan extension is from enhanced mitochondrial homeostasis. Tomatidine, a metabolite of tomatidine, is enriched in unripe tomatoes and has been proved to have beneficial effects in mouse cells for anti-inflammatory (Chiu and Lin 2008), anti-tumorigenic and lowering lipid functions (Fujiwara, Kiyota et al. 2012). In mouse skeletal muscle cells, tomatidine promotes muscle fitness partially through increasing mitochondrial mass (Dyle, Ebert et al. 2014).

Tomatidine can induce moderate ROS, which in turn activates SKN-1/Nrf-2 pathway. The tomatidine induced SKN-1 activation also promotes PINK-1/DCT-1-dependent mitophagy and mitochondrial biogenesis to keep mitochondria healthy. Tomatidine treatment extends worm lifespan, as well as healthspan scored by pharyngeal pumping and swimming movement. The declined swimming movement in aged worms is somehow extended through tomatidine treatment, indicating a rescued mitochondrial function. Pharynx is the important tissue for food intake in *C. elegans*, and the pharyngeal pumping activity is another indicator for worm aging state. Consistently, the subdued pumping during aging is also attenuated upon tomatidine treatment (Fang, Waltz et al. 2017).

This study confirmed the SKN-1 mediated mitochondrial homeostasis via mitophagy and mitochondrial biogenesis is beneficial for *C. elegans* longevity (Fang, Waltz et al. 2017). The small molecules, tomatidine, may provide new approaches to improve healthy aging by activating SKN-1 pathway.

3.4.1.3. Urolithin A induced mitophagy

Urolithin A (UA) is the first identified natural compound that induces mitophagy from *C. elegans* to rat and cultured mammalian cells. Similar to tomatidine, UA can extend lifespan and improve worm fitness depending on mitochondrial function. In young adult (Day1), UA treatment reduces mitochondrial content, accompanied by more fragmented mitochondrial morphology, which depends on mitophagy genes. Likewise, knock down of mitophagy genes (*pink-1*, *dct-1*, *skn-1*) or autophagy genes (*bec-1*, *sqst-1*, *vps-34*) inhibits the lifespan extension following UA exposure. Indeed, UA treatment induces autophagy, which is detected by the increased GFP::LGG-1 puncta in *C. elegans*. In old worms (Day8), UA exposure has no or slight effect on mitochondrial content, whereas results in a higher transcription of respiratory chain subunits, compared with vehicle exposure. It suggests that during the long-term UA treatment, mitochondrial biogenesis is facilitated following the

initial mitophagy induction. The enhanced mitochondrial biogenesis improves mitochondrial function through stabilizing ATP content and increasing the respiration capacity, and therefore leads to prolonged activities of mobility and pharyngeal pumping (*Ryu, Mouchiroud et al. 2016*). It is highly possible that UA induced mitophagy and lifespan extension share the same pathway as tomatidine treatment.

3.4.1.4. Iron-starvation-induced mitophagy

Iron is an essential element for activation of many proteins and enzymes, and the deprivation of iron is often associated with mitochondrial disorder and therefore diseases (*Schiavi, Maglioni et al. 2015*). For instance, severe absence of frataxin, which is a mitochondrial protein encoded by nuclei and regulates the biogenesis of iron-sulfur-cluster (ISC)-containing proteins, results in dysfunction of respiratory chain and pathology in human disease-Friedreich's ataxia (FRDA). Nevertheless, the moderate depletion of frataxin homologue in *C. elegans*, *FRH-1*, has shown to induce beneficial responses for worm longevity (*Ventura and Rea 2007*).

frh-1 RNAi induces lifespan extension depending on specific mitophagy regulators of PDR-1 (homologue of Parkin in *C. elegans*) and PINK-1, since knockdown either of them by RNAi significantly inhibits the frataxin-regulated longevity, whereas the *pink-1* mutant has no effect. The new identified mitophagy receptor, DCT-1, as well as the p62 homologue SQST-1, is also required for *frh-1* RNAi induced lifespan extension. These data highlight that mitophagy may be induced by *frh-1* RNAi and responsible for the lifespan extension. Mitophagosomes that are double positive for DsRed::LGG-1 and mitoGFP, achieved by tools exploited by Tavernarakis' lab, are increased in *frh-1* RNAi worms compared with control (*Palikaras, Lionaki et al. 2015*). (*Schiavi, Maglioni et al. 2015*). Electron microscopic analysis revealed accumulation of electron-dense granules that represent iron deposits within mitochondrial matrix in *frh-1* RNAi worms, which is consistent with results in mouse (*Puccio, Simon et al. 2001*).

frh-1 RNAi mimics a hypoxia state through cytosolic iron deprivation, which activates the hypoxia-inducible factors (HIF), HIF-1 and AHA-1, to modulate longevity (*Zhang, Shao et al. 2009, Schiavi, Maglioni et al. 2015*). Moreover, *frh-1* RNAi can also upregulate the expression of *dct-1* and a series of globin genes, while downregulate the expression of SQST-1. Globin genes encode iron-containing proteins that regulate the cellular redox status in

response to hypoxia (Hoogewijs, Terwilliger et al. 2007). Iron deprivation by feeding a given dose of iron chelator 2,2'-dipyridyl (BP) leads to a mitophagy-dependent longevity, similar to the knockdown of *frh-1*, and the iron deprivation induced mitophagy is not further increased in the background of *frh-1* RNAi, suggesting that the iron deprivation-mediated longevity via is to some degree overlapped with the one mediated by fraxaxin deficiency. However, *frh-1* RNAi induced longevity also relies on EFG-1, HIF-1 prolyl-hydroxylase, and some globins, which are not involved in iron deprivation condition.

Interestingly, the iron-starvation induced mitophagy and therefore lifespan extension are conserved from *C. elegans* to mouse (Ventura and Rea 2007, Schiavi, Maglioni et al. 2015). It suggests that the link between mitophagy and longevity may be at least partially conserved from *C. elegans* to mammals.

3.4.2. The role of mitophagy in neurodegenerative diseases

Mitochondrial quality control via mitophagy has been well proved to associate with neurodegenerative diseases in mammalian systems. In *C. elegans*, autophagy is beneficial to relieve neurodegeneration. Although several neurodegeneration models have been established in *C. elegans*, there are few studies exploring the link of mitophagy and neurodegeneration in *C. elegans*.

A recent paper provided the first evidence for mitophagy's involvement in neurodegenerative diseases in *C. elegans* (Fang, Hou et al. 2019). Taking advantages of the already published papers about mitophagy in *C. elegans*, mitophagy events could be detected in neuron expressing mtRosella or DCT-1::GFP; DsRed::LGG-1 (see the principles on chapter 3.4.1). Upon mitophagy induction by urolithin A (UA), actinonin (AC) or nicotinamide mononucleotide (NMN), the GFP/DsRed fluorescent intensity ratio of mtRosella is decreased, indicating a mitophagy flux. In addition, colocalization between the mitophagy receptor DCT-1 and autophagy marker LGG-1 is increased in DCT-1::GFP; DsRed::LGG-1 transgenic worms. These data indicate that mitophagy is induced in neurons. Moreover, mitophagy in neurons depends on *dct-1*, *pink-1* and *pdr-1*, the mitophagy mediators identified in muscle of *C. elegans*. By contrast, general macroautophagy is not induced. In conclusion, selective mitophagy is stimulated in neurons by UA, AC or NMN. More importantly, the facilitated mitophagy in neuron is beneficial in both two Alzheimer's disease models.

One of the Alzheimer's disease models has been established in transgenic worms by expressing pan-neuronal human A β ₁₋₄₂ (CL2355) protein, and applied for drug screening in *C. elegans* (Fatouros, Pir et al. 2012, Lublin and Link 2013). The *C. elegans* AD model is accompanied by severe cognitive defects, which is evaluated by an aversive olfactory learning chemotaxis assay (Voglis and Tavernarakis 2008). Pharmacological stimulated mitophagy by UA, AC or NMN could ameliorate the cognitive defects in A β ₁₋₄₂ *C. elegans*, and this beneficial effect is abolished by knockdown of *pink-1* or *pdr-1* rather than *dct-1*.

Another *C. elegans* AD model (BR5270) relies on expressing the pro-aggregant F3 Δ K280 tau fragment (Fatouros, Pir et al. 2012) in neuron also shows mitochondrial dysfunction and memory loss. Similar to A β ₁₋₄₂ *C. elegans*, mitophagy stimulation could restore the memory loss in tau nematode. The memory retention induced by NMN requires PINK-1, PDR-1 and DCT-1, while UA is dependent on PINK-1 and PDR-1, and AC is only PINK-1-dependent. The beneficial effects of mitophagy on cognitive improvement and inhibiting phosphorylation of the tau sites have also been proved in AD mice, suggesting a conserved role of mitophagy in neurodegenerative diseases (Fang, Hou et al. 2019).

Julia Samann et al provided an indirect cue for the contribution of mitophagy in neurodegeneration since PINK-1 mutant causes both mitochondrial and neuron dysfunction. Firstly, similar to mammalian system, PINK-1 is localized both in cytoplasm and mitochondria in many tissues including nervous system (Samann, Hegermann et al. 2009). *pink-1* null mutant leads to mitochondrial cristae defect both in muscle and neuron, as well as increased sensitivity to oxidative stresses. Surprisingly, the defects in *pink-1* mutant could be rescued by additional *lrk-1* mutant. *C. elegans lrk-1* is the homologue of familial Parkinsonism gene *PARK8/LRRK2*, which is associated with inherited variants of Parkinson disease (PD) in human (Biskup, Moore et al. 2006, Park, Lee et al. 2006). LRK-1 has been identified to function in axonal-dendritic polarity of synaptic vesicles (SV) localization in neurons of *C. elegans* (Sakaguchi-Nakashima, Meir et al. 2007). Using *C. elegans*, LRK-1 and PINK-1 are shown to act antagonistically in neurite outgrowth.

To investigate neuronal morphological integrity which indicates neuron function, CAN axon pathfinding was visualized by transgenic worms *lqls4 [ceh-10::gfp]* as reported previously (Lundquist, Reddien et al. 2001). Compared with wild type worms, *pink-1* mutant has a defect of CAN axon pathfinding, with premature neurite termination, misguidance, or branching. Although *pink-1* mutant is more sensitive to oxidative stresses, that is not the

cause for its neurite defect, since pre-treatment with paraquat fails to further increase the CAN axon pathfinding defect in *pink-1* mutant (Samann, Hegermann et al. 2009). Considering the role of PINK-1 in mitophagy process, these data suggests that the mitophagy defect in *pink-1* mutant may also be a reason for neuron outgrowth defect. In addition, these studies provide potential tools for exploring the link of mitophagy to neuron functions.

Besides, mitophagy is probably involved in exophers excretion (a process which is associated with neurodegeneration) and human disease-Friedreich's ataxia (a neurodegenerative disease) in *C. elegans*.

During exophers excretion (see [chapter 2.2.5.](#)), one of the main cargos is mitochondrion. Interestingly, both inhibition of autophagy and compromising mitochondrial quality enhance the exophers jettison (Melentijevic, Toth et al. 2017). It is reasonable to speculate that selective mitophagy may also play roles in exophers formation/excretion and neurodegenerative diseases.

In Iron-starvation induced mitophagy, while partially frataxin depletion induces mitophagy, the severe absence of frataxin results in human disease-Friedreich's ataxia (FRDA), a neurodegenerative disease (Campuzano, Montermini et al. 1996). But in this paper, whether mitophagy is also related to Friedreich's ataxia in frataxin complete deletion condition is not explored.

4. The relationship between mitochondrial dynamics and mitophagy

Mitochondria are double membrane organelle originating from an endosymbiotic event whereas an alpha protobacteria has been domesticated by eucaryotic cell. This organelle possesses its own genome (mtDNA). Mitochondria are the key producers of ATP in eucaryotic cells through oxidative phosphorylation. This organelle contains other essential metabolic pathways such as the Krebs cycle and fatty acid oxidation. It is the place where two coupled biochemical reactions of the urea cycle take place. It is also involved in the biosynthesis of heme and lipids and is the major site for reactive oxygen species (ROS) production, which could act either as signalling molecule when produce at low doses or be detrimental for the cells when produced at higher dose. Mitochondria are involved in the early step of the signaling pathway of apoptosis and participate to cell calcium homeostasis.

Mitochondria are highly dynamic organelles that consistently undergo fission, fusion and transport over the cell cytoskeleton tracks. This mitochondrial dynamic is necessary for mitochondrial quantity and quality adaptation to the cell physiology. It serves to propagate variations in calcium concentration, to differentially segregate mtDNA and other matrix components within the mitochondrial reticulum but also to modulate and homogenize its own biochemical activities. It helps to maintain mitochondria in key places where there is special cell energy requirement and finally could modulate the way mitochondria establish contact sites with most of the other organelles. Large mechanochemical GTPases from the Dynamin family are responsible for the mitochondria network reticulum shape fluidity by controlling fusion and fission of mitochondria (*van der Bliek, Shen et al. 2013*). There is a very close relationship between mitophagy and mitochondrial morphology dynamic. Therefore, I will present the main mechanisms underlying the mitochondrial fusion and fission events and discuss how these mechanisms are coupled with mitophagy. I will more specifically expose the ER-mitochondria contact sites structure and composition that are critical for both mitochondrial fission events and autophagosome biogenesis. Finally, I will emphasize the way one of these GTPase, DRP-1, functions at the interface of both mitophagy and mitochondrial fission.

4.1. Mitochondrial fusion

Fusion of two mitochondria requires the cooperation of the independent inner and outer membrane fusion mechanisms. The main mammalian proteins mediating the outer and the inner mitochondrial fusion are mitofusins, Mfn1 and Mfn2, and optic atrophy 1 (Opa1) respectively. Mfn and Opa1 proteins are large GTPases of the Dynamin family that are anchored to the mitochondrial outer membrane and the mitochondrial inner membrane respectively (*Delettre, Lenaers et al. 2000, Chen, Detmer et al. 2003, Santel, Frank et al. 2003*). There are homologues of these genes: the yeast Fzo 1p and the *C. elegans fzo-1* are homologues of mammalian MFN1 and MFN2. The yeast Mgm1 and *C. elegans eat-3* are OPA1 homologues. It is noteworthy that Mfn2, but not Mfn1, is also localized at ER and regulates the mitochondria and ER contact sites (*de Brito and Scorrano 2008*). The proteins involved in mitochondrial fusion are presented in the following **Table 4** (as well as fission proteins).

Table 4. Proteins regulating mitochondrial fission and fusion in yeast, mammals and *C. elegans*

	<i>yeast</i>		<i>mammals</i>	<i>C. elegans</i>
	Functions	Protein	Protein	Protein
Mitochondrial fusion proteins	Mediates the fusion of outer mitochondrial membrane	Fzo1	Mfn2	FZO-1
		Not found	Mfn1	Not found
	Mediates the fusion of inner mitochondrial membrane	Mgm1	OPA1	EAT-3
Mitochondrial fission proteins	Dynamamin-related GTPase for Mitochondrial fission	Dnm1	Drp1	DRP-1
	Required for proper function of Dnm1-containing complex	Mdv1(Fis2)	Not found	Not found
	Required for the assembly and distribution of Dnm1-containing complex	Fis1(Mdv2)	Fis1	FIS-1 and FIS-2
	Adaptor between Dnm1 and Fis1	Caf4	Not found	Not clear
	Mediates Drp1 mitochondrial recruitment	Not found	Mff	MFF-1 and MFF-2
	Mediates Drp1 mitochondrial recruitment	Not found	Mid49/51	Not found
	Acts downstream of Drp1 to execute final scission	Dnm2(Dyn2)	Dnm2(Dyn2)	DYN-1

Basically, the mitochondrial outer membrane fusion process necessitates 3 successive steps: (1) tethering of two mitochondria; (2) docking of two membranes; (3) fusion. The molecular mechanism leading to mitochondrial fusion is still debated. The molecular mechanism explaining fusion process could be different between fungi, yeast Fzo1 has two transmembrane domains and metazoan, Mfn1/Mfn2 have only one transmembrane domain. (Tilokani, Nagashima et al. 2018). I will herein propose the fusion model based on the recently published metazoan fzo1 structure (Mattie, Riemer et al. 2018). Two mitochondria are tethered via trans interactions between the N-terminal domains of mitofusins from two organelles. Probably, GTPase domains and/or hydrophobic heptad repeat coiled-coil domains HR1 trans interactions are involved. The tethering between two mitochondria can be achieved through homophilic (Mfn1-Mfn1 and Mfn2-Mfn2) or heterophilic (Mfn1/Mfn2-

unknown protein) interactions. Afterward, docking and fusion could be achieved by GTPase activity and/or mitofusin oligomerization.

To maintain the structural integrity of inner mitochondrial membrane (IMM), its Opa1-dependent fusion takes place downstream of the OMM fusion and necessitates Mfn1 suggesting that OMM and IMM do communicate during the fusion process. Although, the molecular mechanism governing IMM fusion is unknown it relies on heterotypic interaction of Opa1 with some specific lipid of the IMM in particular cardiolipin (*Ban, Ishihara et al. 2017*). Opa1 contains a unique transmembrane domain with its C-terminal part containing the GTPase domain that localized to the intermembrane space. The C-term region of Opa1 has two proteolytic cleavage sites S1 and S2 for membrane bound protease Oma1 and YME1L respectively. These cleavages sites are localized just after the TM domain and before the GTPase domain. They probably modulate Opa1 activity and make Opa1 cleaved fragment to interact with membrane in a Drp1-like way (*Head, Griparic et al. 2009*). Additionally, deacetylase Sirt3 can promote Opa1 activity by direct deacetylation (*Samant, Zhang et al. 2014*).

The study in *Drosophila* heart tubes demonstrated that: interrupting Mfn-mediated OMM fusion evoked a cardiomyocyte ER stress response that could be rescued by enhancing mitochondrial processing of unfolded proteins, whereas interrupting Opa1-mediated IMM fusion compromised mitochondrial function that was rescued by scavenging reactive oxygen species (*Bhandari, Song et al. 2015*).

4.2. Mitochondrial fission

Mitochondrial fission is a process allowing the division of one mitochondrion into two daughter organelles. The mitochondria fission mechanism employs also two dynamin-related GTPase proteins Drp1/Dnm1 and Dyn2. Unlike, Opa1 and Mfn, these dynamin proteins lack any transmembrane domain or pleckstrin homology (PH) domain and therefore necessitate protein adaptors to be recruited to the OMM. Mitochondrial fission mechanism is composed of five successive steps (**Figure 12**): (1) ER-mediated pre-Drp1 mitochondrial constriction; (2) recruitment of Drp1 by adaptors to the pre-constriction site; (3) maximal constriction by Drp1; (4) recruitment and assembly of dynamin 2 (Dyn2); (5) disassembly (*Kraus and Ryan 2017*). These steps and involved proteins are illustrated in

Figure10 and in this chapter; I will mainly introduce the two dynamin proteins and other regulators during mitochondrial fission process (**Table 4**).

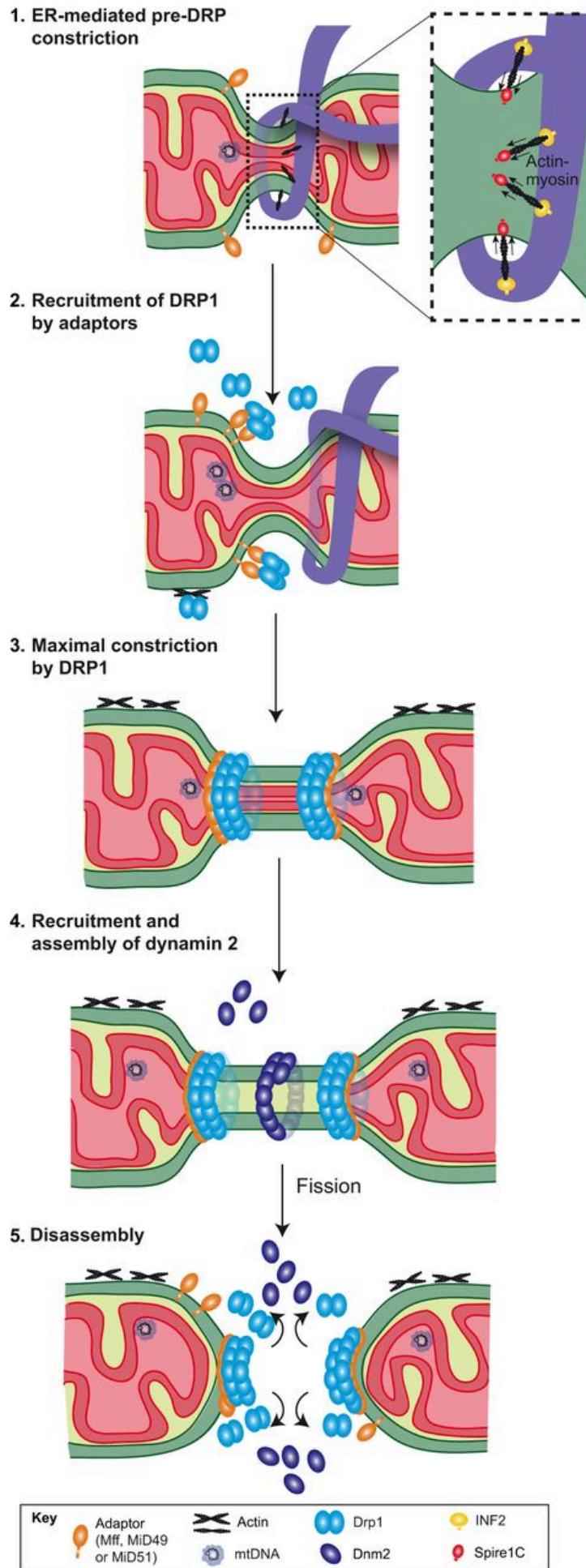


Figure12. Model for mitochondrial fission in animal cells To exert mitochondrial fission, mitochondria are firstly constricted by tubular ER: INF2 localized at ER initiates the nucleation of actin filament, which then, together with myosin, presses outer mitochondrial membrane through interaction with Spire1C. Second, enrichment of Mff, probably as well as MiD49 and/or MiD51, facilitates the recruitment of Drp1 and its assembly around the mitochondrion. Third, the GTPase activity of Drp1 will be triggered to change the conformation, and thereby mediates the constriction of mitochondrion, which also enables the scission of inner mitochondrial membrane. Fourth, after that, Dnm2 is recruited to the constricted mitochondrion to assemble around it. Fifth, further constriction powered by Dnm2 occurs to complete fission, followed by fission machinery disassembly. (*Kraus and Ryan 2017*).

4.2.1. Drp1

Dynamamin-related protein 1, (Drp1 in mammals; Dnm1 in yeast and DRP-1 in *C. elegans*) functions in mitochondrial fission, was initially described in *C. elegans* (*Labrousse, Zappaterra et al. 1999*) and in yeast (*Bleazard, McCaffery et al. 1999*). But here, for convenience, I will unify the writing as Drp1. Drp1 homologues contain four characteristic domains: an N-terminal GTPase domain, a middle domain, a variable domain and a GTPase effector domain (GED) at the C-terminal. This latter domain functions as a GTPase activating protein (GAP) and therefore modulates GTPase activity. The GED domain, together with the middle domain, forms a stalk supporting Drp1 oligomerization (*Praefcke and McMahon 2004, van der Bliek, Shen et al. 2013*). Once Drp1 is recruited to the OMM, oligomerized Drp1s are further assembled into helical ring via the contacts between GTPase domains. Once the ring is completed, GTP hydrolysis drives the Drp1 ring diameter decrease (*Mears, Lackner et al. 2011, Koirala, Guo et al. 2013*) which further enhances membrane constriction. Drp1 is predominantly cytosolically distributed but will be anchored at OMM for mitochondrial fission before oligomerization (*Smirnova, Griparic et al. 2001*).

Mdivi-1 and Dynasore are both drugs that can inhibit Drp1 activity. Mdivi-1 is more specific for Drp1 homologs whereas Dynasore is a general drug which targets broadly Dynamamin proteins (*Cassidy-Stone, Chipuk et al. 2008*). Drp1 dominant-negative mutant (K38A) which blocks its GTP hydrolysis also suppresses the fission events. A395D is defect in mitochondrial targeting and S35A mutant is known to enhance Drp1 GTPase activity by 2-fold compared with WT (*Smirnova, Griparic et al. 2001*).

4.2.2. Drp1 adaptors

Drp1 mediated mitochondrial fission requires co-factors to be recruited to the OMM. There are at least four well-accepted mitochondrial Drp1-receptor proteins at the OMM:

fission 1 (Fis1), mitochondria fission factor (Mff), mitochondrial dynamics protein of 49 kDa (MID49) and MID51 (Ni, Williams et al. 2015). The last three factors are found only in metazoans.

a. Fis1

The first identified Drp1 receptor protein, Mdv1, has been isolated in yeast (Tieu and Nunnari 2000). Mdv1 is a cytosolic protein containing seven tryptophan (W) and aspartic acid (D) repeats, which can form a β propeller structure for binding with Drp1.

In yeast, $\Delta mdv1$ and $\Delta dnm1$ display single interconnected mitochondria net, which is obviously due to the blockage of fission and therefore absence of new tubular mitochondria formation (Tieu and Nunnari 2000). The mitochondrial network morphology in the double mutant $\Delta dnm1 \Delta mdv1$ is highly similar to that observed in the single mutant, indicating that Mdv1 acts in the same pathway as Dnm1. Moreover, either $\Delta mdv1$ or $\Delta dnm1$ efficiently blocks the mitochondrial fragmentation in *fzo-1* cells and generate the single interconnected mitochondria net. Mdv1 is peripherally associated with OMM as puncta with Dnm1. The Mdv1 localization at OMM is independent on Dnm1 whereas its specific localization at OMM to punctate structures requires Dnm1. Interestingly, an Mdv1 paralog, Caf4, was identified in *S.cerevisiae*. It also participates to the mitochondrial fission process. Mdv1 and Caf4 have no homolog in metazoan.

Fis1/Mdv2 is another identified gene required for fission. Fis1 (also called Mdv2) is an integral OMM protein with its N-terminal part exposed to cytoplasm. In absence of Dnm1, Fis1/Mdv2 can mediate the mitochondrial distribution of Mdv1. The $\Delta fis1$ mutant phenotype is similar to the one observed for either $\Delta mdv1$ or $\Delta dnm1$ mutants. Evidences suggest that Fis1 functions in the assembling process of the punctate structures that contain Dnm1 and Mdv1 (Tieu and Nunnari 2000). Much less Dnm1p-containing complexes are formed in $\Delta fis1$ cells, and mitochondrial fission is also inhibited without Fis1. In conclusion, Fis1 is required for the assembly and distribution of Dnm1p-containing complexes, while Mdv1 and Caf4 is important for proper function of the complexes (Mozdy, McCaffery et al. 2000).

Although, Fis1 have homologs in metazoans, its role as a mitochondrial fission factor is debated. Otera et al demonstrated that mammalian Fis1 is dispensable, since neither mitochondrial morphology nor Drp1 recruitment is altered in *Fis1* KO cells (Otera, Wang et

al. 2010). It should also be taken into account that under certain chemical treatment, like antimycin A, Fis1 mutants may have some effect on fission (*Shen, Yamano et al. 2014*). Instead, Otera et al revealed that another Drp1 adaptor, the mitochondrial fission factor (Mff), regulates the recruitment of Drp1 at OMM (*Otera, Wang et al. 2010*).

b. Mff

Mff is an OMM protein that was first identified by RNAi screening for mitochondrial morphology defect in *D. melanogaster*. It contains, a trans-membrane domain (TMD) at the C-terminal for mitochondrial anchoring, a coiled-coil domain just upstream of TMD, and two short amino acid repeats close to N-terminal facing the cytosol (*Gandre-Babbe and van der Bliek 2008*).

Mff down-regulation impairs Drp1 recruitment to mitochondria and therefore alters mitochondrial fission. On the other hand, exogenous Mff induces mitochondrial fragmentation, which is inhibited in a Drp1 dominant (K38A) mutant context. These results suggest that Mff functions in mitochondrial fission by regulating the recruitment of Drp1. Indeed, both the colocalization and the physical interaction between Mff and Drp1 have been observed. Specifically, the Mff N-terminal 50 residues are responsible for Drp1 recruitment (*Otera, Wang et al. 2010*).

In mammals, the knockdown of *Opa1*, which encodes Opa1 protein involved in mitochondrial IMM fusion, causes the cristae disorganization that are more sensitive to apoptotic stimuli. *Mff/Opa1* or *Drp1/Opa1* could reverse the mitochondrial morphology to be elongated but has no effect on cristae. Surprisingly, the cytochrome c release during *Opa1* induced apoptosis is suppressed in *Mff/Opa1* or *Drp1/Opa1*, suggesting that the fission rather than cristae disorganization triggers the release of cytochrome c (*Otera, Wang et al. 2010*).

c. MID49 and MID51

Mid49 and Mid51 are OMM anchored proteins via one TMD localized at their N-terminal part. Mid49/Mid51 form punctate structures around mitochondria but whether this localization is Drp1-dependent is still debated (*Kraus and Ryan 2017*). However, there is no doubt that Mid49/Mid51 can recruit Drp-1 to mitochondrial surface. Knockdown of Mid49 and Mid51 impairs the Drp1 recruitment as well as its associated mitochondrial fission.

Overexpression of Mid49/Mid51 facilitates the Drp1 association with mitochondria but is accompanied with hyper-connected mitochondrial. This unexpected phenotype could be explain by Mid49/Mid51 sequestering Drp1 and prevent Drp1 to be organized into active scission complexes (*Palmer, Osellame et al. 2011*).

How Fis1, Mff and Mid49/51 cooperate and mediate Drp1 recruitment during the fission process? Studies in MEFs showed that both Fis1 and Mff are important for Drp1 recruitment and assembly on mitochondria but Mff is predominant. Knockdown of Mid49/51 causes mitochondrial elongation even in the absence of Fis1 and Mff. In conclusion, each of these four proteins is capable to recruit Drp1 and promote mitochondrial fission (*Loson, Song et al. 2013*).

d. Drp-1 adaptors in *C. elegans*

As in yeast and mammals, Drp1 also serves for mitochondrial fission through the mitochondrial translocation in *C. elegans* (*Labrousse, Zappaterra et al. 1999*). Are there any homologs for Drp1 adaptors in *C. elegans*? Interestingly, in *C. elegans*, there are two homologs of Fis (fis-1 and fis-2) and Mff (mff-1, mff-2). Fis homologs are not required for fission whereas the *mff-1;mff-2* double mutant show fission defect similar to the one seen in *drp-1* mutant (*Shen, Yamano et al. 2014*).

Although *drp-1* deletion completely reverses the mitochondrial fragmentation seen in *fzo-1* RNAi or *eat-3* RNAi worms, *fis1* or *mff* deletions do not induce such phenotype. These results suggest that although Mff has a role for in modulating mitochondrial dynamism, neither Mff nor Fis1 is essential for fission or Drp1 recruitment in *C. elegans* (*Shen, Yamano et al. 2014*).

4.2.3. Drp1 posttranscriptional modifications

Drp1 activity is regulated by multiple post-translational modifications including phosphorylation, ubiquitinylation, SUMOylation, acetylation, o-gluNAcylation (O-N-acetylglucosaminylation) and nitrosylation.

The two most important Drp1 phosphorylated sites are Ser616 and Ser637 that are recognized to be pro-fission site and pro-fusion site respectively. They are both located at the end of the variable domain. Drp1 is phosphorylated by Kinases PKC δ , Rock kinase,

CDK1/Cyclin B or CAMK-II (Ca²⁺-calmodulin dependent kinase II) on Ser616, while protein kinase A (PKA) is responsible for Ser637 phosphorylation (*van der Bliek, Shen et al. 2013*). Since the variable domain is close to OMM, its phosphorylation may alter the interaction of Drp1 with OMM proteins. The p-Ser616 promotes its binding ability with Fis1 and thus fission (*Han, Lu et al. 2008*). Interestingly, Drp1 activation facilitated by p-Ser616 is suppressed by phosphorylation at Ser637 by PKA (*Merrill, Dagda et al. 2011*) which can then be reversed by PP2A, a calcium-activated cytosolic phosphatase (*Cereghetti, Stangherlin et al. 2008*). Ser693, which localized in the GED domain, is a phosphorylation site for GSK3 β , and this phosphorylation significantly reduces the Drp1 GTPase activity. Interestingly, GSK3 β -Drp1 interaction is inhibited by K679A mutant, which abolishes the phosphorylation (*Chou, Lin et al. 2012*). In conclusion, Drp1 phosphorylation status regulates its cycling between cytosol and mitochondria (*van der Bliek, Shen et al. 2013*).

Besides phospho-regulation, Drp1 may also undergo other forms of modifications such as ubiquitylation. The mitochondrial ubiquitin E3 ligase March5 modulates Drp1-dependent mitochondrial fission. MARCH5 RNAi or overexpression of a MARCH5 RING results in a mitochondrial fission defect which is characterised by elongated and interconnected mitochondrial tubules. Interestingly, Drp1 is still recruited to mitochondria in MARCH5 RING mutant cells but it forms big clusters lacking Fis1, suggesting a defect of Drp1 assembling. March5 seems to act downstream of Fis1 for Drp1 constriction, however, whether this regulation is via direct ubiquitination is not clear yet (*Karbowsky, Neutzner et al. 2007*).

Parkin ubiquitinates Drp1 in PINK1/Parkin-mediated mitophagy. Both in HeLa and SH-SY5Y cells, Parkin siRNA leads to a mitochondrial network fragmentation. This altered mitochondrial morphology in Parkin knockdown is concomitantly observed with an increased of Drp1 level while Parkin overexpression induces the Drp1 proteasomal degradation. By contrast, Mfn1/2 and Fis1 protein levels are not modified by Parkin up- or down-regulation. Parkin could directly bind to and promote the ubiquitination of Drp1. The expression of Parkin mutant with impaired E3 ubiquitinase domain or Drp1-binding ability reduces the ubiquitination of Drp1 and consequently its degradation via the proteasome (*Wang, Song et al. 2011*).

Another E3 ligase, MAP1 (or Mulan), is involved in the SUMOylation of Drp1 to stimulate mitochondrial fission. Sumoylation is the term coined for the conjugation of small ubiquitin-like modifier (SUMO) to proteins. Sumoylation has multiple consequences, including the

formation or disassembly of protein complexes, the regulation of protein localization and the modulation of other post-translational modifications such as phosphorylation and ubiquitination (*Geiss-Friedlander and Melchior 2007*). Overexpression of MAP1/Mulan promotes the Drp1 SUMOylation as well as mitochondrial fragmentation. However, Drp1 sumoylation is not essential for Drp1 fission activity (*Braschi, Zunino et al. 2009*).

There are also evidences of a redox regulation of Drp1 activity (*Forkink, Basit et al. 2015*). Specifically, reactive nitrogen species (RNS) such as the nitric oxide (NO) triggers Drp1-dependent mitochondrial fragmentation. Two main mechanisms have been proposed to explain these observations. The NO-dependent nitrosylation of Drp1 on Cys644 could stimulate Drp1 dimerization that stimulate its GTPase activity has been proposed to facilitate mitochondrial fragmentation during neurodegeneration. Preventing Drp1 nitrosylation by cysteine mutation abrogates the neurotoxicity (*Cho, Nakamura et al. 2009*). Alternatively, one can think of one NO-stimulated kinase that could phosphorylate Drp1 on Ser616 and promotes its recruitment to the MOM.

4.2.4. Dnm2

Although Drp1 is the key protein for mitochondrial fission, it has been proposed that Dnm2, rather than Drp1, is essential for the final scission events. Dnm2 (also called Dyn2) belongs to the dynamin family and is best-known for its role in driving endocytosis at the plasma membrane (*Ferguson and De Camilli 2012*). Recently, the lab of Gia Voeltz revealed that Dnm2 is also important for mitochondrial fission (*Lee, Westrate et al. 2016*).

The authors observed that in COS-7 and HeLa cells, both Drp1 and Dyn2 siRNA depletions lead to very similar modification of the mitochondrial network morphology with elongated and hyperconnected mitochondrial tubules. Live-cell images show that Dnm2 is recruited to the Drp1-containing mitochondrial constriction sites (*Lee, Westrate et al. 2016*). However, Dnm2 localization on fission sites is transient and observed just before the division whereas Drp1 ring occurs earlier and persists for a much longer time at the fission sites. Additionally, Drp1 puncta are present at the tips of both daughter mitochondria while Dnm2 only appears on one of the two daughter organelles. These data suggest Dnm2 acts downstream of Drp1. In Dnm2^{KO} cells, Drp1 can still be recruited to mitochondrial surface for assembling at constriction sites, but fails to complete the division. The initial Drp1 signal associated to the dividing mitochondria splits in two and is accompanied by an increase in the length of the

constriction along the longitudinal axis (Lee, Westrate et al. 2016). In addition, fission can still occur but is slightly reduced in the absence of Dnm2 in FRAP-like experiments, indicating there are other proteins cooperating with Drp1 to facilitate mitochondrial division in Dnm2^{KO} cells.

In summary, The ER and the acto-myosin machinery it nucleates around the mitochondria, Drp1 and its mitochondrial adaptors are part of the 'mitochondrial constriction machinery', whereas Dnm2 is the final scission mediator (Kraus and Ryan 2017) and **Figure12**). However, Kamerkar et al (Kamerkar, Kraus et al. 2018) recently challenged this model. Their work questioned the Dnm2 function in scission using a slightly modified approach combining CRISPR mutants (instead of siRNA), different fluorescent constructs and cell lines. The authors concluded that Drp1 is the main dynamin protein responsible for both mitochondrial constriction and scission. There are several proteins been demonstrated to affect mitochondrial fission, including GDAP1 (Barneo-Munoz, Juarez et al. 2015) and March5/MITOL (Karbowski, Neutzner et al. 2007). But it seems that they are independent of Drp1 (Otera, Wang et al. 2010).

4.3. Mitochondrial dynamics in mitophagy process

It is generally accepted that a mitochondrion should be fragmented before engulfment by autophagosome to match the relatively small size of the latter (Tatsuta and Langer 2008, Mao and Klionsky 2013). In this view, multiple studies indicate the Drp-1 mitochondrial fission machinery plays this critical work to adapt the mitochondrial size to the size of the enveloping autophagosome. However, multiple other published works questioned this proposed unique function of the mitochondrial fission apparatus during mitophagy and challenged Drp-1 dependent mitochondrial requirement. In this chapter, I will present an overview of these experimental works.

4.3.1. Drp1-dependent mitophagy

Drp1-mediated mitochondrial fission is considered as necessary for mitochondria engulfment by autophagic structures for degradation. Several cues have shown that depletion of Drp1 causes the fused and elongated mitochondria, which blocks mitophagy.

In yeast, Kanki et al (Kanki, Wang et al. 2009) demonstrate that Dnm1 and mitochondrial fission are required for efficient mitophagy. Later on, Mao et al (Mao, Wang et al. 2013)

indicate that, in yeast, Atg32 and Atg11 mark degrading mitochondria and interact with Dnm1 to recruit it to mitochondria to initiate the fission process. These results support the hypothesis that the fission process is an early step of mitophagy. This result was further confirmed in mammalian cells. In HeLa cells and in MEFs, Drp1-dependent mitochondrial fission defect impaired CCCP induced Pink/Parkin dependent mitophagy (*Tanaka, Cleland et al. 2010*). However, in Parkin/PINK1-mediated mitophagy, the CCCP-induced Parkin recruitment to mitochondria is not affected in Drp1 mutant. It suggests that Drp1-associated mitochondrial fragmentation is not necessary for Parkin translocation (*Narendra, Tanaka et al. 2008*).

Ming Chen et al reveal that Drp1 is also required for FUNDC1-mediated mitophagy (*Chen, Chen et al. 2016*). In normal condition, FUNDC1 interacts with OPA1 via its Lys70 (K70) residue. In mitophagy induced condition, the FUNDC1-OPA1 complex is disassembled. Consequently, FUNDC1 can interact with DNM1L/Drp1, leading to DNM1L/Drp1 translocation to mitochondria. Knockdown of *DNM1L* blocked FUNDC1-mediated mitochondrial fragmentation and mitophagy.

To my knowledge, in *C. elegans*, only three studies deal with the specific role of Drp1 in mitophagy.

Shen et al (*Shen, Yamano et al. 2014*) shows that a *fis-1* depletion and not a *drp-1* inactivation can lead to formation of aggregate between autophagosome and mitochondria in paraquat-induced parkin-dependent mitophagy. *Fis1* depletion and not *drp-1* depletion can disrupt mitophagy in *C. elegans*.

Drp1-depleted worms display disorganized, aggregated and globular mitochondria compared with wild-type worms (*Labrousse, Zappaterra et al. 1999*). Furthermore, mitophagy inhibition using either *dct-1(RNAi)*, *Parkin/pdr-1(RNAi)* or *pink-1(RNAi)* depletion in this mitochondrial morphology defect mutant did not change the mitochondrial structure. This result means that the mitochondrial morphology alteration induced by mitophagy impairment is a secondary event (*Palikaras, Lionaki et al. 2015*).

Moreover, the fission/fusion dynamic of paternal mitochondria can also affect its own engulfment during allophagy. A fission mechanism defect in paternal mitochondria delays its elimination; while by contrast, the fusion defect has a facilitating effect. It goes the same way as in mammalian cells that mitochondrial dynamic is a key factor for mitophagy. It is widely accepted that Drp1 dependent fission of mitochondria is required for its engulfment

and degradation during mitophagy. Even there're few Drp1 independent mitophagy reports, the degradation process is also slowed down. Surprisingly, the dynamic of maternal mitochondria also have an influence on the paternal mitochondrial elimination (PME). In *fzo-1(RNAi)* depleted oocyte, many mitochondria are depolarized and are therefore potentially addressed to mitophagy. Therefore, there is a competition for the autophagy machinery between the very large number of depolarized oocyte mitochondria already present in *fzo-1(RNAi)* mutant oocyte and the few paternal mitochondria to be eliminated (Wang, Zhang et al. 2016).

However, Drp1 function in mitophagy cannot be reduced to a mechanism used by the cell to cut mitochondria in pieces of the right size allowing the autophagosome to wrap them up. Drp1 and the mitochondrial fission mechanism do more than that. Twig et al question the role of the mitochondrial fission process in the context of autophagy. They used cells where either Fis1 was depleted or a dominant negative form of Drp1 (K38A) was expressed. In both conditions, inhibition of the mitochondrial fission results in mitochondrial autophagy reduction (Twig, Elorza et al. 2008).

Furthermore, they demonstrated that fission can produce metabolically different daughter mitochondria: one with normal mitochondrial membrane potential (MMP) and the other with a reduced MMP which is more likely addressed to autophagosome. Indeed, a very recent study reported that Drp1 interacts with Zip1 at the OMM to reduce MMP (Cho, Ryu et al. 2019). This reduced MMP can be restored in healthy mitochondria after fission, whereas those that failed to restore MMP level are removed via mitophagy. In this study, MMP is assessed by tetramethylrhodamine (TMRM) mitochondrial staining. This dye fluorescence is MMP-dependent and therefore, the disappearance of TMRM fluorescence indicates MMP reduction. Dual labelling of mitochondria by TOM20-GFP and TMRM shows that MMP is transiently reduced at Drp1 fission sites just several seconds prior to mitochondrial division. Further data reveals that this transient MMP reduction is triggered by the Drp1 mitochondrial translocation but is Drp1 GTPase activity-independent.

Nevertheless, the GTPase domain of Drp1 is responsible for the binding with Zip1, a Zn^{2+} transporter. Zip1 forms a complex with the inner mitochondrial calcium uniporter (MCU) that promotes the entry of Zn^{2+} into the mitochondrial matrix. The inhibition of mitochondrial Zn^{2+} increase by using a Zn^{2+} chelating agent strongly blocks MMP reduction in Drp1- or Zip1- expressing cells. This result indicates that the role of the Drp1/ZIP1-dependent Zn^{2+}

increase is to mediate MMP reduction (Cho, Ryu et al. 2019). Overexpression of Drp1 or Zip1 reduce MMP, but neither up nor down-regulation of Zip1 alter mitochondrial morphology. These data suggest that Drp1-Zip1 triggered MMP reduction is dispensable for mitochondrial division.

It raises the question what's the function for Drp1-Zip1 mediated MMP reduction? Analysis showed that after fission is completed, all long mitochondria restore MMP within 3 seconds, but a small portion (13.8%) of short mitochondria progressively lost their MMP. During hyperglycemia-induced mitophagy, the daughter mitochondria which fail to restore MMP after division could recruit GFP-Parkin for subsequent autophagic degradation. By contrast, perturbation of Drp1-Zip1 interaction decreases Parkin translocation as well as LC3 puncta (Cho, Ryu et al. 2019). This paper suggests that Drp1 also acts as a mitochondria quality regulator to monitor damaged mitochondria for selective mitophagy. In summary, these data reveals that along with the fission function, Drp1 is also responsible for the selection of mitochondria that are addressed to autophagosome during mitophagy.

Interestingly, another example of an additional non canonical role of Drp1 in mitophagy is given in the paper of Sentelle et al. In this paper, authors show that Drp1 knockdown alters the localization of ceramide on OMM. Consequently, in ceramide stress induced-induced mitophagy, this Drp1 KO prevents mitochondria from targeting to autophagolysosomes mediated by LC3B-II and ceramides (Sentelle, Senkal et al. 2012).

4.3.2. Drp1-independent mitophagy

Although many studies have suggested that Drp1-dependent mitochondrial fission is essential for mitophagy, still several groups demonstrated the existing of DRP1-independent mitophagy.

In yeast, mitophagy induced by rapamycin is inhibited in $\Delta atg11$, $\Delta atg20$ or $\Delta atg24$, while the bulk autophagy is not impaired. Surprisingly, mitochondria are not fragmented upon rapamycin treatment, suggesting that mitochondrial fragmentation does not precede mitophagy in yeast (Mendl, Occhipinti et al. 2011). And the mutant of *mgm1* that is deficient for inner membrane fusion is not sufficient to stimulate mitophagy. Since mitophagy in yeast is not dependent on fission, is Dnm1 (Drp1) still required for mitophagy? To address this question, they induced mitophagy in the absence of Dnm1 and its adaptors, and showed that mitophagy and autophagy could proceed normally, confirming that Dnm1-

mediated mitochondrial fission is not a prerequisite for mitophagy or autophagy in yeast. Although they have observed some mitophagy defect in $\Delta fis1$, it is further proved to result from a secondary mutation in *WHI2* rather than *fis1* itself. *WHI2* encodes a protein consisted of 486 amino acids that is involved in stress responds and Ras-protein kinase A (PKA) signalling pathway (Mendl, Occhipinti et al. 2011). But the inner link between rapamycin-induced mitophagy/autophagy and Whi2 is still unclear.

A study in cultured MEFs also indicated that mitophagy could occur in the absence of Drp1. Drp1-ablated cells show higher LC3-II and mitochondrial p62 accumulation, which suggests an induction of mitophagy, although they may result from the blockade of autophagic fusion. Since mitochondrial contents in Drp1-ablated cells are reduced, indicating an increased mitophagy in Drp1 depletion.

A novel model (shown in **Figure13**) for mitochondrial fission during mitophagy has been recently proposed. With this model, Yamashita and Kanki suggested that mitochondrial fission is concomitant with autophagosome formation, and could occur without Drp1 (Yamashita, Jin et al. 2016, Yamashita and Kanki 2017).

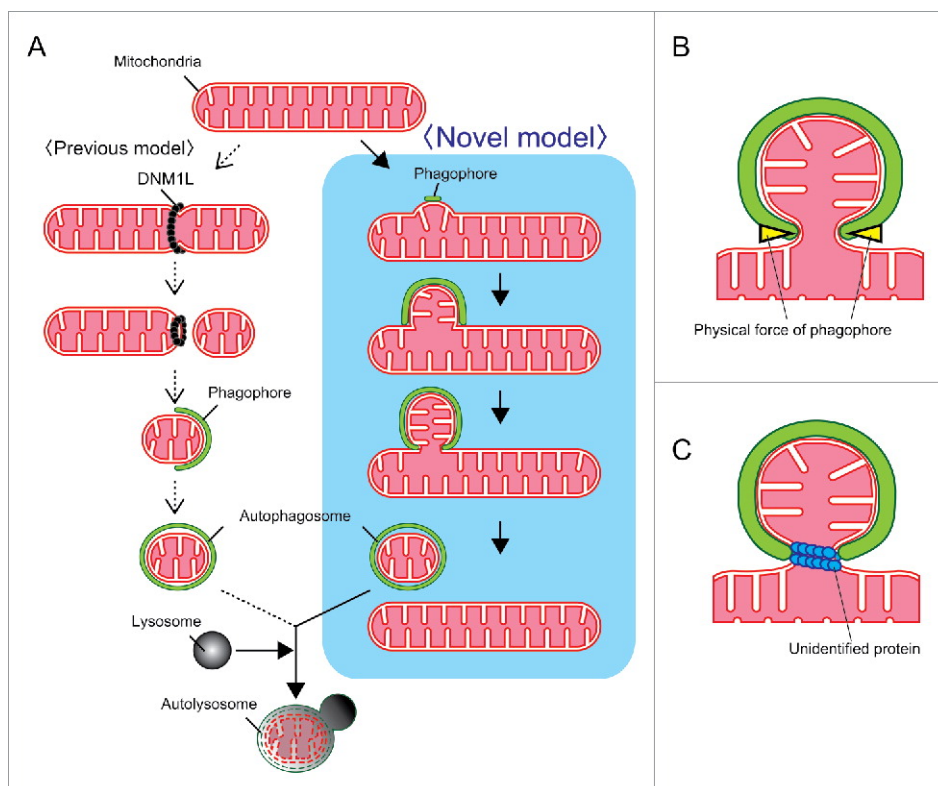


Figure13. Models for mitochondrial division during mitophagy. (A) indicates two possible models to explain how mitochondrial fission occurs during mitophagy. The classical model (left) proposes that, Dnm1L (Drp1)-mediated mitochondrial fission precedes autophagosome assembly. The new model (right) indicates that, mitophagy occurs concomitantly with mitochondrial fission and independently of Dnm1L. The core autophagy machinery assembles before the fission initiation. (B) and (C) provides two hypothesis for how mitochondria are fragmented in the novel model in (A). In the absence of Dnm1L, mitochondrial fission is mediated by the physical force of the expanding phagophore (B) or by a yet unidentified new mitochondrial fission machinery (C). (*Yamashita and Kanki 2017*)

Yamashita *et al* first induced mitophagy in yeast and found that both $\Delta dnm1$ and $\Delta fis1$ underwent mitophagy, whereas the $\Delta atg11$ blocked it (*Yamashita, Jin et al. 2016*). They next analysed the role of Drp1 on hypoxia- or deferiprone (DFP)-induced mitophagy in mammalian cells. Although the majority of mitochondria remain highly fused, small punctate mitochondria are detected in Drp1 KO cells upon mitophagy-induction, especially when fusion between autophagosomes and lysosomes is inhibited by bafilomycin A1. Immunofluorescences and EM results confirmed that small mitochondria are detected within autophagosomes. Compared with WT, Drp1 KO cells could still degrade mitochondria but need a longer time to form mitophagosomes. These results highlight the existence of a Drp1-independent mitophagy mechanism in mammalian cells (*Yamashita, Jin et al. 2016*). Notably, both in WT and Drp1 KO cells, the vast majority of mitophagosomes are formed on tubular or clustered mitochondria (83% in WT and up to 98% in Drp1 KO cells), which is consistent with an independent publication (*Hamasaki, Furuta et al. 2013*). More importantly, sites where mitophagosomes forming are initiated are colocalized with mitochondrial fission sites but are distinct from Drp1 (*Yamashita, Jin et al. 2016*). In summary, mitochondrial division is accomplished concomitantly with autophagosome formation, and is Drp1-independent (*Yamashita, Jin et al. 2016*).

Youle group also demonstrated that Drp1-mediated mitochondrial fission is required for the selection of damaged mitochondria rather than mitochondrial fragmentation during mitophagy (*Burman, Pickles et al. 2017*). Although Drp1 does mediate the fission upon mitophagy induction, mitophagy is not blocked in absence of Drp1. In certain condition, loss of Drp1 may even increase mitophagy flux. However, loss of Drp1 fosters Parkin mitochondrial translocation to entire mitochondria and impairs the selective clearance of mitochondrial subdomains. This work suggests that Drp1 helps to restrict the Pink/parkin module to specific mitochondrial zone (*Burman, Pickles et al. 2017*). This finding is linked to

the recent study published by Cho et al which indicates that Drp1 has a mitochondrial quality control function (*Cho, Ryu et al. 2019*).

4.4. ER and mitochondria contact sites

A small part of mitochondria (2-5% of area) are always in close contact with endoplasmic reticulum (ER). These regions, called mitochondria-associated membranes or MAMs, have key functional roles for ER, mitochondria and other organelles.

4.4.1. The concept and functions of contact sites

ER-mitochondria contact sites are defined as spatially close apposition regions, within 10-30 nm distance, between these two organelles(*Csordas, Renken et al. 2006*). Due to the adjacent distance, the contact sites are usually visualized by electron microscopy (EM) techniques. The close linkage between ER and mitochondria at the contact sites is dynamic and reversible, and does not involved membrane fusion between adjacent organelles (*Vance 2014*).The subcellular fractionation assay helps to isolate a population of ER membranes that are connected and co-purified with mitochondria; it should be distinct from pure ER or pure mitochondria. This fraction represents MAMs (*Wieckowski, Giorgi et al. 2009*). Some of the proteins that are enriched in the MAM fraction have been identified in mammals. These proteins (or called MAM proteins) and their associated functions are shown in **Table 5** (*van Vliet, Verfaillie et al. 2014*).

Table 5. Functional roles of the MAM proteins (van Vliet, Verfaillie et al. 2014).

Function and signaling role of the MAM	MAM resident protein	Specific function(s) of MAM resident proteins
Mitochondrial morphology	Drp1	A member of the dynamin family of proteins of large GTPases involved in mitochondrial fission initiated at the MAMs
	Miro1-2	Mitochondrial GTPase located at OMM connecting mitochondria to the cytoskeleton, and involved in mitochondrial trafficking.
Apoptosis signaling	Mfn2	Mitochondrial outer membrane GTPase and MAM component, which plays a major role in mitochondrial fusion
	PML	A tumor suppressor that by localizing at MAMs regulates IP3R Ca ²⁺ signaling and mitochondrial Ca ²⁺ overload
	S1T	Truncated form of the SERCA pump that leads to pro-apoptotic calcium overload in the mitochondria
Autophagy signaling	PTEN	Tumor suppressor that increases Ca ²⁺ signaling from the ER to the mitochondria through the IP3R
	ATG14L	Early autophagy marker and an essential pro-autophagic protein that relocates to the MAMs to induce autophagosome formation under starvation
Ca ²⁺ transport	ATG5	Important protein in the formation of autophagosomes which associates to the MAMs
	VDAC1	Ca ²⁺ uptake channel in the outer mitochondrial membrane
	IP3R	Main Ca ²⁺ efflux channel of the ER, linked with VDAC1 through Grp75
	Grp75	Chaperone that forms a physical link between IP3R and VDAC1
	Cnx	Ca ²⁺ binding protein and chaperone of the ER
	mTORc2	Mammalian target of rapamycin complex 2 that is able to increase mitochondrial metabolism through regulated Ca ²⁺ increases by localizing at the MAMs
Inflammation signaling	Sigma R1 receptor	Chaperone that interacts with BiP and is involved in IP3R mediated Ca ²⁺ release regulation at the MAMs
	NLRP3	Member of the NOD like receptor (NLR) family forming the multimolecular NLRP3-inflammasome whose assembly is located at the MAMs
Antiviral response	ASC	Adaptor that binds NLRP3 to initiate inflammasome signaling
	MAVS	Mitochondria-localized adaptor molecular serving as important platform in cellular antiviral response
	RII-1	Member of the RLRs family of cytosolic receptors involved in dsRNA sensing it relocates to MAMs after viral infection and binds to MAVS
ER-mitochondrial tethering	Mfn2	Outer membrane GTPase that forms a homodimer and is a crucial mediator in ER-mitochondria connection
	Tpm	A keratin binding protein that plays a role in ER-mitochondria connection and Ca ²⁺ signaling
	PERK	ER stress sensor and Ser/Thr kinase that plays a role in ER-mitochondrial tethering and unfolded protein response signaling
ER stress signaling	Cnx	Ca ²⁺ binding protein and chaperone of the ER
	PACS-2	Cytosolic sorting protein involved in the ER localization of transmembrane proteins and ER vesicle sorting
	Perk	ER stress sensor and Ser/Thr kinase that plays a role in the unfolded protein response and interacts with Mfn2
	Sigma R1 receptor	Chaperone that interacts with BiP and is involved in IP3R mediated Ca ²⁺ release
	Ero1α	An oxidoreductase acting as a major regulator of redox homeostasis in the ER, which associates with MAMs under conditions of ER stress
	Mfn2	Outer mitochondrial membrane GTPase that modulates PERK-mediated UPR signaling
Other processes at the MAM	BiP	Interacts at the MAM with S1R and is an important ER chaperone and Ca ²⁺ buffer
	γ-Secretase	Multisubunit aspartyl protease member of the intramembrane-cleaving protease family, which cleaves the amyloid precursor protein, and found in lipid rafts enriched at the MAMs
	PS1-2	Catalytic components of the γ-secretase complex, regulating ER-mitochondria apposition

Analysis of MAMs protein functions indicate that multiple major roles could be linked to MAMs such as mitochondrial morphology regulation, phospholipid exchange and biosynthesis, Ca²⁺ exchange and signalling, and autophagosome biogenesis (*Giorgi, Missiroli et al. 2015*). In this chapter, I will only focus on MAMs roles in mitochondrial morphology regulation and autophagosome biogenesis.

4.4.2. The role of contact sites in mitochondrial morphology regulation

As I described previously in [chapter 4.2](#), Drp1 and its adaptor proteins regulate mitochondrial fission. However, Drp1 is not capable to initiate mitochondrial fission alone because Drp1 helical construction ring is narrower (around 100-150 nm diameter) than the mitochondrial tubule diameter (around 300 nm diameter). Indeed, contact sites between ER and mitochondria construct, independently of Drp1 and its adaptor proteins, pre-constriction sites that could fit with Drp1 characteristics (*Friedman, Lackner et al. 2011*). ER tubules wrap around and tether the mitochondria to form contact sites where the majority

of fission events (up to 87%) occur. Time-lapse images of triple colours reveal that a large percentage of putative Drp1 subsequently appears at the ER-mitochondria contact sites. The localization of the mitochondrial fission proteins at the regions of ER-mitochondrial contacts indicates a direct role of the contact sites in the process of mitochondrial division. Additionally, Mff protein, which may mark the future sites of mitochondrial Drp1, is also recruited at the regions of ER contact. ER seems to mark the division site via the contact with mitochondria and is likely to be an active participant in the fission process (*Friedman, Lackner et al. 2011*).

In summary, the fission process at the ER-mitochondria contact sites could be described as following: First, mitochondria are constricted by an ER-dependent nucleation of an actin-myosin contractile apparatus at the ER-mitochondria contact zones. Next, Drp1 and its adaptors are recruited and assembled to this pre-constriction site. Lastly, Drp1 further drives the mitochondrial diameter constriction to 50nm allowing Dnm2 to complete the final scission (*Wu, Carvalho et al. 2018*) and **Figure12**.

Surprisingly, the mitochondrial fusion factor, Mfn2, is involved in the tethering of ER-mitochondria contact sites (*de Brito and Scorrano 2008, Filadi, Greotti et al. 2015, Naon, Zaninello et al. 2016*). Olga Martins de Brito *et al* first showed that Mfn2 is enriched at ER where it contacts with mitochondria, and ER as well as mitochondria are fragmented in Mfn2^{-/-} cells. They also demonstrated that MFN2 is a key regulator for ER and mitochondria tethering, and the depletion of Mfn2 in MEFs and HeLa cells loosens ER-mitochondria interactions. Mitochondrial Ca²⁺ uptake is also reduced in response to InsP₃-generated cytosolic Ca²⁺ transients in Mfn2^{-/-} cells.

However, multiple research groups argued that MFN2 regulates negatively rather than positively ER-mitochondria tethering (*Cosson, Marchetti et al. 2012, Ackema, Hench et al. 2014, Filadi, Greotti et al. 2015*). They performed a thorough quantitative EM analysis of close contacts between ER and mitochondria in Mfn2^{-/-} cells and showed an increase, but not a decrease of MAMs, in Mfn2 deficient cells (*Cosson, Marchetti et al. 2012*). Although, Mfn2 exact function in MAMS is still debated one result that can be granted is that MFN2 is a key factor for the crosstalk between ER and mitochondria.

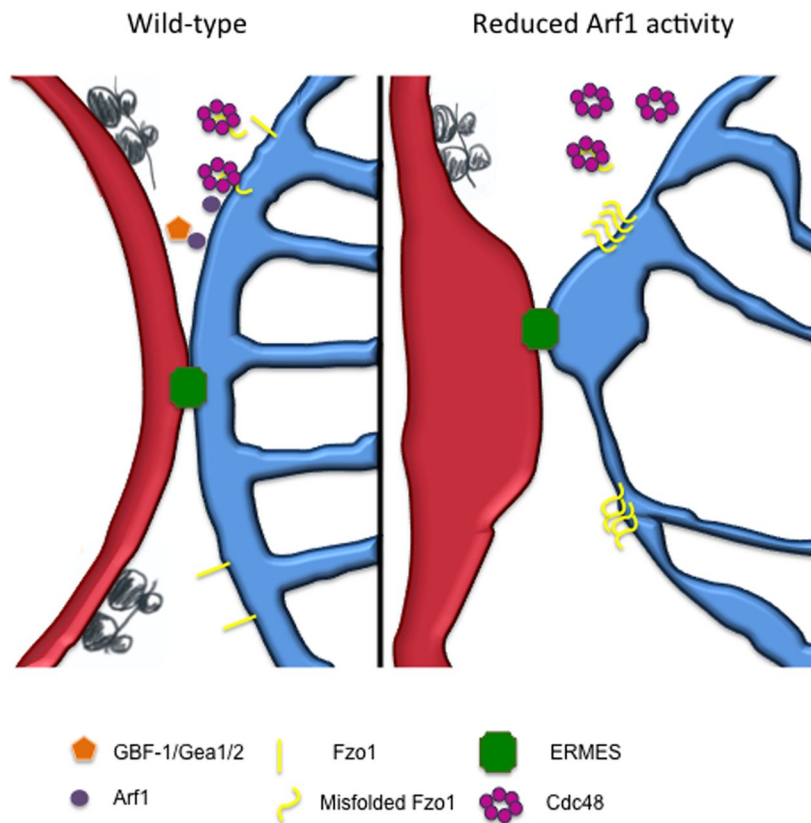


Figure14. Function of Arf1 in mitochondria-related processes The left picture shows the wildtype condition. Arf1 and its GEF, GBF1, contribute to the ER and mitochondrial tethering. The misfolded Fzo1 proteins on mitochondria are removed by Cdc48. The left picture shows the Arf1 knockdown condition. In the absence of Arf1, the ER-mitochondria contact sites are defective, meanwhile, mitochondrial morphology is altered and the misfolded Fzo1 protein accumulate on OMM. (Ackema, Hench et al. 2014).

It has been largely demonstrated that the disruption of ER-mitochondria contact sites alters mitochondrial morphology and function in mammals. In *C. elegans* as well as in other model systems, RNAi against homologs of mammalian or yeast MAM proteins cause hyper-connected mitochondrial network that may be due to fission defect, suggesting the ER-mitochondria connection is also the sites for fission (Ackema, Hench et al. 2014). Recently, Spang lab identified ARF-1.2 as a novel MAM protein in *C. elegans*, ARF-1.2 is a small GTPase, whose homolog in yeast co-fractionated with MAMs. Inactivation of either ARF-1.2 or its guanine nucleotide exchange factors (GEF), GBF-1, results in hyper-fused mitochondria in body wall muscle cells. The altered mitochondrial morphology is similar to that observed in *miro-1* or *vdac-1* knockdown. In addition, the *arf-1.2* RNAi is not sufficient to reverse the fragmented mitochondria phenotype observed in *fzo-1* defect worms. Taken together, ARF-

1.2 regulates mitochondrial morphology and functions via ER-mitochondria contact sites, but does not directly modulate fusion/fission (**Figure14**). However, the contact sites remain but are dysfunctional in ARF-1.2 ablation. In addition, mitophagy is strongly impaired in *arf-1* mutant yeast (*Ackema, Hench et al. 2014*). The role of ER-mitochondria contact sites in autophagy/mitophagy is further discussed in the following section.

4.4.3. The contact sites are also involved in autophagy and mitophagy

The origin of autophagosomal membrane is still a mystery. There are multiple evidences suggesting mitochondria and ER contact sites are involved in autophagosome biogenesis (*Shibutani and Yoshimori 2014*) and **Figure15**.

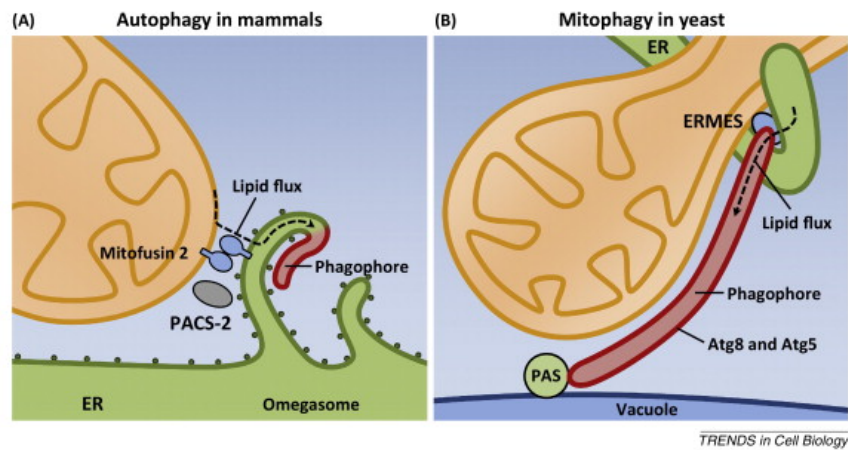


Figure 15. Autophagosome forming at the ER (endoplasmic reticulum)-mitochondria contact sites (A) In mammalian autophagy process, the ER-mitochondria tethering mediated by Mitofusin2 and PACS-2 specify the omegasome site which is the ER region that controls the initiation site for phagophore/autophagosome formation. (B) In yeast system, the ER and mitochondria tethering forms the ERMES complex. The phagophore formation for mitophagy, but not bulk autophagy, is initiated at the ERMES. In yeast, the phagophore initiation site is called PAS. Both in (A) and (B), ER-mitochondria contact sites are critical for the lipid flux between these two organelles. This lipid flux may support the phagophore growth. (*Klecker, Bockler et al. 2014*)

Hailey et al observed that upon starvation-induced autophagy, Atg5, which labels autophagosome initiation sites, appears approximately with mitochondria (*Hailey, Rambold et al. 2010*). Photo-bleaching assay then confirmed the vast majority of the Atg5 puncta is excluded from mitochondrial elements. GFP-LC3 puncta show similar behaviour as Atg5. To address the question whether these autophagosome-mitochondria associations are due to mitophagy events, they labelled the OMM and matrix and observed that they are not overlapped with autophagic structures. It suggests that mitophagy does not underlie the

association of autophagosome with mitochondria. Interestingly, Mitochondrial anchored NBD-Phosphatidylserine (a fluorescent phosphatidylserine lipid) is transferred to newly formed autophagosome, supporting a role for the mitochondrial origin of autophagosome membrane lipids. Moreover, alteration of the ER to mitochondria lipid transfer by Mfn2 depletion almost completely abolishes LC3 puncta formation in starvation-induced autophagy (Hailey, Rambold et al. 2010).

Later on, the role of the ER-mitochondria contact sites in autophagosome formation was further explored (Hamasaki, Furuta et al. 2013). In starvation-induced autophagy, the early autophagic marker Atg14, and Atg5, are localized to the MAMs. MAM fraction is enriched of autophagy proteins such as DFCP1, Atg16, Beclin1 and VPS34, when autophagy is stimulated. Moreover, uncoupling mitochondria from ER by knockdown of key tethering factors PACS-2 or MFN2 leads to attenuated autophagy, including decrease of LC3 lipidation and impaired autophagy machinery on the contact sites. These results suggest that autophagosome formation is initiated at MAMs (Hamasaki, Furuta et al. 2013) and **Figure 15**.

MAMs are also present in yeast, and the protein complex which tethers mitochondria to ER is called ERMES (ER-mitochondria encounter structure) (Kornmann, Currie et al. 2009). The ERMES complex consists of the ER membrane protein Mmm1, the mitochondrial outer membrane proteins Mdm10 and Mdm34, and the soluble factor Mdm12 (Kornmann and Walter 2010). It has been shown that ERMES is important for mitophagy but not bulk autophagy in yeast. ERMES provides lipid for autophagosome formation once the Atg8-Atg32 complex is made on the OMM (Bockler and Westermann 2014). Autophagosomes are also observed to form at ERMES, and BiFC signal assay probes proximity between Mmm1 and Atg8. Upon starvation, GFP-Atg8 can still be delivered to vacuole in ERMES defects, while mitochondria degradation is inhibited. And the mitophagy-specific marker, Atg32, also colocalizes with ERMES. Taken together, mitophagosome originates at the ER-mitochondria contact sites marked by ERMES (Bockler and Westermann 2014) and **Figure 15**.

A study in mammalian cells demonstrated that ER-mitochondria tethering coordinates with autophagosome formation during mitophagy (Gelmetti, De Rosa et al. 2017). In CCCP-induced mitophagy, PINK1, PARK2 and BECN1 levels are increased in the MAM fraction, as well as other early autophagy markers. Either *BECN1* or *PINK1* silencing abolishes the enhanced ER-mitochondria tethering and formation of the omegasome. These results clarify that mitophagosome formation requires the ER-mitochondria contact sites, but on

the other hand, PINK1 and BECN1 function to stabilize the contact sites (*Gelmetti, De Rosa et al. 2017*). DFCP1 recruitment to the omegasome is also dependent on the mitophagy receptor, but whether it happens at the ER-mitochondria contact sites is not explored in this study (*Wong and Holzbaur 2014*).

Although loosened contact between mitochondria and ER is always accompanied by impaired autophagosome/mitophagosome biogenesis, intensive tethering may also lead to autophagy defect. A novel mechanism has been proposed recently for the autophagy regulation by ER-mitochondria tethering. The binding between ER protein VAPB and mitochondrial PTPIP51 tethers two organelles at the contact sites (*Stoica, De Vos et al. 2014, Galmes, Houcine et al. 2016*). Loss of VAPB-PTPIP51 that loosens the ER and mitochondria contact stimulates autophagy. By contrast, increased VAPB-PTPIP51 that tightens the contact inhibits basal or rapamycin-/torin1-induced autophagy. Surprisingly, The VAPB-PTPIP51 regulated autophagy is related to mitochondrial Ca^{2+} transportation. The blockage of autophagy in VAPB-PTPIP51 overexpressing cells is abrogated by siRNAs that repress the IP3-receptor-mediated Ca^{2+} delivery (*Gomez-Suaga, Paillusson et al. 2017*).

VMP1 (EPG-3 in *C. elegans*) regulates the contact of ER with lipid droplets, mitochondria and phagophore (isolation membrane) in metazoan. VMP1 deficiency causes autophagosome formation defect at ER (*Zhao, Chen et al. 2017*) and see [chapter 2.2.2.2 Degradation of SQST-1 during embryogenesis](#)). Whether the tethering between ER and mitochondria is related to the IM formation or release is not explored.

4.5. The roles of Drp1

4.5.1. Drp1 in mitophagy

I have previously described Drp1-dependent and –independent mitophagy. Here I'll present some supplementary information about the role played by Drp1 in mitophagy process.

The mitophagy receptor FUNDC1 has been identified as a novel MAM protein (*Wu, Lin et al. 2016*). Besides binding LC3, FUNDC1 regulates MAM and interacts with DRP1 during mitophagy. In hypoxia-induced mitophagy, FUNDC1 is localized at the ER-mitochondria contact sites via an indirect interaction with calnexin, a MAM protein. Mitochondrial fission upon mitophagy stimuli is significantly inhibited by *FUNDC1* knock-down, implying a role for

FUNDC1 in mitochondrial morphology regulation. FUNDC1 modulates mitochondrial fission via DRP1 since Drp1 translocation sharply decreases in FUNDC1 silencing cells. Co-IP experiments confirm there is a direct interaction between FUNDC1 and Drp1. This study suggests mitochondrial fission and mitophagy are coordinated via the FUNDC1-mediated Drp1 recruitment at ER-mitochondria contact sites (Wu, Lin et al. 2016).

An interesting study indicates that Fis1 mutant could alter mitophagy and generate LC3/LGG-1 aggregation upon mitophagy-induced condition. In this study, they found that overexpression of Fis1 gives rise to grape-like clusters, which are connected by thin tubules of mitochondria, even though Fis1 is not required for fission (Shen, Yamano et al. 2014). These clusters are distinct from that in WT and *drp-1* mutant, but resemble autophagosome intermediates (Yoshii, Kishi et al. 2011). Analysis of *fis1* mutant shows increased LGG-1 clusters, which is further facilitated upon mitophagy induction. These clusters contain LGG-1, Drp1 and portions of mitochondria. The formation of the clusters is dependent on Drp1 and Mff mediated mitochondrial fission, since Mff or Drp1 RNAi suppresses aggregation. In addition, *pink-1* mutant also reduces the LGG-1 clusters in Fis1 mutant worms. By contrast, Drp1 overexpression increases both the number and size of clusters (Shen, Yamano et al. 2014). Taken together, Fis1 mutant induced clusters are products of aberrant mitophagy.

Mammalian Fis1 mutant also forms LC3-containing clusters depending on Drp1, similar to the one seen in *C. elegans*. The studies in cultured cells have explored the mechanism of these clusters: Drp1 is first recruited to mitochondria via Mff, and then into a complex at the ER-mitochondria contact sites for fission. Fis1 is also part of the fission complex but not necessary; however, it mediates the mitochondrial removal after fission in mitophagy-induced conditions (Shen, Yamano et al. 2014).

The autophagic clusters they observed in *fis-1* mutant worms are very similar with what I observed in *drp-1* mutant worms upon heat stress. Both their study and my data indicate a link between mitochondrial fission machinery and autophagy machinery. However, the molecular mechanisms underlying our observations are probably different.

4.5.2. Other functions of DRP-1

Although Drp1 (or DLP1) has been widely proved to regulate mitochondrial fission events, it should be taken into account that Drp1 distributes not only on mitochondria, but also in

cytosol with other organelles. Morphological and biochemical studies (Yoon, Pitts et al. 1998) have shown that Drp1 is predominantly cytosolic, and a substantial portion of cytosolic DLP1 is enriched in a light microsomal fraction which also contains lysosome. In density gradient centrifugation assay, Drp1 was also enriched in light Golgi and ER fraction. A later study found that disruption of Drp1 function dramatically alters the distribution and morphology of both mitochondria and ER (Pitts, Yoon et al. 1999). They demonstrate in these studies that Drp1 is associated with ER tubulars and cytoplasmic vesicles.

The complicated distribution of Drp1 may imply other roles of Drp1 besides regulation of mitochondrial fission.

4.5.2.1. Drp1 is involved in peroxisomal fission

Peroxisomes are ubiquitous cellular organelles that could be observed either as a spherical or tubular organelle. Peroxisome functions usually vary in different cell type. However, most of them have two major and conserved functions: H₂O₂-based respiration and β -oxidation of fatty acid. Other functions include ether lipid (or plasmalogen) synthesis and cholesterol synthesis in animals, glyoxylate cycle in germinating seeds (“glyoxysomes”), photorespiration in leaves, glycolysis in trypanosomes (“glycosomes”), and methanol and/or amine oxidation and assimilation in some yeast (Kawamoto, Tanaka et al. 1979, Tolbert and Essner 1981, Purdue and Lazarow 2001).

Like other organelles, peroxisomes undergo proliferation, multiplication or even degradation in response to nutritional and environmental stimuli (Reddy and Mannaerts 1994). Mammalian Pex11p β regulates peroxisomes biogenesis and morphology, and when overexpressed, Pex11p β promotes peroxisomes proliferation accompanied by elongated morphology (Schrader, Reuber et al. 1998). Drp1 spots are found to localize on these elongated peroxisomes. Moreover, silencing of *DRP1* by siRNA also induces peroxisomal elongation and aggregation resulting from fission inhibition. And two Drp1 adaptors, Fis1 and Mff, which mediates Drp1-dependent mitochondria fission, are also involved in peroxisomes fission (Gandre-Babbe and van der Blik 2008).

These data indicate that Drp1 plays a direct role in peroxisomal fission, in a similar way it plays for mitochondrial fission. But Dnm2, which is the executioner for scission, is only related to mitochondrial but not peroxisomal fission (Kamerkar, Kraus et al. 2018). How Drp-1 mediates this process is largely unknown yet.

4.5.2.2. Drp1 in programmed cell death

A study reported that DRP-1-induced excessive mitochondria fission appears prior to 'refractile corpses' and causes ectopic programmed cell death in *C. elegans* embryos (Jagasia, Grote et al. 2005). Then a following study argued that neither the number nor the kinetics of cell-corpe appearance is affected in *drp-1* null mutant worms. In addition to Drp1, other fusion or fission mutants also show normal programmed cell death (Breckenridge, Kang et al. 2008). The different conclusions between both studies may be due to the off-target effect of the *drp-1* (K40A) used in Jagasia study. *drp-1(tm1108)* or *fis-2(gk363)* weakly enhances the increase of extra cells only in cell-death defect mutants, such as *ced-3* (or *ced-4*) mutant, (Breckenridge, Kang et al. 2008).

Moreover, Drp1 could be cleaved by CED-3 at its amino terminus. This cleavage is important for the proapoptotic function of Drp1, but dispensable for its function in mitochondrial fission, demonstrating that Drp1 has distinct roles in fission and cell death (Breckenridge, Kang et al. 2008).

II RESULTS

1. How *C. elegans* responds to acute heat stress?

2. A preliminary exploration of MAM candidates in *C. elegans*

3. Ether lipid and autophagy (collaborative project)

1. How *C. elegans* responds to acute heat stress?

1.1. Summary

C. elegans, a widely used model animal for the study of autophagy, faces multiply stresses during life cycle. Worms also have developed many mechanisms to adjust and overcome the stress condition. For instance, the starvation stress triggers the dauer formation from L1 stage; dauers are more resistant to the stress from environment and can live several weeks without food. More importantly, dauers can develop into L4 larva once the stress is withdrawn (Golden and Riddle 1984, Hu 2007). Autophagy is important for dauer formation and stress resistance in starved condition (Melendez, Talloczy et al. 2003). In fact, not only under starvation, other stresses, like high temperature (or heat stress), also seem to need the autophagy function for stress resistance and worm survivals. In adult *C. elegans*, it has been shown that heat stress induced autophagy could be beneficial for worm survival and longevity. Tavernarakis group demonstrated that the blockage of mitophagy during severe heat stress (37°C, 7h) impairs the survival of old adult (Palikaras, Lionaki et al. 2015).. Hansen group also showed that a hormetic heat stress (36°C, 1h) applied on young adults is beneficial for worm longevity (Kumsta et al, 2017). However, what are the influences of heat stress on larva? We try to introduce an acute heat stress which would impair but not kill worms, and focus on its effect on larva development. In addition, what is the role of autophagy in worm adjustment to the acute heat stress? These above results are mainly shown in the following manuscript, but I also have an additional part for the extended data to illustrate our work better. The main results of our project are listed here:

- **An acute heat stress (aHS) induces organelles modifications and developmental delay.**

We noticed that an acute heat stress (aHS), 37°C for 1h, on L4 larva could strongly impair worm developing into adult without killing worms. After 24 hours recovery at 20°C, the worms which had been treated by aHS are much smaller and retarded for egg laying compared with the control worms (manuscript **Figure 1A-D, quantifications in E**). DIC pictures showed that the stressed worms were also more transparent, and exhibited various degrees of defect in tissue development, for instance, the blocked fusion of seam cells in epidermis, disordered arrangement in germline and big caves within intestine (**Figure 16**). These obvious development defects were observed after 24h recovery upon aHS, while DIC

pictures suggested that there was no clear impairment on worm tissues soon after aHS (1 to 3 hours recovery). But the transmission electron microscopy (TEM) data revealed that compared with control worms, the organelles within the stressed worms which were recovered in 20°C for 1h, were already been altered. The EM images showed us that aHS to both L4 larva and young adults induced endoplasmic reticulum inflation and mitochondrial fragmentation, the electron dense spots within mitochondrial matrix indicated a severe damage of mitochondria (manuscript Figure 1E-L, E'-L').

- **Mitochondrial function and morphology are transitory altered upon acute HS.**

To better visualize the mitochondrial morphology, we performed the in vivo mitochondrial markers- mitoGFP, Tomm20::mKate2 or immunostaining assay. Both of them proved that aHS could induce mitochondrial network fragmentation in epidermis, muscle and germline (manuscript Figure 2B-E, G-J, quantifications in F, K). Not only the mitochondrial morphology, but also mitochondrial functions were impaired by aHS, confirmed by Seahorse and other assays (manuscript Figure 2A and L). However, the mitochondrial membrane potential did not seem to be affected by aHS, since the MitoTracker™ Red CMXRos, which stains mitochondria depending on mitochondrial membrane potential, could still label mitochondria after aHS treatment (manuscript Figure 2I and J). More important, the fragmented mitochondrial network started to rebuild since 6 hours recovery, the majority of stressed worms had returned to tubular mitochondrial network after 24 hours recovery (manuscript Figure 2M-R).

- **Induction of an autophagy flux protects larvae from acute HS.**

The EM images and in vivo epifluorescences images of autophagic markers showed an increased number of autophagosomes (manuscript Figure 1H-L, H'-L'). To exclude the possibility that the increased autophagosomes are due to the blockage of the fusion with lysosomes, we detected the autophagy in the RNAi of *epg-5*, whose product is required for the fusion between autophagosomes and lysosomes (Wang, Miao et al. 2016), and found that autophagosomes were further accumulated by *epg-5* RNAi upon heat stress (manuscript Figure 3O and P, quantifications in L and Q). This data suggests that acute HS induces an autophagy flux. Both the two homologs of Atg8 family, LGG-1 and LGG-2, participated but had distinguished roles during aHS-induced autophagy (manuscript Figure

3R-V). Autophagy defects had no obvious effect on worm survival from aHS, whereas they led to a more severe developmental delay (**manuscript Figure 3W and X**). Taken together, autophagy is a protective mechanism for worm recovery from acute HS.

- **Mitophagy promotes mitochondrial network rebuilding after acute HS.**

We showed in the manuscript that mitochondria are one of the main cargos during aHS-induced autophagy (**manuscript Figure 4B-E**). Functional autophagy to degrade the damaged mitochondria is important for mitochondrial network rebuilding after aHS (**manuscript Figure 4F-H, quantification in I**). However, the RNAi or mutation of the well-known mitophagy regulators, PINK-1, PDR-1 and DCT-1, had no effect on neither autophagy process nor mitochondrial rebuilding after aHS (**manuscript Figure 4J-L**), suggesting that aHS induced mitophagy at least is partially independent on PINK-1, PDR-1 and DCT-1 (*Palikaras, Lionaki et al. 2015*).

- **DRP-1 is necessary for aHS induced mitochondrial fragmentation.**

It is widely accepted that the DRP-1-mediated mitochondrial fragmentation is a prerequisite for their autophagic engulfment during mitophagy (*Tatsuta and Langer 2008, Mao and Klionsky 2013*), whereas there are also studies demonstrating the existence of DRP-1-independent mitochondrial fission in yeast and mammalian systems (*Mendl, Occhipinti et al. 2011*) (*Hamasaki, Furuta et al. 2013*) (*Yamashita, Jin et al. 2016*). In *C. elegans*, it has been shown by many other groups that DRP-1 is a key fission factor regulating mitochondrial morphology (*Labrousse, Zappaterra et al. 1999*), but is the mitochondrial fragmentation we observed upon aHS also dependent on DRP-1? To address this question, we compared the mitochondrial morphology in *drp-1* null mutant (*Breckenridge, Kang et al. 2008*) under 20°C and aHS. The quantification results showed us that without DRP-1, mitochondrial morphology was slightly modified upon aHS, however, mitochondria were unable to complete fission step (**manuscript Figure 5**).

- **DRP-1 is necessary for autophagosome completion during aHS.**

Although the mitophagy specific regulators are not necessary for aHS-induced autophagy/mitophagy (**manuscript Figure 4J-L**), DRP-1-mediated mitochondrial fission is required for autophagosomes formation. In *drp-1* mutants, there were less

autophagosomes but instead, more autophagic clusters upon aHS, compared with *wild type* worms (manuscript Figure 6A and B, quantifications in C, H-J). These autophagic clusters were confirmed by several autophagy markers, LGG-1, LGG-2 and ATG-18, indicating an abnormal formation of autophagosomes, rather than autolysosomes in *drp-1* mutant worms. In addition, the abnormal autophagic clusters in *drp-1* depletion were always in contact with mitochondria (manuscript Figure 6E, E' and G, G'). Moreover, in wild type worms, autophagosomes were also often in contact with mitochondria, both in basal and aHS condition (manuscript Figure 6D and F), suggesting that mitochondria is the main site for autophagosomes formation. The altered mitochondrial network in *drp-1* mutants may disrupt autophagosomes formation and result in the abnormal clustering.

Autophagy protects *C. elegans* from acute heat-stress-induced development delay through a DRP-1 dependent mitophagy

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Running title: DRP-1 dependent mitophagy

Keywords: acute heat-stress; *Caenorhabditis elegans*; development; autophagy; mitochondrial fission; LGG-1; LGG-2.

Abstract

Stresses, depending on their severity, could affect very differently the cellular response, ranging from a beneficial to a lethal effect. We present here a new paradigm for studying the adaptation of *C. elegans* larvae to acute heat-stress (aHS). aHS induces a strong developmental defect but no lethality or sterility. This developmental stress results in the transitory fragmentation of mitochondria, the formation of aggregates in the matrix and the decrease of mitochondrial respiration. Moreover, an active autophagy flux associated to mitophagy events is triggered in many tissues and in particular in the epidermis. A blockage of the autophagy flux enhances the developmental defect showing that the autophagic response is protective to the animal. However, the depletion of the mitophagy proteins PDR-1, PINK-1 or DCT-1 does not affect the formation of autophagosomes. We discovered that in the epidermis, the mitochondria could be the major site for autophagosome biogenesis both in standard and heat stress conditions. We also report that the depletion of the dynamin related protein DRP-1 affects the HS-induced autophagy process. In the epidermis of *drp-1* animals, the mitochondria are abnormally shaped and connected by thin mitochondrial extensions. Upon acute HS, *drp-1* mitochondria modify their shape but are unable to achieve fragmentation. Autophagy is induced but autophagosomes are abnormally elongated and clustered on mitochondria. This indicates that DRP-1 is essential for quality control of mitochondria by coordinating mitochondrial fission and autophagosomes biogenesis.

Introduction

The effects of heat stress (HS) on lifespan and the mechanisms of adaptation are mainly studied at adulthood but during development, many species could face such aversive condition and should manage to preserve both their cellular homeostasis and developmental programs.

HS response relies on the coordinated activity of both heat shock protein (HSP) chaperone system and autophagy through the activation of the transcription factor HSF1 (Desai *et al*, 2013; Dokladny *et al*, 2013). For instance, in *C. elegans* adults after a mild HS, HSF1 positively controls autophagy gene expression, which participates to a protective hormetic response and life span extension (Kumsta *et al*, 2017).

Numerous studies have further identified HS response pathways specific to particular organelle such as the UPR within mitochondria (UPRmt)(Haynes *et al*, 2013). The mitochondria is central in many aspects of cellular metabolism such as the Krebs cycle, fatty acid oxydation, urea synthesis and the biosynthesis of heme and lipids. It is the major site for ATP production, through OXPHOS complexes, and is also involved in calcium homeostasis and apoptosis. Mitochondriaare also the main producer of reactive oxygen species that could be detrimental for the cell. Recent data have shown that the mitochondria is a central regulator of the decline of HSR during *C. elegans* aging (Labbadia *et al*, 2017).

Mitochondrial quality control is first ensured by UPRmt, an adaptive transcriptional response: that promotes recovery of mitochondrial function (Shpilka & Haynes, 2018), but when dysfunctions are persistent and UPRmt overwhelmed, elimination of the mitochondria can occur by a selective macroautophagy process called mitophagy (Palikaras *et al*, 2018; Pickles *et al*, 2018). Mitophagy engulfs dysfunctional mitochondria in double membrane vesicles, the autophagosomes, which fuse with the lysosome to allow their degradation. Mitophagy occurs at various basal levels depending of cell types but is also induced in stress conditions, such as HS or oxidative stress (Lee *et al*, 2012).

Mitochondria are highly dynamic organelles that reorganize through fusion or fission events, which participate to the mitochondrial homeostasis and can modulate the establishment of contact sites with other cell organelles (Wu *et al*, 2018; Youle & van der Bliek, 2012). Among those, ER-mitochondria contacts are a site of biogenesis of autophagosomes in mammalian cells under starvation (Hamasaki *et al*, 2013). Two members of the Dynamin family, Drp1 and Dnm2, are involved in the mitochondrial fission by a multi-step process including the formation of an endoplasmic reticulum-mediated mitochondrial constriction site (Friedman *et al*, 2011; Labrousse *et al*, 1999; Lee *et al*, 2016). Drp1 oligomerization on the outer mitochondrial membrane (OMM) further increases the constriction and leads to the recruitment of Dnm2 to achieve membrane scission (Ji *et al*, 2015; Kraus & Ryan, 2017). Recent data highlighted a new function for Drp1 in the quality control of mitochondria via an interaction with the mitochondrial Zn²⁺ transporter Zip1 (Cho *et al*, 2019). Impairment of the fission machinery could lead to neurodegenerative diseases (Labbé *et al*, 2014; Wu *et al*, 2018).

C. elegans can develop and reproduce in a range of temperature from 15 to 25°C (Hirsh & Vanderslice, 1976). Adaptation of *C. elegans* to HS has been mainly described in adult animals

in the context of longevity or for genetic screen for resistance (Kumsta *et al*, 2017; Palikaras *et al*, 2015). However, the high heterogeneity in the temperature (28 to 37°C) and the duration of the HS (1 to 6 hours or more) explains the versatility of the effects, ranging from hormesis to lethality. A 1 hour 36°C stress applied to young adult animals has been shown to trigger an hormetic stress response (Kumsta *et al*, 2017) while a 1.5 hours 37°C stress on adults results in 40% lethality (Zevian & Yanowitz, 2014). The transcriptomic analysis of 4th larval stage animals submitted to a 35°C stress revealed a very dynamic change in gene expression in the first two hours of HS but the phenotypes have not been analyzed at the ultrastructural level (Jovic *et al*, 2017).

Recent data on *C. elegans* adult have shown that, in muscle cells, a severe HS induces the fragmentation of the mitochondrial network followed by a DCT-1, PINK-1 and PDR-1 dependent mitophagy (Palikaras *et al*, 2015). Here we analysed the contribution of autophagy to adaptation to stress during the development of *C. elegans*. We developed a novel paradigm to study mitophagy, by submitting early 4th larvae to a sub-lethal acute heat stress (aHS), and characterized the physiological effects on mitochondrial homeostasis and development. aHS induces a rapid fragmentation of mitochondria, the formation of aggregates in the matrix, the decrease of mitochondrial respiration and mitophagy. Stressed animals first present a blockage of development but an autophagic flux enables the rebuilding of the mitochondrial network and the developmental recovery. We report that in the epidermis, the autophagosome biogenesis mainly occurs on mitochondria, in physiological conditions and also upon heat stress. Finally, we discovered that the dynamin related protein DRP-1 is important for autophagosomes formation and could coordinate mitochondrial quality control by mitophagy.

Results

I) Acute heat stress induces organelles modifications and developmental delay.

To analyze whether a sub-lethal HS could induce cellular damages, we submitted either day 1 adults or fourth stage larvae (L4) to 37°C during 60 minutes (see Material and Methods) and perform transmission electron microscopy (TEM). The analysis of four tissues, the epidermis, the muscles, the intestine and the germinal gonad revealed three major subcellular alterations that were detected both in adults (Figure 1) and L4 HS animals (supplementary Figure S1)

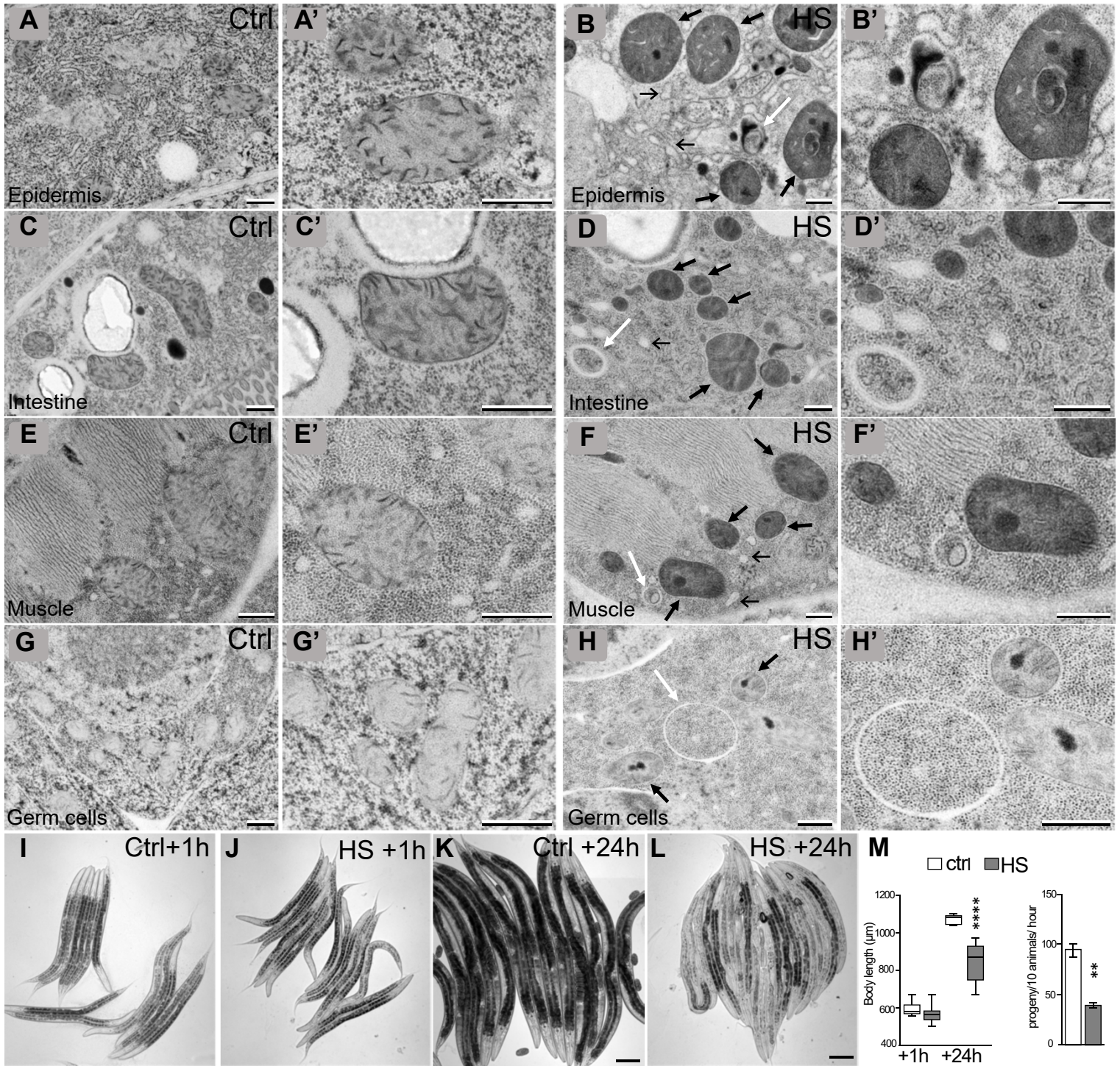


Figure 1: Characterization of cellular and developmental effects of acute heat stress.

A-H) Transmission electron microscopy images of epidermis (A, B) intestine (C,D) muscle (E,F) and germ cells (G,H) in control (A, C, E, G) and HS (B, D, F, H) young adults. Animals were submitted to 37°C during 60 minutes followed by 60 minutes of recovery at 20°C. HS induces a fragmentation and inflation of the ER (open black arrows), the formation of electron dense structures within the mitochondrial matrix (black arrows) and the formation of autophagic vesicles (white arrows). (A'-H') are 2.5-fold magnifications of the boxed areas. The scale bar represents 0.5 μm.

I-M) aHS on L4 animals results in a developmental delay but no lethality. Early L4 animals were submitted to 37°C during 60 minutes and analyzed after 1 hour (I, J) or 24 hours (K, L) recovery at 20°C. M) n=27, 45, 8, 32. T-test ****, p<0.0001; right: 30 worms/treatment, by 4 independent experiments, paired t-test, **, p<0.005

compared to control animals maintained at 20°C. First, the endoplasmic reticulum is fragmented and forms round inflated vesicles compared to the tubular network in control animals (Figure 1A-H). Second, the aspect of mitochondria is altered, they appear darker and the matrix contains irregular electron dense structures, which could correspond to aggregates. Measure of the area and the roundness of the mitochondrial sections in the epidermis indicates smaller but rounder mitochondria (supplementary Figure S1). Third, a number of vesicles that look similar to autophagic structures are present in stressed animals (Figure 1). These data indicate that independently of the stage of the development, several tissues react similarly to HS, which alters both ER and mitochondria morphology, and triggers autophagy. It suggests that such a response to the HS is an intrinsic cellular property. However, the severity in the defects and autophagosomes formation could vary between cell types supporting a tissue-dependent modulation in the response.

We then analyzed whether the effects of HS on L4 larvae could affect their survival, development and fertility. After a 1-hour recovery period at 20°C following the HS, the animals were alive, moving correctly, and presenting no obvious morphological difference with the non-HS controls (Figure 1I, J). Twenty-four hours later, there is no significant difference of survival between HS and control animals (data not shown), but while control worms have reached adulthood and started laying eggs, HS animals are smaller and the start of egg-laying has been delayed (Figure 1K-M). Almost all HS animals become fertile in the next 24 hours (Figure 1M,) and the progeny is viable. These data indicate that a 1-hour 37°C HS on L4 animals results in a developmental delay without affecting the viability and fertility of the worms. The HS parameters, the cellular effects and the developmental consequences on L4 animals, indicate that our conditions correspond to a sub-lethal acute heat stress (aHS) rather than a hormetic stress, and define a good system to analyze the mechanisms of adaptation. For that reason, the rest of the study concentrates on L4 stage to analyze the cellular mechanism of response to aHS in *C. elegans* and focus on the mitochondria phenotype and autophagy.

II) Mitochondrial function and morphology are transitory altered upon aHS

We next address whether the altered mitochondrial morphology and the accumulation of abnormal structures in the matrix is linked with mitochondrial dysfunctions. We used the Seahorse technology to measure the basal and the maximal respiratory capacity in aHS and

control animals. One to two hours after the aHS, both the basal and maximal oxygen consumption of aHS animals are strongly reduced indicating that the mitochondrial respiration is altered (Figure 2A). Because TEM is limited for analyzing the mitochondrial network integrity, we perform immunofluorescence or *in vivo* imaging using various mitochondrial markers expressed in the gonad, the muscle cells, the intestine and the epidermis (Figure 2 and data not shown). Among those, two transgenic strains display an abnormal mitochondrial network at 20°C and were not further used for the study (Supplementary data). Acute HS results in the fragmentation of mitochondria in all tissues analyzed indicating that the stress-mitochondria phenotype observed by EM is accompanied by a complete re-organization of the network. A staining with the matrix mitoGFP (Zhao *et al*, 2017) or the outer mitochondrial membrane Tomm20::mKate2 (Ahier *et al*, 2018), reveals that mitochondria in the epidermis are very strongly affected by aHS, forming numerous small and round vesicular structures instead of the long and reticulated network (Figure 2 D, E, I, J). Quantitative analyses of the branching (aspect ratio) and the circularity (form factor) (Koopman *et al*, 2016; Marchi *et al*, 2017) confirmed the massive fragmentation of the mitochondria (Figure 2F, K).

In the epidermis the co-localization analysis of mitoGFP and CMXRos, a membrane potential dependent mitotracker, shows that most of the fragmented mitochondria maintain a membrane potential (Figure 2I, J). The amount of the epidermal mitoGFP was quantified by western blot in HS and control animals. In control animals, a 25-30kDa major band corresponding to the mitochondrial matrix form after removal of the N-terminal pre-sequence is detected as well as two weak bands corresponding to the precursor forms (Figure 2L). After aHS, the amount of mitochondrial GFP decreases and the precursor forms increased suggesting that the import is somehow affected.

Despite the strong fragmentation of mitochondria, aHS L4 worms are able to resume development and become fertile after 24hours (Figure 1). Therefore, we analyzed the mitochondrial network 3, 6 or 24 hours after aHS. In all animals, the mitochondria are able to rebuild a reticulated network after 24 hours, suggesting a link between mitochondria rebuilding and developmental recovery (Figure 2M-R). Altogether, our data indicate that aHS triggers a rapid mitochondrial fragmentation with functional decrease, followed by a slow recovery of a normal network.

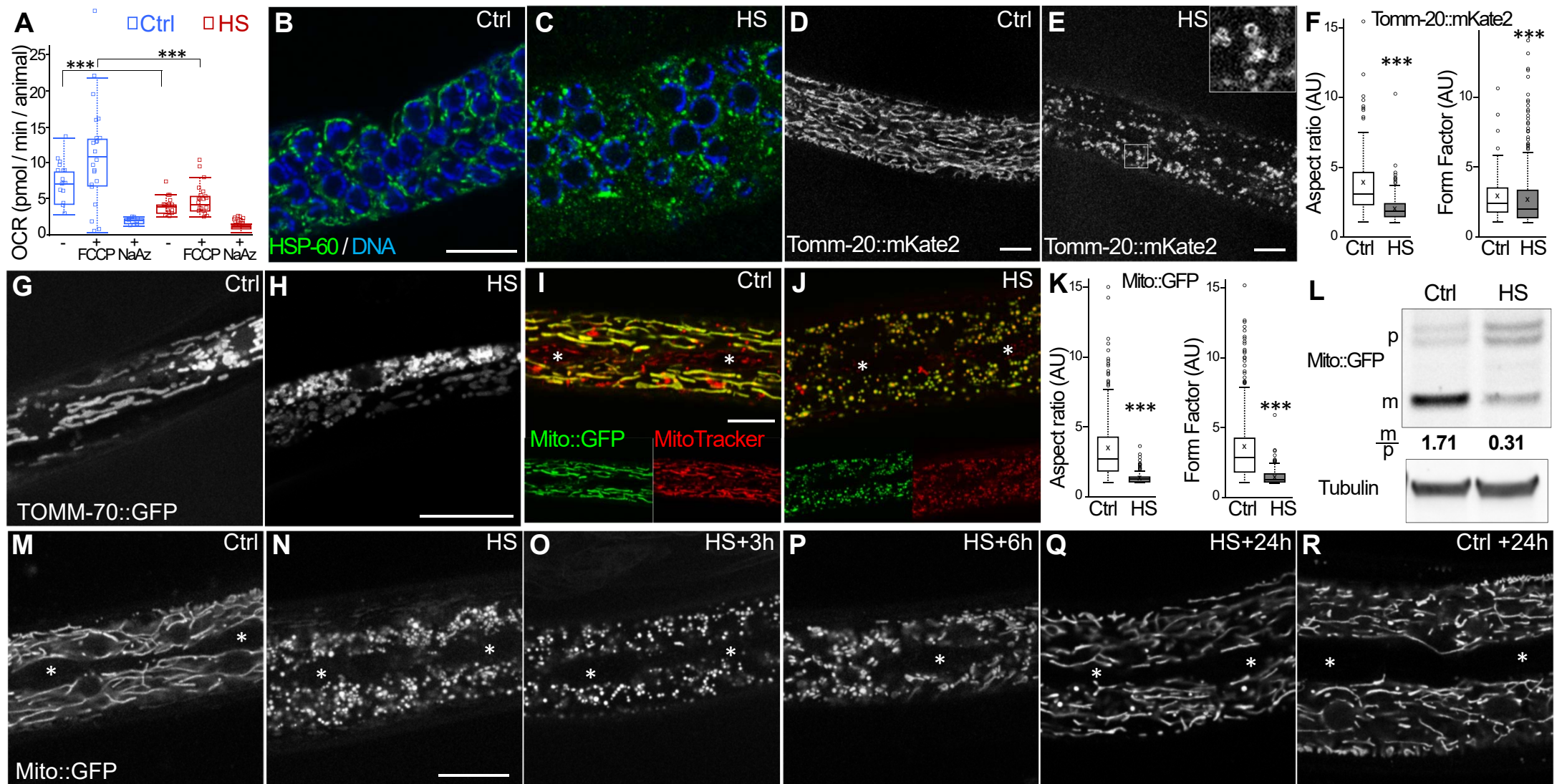


Figure 2: aHS induces transitory fragmentation and dysfunctions of mitochondria

A) Oxygen consumption rate in control and HS animals ($20 < n < 35$; FCCP: Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; NaAz: Sodium azide). Wilcoxon test analysis (***) p-value < 0.001). **B, C**) Confocal images of mitochondrial network in the gonad (anti HSP-60 staining) of control (B) and HS (C) animals. DNA of germ cells is stained with Hoechst (Blue). **D-F**) Confocal images (to check) of the mitochondria in the epidermis, visualized with mKate2 fused with the outer membrane localization domain of Tomm-20 in control (D) and HS (E) animals. Quantification of the mitochondrial shape descriptors, Aspect Ratio and Form Factor using Tomm-20::mKate2 (F). Five animals were analyzed corresponding for a total 200-500 mitochondria. Wilcoxon non-parametric rank test (***) p-value < 0.001). **G, H**) Confocal images of the mitochondria in the muscle, visualized with GFP fused with the outer membrane localization domain of Tomm-70 in control (G) and HS (H) animals. **I-K**) Colocalization of mito::GFP (green) and mitrocker CMXROS (red) in the epidermis of control (I) and HS (J) animals. The white stars shows the position of the specialized seam cells of the epidermis that do not expressed Mito::GFP. Quantification of the mitochondrial shape descriptors, Aspect Ratio and Form Factor using mito::GFP (K). 200-300 mitochondria were measured. Wilcoxon non-parametric rank test (***) p-value < 0.001). **L**) Western blot analysis of epidermal Mito::GFP. The quantification of the cytoplasmic precursor (p) and mitochondrial (m) forms shows a decrease of the import after HS. **M-R**) The recovery of mitochondrial network was analyzed in the epidermis with Mito::GFP in control (M, R) and HS (N-Q) immediately after HS (M, N) and after 3h (O), 6h (P) or 24h (Q, R) recovery. White stars shows seam cell position. The scale bar is 10µm.

III) Induction of an autophagy flux protects larvae from acute HS

Our TEM analyses revealed the presence of autophagic vesicles in various tissues (Figure 1 and supplementary Figure S1). We next wanted to confirm that these structures are *bona fide* autophagosomes and address whether aHS triggers a functional autophagic flux. We first quantify the number of dots positives for GFP::LGG-1, GFP::LGG-2 and ATG-18::GFP, the *C. elegans* homologs of GABARAP, LC3 and WIPI1/2, respectively (Figure 3 A-K) (Alberti *et al*, 2010; Jenzer *et al*, 2014; Jia *et al*, 2007). This confirms that aHS induces an increase in the number of autophagosomes in multiple tissues with a stronger response in the epidermis. The ATG-18 dots, which correspond to initiation of autophagosomes, are detected during aHS, while the increase of LGG-1 and LGG-2 dots is maximum 90 to 120 minutes after aHS (Figure 3 A-H). To exclude the possibility that the dots correspond to GFP aggregates induced by HS, we used the mutated GFP::LGG-1(G116A) and GFP::LGG-2(G130A) that are unable to localize to autophagosomes (Alberti *et al*, 2010; Manil-Ségalen *et al*, 2014) and observed no formation of puncta but a diffuse staining after HS (Figure 3 C, G). Western blot analysis of endogenous LGG-1 confirms the induction of an autophagic flux (Figure 3 L). The last evidence that aHS induces a functional autophagic flux was obtained by a genetic approach. Using mutants and RNAi that block either the formation (*atg-3*, *atg-7*, *lgg-1*) or the degradation (*epg-5*, *lgg-2*) of autophagosomes (Chen *et al*, 2017), we observed respectively a decreased or an increased in the number of autophagosomes (Figure 3M-Q).

We reported previously that the localization of LGG-1 and LGG-2 defines three populations of autophagosomes in *C. elegans* embryo (Manil-Ségalen *et al*, 2014). The *in vivo* analysis of mcherry::LGG-2 (Jenzer *et al*, 2018) and GFP::LGG-1 revealed that aHS induces three types of autophagosomes, LGG-1 only, LGG-2 only and double positive (Figure 3R), and that the colocalization between LGG-1 and 2 increases during recovery (not shown). Moreover, we showed that LGG-1 is essential for biogenesis of autophagosomes and acts upstream of LGG-2, which appears to be involved in autophagosome degradation (Figure 3 S-V). This observation suggests that the sequential functions of LGG-1 and LGG-2, previously reported in the early embryo (Manil-Ségalen *et al*, 2014), are conserved in stress-induced autophagy. We next asked whether the induction of autophagic flux upon aHS could have a protective role for the worm development recovery and survival. We compared the survival and the size of mutant animals for autophagy, 24 hours after aHS, with wild type (Figure 3 W, X). Three hours after HS the *lgg-1*, *atg-3* or *atg-7* animals do not look different to control animals

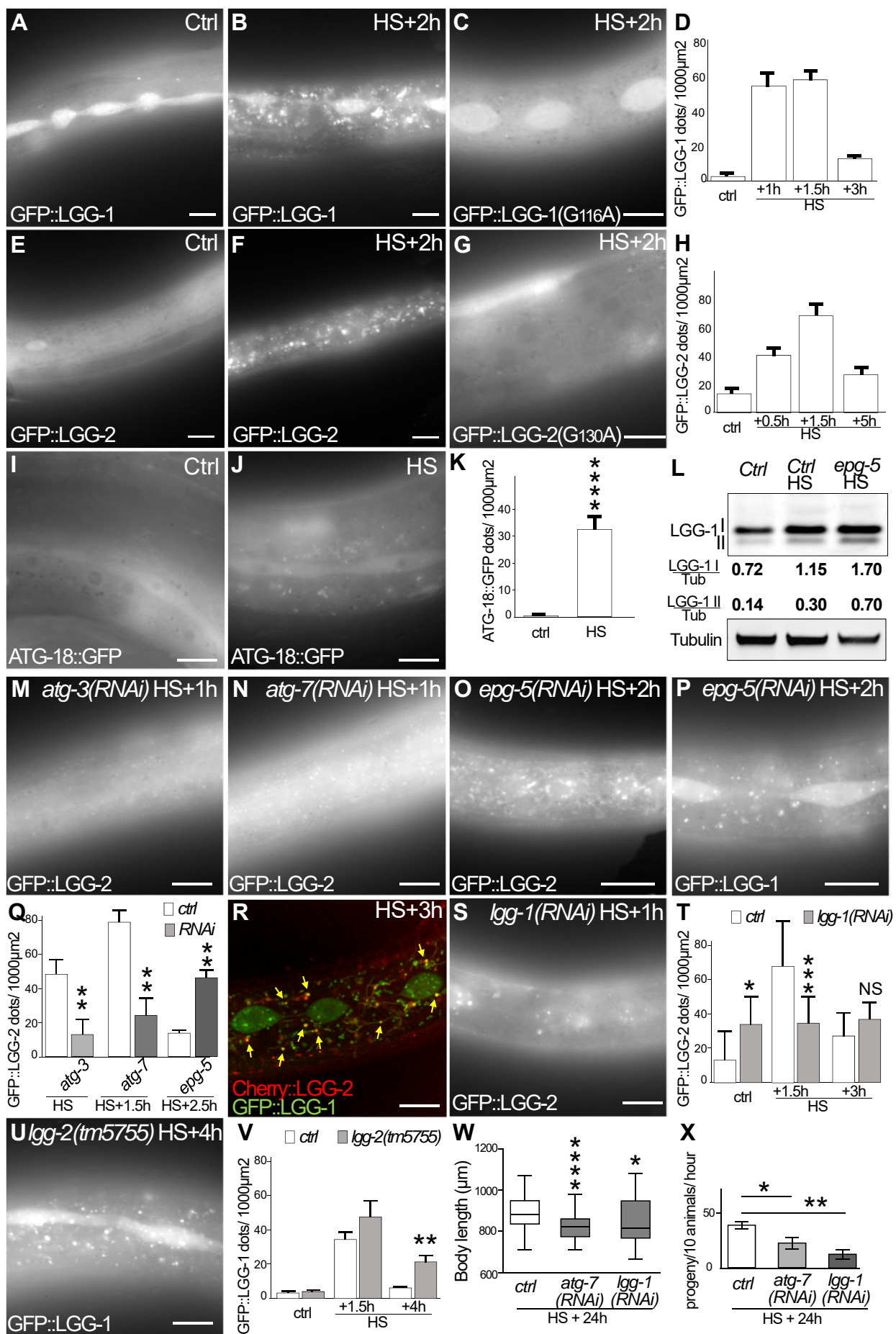


Figure 3: aHS induces a protective autophagy flux

A-D) Autophagosomes are visualized with GFP::LGG-1 in control (A) and HS (C, D) animals and quantified (D Mean + SEM; n=10, 8, 11, 11). The non lipidated GFP::LGG-1 (G116A) does not form puncta after HS (C). **E-H)** Autophagosomes are visualized with GFP::LGG-2 in control (E) and HS (F, G) animals and quantified (H Mean + SEM; n=13, 10, 10, 8). The non lipidated GFP::LGG-2 (G130A) does not form puncta after HS (C). **I-K)** The initiation sites of autophagosomes are visualized with ATG-18::GFP after 30 min treatment in control (I) and HS (J) animals and quantified (K Mean + SEM; n=7, 6). **L)** Western blot of LGG-1 and quantification of the cleaved (I) and lipidated (II) forms shows an increase of the autophagic flux after HS, confirmed by EPG-5 depletion. **M-Q)** The autophagy flux induced by HS was analyzed by imaging of GFP::LGG-2 or GFP::LGG-1 autophagosomes in the autophagy deficient animals *atg-3* (M), *atg-7* (N), *epg-5* (O), and quantified (Q). Mean + SEM; n=5, 5, 5, 5, 8, 5; t-test ** p-value <0.005. **R-V)** LGG-1 acts upstream of LGG-2 during aHS autophagic flux. Three types of autophagosomes are detected after HS, LGG-1 only (green), LGG-2 only (green) and double positive (yellow arrows) (R). The depletion of *lgg-1* (S, T) decreases the number of LGG-2 autophagosomes while the depletion of *lgg-2* (U, V) increases the number of LGG-1 autophagosomes. Mean + SD; n=13, 10, 10, 10, 8, 8; Two-way Anova ** p-value <0.005. **W, X)** The blockage of the autophagy flux exacerbates the developmental phenotypes induced by aHS. 24 h after the HS, the body size (W Box-plot min-max; n=78, 61, 79, t-test, ** p-value <0.005) and the number of progeny (X mean + SEM; paired t-test ** p-value <0.005, **** p-value <0.0001) of *atg-7* and *lgg-1* animals are decreased. The scale bar is 10µm.

suggesting that the autophagic flux is not essential for the immediate survival of animals to aHS. However, 24 hours post aHS, *atg-7* or *lgg-1* RNAi animals are smaller than wild-type animals (Figure 3W). Moreover, autophagy deficient animals start to lay eggs later than the controls (Figure 3X) indicating that the aHS-induced developmental delay is further increased. These data demonstrate that the autophagic flux is protective for the L4 larvae and important for developmental recovery.

IV) Mitophagy promotes mitochondrial network rebuilding after aHS

The correlation between the mitochondrial defects and the induction of an autophagy flux prompted us to investigate mitophagy after aHS. In the epidermis of control animals, the analysis of GFP::LGG-1 and mitoTracker showed no co-localization between mitochondria and autophagosomes (Figure 4A). Interestingly, the measure of the minimal distance between autophagosomes and mitochondria reveals a non-random distribution and a close association of the two organelles (Figure 4A, B). In aHS animals, a fraction of autophagosomes co-localize with fragmented mitochondria suggesting a mitophagy induction (Figure 4 C). Moreover, numerous autophagosomes were also found in close proximity with mitochondria. The measure of the distance between GFP::ATG-18 and mitochondria (Figure 4D, E) confirms that upon aHS, the autophagosome biogenesis is initiated in close proximity to mitochondria. Mitophagy was further confirmed using TEM, by the presence of mitochondria containing dense aggregates within vesicles that look like autophagosomes (Figure 4F). Finally, we also used western blot analysis of Tomm20::mKate2 to monitor the mitophagy flux. Because mKate2 is less sensitive to acidic pH (Shcherbo *et al*, 2009) we postulated that addressing Tomm20::mKate2 to the lysosome should release a mKate2 positive band around 26 kDa (Figure 4G). After aHS, two mKate2 positive bands are detected at 27kDa and 20 kDa, supporting that a fraction of Tomm20::mKate2 is addressed to the lysosome via autophagy. Altogether, our data demonstrate that aHS induces a mitophagy flux.

We then investigated whether the autophagy/mitophagy flux is involved in the rebuilding of the mitochondrial network after aHS. The quantification of mitochondrial rebuilding was performed 24h after aHS in control and autophagy deficient animals (*lgg-1*, *atg-7*). More *lgg-1* and *atg-7* RNAi animals present an altered mitochondrial morphology (Figure 4H-K) demonstrating that autophagy is important for the efficient mitochondrial rebuilding after aHS. Finally, we addressed whether the mitophagy process induced by aHS is dependent of

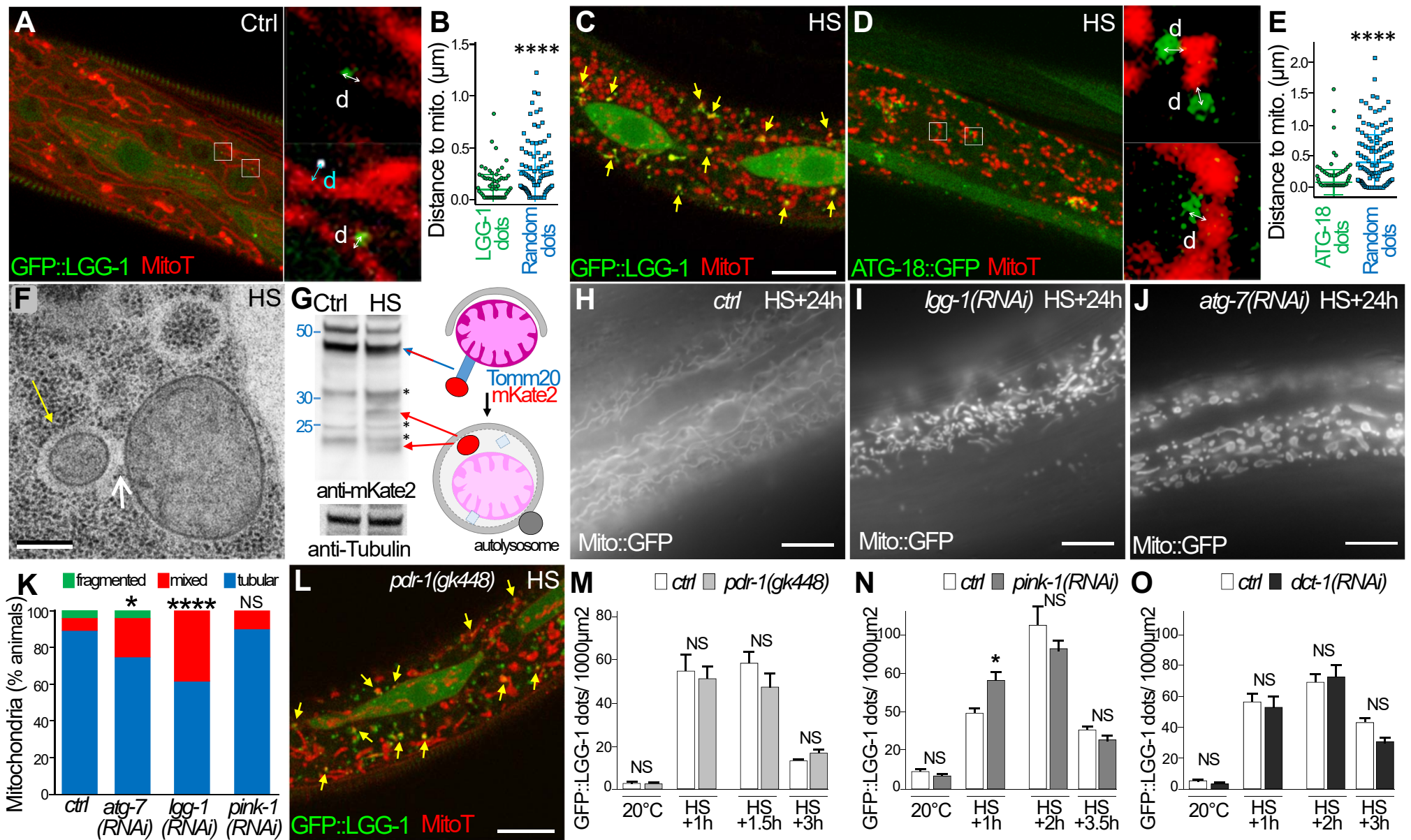


Figure 4 : A mitophagy flux is involved in the mitochondria network rebuilding after aHS

A-E Co-localization analysis of autophagosomes and mitochondria. Confocal images of GFP::LGG-1 (green) and MitoTracker (red) in the epidermis of control animal (A). Insets show the minimal distance (d) between autophagosome and mitochondria and has been compared with random dots (quantification in B n= 89, from 9 animals). In HS animal (C) fragmented mitochondria are co-localizing with autophagosomes (yellow arrows). Confocal images of ATG-18::GFP (green) and MitoTracker (red) in the epidermis of aHS animal (D) showing that initiation of autophagosome occurs in close proximity with mitochondria (insets) compared with random dots (quantification in E n= 151, from 9 animals). The scale bar represents 10 μm . T-test with Welch correction, ****p-value <0.0001. **F** Transmission electron microscopy images of HS animals showing a mitochondria within a vesicle (yellow arrow) still in contact (open white arrow) with a non engulfed mitochondria. Scale bar is 0.2 μm . **G** The western blot analysis of Tomm-20::mKate2, shows the formation of cleaved forms of mKate2 after HS (red arrows). **H-J** The recovery of the mitochondrial network in the epidermis 24h after HS was visualized using Mito::GFP in control (H), *lgg-1* (I) and *atg-7* (J) animals and quantified (K). n=68, 71, 76, 40; Chi-square test, *p-value <0.05, ****p-value <0.0001. **L** aHS induced-mitophagy is present in *pdr-1* mutant animals. **M-N** The depletion of *pdr-1* (I), *pink-1* (J) and *dct-1* (G) does not modify the number of LGG-1 autophagosomes formed after HS. **J** Mean + SEM; n=10, 10, 8, 11, 12, 11, 15; Two-way Anova, NS p-value>0.05. **K** Mean + SEM; n=6, 7, 18, 12, 7, 11, 11, 12; Two-way Anova, *p-value<0.05 NS p-value>0.05. **L** Mean + SEM; n=6, 9, 21, 15, 17, 16, 19, 17; Two-way Anova NS p-value>0.05

either *pink-1/pdr-1* or *dct-1* mitophagy pathways. Mitophagy events are still present (Figure 4L and data not shown) and the number of LGG-1 autophagosomes did not decrease in *pdr-1* mutant animals or in *pink-1* or *dct-1* RNAi (Figure 4M-O), and. Moreover, the mitochondrial network recovery was not affected in *pink-1* RNAi animals (Figure 4K). Altogether, our data show that aHS induces a mitophagy flux at least partially independent of *pink-1/pdr-1* or *dct-1*, and that the autophagy/mitophagy induction is involved in mitochondrial rebuilding. These data also provide the first evidence that mitochondria are the main site for autophagosome biogenesis in the epidermis of *C. elegans*.

V) **DRP-1 is necessary for aHS mitochondrial fragmentation**

Our results indicate that autophagy is involved in mitochondrial network recovery after fragmentation. To address whether the mitochondrial fragmentation is a prerequisite to mitophagy induction we analyzed a null mutant of the dynamin-related protein 1 (*drp-1*), a main actor of mitochondrial fission (Breckenridge *et al*, 2008; Labrousse *et al*, 1999).

DRP-1 function has been mainly studied in the muscle cells of *C. elegans* (Labrousse *et al*, 1999; Scholtes *et al*, 2018; Shen *et al*, 2014) but not well characterized in epidermis. The qualitative and quantitative analyses of the morphology of the mitochondria in *drp-1* animals were performed using mitoGFP and Tomm20::mKate2 (Figure 5). At 20°C, the mitochondrial network of *drp-1* animals in epidermis shows no regular tubular structures but three categories of abnormal mitochondria: the enlarged circular, the filaments-like and the clustered (Figure 5A, and Figure 2M for wild type). The OMM labelling shows that altered mitochondria remain connected (Figure 5C, and Figure 2D for wild type). This data confirms that similarly to muscle cells, maintenance of mitochondria morphology in epidermis is dependent on the fission machinery. However, the oxygen consumption of *drp-1* mutants is not decreased (data not shown) as previously shown (Luz *et al*, 2015). After aHS, the mitochondrial network of *drp-1* animals is no longer detected as small round vesicles contrary to wild-type animals, indicating that the HS-induced fragmentation is DRP-1 dependent (Figure 5B, D, and Figure 2 E, N for wild type). However, the qualitative and quantitative analyses indicate that *drp-1* mitochondrial network is modified after aHS (Figure 5E). The clustering of mitochondria is more obvious and the filament-like are more difficult to observe. To characterize further the phenotypes of mitochondria, we performed a TEM analysis.

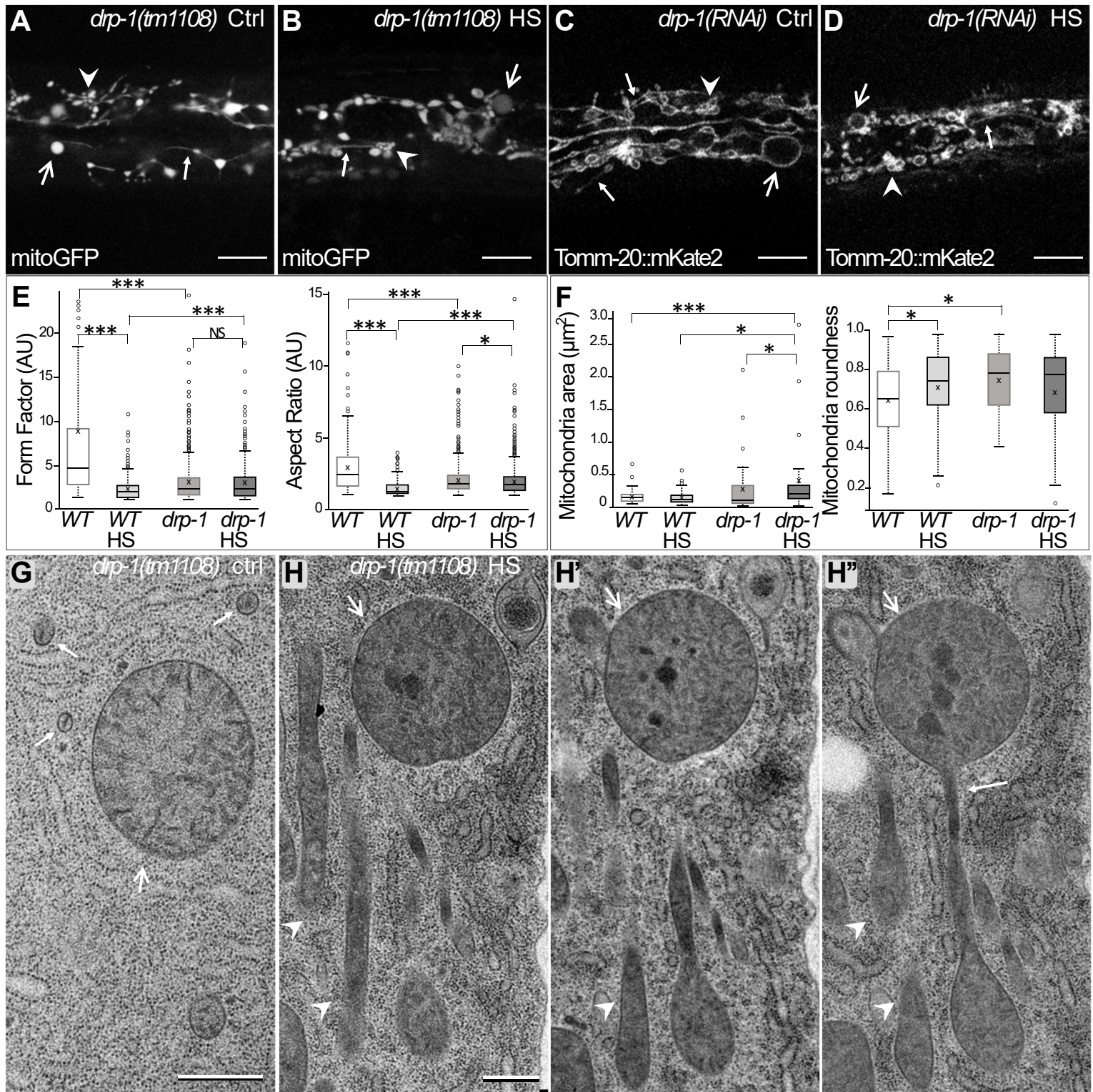


Figure 5: Heat-stress-induced mitochondrial fragmentation is dependent of DRP-1

A-D Confocal images of the mitochondria in the epidermis of *drp-1* animals, visualized with the matrix located mitoGFP (A,B) or outer membrane Tomm-20::mKate2 at 20°C (A, C) or after HS (C, D). Abnormal shaped mitochondria are classified as enlarged (open arrows), clustered (arrowhead) and filament-like (small arrow). **E** Quantification of the mitochondrial shape descriptors, Form Factor and Aspect Ratio using mitoGFP (F). Around 700 mitochondria were measured from 8-9 *drp-1* animals. Wilcoxon non-parametric rank test (p-value *** <0.001). **F** Measures of the area and the roundness of mitochondrial in the epidermis were performed on EM pictures. **G-H** EM pictures of mitochondria in the epidermis of *drp-1* animals, at 20°C (G) or after HS (H). H, H' and H'' are serial sections of stress mitochondria containing dark aggregates and displaying enlarged (open arrows), clustered (arrowhead) and filament-like (small arrow) shape. The scale bar is 10 μm (A-D) or 0.5 μm (G-H).

At 20°C, mitochondria sections are very round but highly variable in size (Figure 5G, quantification in F), confirming the *in vivo* analysis. After aHS, the presence of dark aggregates in the matrix is observed (Figure 5H) and the oxygen consumption is reduced (data not shown), similarly to wild-type animals, but the mitochondria display a very heterogeneous shape and form a highly connected network (Figure 5H quantification in F). Together, our results indicate that in absence of DRP-1, aHS-mitochondria tend to modify their shape but are unsuccessful to achieve fission.

VI) DRP-1 is necessary for autophagosome completion during aHS

We then analyzed whether autophagy/mitophagy is induced upon aHS in absence of fission of the mitochondria. The localization of ATG-18::GFP, GFP::LGG-1 and GFP::LGG-2 was monitored in mutant or RNAi *drp-1* animals at 20°C and after aHS. At 20°C, the three autophagy markers present a pattern similar to wild type (Figure 6A, D, F, quantification in C, H, I). This indicates that DRP-1 is dispensable for the formation of autophagosomes, and its depletion does not result in the induction of autophagy. However, the distribution of autophagosomes is very aberrant after aHS of *drp-1* animals (Figure 6B, E, G compare with Figure 2 for controls). The autophagic markers form vesicular-like structures that cluster in close proximity or even intermingle with mitochondria (Figure 6E', G'). Quantifications of the number and the size of the dots suggests that aHS of *drp-1* animals induces the gathering and/or clustering of autophagic structures. The analysis of the non lipidated GFP::LGG-1(G116A) indicates that the LGG-1 clusters are not protein aggregates (Figure 6K). TEM further confirms the formation of irregular membranous structures in *drp-1* animals after aHS, which engulf stressed mitochondria (Figure 6L).

The localization of autophagic membranes in *drp-1* animals, at 20°C or after aHS, confirms that mitochondria is a favorite site for autophagosome biogenesis in the epidermis. Our data demonstrate that DRP-1 is not necessary for the induction step of autophagy upon aHS. However, the clusters of ATG-18, LGG-1 and LGG-2 positive structures indicate that the induction of autophagosome biogenesis is maintained in absence of DRP-1. Moreover, the abnormal shape of isolation membranes or autophagosomes suggests that the complete biogenesis or closure of autophagosomes is not correctly achieved.

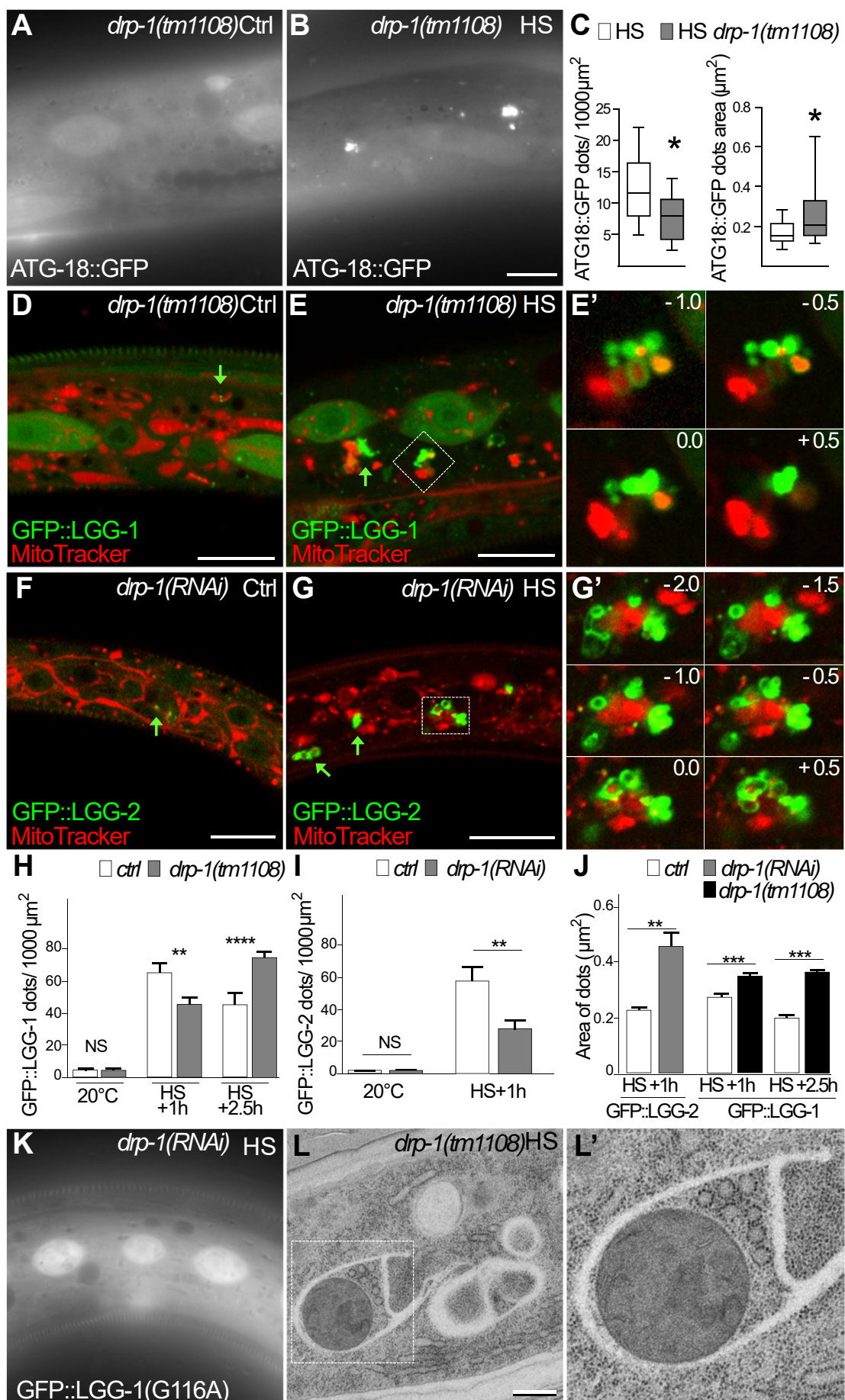


Figure 6: The completion of mitophagosomes is dependent of DRP-1

A-C) The initiation sites of autophagosomes is visualized with ATG-18::GFP in *drp-1* animals at 20°C (A) and after HS (B) and quantified (C). Upon HS autophagosome biogenesis is induced but clustered in *drp-1* animals (compare with figure 3J for a wild-type control). C) Box-plot min-max ; n=13, 14 t-test. **D-J)** Mitochondria are stained with MitoTracker (red) and autophagosomes (green arrows) are visualized with GFP::LGG-1 (D,E) or GFP::LGG-2 (F,G) in *drp-1* animals at 20°C (D, F) and after HS (E, G) and quantified (H-J). Insets in E' and G' are 0.5 μ m Z-series corresponding to the dotted square in E and G. aHS triggers the biogenesis of LGG-1 and LGG-2 positive membrane that clustered and intermingled with mitochondria. H) Mean + SEM, n=19, 16, 12, 11, 10, 10; Two-way Anova, ** p-value <0.005, *** p-value <0.0005). I) Mean + SEM, n=10, 4, 9, 10 Two-way Anova, ** p-value <0.005. J) Mean + SEM, n=9, 10, 12, 11, 10, 10; Two-way Anova** p-value <0.005, **p-value <0.0005). **K)** The non lipidated GFP::LGG-1 (G116A) does not form puncta after HS in *drp-1* animals. **L)** EM pictures of a stress mitochondria engulfed in membranous materials, in the epidermis of *drp-1* animal after HS. The scale bar is 10 μ m (A-K) or 0.5 μ m (L).

Discussion

Stress response pathways help the organism to adapt and survive to changes in environmental conditions. Autophagy is one of the mechanisms used by the cell to resist and adapt to stress conditions. We have characterized a new acute heat stress paradigm for studying the interplay between autophagy and mitochondrial homeostasis. Our data indicate that aHS modifies mitochondrial function, induces a DRP-1 dependent fission of the mitochondrial network and triggers a mitophagic flux.

The aHS (1h at 37°C) during the early 4th larval stage induces a marked developmental delay but no lethality or sterility, confirming previous analyses at 35 or 37°Cs (Jovic *et al*, 2017; Zevian & Yanowitz, 2014). When young adults are submitted to a similar heat stress no obvious anatomical phenotype has been report and worms display a hormetic response and increased lifespan (Kumsta *et al*, 2017). This indicates that during development animals are more sensitive to a short but elevated heat than at adulthood. The adaptation to HS is priority and possibly detrimental to the ongoing developmental program. A transcriptomic analysis revealed a very global shift in expression dynamics during the first 3-4 hours of HS, and identify cuticle synthesis among the major developmental process that are disrupted (Jovic *et al*, 2017). Interestingly, aHS triggers a massive stress response in the epidermis, the main tissue involved in cuticle synthesis during larval stages (Page & Johnstone, 2007).

Our data show that mitochondria are rapidly fragmented upon aHS and present a decrease in oxygen consumption. Moreover, TEM analysis revealed the characteristic presence of dark occlusions in the matrix of heat-stressed mitochondria, which could correspond to protein aggregates due to the temperature-induced misfolding of proteins. Such inclusions have not been observed in the cytosol or in the inflated ER, suggesting that they reflect some specificity of the mitochondria. Noticeably, similar inclusions have been described in sperm-derived mitochondria (Al Rawi *et al*, 2012; Wang *et al*, 2016; Zhou *et al*, 2016). Before their elimination by mitophagy (Al Rawi *et al*, 2012; Sato & Sato, 2011), the small and round paternal mitochondrial present in the fertilized oocyte have also dark spots. A similar phenotype have also been reported in a fraction of mitochondria in *C. elegans* mutants for the fission/fusion genes *eat-3/OPA1* and *fzo-1/mfn1* (Byrne *et al*, 2019), supporting a link between occlusion formation and mitochondrial morphology.

After aHS, the fragmented mitochondria are recovering a tubular shape demonstrating the reversibility of the stressed phenotype. Our data demonstrate that autophagy is important for

the mitochondrial rebuilding and for the developmental delay recovery. Studies on adult worm muscles after various stress, suggest a balance response between PINK-1/PDR-1 dependent mitophagy and mitochondrial biogenesis (Palikaras *et al*, 2015), but did not document the rebuilding of the network. After aHS, we observed a strong mitophagy response in the epidermis but weaker in the muscles confirming a previous report (Kumsta *et al*, 2017). We did not detect an obvious difference in autophagosome formation in *pdr-1* mutant and *pink-1* RNAi animals, suggesting either a different mitophagy pathways or a possible redundancy.

Our study provides the first evidence that autophagosomes are formed close to mitochondria in *C. elegans* epidermis, in basal or aHS-induced autophagy. Hamasaki *et al*. have reported that ER-Mitochondrial contact zones are a major sites for autophagosome biogenesis in mammal cells after a chemical stress (Hamasaki *et al*, 2013). One can suppose that similarly to mammal cells the biogenesis of autophagosomes in *C. elegans* occurs at specific contact sites between ER and mitochondria.

Studies in *Drp-1* mouse models have shown that the mitochondrial phenotype is highly variable between tissues (Kageyama *et al*, 2014; Wakabayashi *et al*, 2009), which could be linked to differences in regards to mitochondrial functions, like ATP production. In *C. elegans*, the phenotype of *drp-1* mutant has been mainly studied in muscles and neurons but not characterized in the epidermis. Our *in vivo* and EM analyses confirm that in the absence of DRP-1, the mitochondria form a highly connected network composed of blebs and thin tubules. This could result from the initial constriction steps for mitochondrial fission, involving ER contact (Elgass *et al*, 2015) and actin filament (Friedman *et al*, 2011; Korobova *et al*, 2013; Kraus & Ryan, 2017), but the impossibility to process further in absence of DRP-1. The modification of the mitochondrial morphology (Figure 6) of *drp-1* animals after heat stress suggests that mitochondria are initiating an unsuccessful fission process.

The autophagy/mitophagy response to aHS is affected in *drp-1* mutant worms, which could be due to the mitochondrial fission or a quality control defect. The selective elimination of mitochondria by mitophagy, has been initially linked to mitochondrial fission (Youle & van der Bliek, 2012). Several reports have shown that a blockage of the fission or an increase of the fusion reduce the mitophagy (Gomes *et al*, 2011; Rambold *et al*, 2011; Twig *et al*, 2008). Fission could allow a “stress test” allowing screening mitochondria and further triggering complete depolarization and mitophagy when a mitochondrion is sub-functional. A recent report

proposed that the coordination of mitochondrial fission and control quality involves the interaction of DRP1 with the Zn²⁺ transporter Zip1 (Cho *et al*, 2019). However, an alternative process has been shown, with a local mitochondrial fission mediated by the extending isolation membrane/phagophore during mitophagy (Yamashita *et al*, 2016). This study performed in hypoxia or DFP-induced mitophagy conditions, showed that most of the autophagosomes are formed on or near mitochondria, and concluded that Drp1 is not essential for this process, despite a decrease in mitophagy efficiency. Our data support a similar mechanism of formation of autophagosomes/mitophagosomes in contact of mitochondria upon aHS in *C. elegans*, but we observed a strong alteration of autophagosomes biogenesis. It is possible that the mechanisms of formation of autophagosomes vary depending on the types of stress and cells used. It is tempting to speculate that in *drp-1* mutant worms, the contact sites between ER and mitochondrial are formed but are maintained for longer time because there is no mitochondrial fission. If these stabilized contact sites are involved in autophagosome biogenesis, a prolonged initiation of isolation membrane would explain the clustering observed upon aHS.

Acknowledgments

The authors would like to thank Hong Zhang, Chonglin Yang, Nektarios Tavernarakis, Steven Zuryn, Sylvie Hermann-Ledenmat, Raynald Cossard and the Caenorhabditis Genetic Center which is funded by the NIH National Center for Research Resources (NCRR), for providing reagents. We are grateful to Legouis lab members for helpful discussions. The present work has benefited from the core facilities of Imagerie-Gif, (<http://www.i2bc.paris-saclay.fr>), member of IBISA (<http://www.ibisa.net>), supported by “France-BioImaging” (ANR-10-INBS-04-01), and the Labex “Saclay Plant Science” (ANR-11-IDEX-0003-02).

This work was supported by the Agence Nationale de la Recherche (project EAT, ANR-12-BSV2-018) and the Association pour la Recherche contre le Cancer (SFI20111203826). YC received a fellowships from the “China Scholarship Council”. None of the authors of this work have a financial interest related to this work.

Materials and Methods

***C. elegans* culture and strains**

Nematode strains were grown on nematode growth media (NGM) plates (autoclaved 1.5g NaCl (Sigma-Aldrich, 60142), 1.5g bacto peptone (Becton-Dickinson, **211677**), 0.5ml cholesterol (Sigma-Aldrich, C8667) 5mg/ml, 1L H₂O, 10g bacto agar (Becton-Dickinson, 214010) supplemented with 500µl CaCl₂ (Sigma-Aldrich, C3306) 1M, 500µl MgSO₄ (Sigma-Aldrich, M5921) 1M, 10ml KH₂PO₄ (Sigma-Aldrich, P5655) 1M, 1650µl K₂HPO₄ (Sigma-Aldrich, 60356) 1M and fed with *Escherichia coli* strain OP50 (Caenorhabditis Genetic Center, E. coli HT115[DE3; <https://cgc.umn.edu/>). The *C. elegans* Bristol N2 strain was used as a wild-type strain. Genotypes of all the strains used in this study are listed in Table S1.

For heat stress, adults worms were allowed to lay eggs for 1 to 2 hours at 20°C, on NGM plates and then removed. NGM plates were maintained at 20° until the progeny reached early L4 stage, and were submitted to 37° for 60 minutes in an incubator (Binder). Plates were then let to recover at 20°C. For egg-laying analysis, 10 worms were placed on NGM-OP50 plates for 1h at 20°C, then removed and the number of eggs immediately scored. Each score was done in triplicate, and reproduced 4 times independently.

For MitoTracker staining, L1-L2 worms were transferred to NGM agar plates containing 3.7 µM of MitoTracker Red CMXRos (Molecular Probes, invitrogen) and incubated for overnight in the dark. Worms were transferred for 1h into a normal NGM plate for lowering background staining.

RNA mediated interference

RNAi by feeding was performed as described (Kamath & Ahringer, 2003; Timmons & Fire, 1998). Fourth-larval stage (L4) animals or embryos were raised onto 1 mM isopropyl-D-β-thiogalactopyranoside (IPTG)-containing nematode growth media (NGM) plates seeded with bacteria (*E. coli* HT115[DE3]) carrying the empty vector L4440 (pPD129.36) as a control or the bacterial clones from the J. Ahringer library, Open Biosystem (lgg-1: JA:C32D5.9, lgg-2: JA:ZK593.6, atg-7: JA:M7.5, epg-5:, pdr-1:, pink-1:, dct-1:, drp-1). RNAi phenotypes of F1 generation embryos were scored one day after the beginning of egg laying by F0 adults.

Light microscopy imaging

Epifluorescence images were captured on an AxioImagerM2 microscope (Zeiss) equipped with Nomarski optics, coupled to a camera (AxioCam506mono). Worms were immobilized by 100mM sodium azide (in M9), and mounted on 2% agarose pads. Considering the effect of

sodium azide on mitochondria respiration, the observation duration after mounting was limited within 10 minutes. Animal length was measured using ImageJ.

Confocal images were captured on a confocal Leica TCS SP8 microscope with serial z sections of 0.5 to 1 μm . Images were analyzed using the ImageJ Cell Counter plugin (<http://imagej.nih.gov/ij>).

Analyses of mitochondrial shape

The Aspect Ratio and the Form Factor, which are indicators of the mitochondrial branching and length, respectively, have been quantified as described before (Koopman *et al*, 2016; Marchi *et al*, 2017). Briefly, on ImageJ software, the images are convolved using the matrix developed by Koopman, then a threshold is applied to isolate mitochondria from background. In the case of *drp-1* mutant, the convolution was not applied because it generated artificial objects. Mitochondria were measured by the implement analyse particles tool with a minimum filter of 15. Form Factor is the reverse of the circularity. For EM pictures the outlines of mitochondria were manually drawn. For figure 4I, the quantification of worms presenting fragmented, mixed or tubular was done by direct observation.

Statistics

All statistical analyses were performed by using either the Prism or the R software (www.r-project.org). The error bars represent the standard deviation (SD). The Shapiro-Wilk's test was used to evaluate the normal distribution of the values and the Hartley Fmax test for similar variance analysis. Data derived from different genetic backgrounds were compared by the Wilcoxon-Mann-Whitney test. The Fisher's exact test and Chi-squared test were used for nominal variables. Manders' coefficients data were compared using the Welch two sample t-test. On boxplots, the bottom and the top of the box are the lower and the upper quartiles, respectively, the black line represents the median and X the mean.

Electron microscopy

Larvae L4 stages were transferred to M9 20% BSA (Sigma-Aldrich, A7030) on 200 μm -deep flat carriers (Leica Biosystems), followed by cryo-immobilization in the EMPACT-2 HPF apparatus (Leica Microsystems; Vienna Austria) as described (Largeau & Legouis, 2019). Cryo-

substitution was performed using an Automated Freeze-substitution System (AFS2) with integrated binocular lens and incubating chamber (Leica Microsystems; Wetzlar, Germany) with acetone. Blocks were infiltrated with 100% EPON and embedded in fresh EPON (Agar Scientific, R1165). Ultrathin sections of 80 nm were cut on a ultramicrotome (Leica Microsystems, EM UC7) and collected on a formvar and carbon-coated copper slot grid (LFG, FCF-2010-CU-50). Sections were contrasted with 0,05% Oolong tea extract (OTE) for 30 minutes and 0.08 M lead citrate (Sigma-Aldrich, 15326) for 8 minutes. Sections were observed with a Jeol 1400 TEM at 120 kV and images acquired with a Gatan 11 Mpixels SC1000 Orius CCD camera.

Oxygen consumption measurements

Synchronized eL4 worms were recover in M9 buffer (Brenner, 1974) and washed three times in EPA (60mg MgSO₄-7 H₂O, 60mg CaSO₄ 2H₂O, 4mg KCl per liter)(Luz *et al*, 2015) separated by 2min centrifugation at 100g at 21°C. Worms were transferred in SeaHorse XF plate wells, in a final 180µl volume and six measurements were performed with a setting corresponding to 2 min mix, 30s wait, 2min measure. 15 µM FCCP (Sigma, C2920) and 40mM sodium azide were used to determine spare activity and non mitochondrial oxygen consumption. The number of animals (15 to 30 per well) was determined to normalize the OCR calculated by the Wave software (<http://www.agilent.com/en/products/cell-analysis/software-download-for-wave-desktop>). Experiments were performed in triplicates.

Western blot

Total protein extracts were prepared from a synchronized population of eL4 on the ubiquitous Tomm20:: mKate2::HA strain. The worms were collected after centrifugation and then mixed with the lysis buffer (PBS Triton 2%) containing glass beads. They were then denatured using Precellys 24 machine at 6000rpm with incubation for about 5 min twice to cool down the sample. The protein extracts are then centrifuged at 15000 rpm and separated on a NuPAGE 4%-12% Bis- Tris gel (Life Technologies, ref). The non-specific sites are then blocked after the incubation for one hour with TBS Tween 1X (Tris Base NaCl, Tween20) milk 5%. Blots were probed with anti-GFP, anti-RFP (targets mKate2, 1:5000; ThermoFischer Scientific; R10367) or anti-Tubulin (1:2000; Sigma, ref) and revealed using HRP-conjugated antibodies (1:1000,

company, ref) and the Super Signal Pico Chemiluminescent Substrate, Pierce. Signals were revealed on a Las3000 photoimageer (Fuji) and quantified with imageJ.

Table1: strains used in the study

Strain name	Description
DA2123	<i>adls2122[Plgg-1::gfp::lgg-1; rol-6 (df)] II</i>
VIG09	<i>unc119(ed3)III; IS[unc-119(+); Plgg-2::gfp::lgg-2]</i>
RD202	<i>unc119(ed3)III; IS[unc-119(+); Plgg-1::gfp::lgg-1(G116A)]</i>
RD155	<i>unc119(ed3)III; Ex[unc-119(+); Plgg-2::gfp::lgg-2(G130A)]</i>
RD204	<i>unc119(ed3)III; IS[unc-119(+); Ppie-1::gfp::lgg-1]</i>
CU6372	<i>drp-1 (tm1108) IV</i>
YC1-1	<i>N2; Ex[Pmyo-3::mito::Timer ; rol-6(su1006)]</i>
SJZ328	<i>[eft-3p:: tomm-20::mKate2::HA::tbb-2 3' UTR]</i>
?	<i>drp-1(tm1108);Ex[pmyo-3::mito::Timer; rol-6(su1006)]</i>
EU2917	<i>drp-1(or1941[drp-1::GFP]) IV.</i>
IR1631	<i>N2;EX003[P_{myo-3}TOMM-20::mitoRosella]</i>
IR1284	<i>N2;IS[Pmyo-3mitoGFP]; Ex011[P_{lgg-1}DsRed::LGG-1]</i>
?	<i>P_{myo-3}Tom70::GFP</i>
FU2565	<i>yqis158[Py37alb.5::mito::GFP+unc-76]</i>
YC130	<i>unc119(ed3)III; Ex[unc-119(+); Ppie-1::HRP-KDEL]</i>
MAH 247	<i>sqli25[atg-18p::atg-18::gfp+rol-6(su1006)]</i>
LIU	<i>ldrls2[Pmtd-28::mdt-28::mCherry+unc-76]</i>
VC1024	<i>pdr-1(gk448) III</i>
BX259	<i>acl-7 (wa20) II</i>
RD380	<i>RET-1::GFP</i>
RD393	<i>mCherry::LGG-2; Plgg-1::GFP::LGG-1</i>
RD209	<i>unc119(ed3)III; IS[unc-119(+); Ppie-1::mCherry::lgg-1]</i>

List of supplementary figures

Supplementary Figure S1: TEM characterization of aHS in L4 animals

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Supplementary materials and methods

C. elegans strains

DA2123 *adls2122*[*Plgg-1::gfp::lgg-1*; *rol-6* (*df*)] II

This strain is a gift from C. Kang (*Melendez, Tallochy et al. 2003*). Wild type N2 worms were injected with a transgene expressing the N-terminal GFP tagged LGG-1 under the control of the *lgg-1* promoter and a marker plasmid, PRF4 [*rol-6*(su1006)] expressing a dominant mutant allele of *rol-6* which confers a roller phenotype ([Rol]). Transgenes have been subsequently integrated in the *C. elegans* chromosome II by gamma rays irradiation. Therefore, 100% of worms are [Rol] and expressed the GFP tagged version of LGG-1.

VIG09 *unc-119(ed3)III*; *Is*[*unc-119(+)*; *Plgg-2::gfp::lgg-2*]

This strain is a gift from Vincent Galy (*Al Rawi et al, 2012*). *unc-119(ed3)* mutant worms were transformed by biolistic using an N-terminal GFP tagged version of LGG-2, driven by the *lgg-2* promoter and the selection plasmid expressing the wild type version of *unc-119* gene. Transgenes are integrated in one *C. elegans* chromosome.

RD372 (*lgg-2*(*pp112*)[*mCherry::lgg-2*])

This strain has been constructed in R. Legouis laboratory (*Jenzer, Simionato et al. 2019*). This strain was obtained by a CRISPR-CAS9 (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-associated 9) approach, using a *dpy-10* co-CRISPR protocol (*Paix, Folkmann et al. 2015*). mCherry was inserted in-frame 13 nucleotides after the beginning of exon 1.

RD393 *mCherry::LGG-2*; *Plgg-1::GFP::LGG-1*

This strain was generated by crossing RD372 mCherry::LGG-2 males with DA2123 hermaphrodite. The crossing has been made by Roman Leboutet (unpublished results).

SJZ328 *foxSi75* [*eft-3p::tomm-20::mKate2::HA::tbb-2 3' UTR* (*oxti185*)]

This strain is a gift from Arnaud Ahier (*Ahier, Dai et al. 2018*). This nematode strain expresses ubiquitously the mkate2 fluorescent protein addressed to the outer mitochondrial membrane. It is an integrated line. It was generated by amplifying the *eft-3p* promoter sequence from pSZ115 using primers SZ23 and CD5, *tomm-20* genomic DNA was amplified from N2 worms and the *mKate2* sequence was amplified from pDD287.

FU2565 *yqis158*[*Py37alb.5::mito::GFP+unc-76*]

This strain is a gift from Chonglin Yang (*Zhou, Wang et al. 2018*). This strain expressed and addressed GFP to the mitochondrial matrix under the control of the epidermis specific promoter of *y37alb.5* gene.

RD155 *unc-119(ed3)III*; *Ex*[*unc-119(+)*; *Plgg-2::gfp::lgg-2*(G130A)]

This strain has been constructed in R. Legouis laboratory (*Alberti et al, 2010*). *unc-119(ed3)* mutant worms were transformed by biolistic using an N-terminal GFP tagged version of the mutated version (G130A) of LGG-2, driven by the *lgg-2* promoter, and the selection plasmid expressing the wild type version of *unc-119* gene. This is a non-integrated line.

RD204 *unc-119(ed3)III*; *Is*[*unc-119(+)*; *Ppie-1::gfp::lgg-1*]

This strain has been constructed in R. Legouis laboratory (*Manil-Ségalen et al, 2014*). Integrated transgenic lines was generated by microparticle bombardment of *unc-119(ed3)* mutant worms using an N-terminal GFP tagged version of the mutated version (G130A) of LGG-2, driven by the *lgg-2*

promoter, and the selection plasmid expressing the wild type version of *unc-119* gene. This is a non-integrated line.

RD202 *unc-119(ed3)III; Is202[unc-119(+); Plgg-1::gfp::lgg-1(G116A)]*

This strain has been constructed in R. Legouis laboratory (Manil-Ségalen et al, 2014). *unc-119(ed3)* mutant worms were transformed by biolistic using an N-terminal GFP tagged version of the mutated version (G130A) of LGG-2, driven by the *lgg-2* promoter, and the selection plasmid expressing the wild type version of *unc-119* gene. This is a non-integrated line.

CU6372 *drp-1(tm1108) IV*

This strain has been obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA). This homozygous strain is viable but has a slow growth. This mutant strain has been first described in Breckenridge et al (Breckenridge, Kang et al. 2008).

RD415 *drp-1(tm1108);Ex[pmyo-3::mit::Timer; Ex rol-6(su1006)]*

RD415 was constructed by crossing *drp-1(tm1108)* males with YC1-1 strain hermaphrodite. F1 progeny with MitoTimer signal and Rol phenotype were subsequently selected, isolated and genotyped by PCR (Polymerase Chain Reaction) for the presence of one *drp-1* deletion allele. F2 animals from such mother were then subsequently isolated and PCR genotyped once they have started to lay eggs to select lines harbouring a [Rol] phenotype.

EU2917 *drp-1(or1941[drp-1::GFP]) IV*

This strain has been obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA). The endogenous *drp-1* locus was tagged with GFP at its N-terminal using CRISPR/Cas9. This mutant strain has been first described in Lowry J, et al. (Lowry, Yochem et al. 2015)

IR1631 *N2;Ex003[Pmyo-3::TOMM-20::mitoRosella]*

This strain is a gift from Nektarios Tavernarakis (Palikaras, Lionaki et al. 2015). This strain is used to monitor mitophagy. Rosella probe combines DsRed (the coral *Discosoma* red fluorescent protein) which emits a pH-insensitive red fluorescence and GFP which emits a pH-sensitive green fluorescent.

IR1284 *N2;Is[Pmyo-3::mito::GFP];Ex011[Plgg-1::DsRed::LGG-1]*

This strain is a gift from Nektarios Tavernarakis (Palikaras, Lionaki et al. 2015). It expresses a dual fluorescent reporter system combining a mitochondria marker, the mitochondrial-targeted GFP with a DsRed autophagosome marker.

VC1024 *pdr-1(gk448) III*

This mutant strain has been obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA). This strain was made by the *C. elegans* Reverse Genetics Core Facility at the University of British Columbia, which is part of the international *C. elegans* Gene Knockout Consortium. This strain is homozygous for a deletion allele of the *pdr-1/K08E3.7* gene. The mutant is superficially wild type (Funatsu et al. 2012).

BX259 *acl-7(wa20) II*

This strain has been obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA). This homozygous mutant strain is deficient in ether-linked lipids synthesis with high content of saturated fatty acids. This mutant strain has been first described in Shi X et al. (Shi, Tarazona et al. 2016)

RD380 *ret-1(pp120)[ret-1::GFP]*

This strain has been constructed in R. Legouis laboratory (Scarcelli et al. manuscript in preparation). GFP was inserted at the endogenous *ret-1* locus by a CRISPR-CAS9 (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-associated 9) approach, using a *dpy-10* co-CRISPR protocol (Paix,

Folkmann et al. 2015). GFP was inserted in-frame at the C-terminus in order to label all RET-1 isoforms.

RD209 *unc-119(ed3)III; Is[unc-119(+); Ppie-1::mCherry::lgg-1]*

This strain has been constructed in R. Legouis laboratory (Manil-Ségalen et al, 2014). This integrated transgenic line was generated by microparticle bombardment of *unc-119(ed3)* mutant worms using an N-terminal mCherry tagged version of the mutated version of LGG-1, driven by the *pie-1* promoter, and the selection plasmid expressing the wild type version of *unc-119* gene.

YC1-1 N2; Ex[Pmyo-3::mito::Timer ; rol-6(su1006)]

This strain was provided by Agnes Delahodde (I2BC, unpublished result). Wild type N2 worms were injected with a transgene expressing the Timer protein addressed to the mitochondrial matrix under the control of the *myo-3* promoter (muscle specific expression) and a marker plasmid, PRF4 [rol-6(su1006)] expressing a dominant mutant allele of *rol-6* which confers a roller phenotype ([Rol]). This is a nonintegrated line.

RD415 *drp-1(tm1108); Ex[Pmyo-3::mito::Timer ; rol-6(su1006)]*

To generate RD415, we crossed *drp-1* mutant males with RD415 hermaphrodites with a roller phenotype. [Rol] F1 progeny were isolated and genotyped by PCR for the presence of one *drp-1* deletion allele. [Rol] F2 animals were then subsequently isolated and genotyped once they have started to lay eggs. Then, the presence of mitoTimer signal was tested using fluorescence microscopy. We repeated this double selection until we selected one line giving roller animals, expressing mitoTimer and homozygous for the *drp-1(tm1108)* deletion allele.

RD416 *drp-1(tm1108); foxSi75 [eft-3p::tomm-20::mKate2::HA::tbb-2 3' UTR (oxti185)]*

To generate RD416, we crossed *drp-1* mutant males with SJ2328 hermaphrodites. F1 progeny were isolated and genotyped by PCR for the presence of one *drp-1* deletion allele. F2 animals were then subsequently isolated and genotyped once they have started to lay eggs. Then, the presence of mKate2 signal was tested using fluorescence microscopy, progeny with a weak mkate2 signal was then isolated. We repeated this double selection until we selected one line with strong mkate2 and homozygous for *drp-1* deletion allele.

RD 417 N2; Ex[Pmyo-3::Tom70::GFP ; rol-6(su1006)]

This strain is a gift from Ann Spang (Ackema, Hench et al. 2014). The molecular construction has been made by Arnaud Labrousse (Labrousse, Zappaterra et al. 1999). The *pmyo-3::TOM70::GFP* plasmid (Labrousse, Zappaterra et al. 1999) was co-injected with pRF4 [rol-6(su1006)] (Mello, Kramer, 1991). This mitochondrial outer membrane marker has the N-terminal 30 amino acids of TOM70 amplified from yeast genomic. This is a non-integrated line highly instable.

RD408 *unc-119(ed3)III; Ex[unc-119(+); Ppie-1::HSP-3::HRP::KDEL]*

This integrated transgenic lines was generated by microparticle bombardment of *unc-119(ed3)* mutant worms using the HRP cDNA fused with the ER addressing signal and ER retention signal of *hsp-3* gene at the N-terminus and C-terminus respectively, driven by the *pie-1* promoter, and the selection plasmid expressing the wild type version of *unc-119* gene.

MAH 247 *sqIs25[atg-18p::atg-18::gfp+rol-6(su1006)]*

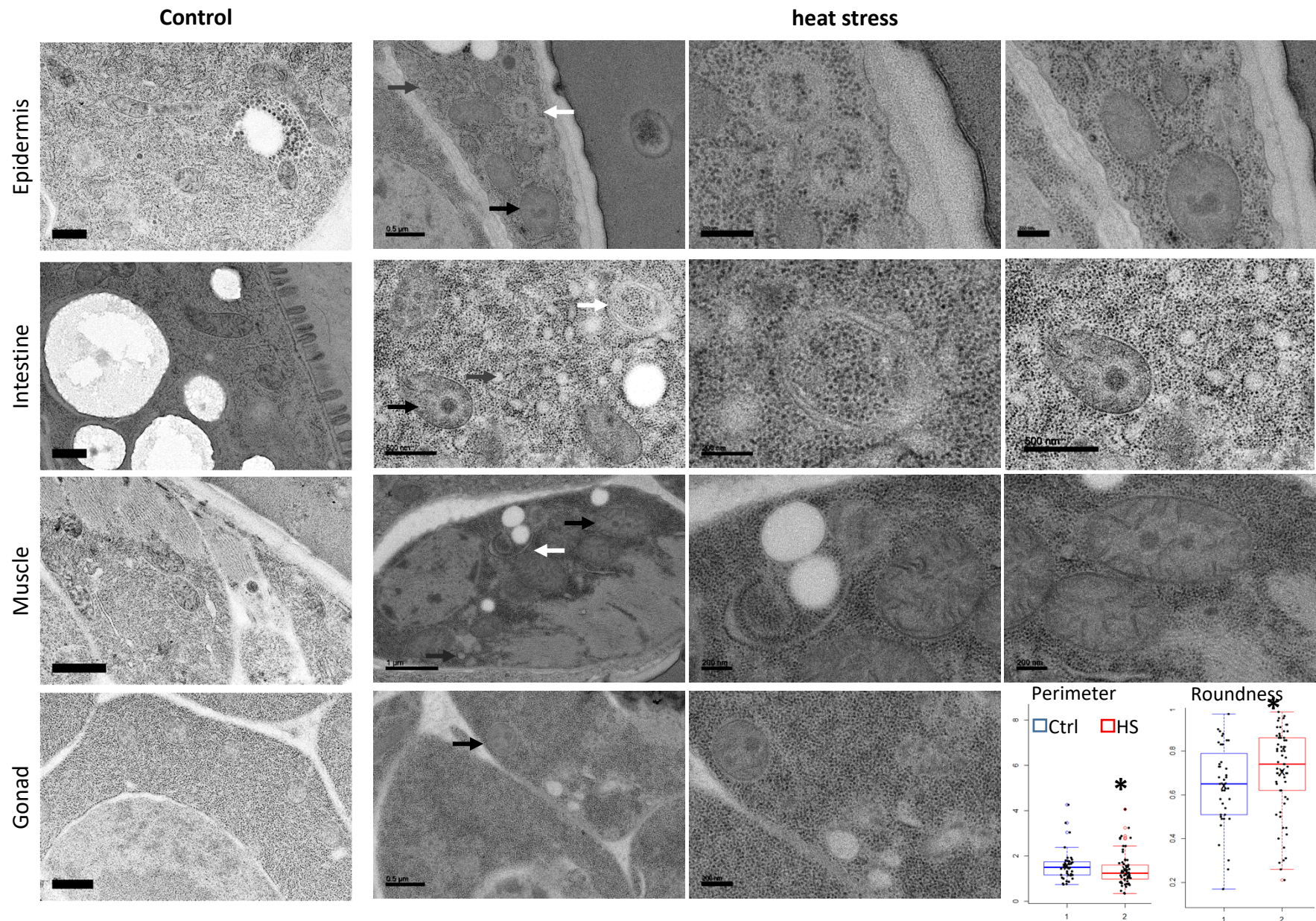
This strain has been obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA). Wild type N2 worms were injected with a transgene expressing the C-terminal GFP tagged ATG-18 under the control of the *atg-18* promoter and a marker plasmid, PRF4 [rol-6(su1006)] expressing a dominant mutant allele of *rol-6* which confers a roller phenotype ([Rol]). Transgenes have been subsequently integrated in one *C. elegans* chromosome. Therefore, 100% of worms are

[Rol] and expressed the GFP tagged version of ATG-18. This strain has been first described in McQuary et al. (*McQuary, Liao, 2016*).

LIU2 Idrls2[Pmtd-28::mdt-28::mCherry+unc-76]

This strain is a gift from Zhang Hong and constructed by Liu Pingsheng (*Na, Zhang, 2015*).

Supplementary Figure 1 Chen et al



1.3. Extended data

1.3.1. Phenotypic characterizations

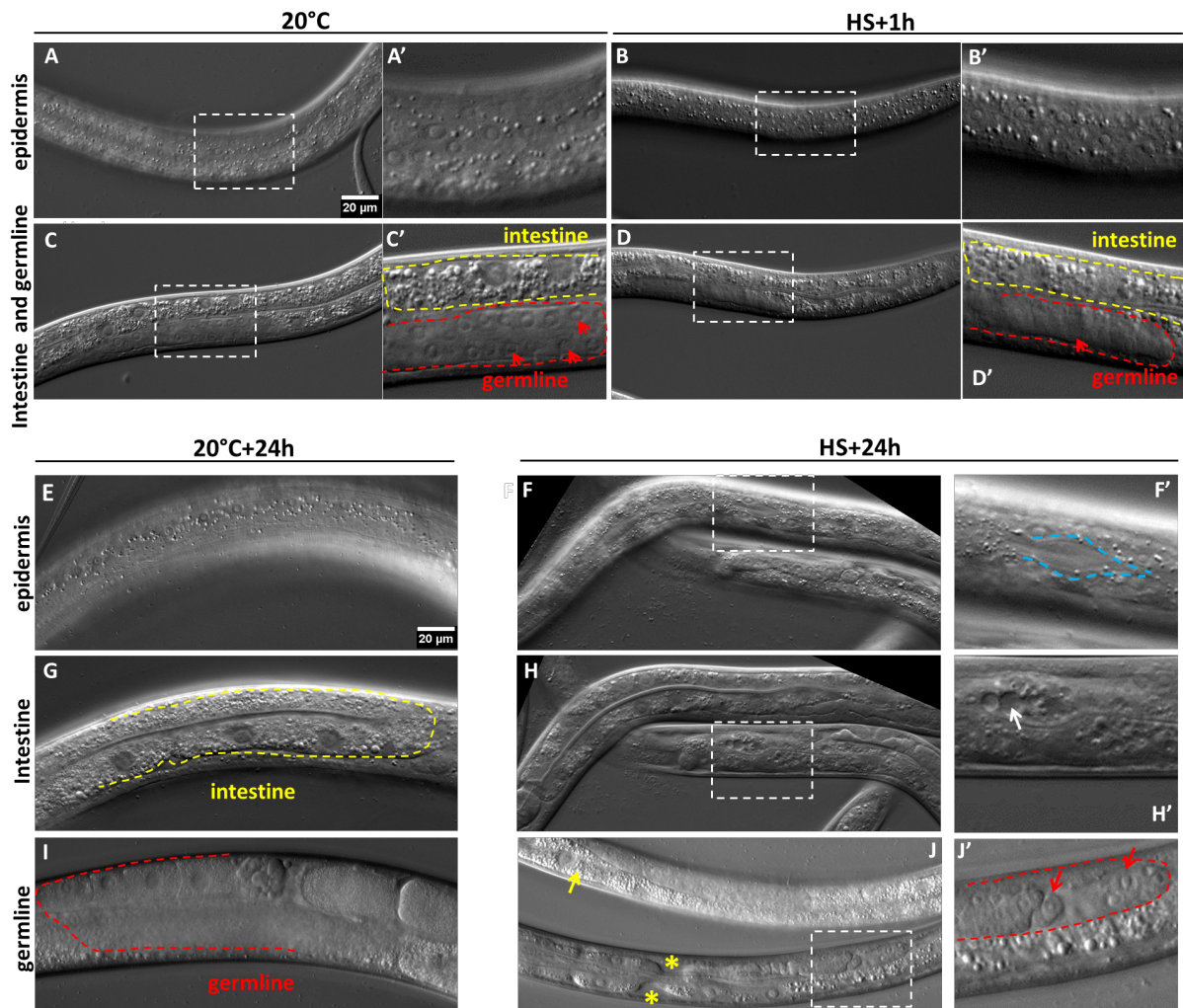


Figure 16| Heat stress impairs the tissue morphologies during development. (A to D), DIC pictures of N2 worms from control (A, C) or 1h recovery after heat stress (B, D) show epidermis (A, B) and intestine and germline (C and D). The A'-D' are the amplified pictures from the white frames of the corresponding area of A-D. (E to J), DIC pictures of N2 worms from control (E, G, I) or 24h recovery after heat stress (F, H, J). Tissues shown here are epidermis (E, F), intestine (G, H) and germline (I, J). F', H' and J' are the amplified pictures of F, H and J. Intestine and germline are indicated in (C', D', G, I, J')_by the yellow and red dashed line, and blue lines in F' represents the seam cell. The red and yellow arrows, and yellow asterisks indicate germline cells, intestine cell, and the abnormal intestine construction respectively. Scale bar: 20 μ m.

In the manuscript Chen et al., we have demonstrated that the heat stress treatment inhibits worm development and shortens worm body size (manuscript Figure 1A-E). But we were also curious about what are the influences of heat stress on the tissue level. Considering distinguishing these tissues under DIC, we chose the epidermis, muscle and germline as the

tissues for observation. Firstly, we detected the tissues morphologies soon after heat stress. Compared with the well-arranged germline that was indicated by the clear nuclei within the worms growing at 20°C (**Figure 16C and C'**), the stressed worms had a fuzzy nuclear morphology which might indicate the disordered germline (**Figure 16D and D'**). Meanwhile, the epidermis and intestine had no obvious defect in the stressed worms (**Figure 16B and B'**). By contrast, the stressed worms were heterogeneous after 24h recovery. While the major worms are well recovered from the aHS, in some stressed worms, the tissues are strongly damaged. For instance, the seam cells of epidermis failed to fuse together (**Figure 16F and F'**) and the construction of intestine was abnormal (**Figure 16H, H' and J, J'**). Compared with the well-turned gonad arm which was consisted of the meiotic and mitotic part, loop part, oocytes and sperms in the control worms, some of the stressed worms had shortened germline by different degrees, and one of the main defects was the severe disorder of the meiotic or mitotic part (**Figure 16J'**). To sum up, heat stress treatment has only a slight influence on worms soon after the treatment but leads to a further defect of tissues development.

1.3.2. Autophagy/mitophagy

Table 6. Autophagy responds differently in muscle and epidermis

		GFP::LGG-1 dots (DA2123, L3-L4)			
Stress time		37°1h	37°1.5h*	37°2h*	37°3h*
tissue		epidermis	muscle	Both epidermis and muscle	
Recovery time	0h	X		X	X
	1h	↑↑	X	X	X
	2h	↑↑↑	X	X	X
	4h	↑	↑	X	X
	5h	↑	↑↑		
	6h	X	↑↑		
	24h	No lethality		variable survivals	

X --indicates no obvious change of GFP::LGG-1 dots

↑--indicates an increase of GFP::LGG-1 dots

The number of the blue arrows indicates the population of dots

↑--indicates a weak increase of GFP::LGG-1 dots

* --from only 1-2experiments

To search for the most appropriate heat stress that induces autophagy in *C. elegans*, we examined autophagy events by measuring GFP::LGG-1 dots in response to different treatment (Table 6). A heat stress longer than 1h was able to induce autophagy in L3-L4 worms, while too long-time treatment (3h) led to lethality. It is interesting that the recovery at normal temperature after 37°C treatment is important for autophagy induction since the HS1h and recovery 1h rather than the HS 2h recovery 0h was able to increase the GFP::LGG-1 dots. But why recovery is required for autophagy induction is unclear yet. We also observed a tissue-specific response upon heat stress. Autophagy in epidermis occurs before that in muscle. GFP::LGG-1 dots started to increase after 1h recovery in epidermis, while their appearance in muscle cells took at least 4 hours of recovery. This result is corresponding with Hansen's team work, in which the hormetic heat stress (36°C, 1h) induces autophagy in epidermis prior to muscle in young adults (Kumsta, Chang et al. 2017). In general, the heat stress induced autophagy in epidermis starts from 1h recovery and ends after 5-6h recovery, and the number of autophagosomes peak at 2h recovery after heat stress. On the other hand, the increasing of autophagosomes in muscle begins after 4h recovery and is maximum one hour later.

However, in our heat stress condition (37°C, 1h) in L3-L4 larva, most of the increased GFP::LGG-1 dots or clusters in muscle did not colocalize with the fragmented mitochondria (Figure 17C and C').

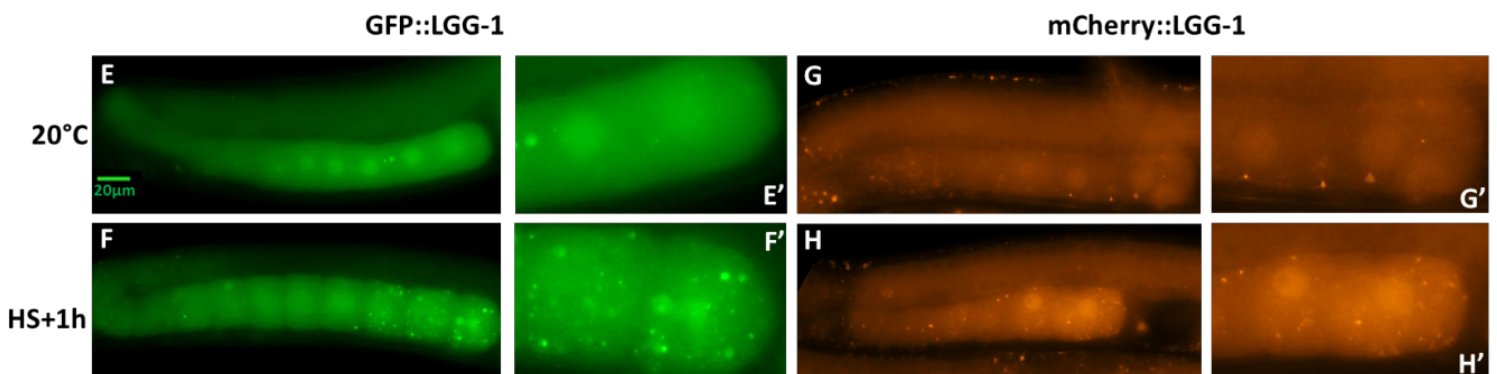
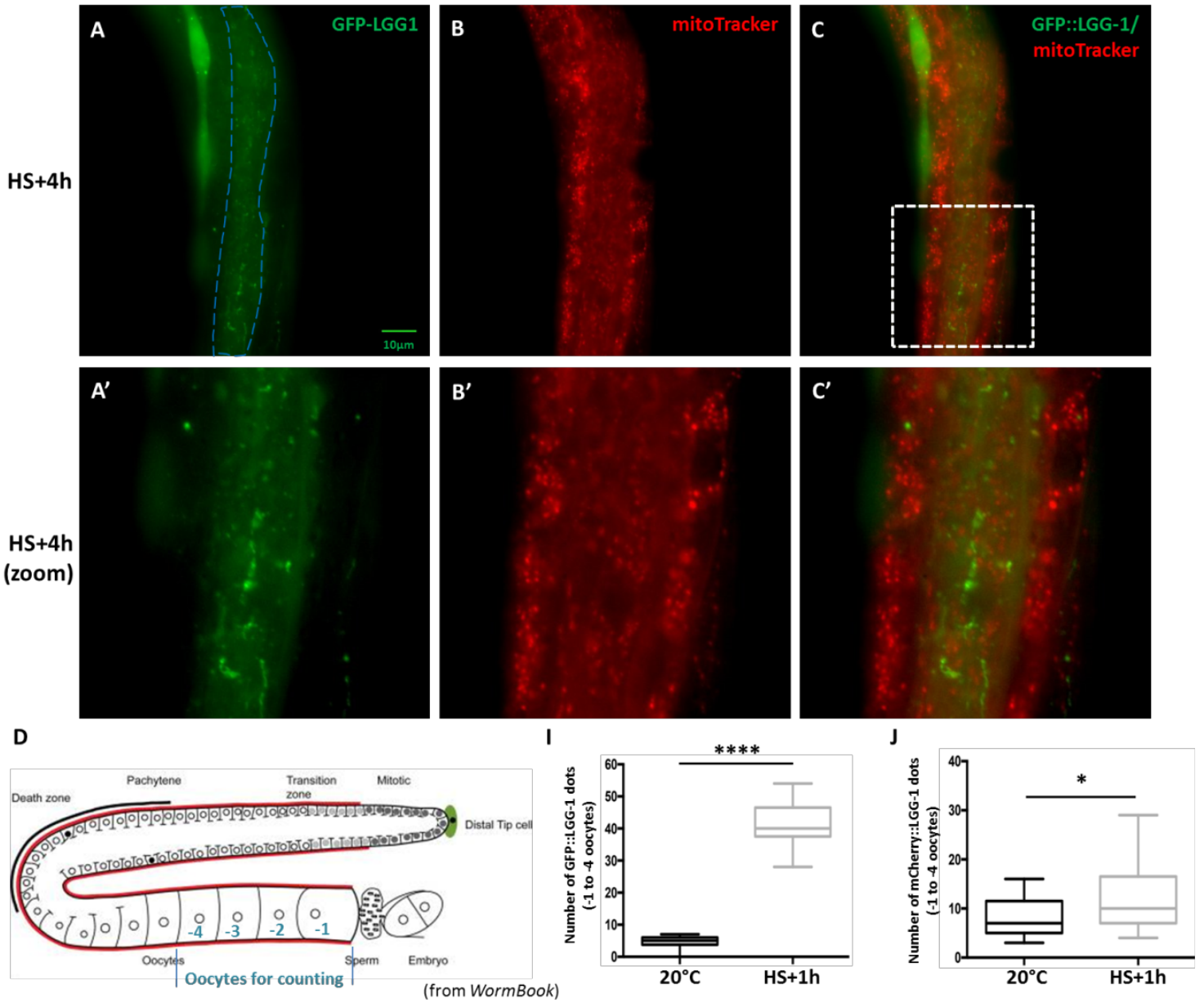


Figure 17| Heat stress also induces autophagy in the muscle and germline. (A-C), autophagy, rather than mitophagy, is stimulated by heat stress in muscle. Worms expressing GFP::LGG-1 were treated with aHS for 1h, followed by 4h of recovery. Mitochondria were stained with MitoTracker™ Red CMXRos (B, B'). Autophagy (A, A') was detected in muscle, as indicated by blue dashed lines. Merged images of GFP::LGG-1 and mitochondria are shown in (C, C'). (D) Schematic diagram of structure of germline. (E-J), Worms were engineered with Ppie-1::GFP::LGG-1 (E, F, E', F', I) or Ppie-1::mCherry::LGG-1 (G, H, G', H', J) to express LGG1 in germline, and treated with aHS for 1h and then recovered for 1h. GFP::LGG-1 and mCherry::LGG-1 dots number were counted in (I, J). n= 11, 10 (I) and 17, 14 (J). Mann-whitney test, ** p<0.005; **** p<0.0001. Representative images are shown in (E-H, E'-H').

The germline is another potential model for studying heat stress induced autophagy and a previous published paper has demonstrated that the elevated autophagy helps to inhibit tumour growth in germline (*Gomes, Odedra et al. 2016*). For this reason, we decided to analyse the autophagy in germline both under basal and heat stress conditions.

Since the oocytes and spermatozoan are not completely formed in larva, we used the young adult, which has the mature germline to perform heat stress assay. For *in vivo* observation of autophagosomes, we applied the strain RD204 expressing GFP::LGG-1 under the *pie-1* promoter which drives the gene expressing in germline cells (*Manil-Segalen, Lefebvre et al. 2014*). LGG-1 dots, visualized by both markers of GFP and mCherry, dramatically increase in the oocytes upon heat stress, while there were few LGG-1 dots under basal condition (**Figure 17E-H, E'-H', quantifications in I and J**). Moreover, the major region that responded to the heat stress is the approximate oocytes (-1 to -4), which are soon to go through fertilization. One possible explanation is that the approximate oocytes are more sensitive to stress condition, suggesting that the strongly triggered autophagy in approximate oocytes helps to clear the damaged material within cells and prevent these damages from the offspring.

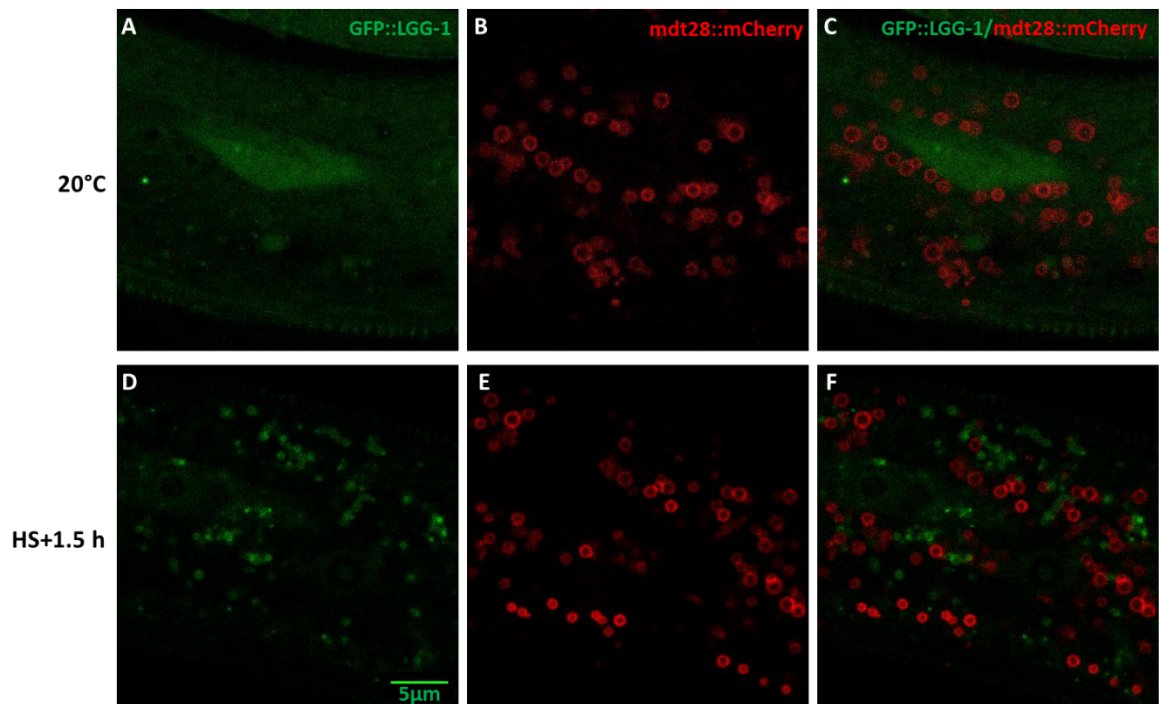


Figure 18| Lipophagy is not induced after heat stress. Worms expressing both GFP::LGG-1 and mdt-28::mCherry (marks lipid droplets) were treated with heat stress for 1h and then recovered for 1.5h (D-F), or untreated as control (A-C). Autophagy (A, D) and lipid droplets (B, E) were detected in epidermis by confocal microscopy. Merged images of GFP::LGG-1 and mdt28::mCherry are shown in (C, F), which indicate that LGG1 dots do not colocalize with lipid droplets. Scale bar: 5µm.

Mitochondria are one of the main cargos of heat-stress induced autophagy. At the same time, the EM pictures showed that heat stress also causes damages to other organelles, for instance, parts of ER were inflated or fragmented upon heat stress (manuscript Figure 1F-L). However, due to the technique limitations, we are unable to detect the ERphagy events in epidermis. In starvation-induced autophagy, genetic evidences suggest that several lipolytic enzymes, as *lipl-1* and *lipl-3*, are involved in the degradation of lipid droplets, or lipophagy (O'Rourke and Ruvkun 2013). In our aHS system, the lipid droplets that are marked by mdt28-mCherry (Zhao, Chen et al. 2017) had almost no colocalization or contacts with the enriched autophagosomes (Figure 18F). The morphology and the number of lipid droplets were not altered by heat stress too (data not shown). Taken together, we got the preliminary conclusion that lipophagy is not induced by heat stress. However, to completely confirm that lipophagy is not triggered by heat stress needs more experiments.

1.3.3. DRP-1

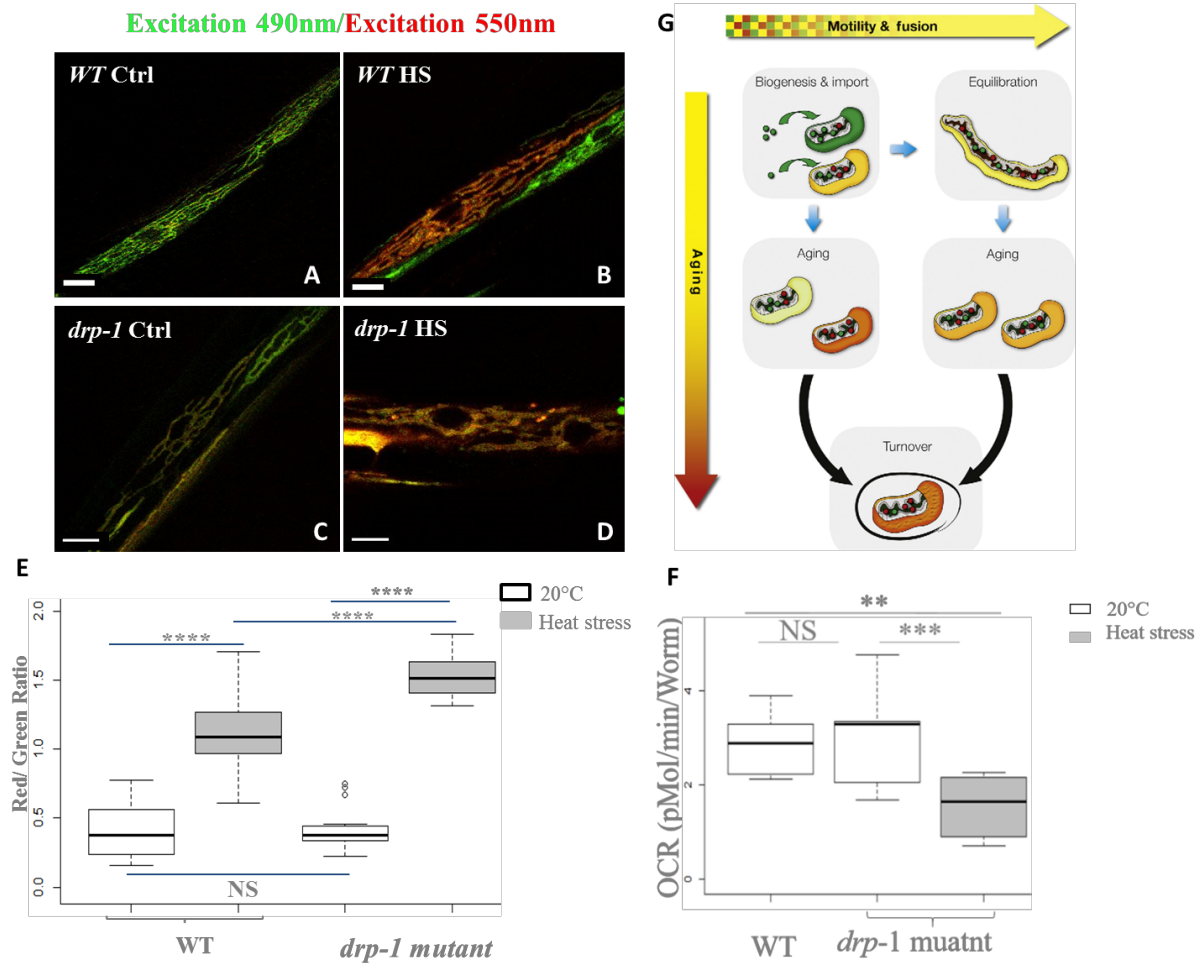


Figure 19| *drp-1* deletion predisposes mitochondria to damage upon heat stress. (Results from Siham Zentout). (A-D) , mitoTimer labelled mitochondria in the body wall muscle cells, mitoTimer is the merge of pictures acquired separately by excitation 490nm and excitation 550nm of confocal. E, Representative graphs for the red/ green ratio (a.u.) in different conditions. (F) Basal OCR per animal for wild type and *drp-1* (*tm1108*) at 20°C and after the Heat stress (number of animals: 20-40). t-test, NS, $p > 0.05$; ** $p < 0.005$; *** $p < 0.0005$; **** $p < 0.0001$. (G) MitoTimer readout is affected by mitochondrial biogenesis and motility&fusion. The newly imported mitoTIMER into mitochondria is green, and then fluorescence converts green-to-red during aging. Lastly, turnover of mainly old MitoTimer will influence levels of red fluorescence. The X-axis indicates that fusion of mitochondria equilibrates new and old MitoTimer protein. Adapted from Kyle Marvin Trudeau *et al.*

MitoTimer genetic tool, which has been previously constructed to monitor mitochondrial biogenesis and mitophagy (Figure 19G), could be a useful tool for our heat stress study (Laker, Xu *et al.* 2014).

But for some technical reasons, we firstly constructed a muscle-specific mitoTIMER transgenic nematode which expresses a Timer fluorescent protein into the mitochondrial matrix. If this mitoTIMER is well functional in muscles, we could later express it in worm epidermis for further studies (Terskikh, Fradkov *et al.* 2000, Hernandez, Thornton *et al.* 2013, Trudeau, Gottlieb *et al.* 2014). In wild type worms, the increased Red/Green ratio indicated

damages within heat stressed mitochondria, and this was corresponding with our EM and Seahorse results. These results together illustrated that heat stress impairs mitochondrial homeostasis and functions (**Figure 19A, B and E**). *drp-1 mutant* worms showed a higher Red/Green ratio comparing with wild type worms in heat stress condition, while there was no differences between *wild type* and *drp-1* mutant in basal condition, suggesting that *drp-1* depletion is important for mitochondrial homeostasis in stress condition but not in basal condition (**Figure 19C, D and E**). Seahorse assay also revealed that although mitochondrial network is highly fused in *drp-1* mutants, its respiratory activity was not affected in basal condition, while heat stress significantly reduced the mitochondrial OCR in *drp-1* mutants (**Figure 19F**).

To sum up, DRP-1 mediated mitochondrial fission does not seem essential for mitochondrial homeostasis in basal condition; however, DRP-1 is important for both mitochondrial morphology regulation and mitochondrial homeostasis maintenance in stressed condition.

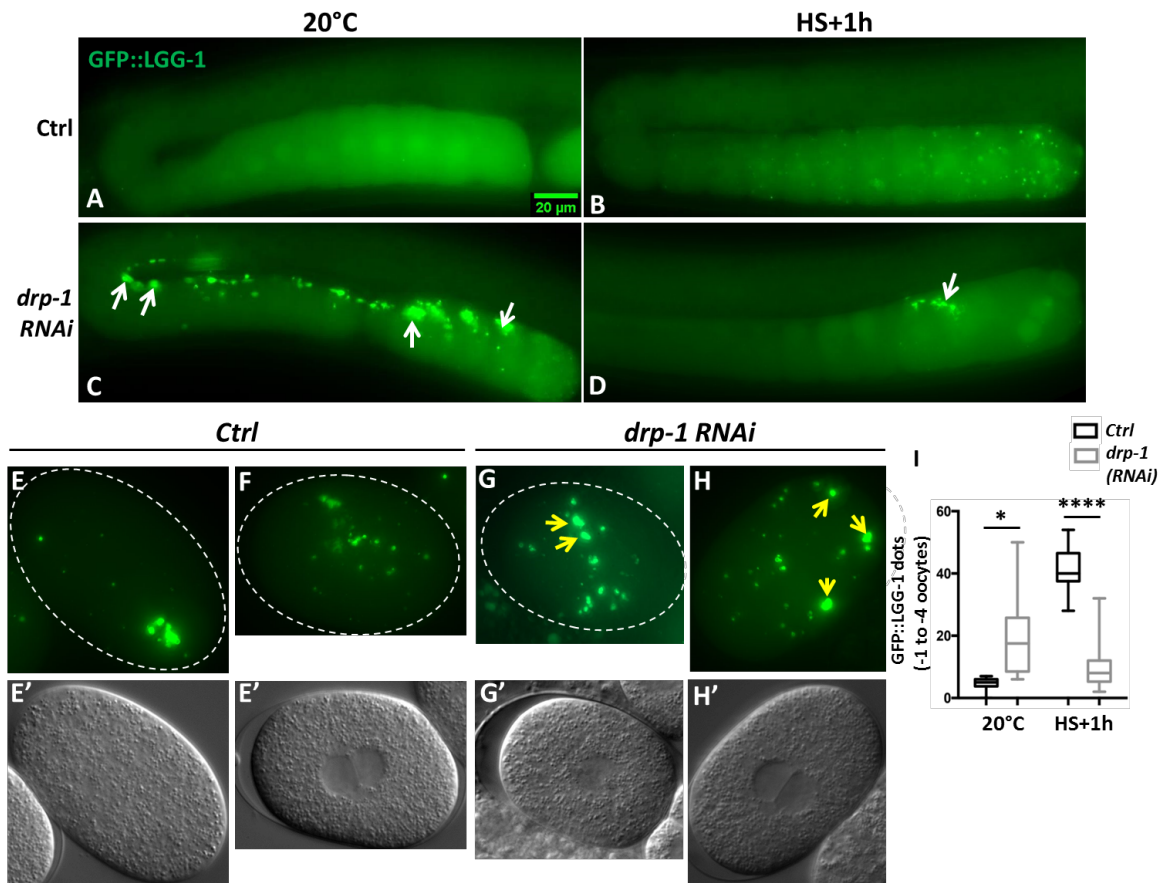


Figure 20| *drp-1 RNAi* also induces autophagic clusters in germline and early embryo. (A-D) Worms expressing GFP::LGG-1 specifically in germline (RD204) were submitted to heat stress for 1h, followed by 1h recovery (B, D), or left untreated (A, C). GFP::LGG1 dots were detected in control (A, B) or *drp-1 RNAi* (C, D) worms. White arrows indicate GFP::LGG-1 clusters in germline. (E-H) GFP::LGG-1 puncta or clusters were monitored in pronuclear formation-stage embryos (E, E') or during first cell division (G, G', F, F', H, H') in control or *drp-1 RNAi* worms (RD204). E' to H' are the DIC pictures of E to H, respectively. Yellow arrows indicate bigger clusters. (I), Quantification of numbers of GFP::LGG-1 in germlines of A to D (n=11,10; 9,12), Two-way anova, * p<0.05; **** p<0.0001. Scale bar: 20µm.

We have shown that DRP-1 is essential for autophagosome formation upon heat stress (Chen et al. [manuscript Figure 6](#)). But is DRP-1 specific for heat stress induced autophagy or also involved in the autophagosome formation induced by other stimulator? To answer this question, we compared the autophagy processes between *drp-1 RNAi* and *wild type* embryos. In *wild type* embryos, GFP::LGG-1 formed clusters ([Figure 20E](#)) to rapidly degrade paternal mitochondria and MOs after fertilization, which had been reported previously (*Al Rawi, Louvet-Vallee et al. 2011, Sato and Sato 2011*). Similar phenotype was also observed in *drp-1 RNAi* embryos (data not shown). During the first cell division, the aggregated GFP::LGG-1 was dispersed to smaller clusters ([Figure 20F](#)). By contrast, the *drp-1 RNAi* embryos exhibited much bigger dispersed GFP::LGG-1 clusters (yellow arrows in [Figure 20G,H](#)). These

abnormal autophagic structures suggest a defect of allophagy in *drp-1* deprived embryos. But we have not yet performed any experiments to check whether the degradation of paternal mitochondria is affected in *drp-1 RNAi* condition. It is interesting that *drp-1 RNAi* caused GFP::LGG-1 clustering in germline even in basal condition (**Figure 20C**), in which autophagy level was considered to be low (Manil-Segalen, Lefebvre et al. 2014). It seems that the heat stress could reduce the GFP::LGG-1 clusters in *drp-1 RNAi* germline (**Figure 20C and D, quantification in I**), which is opposite to what we observed in epidermis. This may imply that autophagy mechanisms between epidermis and germline are not the same. The existence of the clusters in *drp-1 RNAi* germline also raises another question: the abnormal aggregates during allophagy may originate from the GFP::LGG-1 clusters in germline or oocytes, while *drp-1 RNAi* itself may have no influence on allophagy.

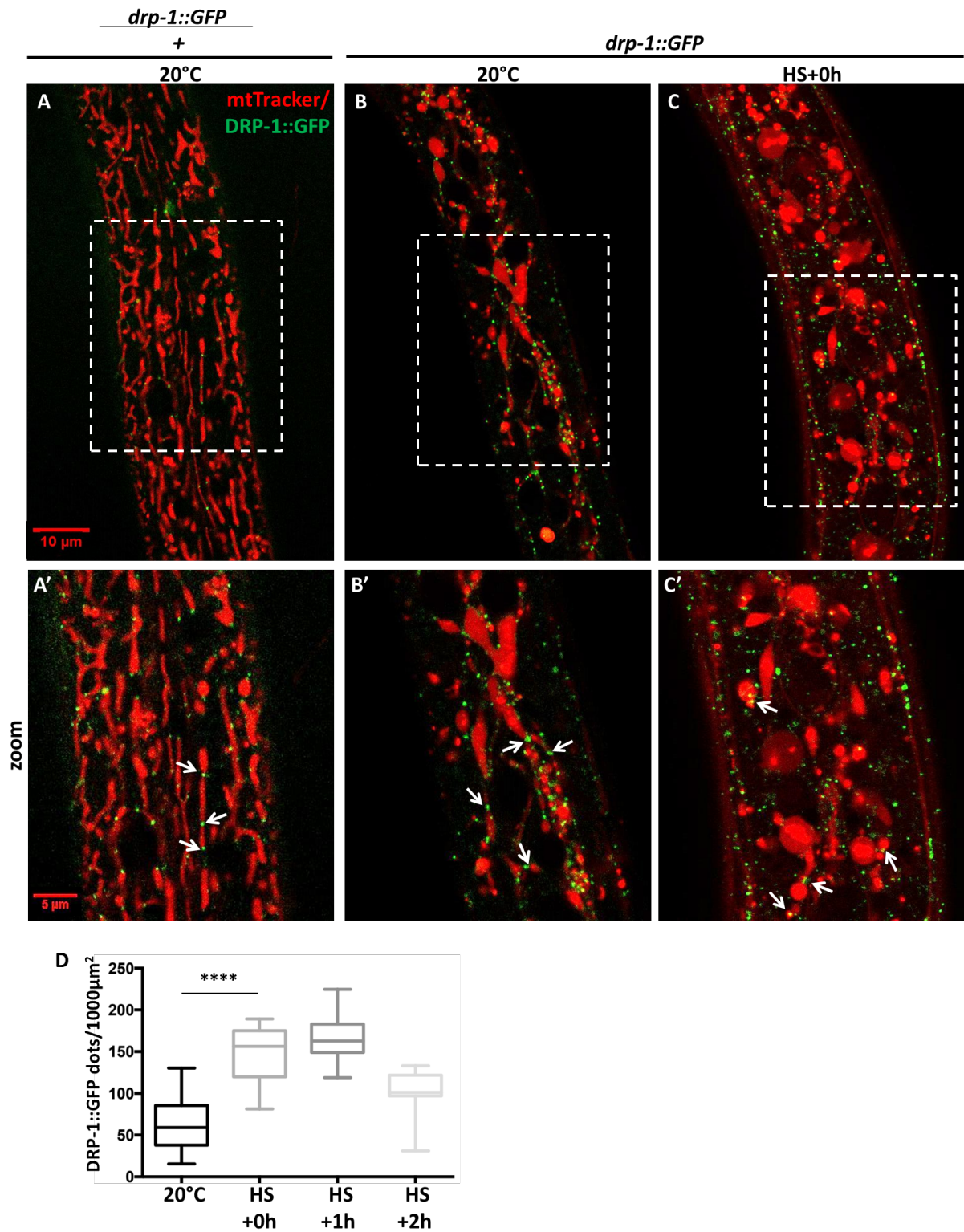


Figure 21| DRP-1 responds to heat stress. (A-C, A'-C') Worms expressing GFP::DRP-1 were stained with mitoTracker to label mitochondria and then cultured at 20°C (A, A', B, B') or in heat stress condition (C, C'). A' to C' are zoom images of the white frames from A to C, respectively. White arrows indicate the DRP-1 recruited sites where mitochondria are fragmented. (D) Quantification of numbers of GFP::DRP-1 dots in the homozygous under indicated conditions. (n=9,8,13,11) t-test, **** p<0.0001.

We have also detected the endogenous DRP-1 in the presence or absence of heat stress. In the heterozygous *gfp::drp-1/+* worms, GFP::DRP-1 dots were present on mitochondrial surface or even on the tips of the fragmented mitochondria, as well as what had been described previously (**Figure 21A and A'**, indicated by arrows) (Labrousse, Zappaterra et al. 1999). It suggests that the endogenous GFP::DRP-1 was correctly localized. Heat stress increased the number of endogenous GFP::DRP-1 dots soon after the treatment (**Figure 21D**), and this increasing was corresponding with the behaviour of mitochondrial fragmentation. However, even in basal condition, mitochondria in the homozygote GFP::DRP-1 were elongated but some of them were blebed (**Figure 21B and B'**), a phenotype similar to the one of *drp-1 null mutant*. The endogenous GFP::DRP-1 failed to separate mitochondria, although the dots were still present on mitochondria (**Figure 21B-C, B'-C'** by white arrows). These evidences suggest that the endogenous GFP::DRP-1 is non-functional but its localization on mitochondria is not affected. Upon heat stress, the increased GFP::DRP-1 dots could also be recruited onto mitochondrial surface, however, no DRP-1 mediated fission was observed. In summary, DRP-1 is required for mitochondrial fission both in basal and heat stress conditions.

1.3.4. Discarded tools

At the beginning of my PhD study, we had received two strains, IR1631: N2;EX003[P_{myo-3}TOMM-20::mitoRosella] and IR1284: N2;/s[P_{myo-3}mitoGFP]; Ex011[P_{lgg-1}DsRed::LGG-1], from Tavernarakis lab to detect mitophagy in muscle. Since I focused on epidermis rather than muscles later, I did not use these strains in my manuscript. However, it could be useful to make a memo about our observations in these worms.

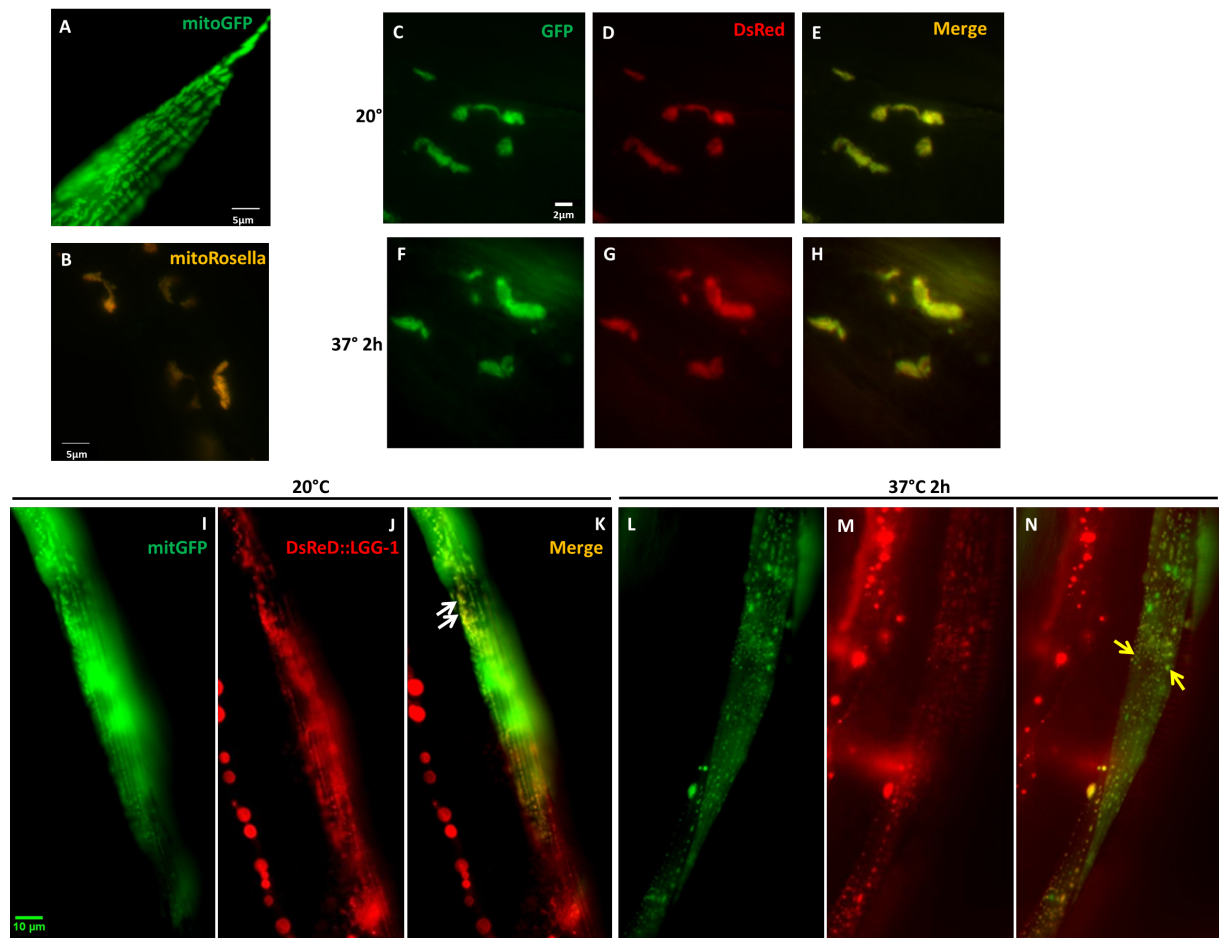


Figure 22 | The discarded tools for monitoring mitophagy in *C. elegans* muscle. (A-H) Worms were engineered with mitochondrial localized GFP (mitoGFP) or GFP and DsRed (mitoRosella). The mitochondrial morphology is severely distorted in mitoRosella nematodes (B) compared with mitoGFP (A). (C-H) mitoRosella worms were cultured at 20°C (C-E) or 37°C (F-H). Mitochondrial morphology was checked by microscopy. (I-N) Worms expressing both mitoGFP(I, L) and DsRed::LGG-1 (J, M) were maintained at 20°C (I-K) or 37°C for 2h (L-N). Colocalization between mitoGFP and DsRed::LGG1 was checked (K, N). Scale bars: A-H, 5µm; I to N, 10µm.

mitoRosella consists of mitochondria targeting peptide (a segment of Tom20), a pH-sensitive GFP, and a pH-insensitive DsRed, which is expressed under the muscle specific promoter *myo-3*. The GFP in the mitoRosella biosensor is quenched in the acidic autolysosomes while the DsRed fluorophore is resistant. For this reason, the reduced GFP/DsRed ratio of mitoRosella is supposed to represent mitophagy events (Palikaras, Lionaki et al. 2015). In the published paper in 2015, these two strains responded to several mitophagy stimulators and were able to indicate mitophagy levels (Palikaras, Lionaki et al. 2015). However, we met some difficulties when using these two strains.

For mitoRosella strain, the mitochondria were irregular and aggregated, which were completely different from the normal tubular mitochondria (Figure 22A and B). We presumed that the altered mitochondria morphology might result from the overexpression

of Rosella on mitochondrial surface. In addition, we were unable to reproduce the GFP quenching effect by 37°C, 2h treatment (**Figure 22C to H**), which was recommended by Tavernarakis lab. Taken together, we think the mitoRosella is not a good tool to study mitophagy in our hand.

Although heat stress at 37°C for 2h could fragment mitochondria that were colocalized with the increased DsRed::LGG-1 in the IR1284 strain, there were also a lot of colocalization events between the tubular mitoGFP and DsRed::LGG-1 under basal condition (**Figure 22I to N**). This strange behaviour of the DsRed::LGG-1 makes this strain difficult to be used for mitophagy study. In conclusion, we decided to give up both strains and to develop other tools to monitor mitophagy events in *C. elegans*.

2. A preliminary exploration of MAM candidates in *C. elegans*

2.1. Summary

It has been shown in mammalian cells that ER-mitochondria contact sites is one of the sites for autophagosome biogenesis. Atg5, Atg14 and other early autophagy markers are present at the ER-mitochondria contact sites for autophagosome assembly (Hailey, Rambold *et al.* 2010, Hamasaki, Furuta *et al.* 2013). In yeast, however, it is considered that the ER-mitochondria contact sites are required for specific mitophagy process rather than general autophagy (Bockler and Westermann 2014). It is interesting that in control or stress-induced conditions, we could always detect the autophagosomes in close proximity to mitochondria. Interestingly, the recent study by Vincent SCARCELLI in our lab revealed that in embryo, autophagosomes also locate close to ER (unpublished data). All these results indicate that autophagosomes may form at the ER-mitochondria contact sites in *C. elegans*. In addition, a recent study in *C. elegans* showed that the deletion of *epg-3* blocks the release of phagophore from ER and later autophagosome maturation (Zhao, Chen *et al.* 2017). To our knowledge, there is no study exploring the role of ER-mitochondria contact sites in autophagy process in *C. elegans* yet.

Since DRP-1 is recruited to the ER-mitochondria contact sites for fission (See [Introduction 4.2 and 4.4.2](#)), we hypothesize that the *drp-1* RNAi or null mutation induced autophagic clusters are related to the ER-mitochondria contact sites, and *drp-1* depletion may result in the abnormal interaction of ER and mitochondria, which could further disrupts the autophagosome biogenesis. To test this hypothesis, we tried to interrupt the ER-mitochondria contact sites by the RNAi of MAM (mitochondria-associated membranes) candidates in *C. elegans*, and then detected their roles in both mitochondrial fission and heat stress induced autophagy/mitophagy. The results are as followings:

- **The potential MAM proteins in *C. elegans*.**

First of all, based on the reported MAM proteins in mammalian system (van Vliet, Verfaillie *et al.* 2014, Lopez-Crisosto, Bravo-Sagua *et al.* 2015), by BLAST assay, we listed the MAM homologs in *C. elegans* ([Table 7](#)). These MAM candidates, together with the identified MAM protein in *C. elegans*, ARF-1.2 (Ackema, Hench *et al.* 2014), were not all involved in

mitochondria morphology regulation. We found that only the RNAi against *arf-1.2*, *vdac-1* and *miro-1* could impair mitochondrial network, suggesting their encoded products are the potential MAM proteins.

- **The effect of RNAi against MAM candidates in basal and heat stress-induced autophagy process.**

Whether ER-mitochondria contact sites are required for autophagosome formation was then tested through the RNAi of the MAM candidates, which were listed in [Table 6](#). Unfortunately, none of the RNAi showed significantly altered autophagy both in basal and heat stress condition. More importantly, the autophagic clusters in *drp-1* RNAi or mutant worms had never been found when these MAM candidates were knocked down. It may give us a clue that the *drp-1* depletion induced autophagy defect is not due to the disrupt ER and mitochondria tethering.

- **Autophagy/mitophagy is altered by *fzo-1* RNAi.**

FZO-1 is the homolog of mammalian Mfn2, which plays a key role in tethering ER to mitochondria at the contact site (*de Brito and Scorrano 2008, Filadi, Greotti et al. 2015, Naon, Zaninello et al. 2016*). The RNAi of *fzo-1*, as well as other MAM candidates, was unable to impair autophagosome formation induced by heat stress. However, in basal condition (20°C), *fzo-1* RNAi worms had more GFP::LGG-1 puncta, which were often colocalized with mitochondria in epidermis. The hypothesis is that *fzo-1* RNAi induces more fragmented mitochondria, and then triggers mitophagy for degradation.

- **New tools for studying ER-mitochondria contact sites.**

In the end, we tried to develop new approaches to directly detect the ER-mitochondria contact sites. We firstly tried to detect ER-mitochondria contact sites by confocal microscopy. To achieve this, an ER protein RET-1 was tagged with GFP and mitochondria were stained with mitoTracker CMXRos. This approach allowed the *in vivo* observation, but both ER and mitochondria networks were very dense, which made it difficult to detect the contact zones. The second try was to construct a strain expressing HRP-KDEL by EM approach (*Schikorski, Young et al. 2007, Giordano, Saheki et al. 2013*).

In summary, here we explored the roles of MAM proteins in our aHS-induced autophagy/mitophagy, and these data suggested that these MAM candidates are not required for autophagosome formation in heat stress-induced autophagy/mitophagy. To further study the MAM proteins or ER-mitochondria contact sites in *C. elegans*, we have developed the first HRP-KDEL strain in *C. elegans* to detect the contact sites under EM. We hope this novel tool will help us for the further study of the relationship between ER-mitochondria contact sites, autophagosome formation and mitochondria fission in *C. elegans*.

2.2. Results

2.2.1. The potential MAM proteins in *C. elegans*

The contact sites that the endoplasmic reticulum (ER) forms with mitochondria, called mitochondria-associated membranes (MAMs), a large number of proteins have been found enriched in MAMs forming the basic components of MAM, are called MAM proteins (*Rusinol, Cui et al. 1994, van Vliet, Verfaillie et al. 2014*). One difficulty to study MAM proteins is to distinguish their MAM-associated and –none associated functions. For instance, the mammalian Mfn2, a mitochondrial fusion factor, has been proved to be a key factor to direct tether mitochondria with ER (*de Brito and Scorrano 2008, Filadi, Greotti et al. 2015, Naon, Zaninello et al. 2016*). EM data showed that the ER-mitochondria contact sites are affected (increased or decreased, on debate) in the absence of Mfn2 (*Naon, Zaninello et al. 2016*). It is the same case for DRP-1. As a key fission protein for mitochondria, DRP-1 is also a MAM element (**Table 7**).

To study the ER and mitochondria contact sites, firstly, we used the BLAST on NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and identified the homologs of the mammalian MAM proteins in *C. elegans* (**Table 7**). Besides the well-known MAM protein like MIRO-1, VDAC-1, CNX-1 and DRP-1, FZO-1, we also listed here TAG-232 and ACS-4, 13, 17, which were not well characterized in *C. elegans*. In addition, the homologue of Atg14L, which is recruited to the ER-mitochondria contact sites to promote autophagosome assembling, is EPG-8 in worms (*Yang and Zhang 2011*). The only validated MAM candidate in *C. elegans* is ARF-1.2 (*Ackema, Hench et al. 2014*).

Table 7. The potential MAM proteins in *C. elegans* and their homologs in mammals (adapted from Alexander R. van Vliet, 2014, BBA and Camila López-Crisosto, BBA, 2015)

Category of functions	MAM proteins in mammals	detail functions	Homologue in <i>C. elegans</i>
Mitochondria morphology	Drp1	Mitochondrial fission protein	DRP-1
	Miro1	GTPase connecting mitochondria with cytoskeleton	MIRO-1
Autophagy signaling	ATG14L	early autophagy assembling	EPG-8
Ca ²⁺ transport	VDAC1	Ca ²⁺ uptake channel in the outer mitochondrial membrane	VDAC-1
	Calnexin (Cnx)	Ca ²⁺ binding protein and chaperone of the ER	CNX-1
ER-mito tethering	Mfn2	Major in mitochondrial fusion, also regulates ER-mitochondrial tethering	FZO-1
ER stress signaling	PACS-2	Cytosolic sorting protein involved in the ER localization of transmembrane proteins and ER vesicle sorting	TAG-232
Lipid transport	FACL4	Involved in lipid synthesis and degradation, is frequently used as a marker during subcellular fractionations	ACS-4,13,17

Next, we asked whether these homologs are also involved in the ER-mitochondria contact sites in *C. elegans*. The limit distance of ER-mitochondria contact sites is within 10-30nm (Csordas, Renken et al. 2006), making it difficult to discriminate them under the common or confocal microscopy. Since one of the major functions for ER-mitochondria contact sites is regulating mitochondrial morphology, and the defect of ER-mitochondrial tethering is also coordinated with the alteration of mitochondrial morphology (Giorgi, Missiroli et al. 2015), as a first attempt, we decided to use the mitochondrial network in body wall muscle cells as an indicator to evaluate the ER-mitochondria tethering.

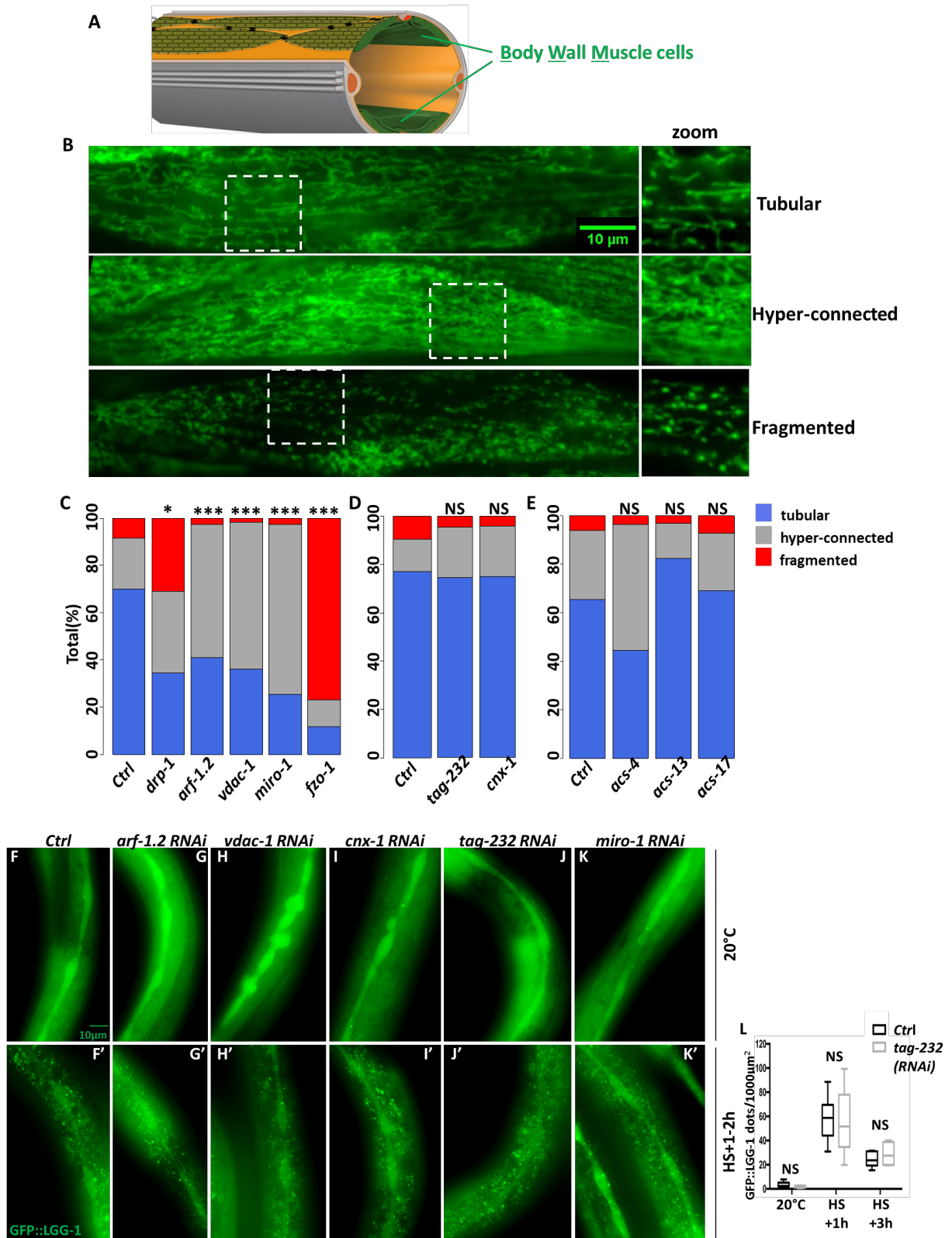


Figure 23| The roles of MAM candidates in mitochondrial morphology regulation and heat stress-induced autophagy. (A) A schematic diagram showing the body wall muscle cells in green. (B) Representative images showing three main mitochondrial morphology labelled by Tom70::GFP within body wall muscle cells: tubular, hyper-connected, and fragmented. Strain used is $P_{myo-3}Tom70::GFP$. (C-E) MAM candidates RNAi in $P_{myo-3}Tom70::GFP$ worms as indicated in the graph, and three types of mitochondrial morphology were quantified. $n=47,29,81,56,39,35$ (C); $52,43,81,35$ (D) and $35,29,34,29$ (E). Fisher test; * $p<0.05$, *** $p<0.0005$. (F-L, F'-K') *arf-1.2* (G, G'), *vdac-1* (H, H'), *vdac-1* (I, I'), *tag-232* (J, J'), *miro-1* (K, K') were knocked down by RNAi in

GFP::LGG1 worms, control RNAi: L4440. then the worms were cultured at 20°C (F-K) or 37°C for 1h, followed by 20°C for 2h (F'-K'). Quantification of *tag-232* RNAi compared with control at basal and heat stress conditions is shown in (L) (n=13,7; 13,13; 5,4. Two-way anova, p>0.05).

The mitochondrial morphology in body wall muscle cells was classified into three categories: tubular, hyper-connected and fragmented (Ackema, Hensch et al. 2014). We observed obvious mitochondrial morphology changes in the *arf-1.2*, *vdac-1*, *miro-1* and *fzo-1* RNAi worms, similar to the published paper (Ackema, Hensch et al. 2014). The knockdown of *arf-1.2*, *vdac-1* and *miro-1* increased the mitochondrial connection and led to a hyper-connected phenotype as well as the *drp-1* RNAi (Figure 23C). FZO-1 is the mitochondrial fusion protein, its deprivation results to the fusion defect and therefore more fragmented mitochondria (Breckenridge, Kang et al. 2008). Our results also confirmed the role of *fzo-1* in mitochondrial fusion (Figure 23C). However, the other candidates showed no obvious effect on mitochondrial morphology (Figure 23D, E). There are three possible explanations for it: firstly, the RNAi efficiency varies due to different target genes, and the MAM candidates whose knockdown showed no defect might result from a low RNAi efficiency, a mutant should be used instead; Secondly, the MAM candidates we tested here may had other function rather than tethering at the ER and mitochondria contact sites; thirdly, perhaps the composition for ER-mitochondria contact sites in *C. elegans* differs from that in mammals, and the homologs of the mammalian MAMs are not all present at the ER-mitochondria contact sites in *C. elegans*. Although our results and the published paper both indicated that *arf-1.2*, *vdac-1* and *miro-1* are the MAM candidates in *C. elegans* (Ackema, Hensch et al. 2014), to better understand the function of MAMs in *C. elegans*, we decided to test the role of these MAM candidates in basal and stress-induced autophagy.

2.2.2. The role of ER-mitochondria contact sites in heat stress-induced autophagy

Studies in mammalian cells demonstrated that disruption the ER-mitochondria contact sites via knocking down of PACS-2 and Mfn2, two MAM proteins, reduces the autophagosome formation (Hamasaki, Furuta et al. 2013). However, to our surprise, neither in normal nor heat stress conditions, none of the MAM candidates RNAi led to obvious changes of autophagy process, which was indicated by GFP::LGG-1 dots (Figure 23F-K; F'-K'). The statistical analysis of GFP::LGG-1 puncta of the RNAi against *tag-232*, whose mammalian product is required for the tethering of ER and mitochondria (Simmen, Aslan et al. 2005),

clearly showed no significant differences compared with control (**Figure 23L**). There are two possibilities. Since none of the MAM candidates depletion could affect autophagosome formation, one possibility is that the ER-mitochondria contact sites are probably not the main place. Another possibility is that even one MAM protein is absent, the ER-mitochondria contact sites were still kept in epidermis. Other clues support the second possibility, for instance, the autophagy markers (GFP::LGG-1, GFP::LGG-2 and ATG18::GFP) were often in contact with mitochondria, both in basal and stress-induced autophagy. In addition, Hong ZHANG lab had proved that the isolation membrane (IM) is initiated at ER, and the EPG-3/VMP1 mediated ER-IM separation is important for the correct formation of autophagosomes in embryo. The *VMP1* KO in cultured cells led to an abnormal tight contact between ER and other organelles, including mitochondria (*Zhao, Chen et al. 2017*).

Moreover, we had never seen autophagic clusters in the RNAi worms of any MAM candidates upon heat stress except *drp-1* (**Figure 23F'-K'**). Until now, we have no clear evidence if ER-mitochondria contact sites remained in *drp-1* mutant. It is difficult to conclude whether the autophagic clusters dependent on the contact sites or not.

2.2.3. Autophagy/mitophagy is altered by *fzo-1* RNAi

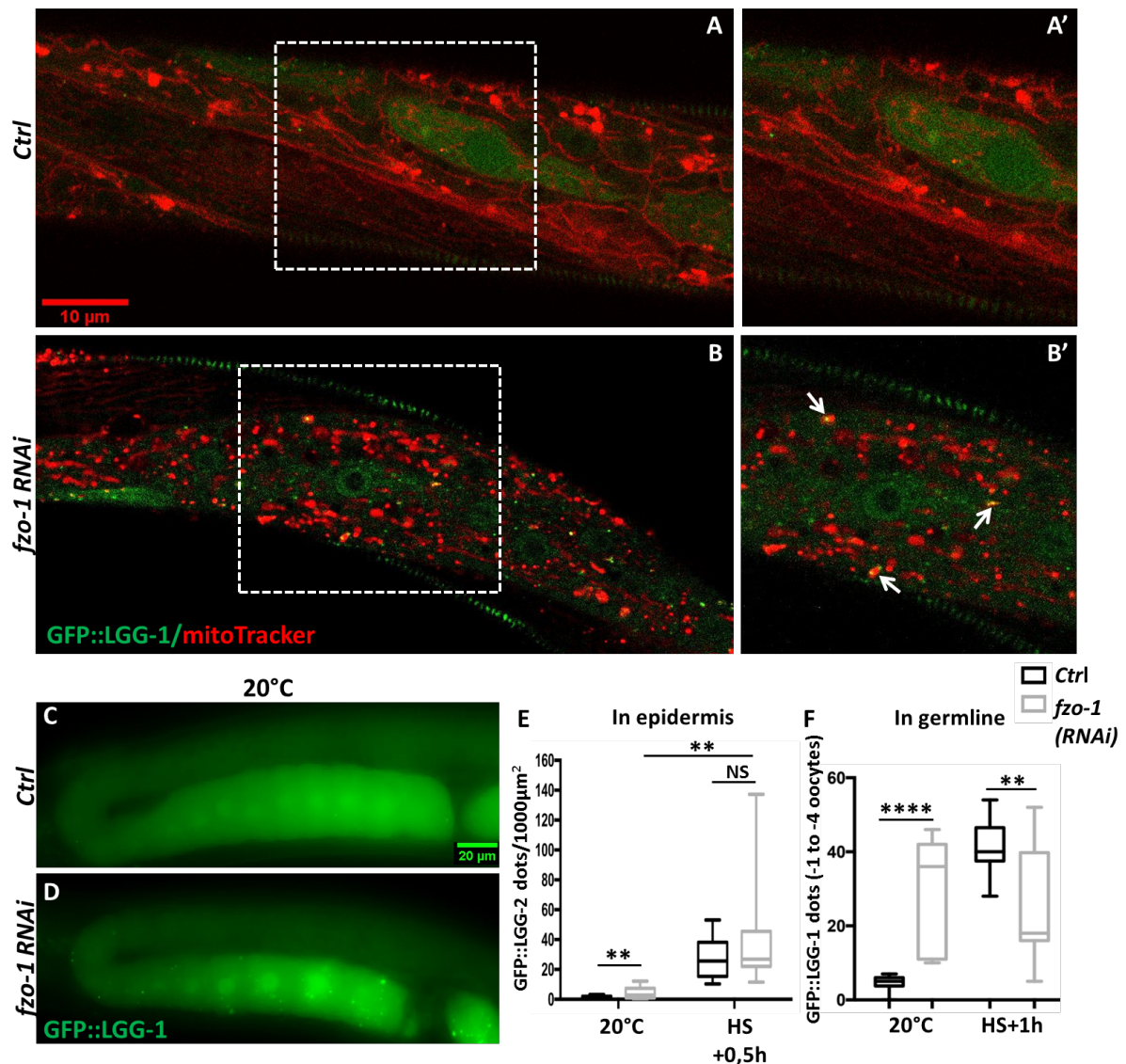


Figure 24 | Autophagy/mitophagy is altered by *fzo-1* RNAi. (A, B, A', B') GFP::LGG1 worms were interfered by *fzo-1* RNAi (B, B') or control bacteria (A, A'), and then stained with mitoTracker CMXRos to mark mitochondria. The colocalization between mitochondria and GFP::LGG1 dots was checked by confocal microscopy. A' and B' are the zoom pictures of the white frames in A and B, respectively. The white arrows in B' indicate the colocalization between GFP::LGG-1 and mitochondria. (C, D, F) *fzo-1* (D) was knocked down in GFP::LGG1 worms. And GFP::LGG1 dots in the approximate oocytes (-1 to -4) of control (C) and *fzo-1* RNAi worms were counted in (F) (n= 11,7; 10,10. t-test, **** p<0.0001; * p<0.05). (E) *fzo-1* RNAi in GFP::LGG2 worms, and treated with heat stress, followed by 0.5 h recovery, or left untreated (20°C). GFP::LGG-2 dots in epidermis of control and *fzo-1* RNAi worms were quantified in (E) (n= 10,17; 9,13. t-test, ** p<0.005; NS p>0.05).

In mammals, it has been proved by several groups that MFN2 is a MAM protein, and more importantly, it is a key factor regulating the ER and mitochondria tethering. MFN2 mutation or depletion increases (or decreases, on debate) ER-mitochondria contact sites (*de Brito and Scorrano 2008, Filadi, Greotti et al. 2015, Naon, Zaninello et al. 2016*). But whether its homolog in *C. elegans*, FZO-1, is also involved in ER-mitochondria contact sites has not been

studied yet. FZO-1 mediates mitochondrial fusion in *C. elegans* and its deletion led to a fragmented mitochondrial network (Figure 24B). The basal levels of GFP::LGG-1 and GFP::LGG-2 puncta were elevated by *fzo-1* RNAi both in epidermis and germline (Figure 24B, D and quantifications in E, F). Heat stress could further augment the epidermis GFP::LGG-2 puncta in *fzo-1* RNAi worms (Figure 24E). These data indicate that the MAM candidate, *fzo-1*, is not required for autophagosome formation both in basal and heat stress conditions in epidermis. Moreover, parts of GFP::LGG-1 puncta induced by *fzo-1* RNAi in basal condition had overlap with mitochondria (Figure 24B', white arrows), suggesting the induction of mitophagy by mitochondrial fragmentation.

2.2.4. New tools for studying MAM

Until now, the RNAi against several MAM candidates showed no defect on autophagosome formation in heat stress-induced autophagy process, although the location of autophagy markers close to mitochondria reminded us of a potential link between mitochondria and autophagosome biogenesis. We could not get the conclusion that the ER-mitochondria contact sites are not involved in autophagy process, since we have no evidences showing the ER-mitochondria tethering is altered by the RNAi of MAM candidates in *C. elegans*. To further confirm the relationship between ER-mitochondria contact sites and heat stress-induced autophagy/mitophagy, we tried to develop tools to directly detect the contact sites.

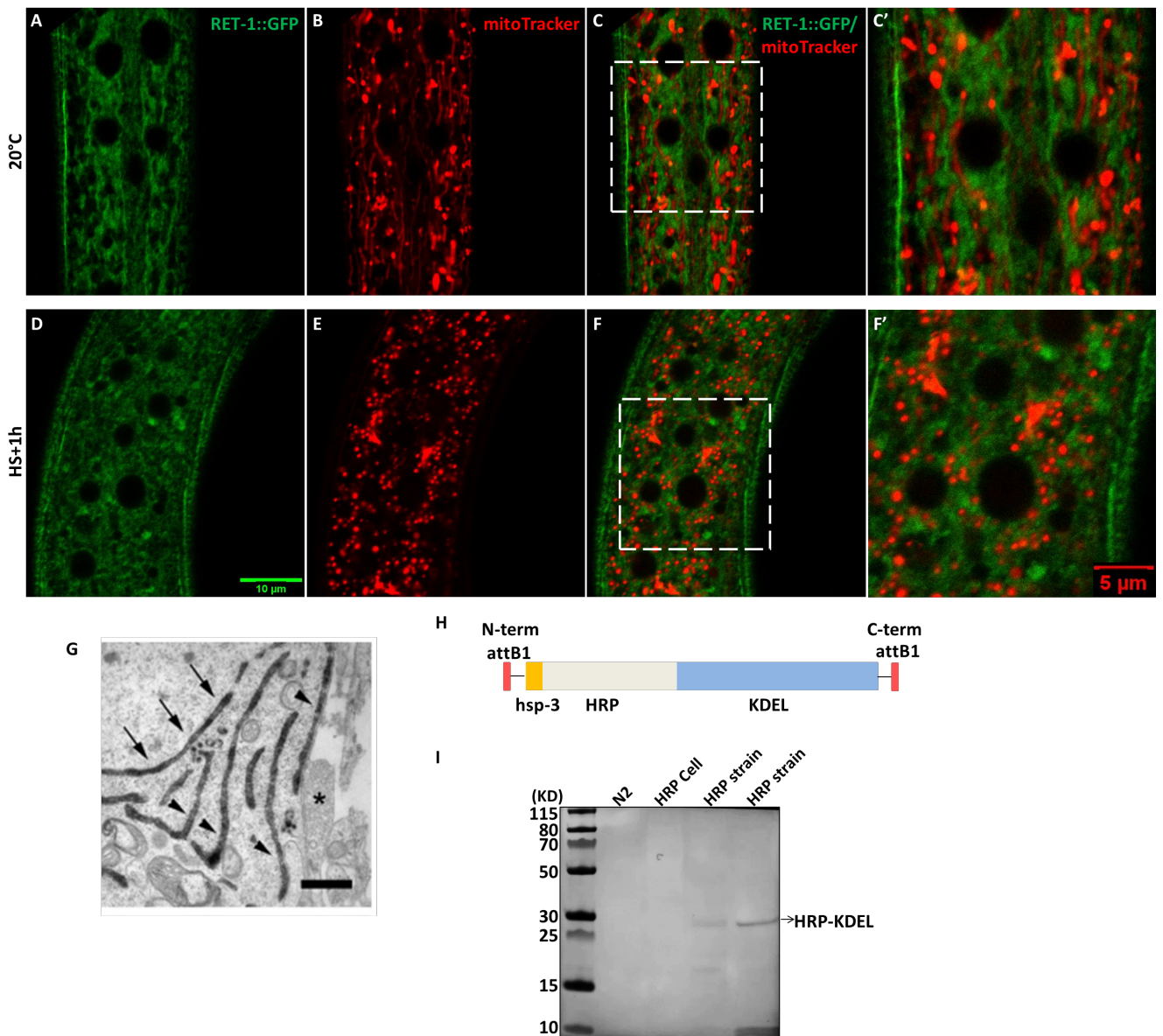


Figure 25 | Tools for studying ER-mitochondria contact sites. (A-F, C', F') Worms expressing RET-1::GFP (A, C), which marks ER, were stained with MitoTracker™ Red CMXRos to label mitochondria (B, E). The worms were then administrated with heat stress for 1h, followed by 1h recovery (D-F, F'), or left untreated (20°C) (A-C, C'). Colocalization between mitochondria and ER was detected by confocal microscopy (C,F). Zoom in pictures of C and F are shown in C' and F'. (G) EM picture of HRP-labelled ER structure (black arrows). (from Schikorski T, Young SM Jr. *Hu Y*; 2007). (H) A schematic map of the combined HRP genes for constructing transgenic worms. (I), Western blot detected by Anti-HRP antibody (result from Céline Largeau). Line1-4 (from left to right) are samples from N2 worms (Line1), HeLa cells overexpressing HRP-KDEL (Line2, a gift from Francesca GIORDANO), and two HRP expressing strains we obtained by biolistic (Line3,4).

The first and also the easiest way we thought to observe the contact sites is by *in vivo* observation of ER and mitochondria, which were labelled by RET-1::GFP and MitoTracker™ Red CMXRos, respectively. Unfortunately, there were dense of ER and mitochondria in epidermis, and these two organelles were always closed, which made it difficult to identify the real contact zones (Figure 25C, C' and F, F'). Another information we obtained from the

RET-1::GFP worms was that heat stress caused a change of the ER morphology, and the ER structure seemed to loosen and separate upon heat stress treatment (Figure 25A, D). Sometimes we could even detect big ER patches in the stressed worms (Figure 25D), but we have no clues what this phenotype means.

Then we decided to switch to the EM tools to observe the ER-mitochondria contact sites, which are potential sites for autophagosome formation, as well as for DRP-1 mediated mitochondrial fission. EM analysis of contact sites between both organelles is still the best approach to make a qualitative and quantitative analysis of MAMs. As previously published, in order to best visualize the ER, we decided to create *C. elegans* transgenic lines expressing the horseradish peroxidase enzyme (HRP) within the ER. HRP catalyzes an enzymatic reaction using hydrogen peroxidase (H₂O₂) and 3-3' diaminobenzidine (DAB) that yields an insoluble oxidized DAB which, forms a brown precipitate at the location of the HRP. This precipitate is made electron dense with the addition of osmium. Therefore, it is visualized as dark structure under EM (Schikorski, Young et al. 2007, Giordano, Saheki et al. 2013). The ER, containing HRP, can be more easily identified via this electron dense dark structure, facilitating visualisation of its contact sites with mitochondria.

Together with Emmanuel Culetto, we have designed the cloning strategy to make a construct to express HRP in the germline cells of *C. elegans*. This strategy is based on both Francesca Giordano's papers and research work performed by Monica Gotta laboratory.

It is important that HRP should be addressed to the ER luminal part (F.Giordano, personal communication). One paper from Gotta lab describe how to synthetise one gene to express an ER luminal GFP protein reporter in *C. elegans* (Compartmentalization of the endoplasmic reticulum in the early *C. elegans* embryos (Lee, Prouteau et al. 2016). In this paper they successfully expressed and addressed the GFP protein to the ER lumen by using the ER addressing signal and ER retention signal of *hsp-3* gene. We followed the strategy described in this paper with the 5 following steps: (1) fuse in frame at the N-term a signal peptide to address the HRP to the ER; (2) fuse at the C-term a KDEL sequence to sequestrate the protein within the ER; (3) modify the sequence to allow cloning with the gateway procedure; (4) clone the cDNA ER-HRP-KDEL within Pdonr201 by a BP recombination reaction (Invitrogen) (gateway system) to give pENTR-ER-HRP-KDEL ; (5) transfer DNA to the final destination vector (Pid2.02) by performing a LR reaction (gateway system) between the

entry clone pENTR-ER-HRP-KDEL and the *pie-1* destination vector to get the full construction under the control of the *pie-1* promoter: pid2.02-ER-HRP-KDEL.

Pid2.02 plasmid contains the *pie-1* promoter which allows the expression of HRP-KDEL in germline and early embryos. The pid2.02-ER-HRP-KDEL vector was introduced into *unc-119(ed3)* worms by biolistic bombardment using a PDS-1000/HE system (BioRad), as described (Praitis, Casey *et al.* 2001), to create transgenic lines. One month later, while most of the *unc-119* worms were dead because of starvation, transgenic worms were alive and moved normally. HRP transgenic construct presence and HRP expression in the transgenic strain were further confirmed by performing both PCR and Western Blot assay respectively (Figure 25I).

The HRP-KDEL strain would be further employed to study the ER-mitochondria contact sites in mitochondrial fission and autophagosome formation in various environmental conditions and genetic contexts not only in germline but also in epidermis.

3. Ether lipid and autophagy (collaborative project)

3.1. Summary

Phospholipids are the main components of cellular membranes, and most of the phospholipids consist of the hydrophobic fatty acids attached to a hydrophilic phosphoglycerol head group through an ester bond. However, there is a type of phospholipids in which the long chain fatty alcohols are connected to the phosphoglycerol by an ether bond (*Fahy, Subramaniam et al. 2005*). This type of phospholipids is termed as ether lipids, constituting approximately 20% of the total phospholipid pool in mammals (*Dean and Lodhi 2018*). Ether lipids are important for the maintenance of membrane physical properties, including membrane fluidity, promotion of membrane fusion and contributing to lipid microdomains (*Wallner and Schmitz 2011*). Moreover, ether lipids are required for the proper functions of integral membrane proteins, and the defect of ether lipids is associated with many human diseases (*Braverman and Moser 2012*).

During the ether lipids synthesis, which starts in peroxisomes and finishes in ER (**Figure 26A**), three genes, *acl-7*, *ads-1* and *fard-1*, have been identified in *C. elegans* to be essential. The loss-of-function of either of these genes shows a remarkable decreased level of 18:0 DMA and a high saturated level of fat, suggesting the lack of ether lipid biosynthetic capability. The encoded proteins of *acl-7*, *ads-1* and *fard-1* are all required in the initial step of ether lipid synthesis in peroxisomes, and their corresponding homologs in mammals have the similar functions as in *C. elegans* (shown in **Figure 26A**). Whereas different from the high infertility and other severe developmental defect resulted from the deficiency of ether lipids in mice, *C. elegans* is able to grow similarly as wild type in normal condition, despite a shortened lifespan and a reduced brood size (*Shi, Tarazona et al. 2016*). This advantage makes *C. elegans* a good alternative tool for exploring the roles of ether lipid in cell metabolism, especially in stress conditions.

Autophagy provides a degradation pathway for the regulation of lipid metabolism, which is termed as lipophagy. On the other hand, lipids could in reverse regulate autophagy activity, but the mechanism is complex (*Jaishy and Abel 2016*). As we have already established a good tool to induce autophagy by the acute heat stress, in the contexts of the collaboration with a team working in autophagy in Dijon, we next detect whether the deficiency of ether

lipids alters the autophagy responses in *C. elegans*. Our results indicate that the impaired ether lipids biogenesis caused a defect of autophagy and reduced worm survival under starvation.

3.2. Results

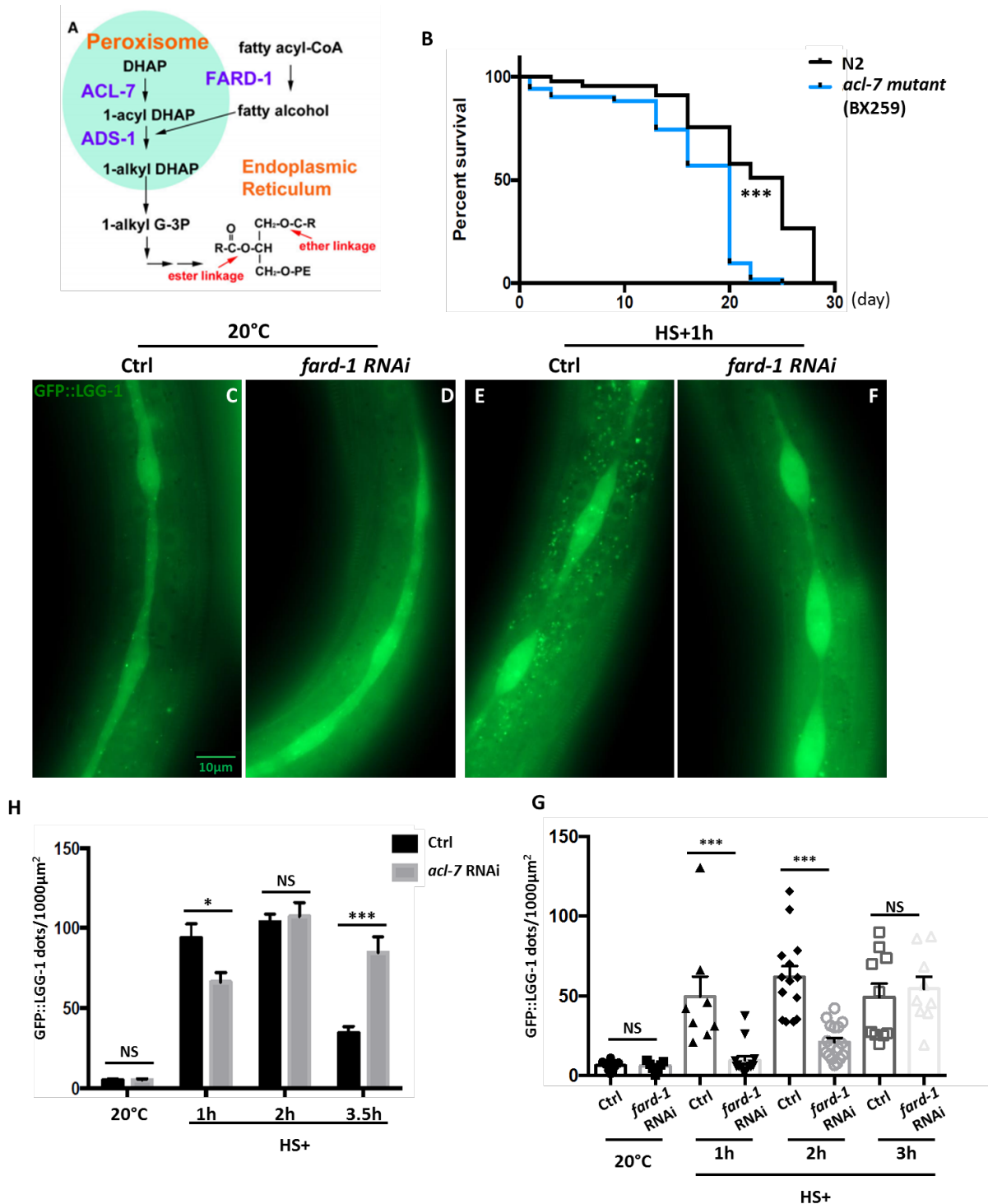


Figure 26 | ether lipids biosynthesis is important for autophagy in *C. elegans*. (A) A schematic diagram showing ether lipid synthesis process. Ether lipid synthesis begins in peroxisome and ends in endoplasmic reticulum. The three identified proteins involved in ether lipids synthesis pathway is indicated in this schematic: ACL-7 is the acyltransferase for DHAP; FARD-1 is associated with the peroxisome membrane to function as the fatty acyl-CoA reductase, ADS-1 replaces the acyl group with the alkyl group and forms 1-alkyl DHAP. (B) Starvation survival curves of first stage larvae in N2 and *acl-7* mutant worms, at 20°C. Log-Rank test, *** p < 0.001. (C-F) worms expressing GFP::LGG-1 under control or *fard-1* RNAi background are

subjected to 20°C or 37°C 1h, and then images are taken after 1h recovery at 20°C. (H,G) Quantifications of GFP::LGG-1 puncta of control and *fard-1* RNAi (G) or control and *acl-7* RNAi (H). (H) Two-way anova, NS $p>0.05$; * $p<0.05$; *** $p<0.005$. $n>10$. (G) t-test, NS $p>0.05$; *** $p<0.005$. $n>10$.

In heat stress induced autophagy, the knockdown of either *fard-1* or *acl-7*, which had been shown to be essential for ether lipids synthesis, strongly reduced GFP::LGG-1 puncta (**Figure 26E,F and quantifications in H, G**). Whereas the basal level of autophagy in epidermis was not impaired by *fard-1* or *acl-7* RNAi (**Figure 26C, D and quantifications in H, G**). These results suggest that ether lipids are required for autophagy upon heat stress condition. It is worth noting that the impaired autophagy in *fard-1* or *acl-7* RNAi worms could somehow be rescued when worms were recovered for longer time after aHS (**Figure 26H**). It may indicate that there is a compensatory effect of autophagy when ether lipids synthesis is inhibited.

Moreover, the *acl-7* loss-of-function mutant, whose ether lipids biosynthesis is blocked at the beginning step, was more sensitive to starvation compared with wild type worms (**Figure 26B**). Since several groups have proved that under starvation, autophagy is elevated and beneficial for newly hatch L1 worms. By contrast, autophagy deficiency decreases L1 survival under starvation (*Kang, You et al. 2007*). Our data suggest that autophagy may be impaired by the reduced ether lipids.

III DISCUSSION AND PERSPECTIVES

1. The role of autophagy during heat stress
2. ER-mitochondria contact sites in *C. elegans*
3. The autophagy defect in *drp-1* mutant worms

1. The role of autophagy during heat stress

1.1. Heat stress studies

Heat stress is a common stress for organisms, from bacteria, plants, and mammals. When organisms are exposed to an environmental change where the temperature is high, their cellular metabolisms changed rapidly to adapt to the heat stress. For instance, the expression level of heat shock proteins (HSPs) is elevated during heat shock, to avoid the formation of protein aggregates and maintain proteostasis.

C. elegans is a powerful model animal to explore the effects of heat stress on development and longevity. *C. elegans* can grow and reproduce at temperatures from 12°C to 26°C. However, the growth rate is more than 2-fold at 25°C compared with that at 16°C (Hirsh, Oppenheim *et al.* 1976). Long exposure to temperature higher than 25°C causes sterility which is an obstacle for substantial maintenance (*wormbook*), but exposure at high temperature for a short time is not always detrimental for *C. elegans*. It is known that the effects of heat stress vary due to the different temperature, duration and stage of worms (Zevian and Yanowitz 2014). It has been surprising to discover that worms are sensitive to a slight change of temperature and an increase from 26.0 °C to 26.2 °C could be sensed by worms to adjust their behaviours (Dawe, Smith *et al.* 2006). For this reason, the heat stress treatment should be carefully chosen according to the aim of experiments.

In recent years, heat stress has been applied as a simple tool to induce autophagy in *C. elegans*, but the methodology to realize the heat stress used in different studies lead to very distinct effects. In our study, we have implemented heat stress at 37°C for 1 hour on L4 larvae. We qualify treatment as an acute heat stress (aHS for short) able to trigger autophagy but without worm lethality. In the bibliography, we also noticed other heat stress treatments applied by other groups in autophagy studies in *C. elegans*. For instance, Tavernerakis group was the first to reveal that heat stress could induce mitophagy (Palikaras, Lionaki *et al.* 2015), while Hansen group applied a hormetic heat stress to induce autophagy in *C. elegans* (Kumsta, Chang *et al.* 2017). In this section, I will compare the different heat stress treatments and effects between our study and others (Table 8).

Table 8. A comparison of different heat stress in the study of autophagy in *C. elegans*

	Tavernarakis	Hansen	Me
Worm stage	4-day-old Adults	1-day-old Adult	L3-L4 larvae
Treatment	37°C 2h	36°C 1h (hormetic heat stress)	37°C 1h (acute heat stress)
The effect of heat stress	Causes about 25% lethality	Benifical	Damage, but not lethal
Observation	Mainly in muscle	Different tissues	Mainly in epidermis
Autophagy cargoes	mitochondria	No claim	mitochondria

a. Different heat stress treatments

Among the different procedures and conditions to achieve heat stress in our study and others, the first difference concerns the worm stage. In Tavernarakis paper, 4-day-old adults which had completed egg-laying were used, while 1-day-old adults were chosen in Hansen paper. It has been shown that the 4-day-old adults are more resistant to heat stress (37°C, 1.5h) compared with 1-day adult (*Zevian and Yanowitz 2014*). But neither Tavernarakis nor Hansen used heat stress that induces a severe lethality. One of the advantages of adults is that the tissues have fully grown and differentiated and therefore the observation in different tissues is easy. However, it is also very interesting to address the effect of heat stress on larvae development. Besides, we finally chose the L4 larvae as it offer a good compromise and have already been used in other studies (*Jovic, Sterken et al. 2017*).

The second difference is the temperature and duration. Tavernarakis group treated worms at 37°C for 2 or 4 hours, whereas Hansen group used a hormetic heat stress being 36°C, 1h. We applied a protocol of 37°C for 1h, which we call it acute heat stress since the temperature is very high with a short duration. The high temperature is able to induce sufficient responses whereas the short duration avoids severe damages. However, to evaluate worm thermotolerance, both studies used a longer duration: Tavernarakis group treated worms at 37°C up to 7 hours and Hansen group also prolonged the treatment time from 1 hour to 8 hours. A longer heat stress is applied in thermotolerance study to test the adaptation and quantify the worm lethality, in control and autophagy deficient animals. However, in our hand we doubt that autophagy could still respond to such long time heat stress, in L4 animals, because at 37°C for 2h, autophagosomes are not increased.

b. Worms responses under different heat stress conditions

Comparison of the three studies demonstrated that autophagy could be induced by different heat stress, but different autophagy responses and physiological changes are triggered according to the treatments.

The first difference concerns the autophagic cargos that have been analyzed and the question of selective vs bulk autophagy. Tavernarakis group illustrates an abundant mitophagy induction in muscle cells after heat stress, whereas our aHS seems to have no effect on mitophagy in muscle cells. Instead, a portion of mitochondria in epidermis are degraded via mitophagy, suggesting that autophagy cargos also vary due to different heat stress treatments. We also tested another potential cargo for autophagy, the lipid droplets (**Figure 18**), and our result suggest that lipid droplets are not one of the main cargos upon heat stress-induced autophagy. In addition, we have observed an inflated ER that suggests a stress of the ER but we have not performed further experiments to test ERphagy yet. Hansen publication did not report any characterization of cargos in hormetic heat stress-induced autophagy.

The second difference is the tissue specificity of the autophagy responses. Hansen group observed an increased autophagy in multiple tissues, including epidermis, muscle, nerve ring and intestine. Although the temperature and worm stage we used are different from Hansen group, we obtained similar conclusions. Firstly, they noticed that upon hormetic heat stress, autophagy responds diversely from tissue to tissue. For instance, the GFP::LGG-1 labelled autophagosomes in epidermis and body wall muscle cells began to increase immediately after heat stress, whereas the response was delayed to 2-4 h in the nerve ring and intestine. On the other hand, the duration of autophagy also showed a tissue specificity. These data are partially consistent with our results. In our study, aHS induced autophagy is firstly observed in epidermis, whereas the increase of autophagic puncta in body wall muscle cells needs 4 hours recovery at 20°C. In addition, our results are the first report of a tissue-specific size of autophagosomes in *C. elegans*. Indeed, both fluorescent images and EM images show that autophagosomes in intestine are bigger than in other tissues.

The third difference observed between the three studies concerns the effects of heat stress on the animals. In all studies the autophagy is a beneficial mechanism upon heat stress, but at different levels. Tavernarakis data shows that the 7h heat stress of 37°C, results in about 25% lethality in N2 worms but up to 75% lethality in mitophagy deficient worms (*Palikaras, Lionaki et al. 2015*), suggesting that mitophagy allows a partial resistance

of worms to particularly harsh heat stress. The main effect of heat stress in Hansen study is the existence of a hormetic response beneficial for the animal. One evidence to support their conclusion is that heat stress treated worms survive longer in an autophagy-dependent manner. Another evidence is that animals subjected to mild heat stress on day 1 of adulthood were indeed more resistant to a second thermal stress later in life (day 4 and 5 of adulthood) and had less protein aggregates compared with the none-stressed worms. My experiments have rather focus on the role of autophagy/mitophagy for worm recovery after the acute heat stress since there is no lethality during or soon after heat stress. In autophagy-deficient worms, the developmental delay is more severe and the mitochondrial network rebuilding is impaired. This indicates that autophagy is beneficial for the mitochondrial homeostasis and important for resuming the developmental program. We could not conclude whether there is a direct link between the developmental delays with mitochondrial network remodeling.

To sum up, our data have shown that heat stress is a powerful tool to stimulate autophagy in *C. elegans*, which is consistent with the two other groups. We also provide an approach to understand the impact of heat stress on cellular homeostasis and worm development.

1.2. How autophagy affects worm recovery from heat stress?

It is interesting that although the organelles are strongly altered by the aHS, most of worms still could recover from the damage and develop into adults. Our results show that autophagy is a beneficial factor for worm recovery. We offer one possibility that autophagy helps to eliminate the damaged materials and organelles which are toxic to the cellular homeostasis. Taken mitochondria as an example, upon heat stress, mitochondrial morphology and functions are both damaged, so autophagy is stimulated to clear these damaged mitochondria. However, inhibiting mitophagy by *lgg-1* or *atg-7* RNAi further impairs mitochondrial network rebuilding during worm recovery. As mitochondria are the main organelles to provide energy and associated with many physiology processes, the correct rebuilding of mitochondrial network is essential for worm recovery from the heat stress induced damage.

Tavernarakis group demonstrate a transcriptional regulation that coordinates mitophagy with mitochondrial biogenesis under heat stress condition. They found that mitophagy

inhibition specifically activates SKN-1, which is required for the expression of several mitochondrial biogenesis genes as well as the mitophagy mediator *dct-1*.

Indeed, the most studied heat stress response is via transcriptional regulation. Genome wide gene expression analysis in *C. elegans* shows that a heat stress at 35°C for 2h affects the expression of plenty of genes that are involved in development, reproduction and metabolism (Jovic, Sterken et al. 2017). For instance, genes involved in cuticle synthesis and metabolic processes were down-regulated by heat stress, which may partially explain the development delay (Jovic, Sterken et al. 2017). Moreover, the expression of these genes is dependent on HSF-1 (Heat Stress Factor 1) (Brunquell, Morris et al. 2016). Then what is the link between autophagy and HSF-1-mediated genes regulation? Hansen group revealed that autophagy genes are elevated through HSF-1 regulation in *C. elegans*. The overexpression of HSF-1 alone is sufficient to trigger autophagy, while the *hsf-1* RNAi prevents the increasing of autophagosomes upon heat stress. Further studies found that many of the autophagy-related genes induced by heat shock contain at least one putative HSE in their promoter regions, but whether HSF-1 regulates autophagy directly or whether other transcriptional regulators besides HSF-1 are involved in the upregulation of autophagy genes upon heat stress needs more evidences. In addition, *hlh-30*, the orthologue of mammalian transcription factor EB, a conserved regulator of multiple autophagy-related and lysosomal genes (Lapierre, De Magalhaes Filho et al. 2013), is also required for the up-regulation of several autophagy genes upon heat shock.

Here we have listed several mechanisms of how autophagy protects worm from heat stress, heat-shock response pathway seems to be the prominent in this process. However, since the heat stress leads to many cellular changes, other pathways may be also important for worms' resistance under heat stress conditions.

1.3. Mitophagy mediators

Both the in vivo colocalization assay and western blot assay indicate mitophagy events during acute heat stress-induced autophagy in epidermis. The study by Tarvenarakis (Palikaras, Lionaki et al. 2015) has shown that the increased of LGG-1 puncta is blocked by the RNAi of *pink-1* or *dct-1*, indicating that heat stress-induced mitophagy in muscle cells depends on both *pink-1/pdr-1* pathway and mitophagy receptor *dct-1*. However, in our hand, neither depletion of *pink-1/pdr-1* nor *dct-1* reduces the number of LGG-1

autophagosomes in epidermis, and the colocalization images also suggest that mitophagy still exists in *pdr-1* mutant (data not shown). Based on these results, we got the conclusion that *pink-1/pdr-1* or *dct-1* is not required for aHS induced mitophagy in epidermis. However, the efficiency of *pink-1* or *dct-1* RNAi was not confirmed in my study yet. This discrepancy is intriguing, but one possibility is that our conditions of heat stress are less effective to induce a PINK-1/PDR-1 response or that it is a tissues specific response. Other proteins may mediate mitophagy in *C. elegans*, for instance, PHB-2 that has been identified as a novel mitophagy receptor recently. PHB-2 is an IMM protein both in mammals and *C. elegans*. In *C. elegans*, PHB-2 and ALLO-1, but not PINK-1/PDR-1, are involved in allophagy process (Sato, Sato et al. 2018) (Wei, Chiang et al. 2017). The clearance of paternal mitochondria upon fertilization is delayed by sperm-derived *phb-2* RNAi. Of note, Tavernarakis data show that the increased mitochondrial mass in intestine is only observed by *pink-1/pdr-1* or *dct-1* RNAi, rather than *phb-2* RNAi, suggesting mitophagy is not affected by *phb-2* RNAi in the intestine of aged animals. These results hint the existence of different mitophagy mechanisms in different tissues and by different inducers. In mammals, several mitophagy mediators have been identified, such as PINK1/Parkin, NIX, FUNDC1 etc..., and their involvement in mitophagy varies according to different cell types, different inductions. Perhaps in the epidermis of *C. elegans*, aHS-induced mitophagy is mediated by other mitophagy proteins which have not been identified yet. We have noticed that in *C. elegans*, there is a homolog of FUNDC1, the mammalian mitophagy receptor, which may be a potential mitophagy protein. In addition, the PGAM5 protein, which regulates activity of FUNDC1, as well as the PINK1/Parkin pathway, also has a homolog in *C. elegans*. It would be worth to further analyze the roles of FUNDC1 and PGAM5 during mitophagy in *C. elegans*, and identifying other mitophagy mechanisms in *C. elegans*.

2. ER-mitochondria contact sites in *C. elegans*

2.1. The study of ER-mitochondria is an emerging field in *C. elegans*

ER and mitochondria contact sites have been widely studied in mammals and yeast, and the contact site is a functional region engaging in Ca^{2+} signalling, mitochondrial morphology regulation, lipid biosynthesis, autophagosome biogenesis ... (Giorgi, Missiroli et al. 2015). Whereas to our knowledge, there are few studies investigating the roles of ER-mitochondria contact sites in *C. elegans*. In fact, until now, there are no validated MAM proteins in *C. elegans*. How to detect the ER-mitochondria contact and identify the MAM fractions has not yet been studied in *C. elegans*.

In 2014 a paper combined the tools for detecting ER-mitochondria contact sites in yeast and identified ARF-1.2 as a potential MAM protein in *C. elegans* (Ackema, Hench et al. 2014). The authors observed a defect of mitochondrial morphology and functions in *arf-1.2* depletion, in yeast, *C. elegans* and mammalian cells. Gradient centrifugation assay revealed that ARF1 is enriched in the ER and mitochondria co-fraction, suggesting it is a MAM protein. Moreover, the depletion of *arf-1.2*, as well as other homologs of MAM protein, leads to a hyper-connected mitochondrial network in *C. elegans*. By measuring the mitochondrial morphology, in our study, we have identified several potential MAM candidates whose RNAi causes highly-fused mitochondria. It should be noted that in yeast, the ER and mitochondria tethering is still present in the *arf1-11Δarf2*, the mutant of the *arf-1.2* homologue in yeast, suggesting that ARF-1.2 is not required for the physical tethering of ER and mitochondria (Ackema, Hench et al. 2014). They did not either explore what would happen to the ER-mitochondria contact sites in *C. elegans*. Their results remind us that the depletion of MAM protein not always results to the disruption of ER-mitochondria interaction. More important, the ER and mitochondria tethering in *C. elegans* may differ from yeast. Although we could observe the altered mitochondrial morphology in the RNAi of several MAM candidates, we still ask the question: whether this alteration is due to the defect of ER-mitochondria contacts sites or not? To answer it, we plan to detect the ER and mitochondria tethering in worms. As well as, new tools for detecting ER-mitochondria contact sites are needed in *C. elegans*.

2.2. Tools for studying ER-mitochondria contact sites

The association between the ER and mitochondria was first visualized in the 1970s with the help of electron microscopy. Up to now, different approaches have been developed to study ER-mitochondria interactions in yeast and mammals. One of the useful tools for the analysis of MAM is based on the subcellular fractionation. It allows the isolation of MAM fractions by differential ultracentrifugation coupled to a Percoll gradient (*Wieckowski, Giorgi et al. 2009*). By this assay, hundreds of proteins have been identified in MAM fractions, and these proteins are called MAM proteins. However, the purified fractions may be much less than the real MAMs. Moreover, the MAMs proteins are not restricted to contact sites and are found in ER or Mitochondria out of the contact sites. A new bifluorescence complementation (BiFC) method that labels a subset of ER-mitochondrial associations in fixed and living cells has also been described (*Harmon, Larkman et al. 2017*). Besides, immuno-localization of ER and mitochondria-specific fluorophores, fluorescent proteins were also classically used to study ER-mitochondria interactions (*Rizzuto, Pinton et al. 1998*), however, the resolution of confocal is too large for the contact zones (10-30nm)(*Csordas, Renken et al. 2006*). The transmission electron microscopy has high resolution, which makes it a convincing tool to show the ER-mitochondria tethering (*Cosson, Marchetti et al. 2012*). Especially the recent studies have combined the EM with the HRP labelling of ER, providing a powerful tool to detect the ER-mitochondria contact sites under EM, and this approach has been widely used now (*Giordano, Saheki et al. 2013*).

In *C. elegans*, there is no tool for studying ER-mitochondria contact sites yet, and even no evidence showed whether the ER-mitochondria tethering is similar to yeast or mammalian system. The newly generated transgenic worms expressing HRP-KDEL, which allows measuring the ER-mitochondria contact sites under EM, could be a useful tool to promote the understanding of ER interaction with mitochondria.

3. The autophagy defect in *drp-1* mutant worms

We have shown autophagic clusters in *drp-1* mutant upon heat stress. Evidences suggest that these clusters are due to the defect during the elongation of isolation membrane. How *drp-1* depletion affects autophagosome formation needs further studies, but I proposed here several hypotheses to explain it.

Hypothesis1: Autophagic clusters in *drp-1* mutant result from the incorrect engulfment during mitophagy (a secondary effect from the fission defect of *drp-1*)

Our manuscript demonstrates that DRP-1 is required for mitochondrial fission in heat stress condition. In the absence of DRP-1, mitochondria are still blebbed and maintaining in connection in *C. elegans* (manuscript Figure6). Both in yeast and mammals, DRP-1 is recruited to the ER-mitochondria contact sites to assemble around mitochondria. More importantly, ERs pre-constrict mitochondria on these sites to enable the DRP-1 assembly. It is generally perceived that DRP-1-mediated mitochondrial fission is a prerequisite for autophagic engulfment during mitophagy (Tatsuta and Langer 2008, Mao and Klionsky 2013). For this reason, the huge mitochondria in *drp-1* mutant could be the obstacle for mitophagosome formation and lead to the clustering of isolation membranes. The observed autophagosomes are normally within 300-900nm diameter in cultured cells, suggesting that the size of autophagosomes is controlled by some mechanism to avoid the overgrowth. Alive confocal images show that GFP::LGG-1/-2 structures intermingle around mitochondria in *drp-1* mutant, while the stacks clearly show the autophagic vesicle around mitochondria is incomplete. These data support our hypothesis that during heat stress induced mitophagy, isolation membranes are elongated around a portion of mitochondria, whereas in the absence of *drp-1*, autophagosomes which fail to enclose the huge mitochondria over-grow into clusters. Another study also revealed that during allophagy, the morphology of paternal mitochondria affects their degradation ((Wang, Zhang et al. 2016). A defect of fission in paternal mitochondria delays their elimination; while by contrast, the fusion defect has a facilitating effect. It goes the same way as in mammalian cells that mitochondrial dynamic is a key factor for mitophagy.

Hypothesis2: Autophagic clusters in *drp-1* mutant result from the disrupted ER-mitochondria contact sites where autophagosome is forming

It is interesting that both DRP-1-mediated mitochondrial fission and autophagosome initiation can take place at the ER-mitochondria contact sites. Both Mfn2 and Drp1, two key factors for mitochondrial fusion and fission, are MAM proteins that are enriched in the MAM co-fractions. Perhaps DRP-1 could function as a MAM protein regulating ER and mitochondria interaction and therefore autophagosome biogenesis.

Studies in mammals have shown that several autophagic markers, including the phagophore markers, appear at the ER-mitochondria contact zone and are enriched in MAMs when autophagy is stimulated. Uncoupling mitochondria from ER by knockdown of PACS-2 or MFN2 leads to attenuated autophagy, including decrease of LC3 lipidation and impaired autophagy machinery on the contact sites (Hamasaki, Furuta et al. 2013). In our study, *drp-1* mutant also leads to reduced LGG-1 autophagosomes, similar to the autophagy defect in ER-mitochondria uncoupled cells. In addition, autophagosomes (labelled by GFP::LGG-1 or GFP::LGG-2) often appears in proximity to mitochondria in basal and stimulated autophagy. There is almost no study about the autophagosome origin in *C. elegans* yet, except one suggests that ER could be associated with the isolate membrane during embryogenesis. They found that the ER-localized protein EPG-3 (homologue of VMP1 in mammals) regulates autophagosome formation at the ER-isolation membrane (ER-IM) contact sites, and the loss of EPG-3 suppresses the release of IM for autophagosome closure and leads to autophagic clustering (Tian, Li et al. 2010, Zhao, Chen et al. 2017). Whereas the involvement of mitochondria in this process is not clear. To test whether these autophagosomes are close to the ER-mitochondria contact zones, we are planning to do colocalization analyses between ER, mitochondria and autophagosomes in worms.

Another question is that whether the autophagic cluster in *drp-1* mutant is specific for mitophagy or not? Studies in yeast indicate that ERMES is specifically required for selective mitophagy but not bulk autophagy, which is different from mammals. But in *C. elegans*, we have no preferred answer for this question yet.

Taken together of the hypothesis1 and hypothesis2, mitochondria fission and autophagosome initiation may be coordinated at the ER-mitochondria contact sites for proper mitophagy. In mammals, the mitophagy receptor FUNDC1, is a novel MAM protein mediating mitochondrial fission and fusion via the direct binding with Drp1 and Opa1. CCCP triggered FUNDC1 dephosphorylation, which promotes the FUNDC1-Opa1 disassembly. Then the released FUNDC1 from FUNDC1-Opa1 complex could bind with Drp1 and promote the recruitment on mitochondria. In turn, the knockdown of *DNM1L* (or *DRP1*) blocked FUNDC1-mediated mitochondrial fragmentation and further mitophagy (Chen, Chen et al. 2016). Youle and Blik revealed an autophagic aggregate that similar as ours in the mutant of Fis1 both in cultured cells and in *C. elegans* muscle. Fis1 was identified as an interaction

protein of DRP-1 and mediates mitochondrial fission in yeast; however, *fis-1 mutant* in *C. elegans* has no alteration of mitochondrial morphology. They further confirmed these autophagic clusters as excessive isolation membranes. They demonstrated that TBC1D15, LC3, Fis1 and Rab7 coordinate to shape the nascent autophagosome isolation membrane during mitophagy (Yamano, Fogel et al. 2014) (Shen, Yamano et al. 2014). It is possible that in *C. elegans*, mitochondrial fission and autophagy pathway are also somehow coordinated, and the ER-mitochondria contact sites are reasonable choices for their coordination. A recent study suggests that Drp1 also acts as a regulator to monitor damaged mitochondria for the selection of mitophagy (see [introduction 4.3.1](#)) (Cho, Ryu et al. 2019). The significant reduction of autophagosomes we observed in *drp-1* mutant may be a feedback from the mitochondrial fission defect.

From the published papers, we also noticed that mitochondrial fission and mitophagy could occur in the absence of DRP-1 in yeast and mammals. Yamashita and Kanki proposed a novel model of mitochondrial fission that is mediated by the extending isolation membrane/phagophore during mitophagy (Yamashita, Jin et al. 2016, Yamashita and Kanki 2017). They showed that autophagosomes were always formed on or near mitochondria, which is similar as what we observed both in basal and aHS in *C. elegans*. However, in their model, DRP1 is dispensable for mitophagy, and the mitochondrial DRP1 is not colocalized with autophagy markers (Yamashita, Jin et al. 2016, Yamashita and Kanki 2017). It could be possible that the DRP-1 associated mitochondrial fission and autophagosome biogenesis are taken place at different ER-mitochondria contact sites. The involvement of DRP-1 during mitophagy is poorly understood in *C. elegans*. We have proved that the heat stress induced mitochondrial fragmentation is DRP-1-dependent; we will next perform western blot to check whether mitophagy is blocked by *drp-1* mutant.

Hypothesis3: Other roles of DRP-1 in autophagy

The best-known role of DRP-1 is regulating the fission events of mitochondria but also in peroxisomes. However, in the density gradient centrifugation assay, DRP1 is predominantly cytosolic, but also enriched in light Golgi and ER fraction (Yoon, Pitts et al. 1998). The distribution of DRP1 also varies in different tissues. They demonstrate that DRP1 is associated with ER tubulars and cytoplasmic vesicles. We wonder the role of the DRP1 portion which is located in ER tubulars and cytoplasmic vesicles, and whether DRP-1 has

other undiscovered roles, especially during autophagy process. For instance, DRP-1 may be involved in the Atg9 traffic between phagophore and other vesicles, and the *drp-1* depletion affects the expansion of phagophore. This may also explain the reduced autophagosomes in *drp-1* mutant worms upon heat stress.

IV ANNEXES

Review

Approaches for Studying Autophagy in *Caenorhabditis elegans*

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Received: 25 July 2017; Accepted: 26 August 2017; Published: 30 August 2017

Abstract: Macroautophagy (hereafter referred to as autophagy) is an intracellular degradative process, well conserved among eukaryotes. By engulfing cytoplasmic constituents into the autophagosome for degradation, this process is involved in the maintenance of cellular homeostasis. Autophagy induction triggers the formation of a cup-shaped double membrane structure, the phagophore, which progressively elongates and encloses materials to be removed. This double membrane vesicle, which is called an autophagosome, fuses with lysosome and forms the autolysosome. The inner membrane of the autophagosome, along with engulfed compounds, are degraded by lysosomal enzymes, which enables the recycling of carbohydrates, amino acids, nucleotides, and lipids. In response to various factors, autophagy can be induced for non-selective degradation of bulk cytoplasm. Autophagy is also able to selectively target cargoes and organelles such as mitochondria or peroxisome, functioning as a quality control system. The modification of autophagy flux is involved in developmental processes such as resistance to stress conditions, aging, cell death, and multiple pathologies. So, the use of animal models is essential for understanding these processes in the context of different cell types throughout the entire lifespan. For almost 15 years, the nematode *Caenorhabditis elegans* has emerged as a powerful model to analyze autophagy in physiological or pathological contexts. This review presents a rapid overview of physiological processes involving autophagy in *Caenorhabditis elegans*, the different assays used to monitor autophagy, their drawbacks, and specific tools for the analyses of selective autophagy.

Keywords: *C. elegans*; genetics; in vivo imaging; electron microscopy; mitophagy; aggrephagy; LGG-1; LGG-2

1. *C. elegans*, an Animal Model to Study Autophagy

1.1. General Experimental Advantages

A *Caenorhabditis elegans* adult is an approximately 1 mm, transparent nematode, predominantly found as a self-fertilizing hermaphrodite. *C. elegans* males emerge spontaneously at low frequency and are able to fertilize hermaphrodites, which allows for all genetic approaches. The life cycle of *C. elegans* includes an embryonic phase, four consecutive larval stages (L1–L4) separated by cuticle sloughing, and the adult stage, during which the worm is reproductively mature. In favorable conditions, the lifespan of wild-type *C. elegans* is approximately two to three weeks. In response to harsh environments, early larvae can enter an alternative larval stage called dauer, which enables them to survive for several months. *C. elegans* has a rapid development, three days, and is highly fertile, with a progeny of a few hundred for a single hermaphrodite. Its cell lineage, 959 somatic nuclei in the adult hermaphrodite, is invariant, and its stereotypical development leads to the differentiation

of various cell types and tissues (epidermis, intestine, muscle, neurons ...). Its small size and transparency are convenient for diverse microscopy approaches, and populations can be synchronized and cultured in liquid medium to obtain a large quantity of biological material. Classical genetic tools are available in *C. elegans*, and transgenesis or RNA interference (RNAi) are commonly performed. Moreover, the genomic engineering by CRISPR-Cas9 has been recently adapted to *C. elegans* to efficiently create new transgenic or mutated strains [1]. All these experimental advantages established *C. elegans* as a good model, which has been key in the understanding of major biological processes such as the apoptotic cell death or the discovery of RNAi.

1.2. Physiological Processes Involving Autophagy in *C. elegans*

The *Atg* (autophagy related) genes, which are involved in various aspects of autophagy, were first identified in yeast, but are mostly conserved in other eukaryotes (Table 1). In mammals, numerous orthologs emerged from genetic amplifications, but in *C. elegans*, most *atg* genes have one single ortholog. However, two homologs exist for *Atg4*, *Atg8*, and *Atg16* (Table 1). Interestingly, each homolog of the ubiquitin-like protein ATG8, named LGG-1 and LGG-2, corresponds to a specific member of the GABARAP and LC3 families, respectively. Both LGG-1 and LGG-2 are conjugated to a phosphatidylethanolamine (PE) lipid present in the membrane of phagophore and autophagosome [2]. Although *lgg-1* is essential for development and fertility, *lgg-2* null mutants do not present a gross developmental or morphological phenotype. In regards to the limited genetic duplications and the simplicity of the animal, *C. elegans* has emerged as a powerful alternative model for exploring autophagy in the context of tissue-specificity and developmental processes [3–5].

Table 1. Autophagy genes in *C. elegans*, and homologs.

<i>C. elegans</i> Genes	Mutant Alleles or RNAi	Mammalian Homologs	Yeast Genes	References
<i>atg-2</i>	<i>bp576</i>	<i>ATG2</i>	<i>ATG2</i>	[6]
<i>atg-3</i>	<i>bp412/RNAi</i>	<i>ATG3</i>	<i>ATG3</i>	[7]
<i>atg-4.1</i>	<i>bp410</i>	<i>ATG4A, ATG4B</i>	<i>ATG4</i>	[2]
<i>atg-4.2</i>	<i>tm3948</i>	<i>ATG4C, ATG4D</i>	<i>ATG4</i>	[2]
<i>atg-5</i>	<i>bp546/RNAi</i>	<i>ATG5</i>	<i>ATG5</i>	[8]
<i>atg-7</i>	<i>bp422/RNAi</i>	<i>ATG7</i>	<i>ATG7</i>	[7]
<i>atg-9</i>	<i>bp564/RNAi</i>	<i>ATG9</i>	<i>ATG9</i>	[6]
<i>atg-10</i>	<i>bp421, bp588/RNAi</i>	<i>ATG10</i>	<i>ATG10</i>	[7]
<i>atg-16.1</i>	<i>gk668615d</i>	<i>ATG16L1</i>	<i>ATG16</i>	[8]
<i>atg-16.2</i>	<i>bp636, ok3224</i>	<i>ATG16L2</i>		[8]
<i>atg-18</i>	<i>gk378/RNAi</i>	<i>WIPI1, WIPI2</i>	<i>ATG18</i>	[9,10]
<i>bec-1</i>	<i>ok691, bp613, ok700/RNAi</i>	<i>BECN1</i>	<i>VPS30/ATG6</i>	[10,11]
<i>epg-1</i>	<i>bp414</i>	<i>KIAA0652</i>	<i>ATG13</i>	[12]
<i>epg-2</i>	<i>bp444/RNAi</i>	?	<i>VPS34</i>	[9]
<i>epg-3</i>	<i>bp405/RNAi</i>	<i>VPM1</i>	<i>VPS34</i>	[9]
<i>epg-4</i>	<i>bp425/RNAi</i>	<i>EI24</i>	<i>VPS34</i>	[9]
<i>epg-5</i>	<i>tm3425/RNAi</i>	<i>EPG5</i>	<i>VPS34</i>	[9]
<i>epg-6</i>	<i>bp242</i>	<i>WIPI3, WIPI4</i>		[6]
<i>epg-7</i>	<i>tm2508</i>	<i>FIP200</i>	<i>VPS34</i>	[13]
<i>epg-8</i>	<i>bp251</i>	<i>ATG14</i>	<i>ATG14</i>	[14]
<i>epg-9</i>	<i>bp320</i>	<i>ATG101</i>	<i>VPS34</i>	[15]
<i>let-363</i>	<i>h98/RNAi</i>	<i>TOR</i>		[16–18]
<i>lgg-1</i>	<i>tm348/RNAi</i>	<i>GABARAP</i>	<i>ATG8</i>	[7,9,18–25]
<i>lgg-2</i>	<i>tm5755/RNAi</i>	<i>LC3</i>	<i>ATG8</i>	[22,24,26]
<i>lgg-3</i>	<i>RNAi</i>	<i>ATG12</i>	<i>ATG12</i>	[7,22]
<i>pgl-3</i>	<i>bp439</i>	?		[12]
<i>rab-7</i>	<i>ok511/RNAi</i>	<i>RAB7</i>		[19,23–25]
<i>sepa-1</i>	<i>bp456</i>	?		[9,12]
<i>sqst-1</i>	<i>ok2892</i>	<i>SQSTM1/p62</i>		[13]
<i>unc-51</i>	<i>e369/RNAi</i>	<i>ULK1</i>	<i>ATG1</i>	[18,27,28]
<i>vps-34</i>	<i>h741</i>	<i>VPS34</i>	<i>VPS34</i>	[29]
<i>vps-39</i>	<i>tm2253</i>	<i>VPS39</i>		[25]
<i>vps-41</i>	<i>ep402</i>	<i>VPS4</i>		[25]

During the last 15 years, numerous studies on *C. elegans* have shown that autophagy is involved in multiple processes through embryonic and larval development. Upon fertilization, the degradation of several paternal organelles is dependent of the formation of autophagosomes. This fast process, called allophagy (allogenic organelles autophagy), leads to the degradation of the sperm components by selective autophagy, and is linked to the polyubiquitination of these organelles [19,24]. In this particular process, it has been shown that LGG-1 acts upstream of LGG-2, with LGG-1 being involved in the early steps of autophagy, and LGG-2 in the maturation of autophagosomes [25]. Later during embryogenesis, a selective autophagy process called aggrephagy is involved in the removal of proteins that are prone to form aggregates (see below Section 3.1.1). During this process, different maternally inherited germline-specific components are selectively eliminated by aggrephagy in somatic cells, allowing their restriction to the germline precursor cells. The depletion of various autophagy proteins results in a late embryonic lethality demonstrating that autophagy also plays a role in differentiation and organogenesis [30]. During larval development, autophagy acts as a response to unfavorable environmental conditions. For instance, autophagy allows newly hatched L1 larvae to survive one to two weeks without nutrients [20]. Autophagy also has a critical role for the epidermal differentiation of the dauer larvae stage, which enables survival in harsh environments for several months [31].

Autophagy is also involved in the response to various kinds of stress such as osmotic stress, oxidative stress, starvation, and resistance to pathogens. The inhibition of autophagy in *C. elegans* leads to a reduced survival under particular stress conditions [20,21,32–34]. For instance, inactivation by RNAi of autophagy genes (*atg-7*, *bec-1*, *lgg-1*) decreases the survival rates in *S. typhimurium*-infected animals and suppresses the resistance conferred by the insulin-like signaling pathway. Hormetic heat stress induces autophagy, and thus improves survival and proteostasis in the nematode [35].

Using genetic contexts where the lifespan of the worm is extended (*eat-2*, *daf-2*, *let-363*, among others), studies have shown that aging and longevity are dependent on autophagy [16]. Indeed, the long-lived phenotype can be suppressed when several autophagic genes are inactivated (*lgg-1*, *atg-18*, *bec-1*, *atg-7*, *atg-9*, *vps-34*) [36]. Several pieces of evidence link autophagy and longevity in *C. elegans* [16,23], notably through the insulin pathway, which is consistent with knowledge on other species.

Cell death is crucial for many aspects of normal development, and can occur by apoptosis, necrosis, or other processes. *C. elegans* is a well-established model to study cell death, and more than 10% of cells undergo programmed cell death during embryonic and larval development [37]. Autophagy genes are involved in apoptotic cell degradation during development, and in germline cells. Dying cells lead to the formation of cell corpses, and autophagy proteins (BEC-1, UNC-51, ATG-18) were shown to be involved in the removal of those corpses in germline cells. In addition, the inactivation of *bec-1*, the ortholog of human Beclin-1 that interacts with the apoptotic factor CED-9/BCL2, causes an augmentation of the number of apoptotic cell corpses in embryo [38]. Moreover, some classical autophagy proteins are implicated in the so-called LC3-associated phagocytosis (LAP), which is involved in apoptotic cell removal. LAP is distinct from autophagy because it does not require the whole autophagy machinery, and LC3 is recruited to a single membrane vesicle. BEC-1 is critical for the removal of cell corpses in germline cells by a LAP process. Recently, a LAP-dependent degradation, requiring BEC-1, LGG-1 and LGG-2, but not UNC-51 and EPG-8, has been demonstrated for mid-bodies in *C. elegans* embryo [39]. Using a model of necrosis for touch neurons, some studies have shown that autophagy is upregulated in early phases of necrotic cell death, and synergizes with the lysosomal pathway [18,40].

Until 2016, the relationship between autophagy and tumor cells has not been addressed using available models of *C. elegans*. However, a first report demonstrated that the upregulation of autophagy has a role in limiting the growth of heterogeneous tumors in the gonad [41].

2. Methods to Monitor Autophagy in *C. elegans*

2.1. Imaging Autophagy

One of the most common ways to monitor and study autophagy consists of imaging autophagic structures, receptors and cargoes. The transparency of *C. elegans* has facilitated the development of light microscopy approaches, either in vivo or on fixed material. Additionally, different protocols have been optimized for analyzing autophagy by electron microscopy in *C. elegans*.

2.1.1. In Vivo Imaging

Similarly to Atg8/LC3, which are widely used to monitor autophagy [42], LGG-1 and LGG-2 are the most frequent markers to localize autophagosomes in *C. elegans*, because they are recruited to autophagosome through the lipidation of their C-terminus. Several strains expressing fluorescent fusion proteins with LGG-1 and LGG-2 are available (Table 2). In 2003, Melendez and colleagues generated the first strain expressing GFP::LGG-1, driven by its own promoter [31], which rapidly became the most common way to localize autophagosomes in *C. elegans* (strain DA2123 [20]). GFP::LGG-1 shows a dynamic pattern of dots during embryogenesis, which appears along with a diffuse signal in some cells that can be reduced by confocal imaging (Figure 1A). In adult and larva, in nutrient-rich conditions, GFP::LGG-1 is diffused in various tissues. Upon starvation, the number of GFP::LGG-1 dots increases mostly in hypodermis, seam cells, and intestinal cells, corresponding to an activation of autophagy [4]. The quantification of autophagy has been performed by counting GFP::LGG-1 dots at most stages of development and in most tissues. However, care should be taken, since some cells present a high cytosolic diffuse signal, and because GFP::LGG-1 is a multiple-copies transgene with an overexpression that might affect the number of autophagosomes, and possibly forms aggregates in particular conditions. Moreover, despite an expression under LGG-1's own promoter, DA2123, GFP::LGG-1 is silenced in the germline and the very early embryo. The use of a stage-specific promoter, *Ppie-1*, has overcome this difficulty (strain RD204 [25]). DsRed::LGG-1 and mCherry::LGG-1 have been generated, although they have been less extensively used and are more prone to aggregation, especially in nutrient deprivation conditions [5,20,43]. Interestingly, a tandem fusion GFP::mCherry::LGG-1 has been created, which allows for the monitoring of the autophagic flux in vivo (Figure 1D) [25]. GFP and mCherry have different sensitivities to the acidic pH of the lysosome, which enables autolysosomes (mCherry-only positive dots) to be distinguished from autophagosomes (GFP and mCherry positive dots). However, due to the *pie-1* promoter-driven expression, this tool can only be used in the early embryo and germline cells [25].

LGG-2 has been less characterized than LGG-1, but GFP::LGG-2 can also be used as an alternative marker to monitor autophagy activity during development, because its localization at phagophores and autophagosomes has been validated by immunoelectron microscopy [25]. Of note, two particular constructs have been generated, GFP::LGG-1(G116A) and GFP::LGG-2(G130A), which inhibit their lipidation and can be used as controls to exclude aggregation artefacts (Figure 1C).

Another caution about the interpretation of LGG-1 and LGG-2 patterns arises from recent studies that show that in starved mammalian cells, the LC3 family is dispensable for autophagosome formation [44]. This finding supports the idea that an autophagosomal-like structure can be formed in the absence of the Atg conjugation system [45]. Thus, it is possible that LGG-1 and LGG-2 may not be involved in all autophagy processes. To overcome these potential limitations, alternative and reliable markers of autophagosomes should be investigated in *C. elegans*. One interesting possibility would be to use the syntaxin 17 (STX17), which labels closed autophagosomes in mammals [46]. Unfortunately, there is no obvious homolog of *stx17* in the *C. elegans* genome. Alternatively, the identification of other *C. elegans* ATG orthologous genes enabled the generation of several tools to visualize autophagy in vivo. Fluorescent reporters such as GFP::BEC-1, GFP::ATG-4.1, GFP::ATG-9, and GFP::ATG-18 have been generated, and their specific expression patterns have been described [2,6,38,47]. Those reporters have not been frequently used, but should emerge as powerful complementary tools to further visualize

and analyze the autophagy process, especially for early steps of phagophore formation. Additionally, the analysis of aggrephagy by Zhang et al. led them to develop several interesting tools [4,7], which are not restricted to this particular autophagy process. For instance, several GFP-tagged proteins can be used to analyze the degradation or visualize specific cargoes, such as the SEPA family and the autophagy receptor SQST-1 (see Section 3.1).

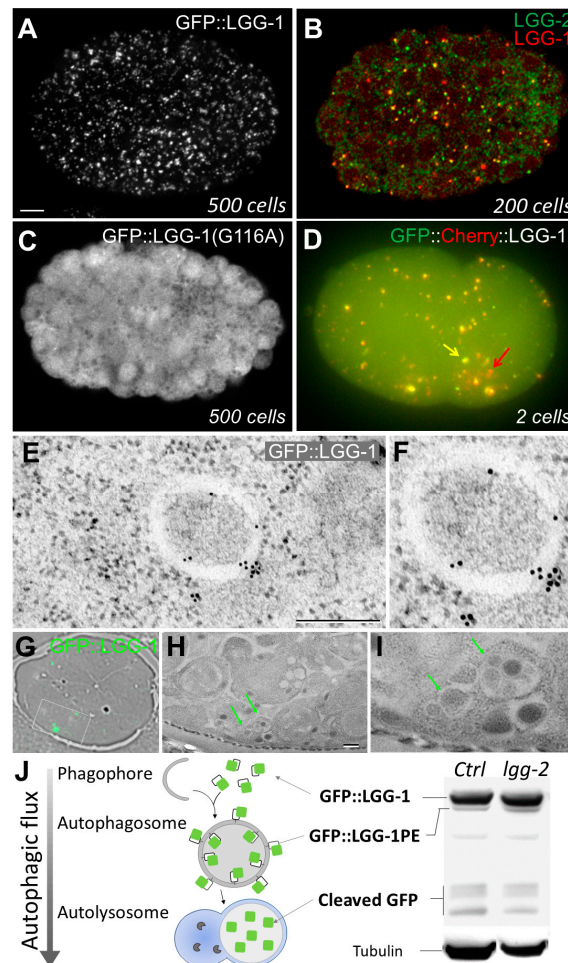


Figure 1. Use of LGG-1 and LGG-2 to monitor autophagy in *C. elegans*. (A) In vivo confocal picture of GFP::LGG-1 in a 500-cell embryo. GFP::LGG-1 puncta correspond to autophagosomes; (B) Merge confocal picture of a co-immunostaining of LGG-1 and LGG-2 in 200-cell embryo. Three types of puncta can be distinguished: LGG-1 positive (red), LGG-2 positive (green), and double positive (yellow); (C) In vivo confocal picture of GFP::LGG-1(G116A) form in a 500-cell embryo. This non-lipidated form presents a diffuse localization pattern with no puncta; (D) Epifluorescence merge picture of the tandem GFP::mCherry::LGG-1 in a two-cell embryo. The yellow arrow indicates an autophagosome (yellow resulting from GFP and mCherry fluorescences), whereas the red arrow shows an autolysosome (only mCherry fluorescence in acidic compartment); (E,F) Electron micrographs of GFP::LGG-1 embryos incubated with antibodies coupled to gold beads, revealing autophagosomal structures; (G–I) Correlative light and electron microscopy (CLEM) analysis of GFP::LGG-1; (G) Merge between bright field and fluorescence images of an ultrathin section of an embryo; (H,I) Electron micrographs of the boxed region in G. Green arrows indicate GFP::LGG-1 positive autophagosomes; (J) Schematic representation of autophagic flux and Western blot analysis of GFP::LGG-1 using GFP antibodies. The cleaved GFP correspond to a product of degradation in autolysosomes. The relative quantity of GFP::LGG-1 and the phosphatidylethanolamine (PE) conjugated form allows the measurement of the autophagic flux. Tubulin is used for normalization. Scale bar: 5 μm (A); 2 μm (E); 1 μm (H).

2.1.2. Immunostaining of Autophagy Proteins

Additionally to fluorescent reporters, antibodies against several autophagy proteins have been produced and used in *C. elegans* (Table 2). Immunostaining of the endogenous LGG-1 and LGG-2 proteins revealed dots that are similar to the GFP::LGG-1 dots in transgenic worms [6,24]. While GFP::LGG-1/LGG-2 reporters are useful for observations of live animals, immunostaining is essential to reflect the endogenous protein localization. Anti-LGG-1 and LGG-2 antibodies confirmed that the basal level of autophagy is not constant during embryonic development, with a maximum puncta between 100-cell and 200-cell stages [4]. In different autophagy mutant contexts, the pattern of LGG-1 is drastically modified. For instance, in *atg-3* and *atg-7* mutants, both required for LGG-1 conjugation to PE, no LGG-1 puncta are detected [9]. In *atg-2*, *atg-18* and *epg* mutants, the progression of the autophagy flux is impaired, resulting in the accumulation of LGG-1 puncta [6,9]. Co-localization approaches using anti-LGG-1 and anti-LGG-2 antibodies allowed researchers to highlight the existence of three distinct populations of autophagosomes: LGG-1 only, LGG-2 only, or double positive (Figure 1B) [25]. The co-localization approaches between LGG-1/LGG-2 and other proteins, for instance the autophagy substrate SEPA-1, are easy to perform by immunostaining in the embryo, but this approach is more complicated in larva or adult, especially for some tissues. Because anti-LGG-1 and anti-LGG-2 antibodies are limited resources and not commercially available, some studies used anti-GFP and anti-mCherry antibodies in reporter-expressing strains to perform co-localization analyses.

Table 2. List of autophagic reporters and targets of antibodies in *C. elegans*.

<i>C. elegans</i> Protein	Tools	Localization Pattern	References
LGG-1	GFP, DsRed, GFP::Cherry, Cherry, mRFP; antibody	Puncta	[9,25,28,31,40,48]
LGG-2	GFP::LGG-1(G116A) GFP; antibody	Diffuse Puncta	[25] [24]
BEC-1	GFP::LGG-2(G130A)	Diffuse	[23]
ATG-4.1	GFP, mRFP	Puncta/Patch	[28,38]
ATG-9	GFP	Diffuse	[2]
ATG-18	GFP	Diffuse	[6]
EPG-1	GFP	Puncta	[47]
EPG-2	GFP	Diffuse	[12]
EPG-3	Antibody	Puncta	[9]
EPG-4	GFP	Diffuse	[9]
EPG-5	GFP	Diffuse	[9]
EPG-6	GFP	Diffuse	[6]
EPG-7	GFP	Puncta	[13]
EPG-8	GFP	Diffuse	[14]
LMP-1	GFP	Puncta	[11]
SEPA-1	GFP, RFP; antibody	Puncta/Patch	[7,12]
SQST-1	GFP	Puncta/Patch	[9]
PGL-1	GFP; antibody	Puncta/Patch	[7]
PGL-3	Antibody	Puncta/Patch	[7]

2.1.3. Electron Microscopy

At the end of 1950s, electron microscopy (EM) allowed researchers to observe double-membrane vesicles, which were first coined as “autophagosomes”, by C. de Duve in 1963 [49,50]. The three main steps of the autophagy flux were described first on a morphologic basis: phagophore, autophagosome, and autolysosome. EM is still considered to be one of the most reliable approaches in the study of autophagy, as it allows the anatomic observation of the different autophagic structures without labelling. It is also a very unique method for analyzing many detailed aspects, from the sub-cellular

compartments to the tissue organization when autophagy is impaired. EM approaches have been applied and optimized for *C. elegans* [51]. When *C. elegans* is grown in starvation conditions, it has been observed through EM that the relative volume of autophagic structures increases 10-fold [34]. Autophagic structures have also been shown by EM analysis to accumulate under oxidative stress, and in mutants such as *glp-1* and *daf-2* [17,31]. In the *epg-3* or *epg-4* mutants, an abnormal accumulation of phagophore is observed, which supports the idea of a role for these proteins in the early steps of autophagosome formation [9]. Therefore, the anatomic analysis of autophagic structures by EM, combined with a quantitative analysis, allows for a good characterization of the autophagy flux. Moreover, combining EM with gold immunostaining can create a more specific analysis of various autophagy proteins or processes. Indeed, the presence of LGG-1 and LGG-2 at the membranes of phagophores and autophagosomes were confirmed with this technique (Figure 1E). As an alternative approach to immunogold labelling, correlative light and electron microscopy (CLEM) allows researchers to study the distribution of an autophagy protein when antibodies are not available. The principle is to combine EM with the observation of the fluorescence of a reporter by using fixation conditions and embedding resin that preserves the fluorescence. Such a technic has been efficiently used for GFP::LGG-1 and GFP::LGG-2, allowing researchers to correlate fluorescent puncta with vesicular structures (Figure 1G) [25].

EM-based approaches present the advantages of being anatomical, very resolutive, and independent of any markers, so they are often essential to further confirm light microscopy observations. Although EM approaches are very informative, they can only be performed on fixed tissue, which can generate artefacts; they are time and resources consuming; and they require a high level of expertise. Moreover, rare events could be very difficult to identify, and 3D reconstitutions are complicated to perform in routine. Therefore, EM and light microscopy analysis are very complementary approaches.

2.2. Molecular Approaches

Autophagy is a very dynamic process whose regulation relies on multiple parameters. Although imaging autophagy reveals insights about important features of the process, it is not sufficient to address all aspects. In order to monitor the level of autophagy and/or the completion of the different steps of the autophagic process, different molecular tools are available that enable the quantitative analysis of protein modifications.

2.2.1. Monitoring Autophagic Flux by Western Blotting

LGG-1 is synthesized as a precursor protein and immediately cleaved at position 116 by the protease ATG-4.1 to generate LGG-1-I, a protein diffuse in the cytosol with a C-terminal exposed glycine residue. By a conjugation mechanism involving several ATG proteins, LGG-1-I is lipidated to PE, thereafter called LGG-1-II, and associated at the membrane of the phagophore and autophagosome [25]. Due to their differences in molecular weight and lipid modification status, LGG-1 forms can be separated on a SDS-page gel, with LGG-1-II migrating slightly faster than LGG-1-I, and revealed by Western blotting (WB). The relative quantity of the LGG-1-II form compared with LGG-1-I can generally be correlated with the number of autophagosomes. Measuring the ratio of LGG-1 conjugated to autophagosomes is a way to quantify autophagy activity in different contexts [4,52]. For example, in embryos, the LGG-1 precursor is not visible, and LGG-1-II is in small minority compared with LGG-1-I. When autophagy flux is blocked, either during autophagosome formation or maturation (*epg-3*, *epg-5*), both LGG-1-I and LGG-1-II levels increase. In contrast, when LGG-1 cleavage is impaired (*atg-4.1* mutant), a significant accumulation of LGG-1 precursor is observed, and LGG-1-I becomes undetectable. When conjugation is blocked (*atg-3*), LGG-1-I is accumulated and the lipidated form is not present. More generally, an increased level of lipidated LGG-1-II reflects an increase in the number of autophagic structures, which can result from both an increase of the autophagic flux or a blockage of autophagosomal maturation. The usage of lysosomal inhibitors can help distinguish between those

cases [52]. An alternative approach consists of using GFP::LGG-1-expressing strains. Within these constructs, WB using an anti-GFP allows the identification of the lipidated and non-lipidated forms, and an additional lower band that corresponds to the cleaved GFP (Figure 1J). GFP::LGG-2 can also be used for monitoring autophagy through WB, although it has been less extensively characterized and used. When autophagy is functional, a major band for GFP::LGG-2 is observed along with two minor bands, one slightly higher and one slightly lower, which could correspond to post-translational modifications. The lipidated form has not been strictly identified yet with the non-lipidated form GFP::LGG-2(G130A); only the major band is observed [23]. Similarly to LGG-1, a cleaved GFP appears upon formation of the autolysosome, and is very convenient for measuring the autophagic flux. For example, in a context where the formation of the autolysosome is impaired, the amount of the cleaved GFP is decreased, while GFP::LGG-1-I and GFP::LGG-1-II forms are increased.

If WB has been predominantly performed on LGG-1 and LGG-2, the quantification of known cargoes and receptors of autophagy also has been punctually used. In particular, the amount of the autophagy cargo PGL-3 and the receptor SQST-1 have been used as indicators of autophagy activity by WB [7,53].

Western blotting approaches are useful to evaluate and quantify autophagy in different contexts. They are also relatively easy to perform, but some limitations should be mentioned. Since LGG-1 expression highly depends on the developmental stage, the population of worms or embryos needs to be synchronized, and the material must be in sufficient quantity. Additionally, the sensitivity of this technique is limited, and subtle changes of autophagic flux state might be not observed. In particular, a modification of the autophagic flux limited to a specific tissue could potentially be very difficult to detect with this technique.

2.2.2. Monitoring Autophagy Genes Expression by RT-qPCR

While the post-translational modification of several autophagy proteins is very important for autophagy, analyzing the transcriptional level could be a good indication of an autophagy induction/repression. Measuring the transcription level of autophagy genes by RT-qPCR has been an efficient way to detect the induction of autophagy. In the context of starvation or the inhibition of the LET-363/TOR signaling pathway, this technique revealed an increase of mRNA levels of several autophagy genes (*lgg-1*, *atg-18* . . .). This approach allowed the identification of new actors involved in the regulation of autophagy gene expression in *C. elegans*, and particularly the transcription factor HLH-30, ortholog of mammalian TFEB [54]. In a similar way, in the long-lived insulin receptor *daf-2* mutants, mRNA levels of some autophagy genes are increased, which supports the link between autophagic activity and lifespan expansion [55]. For this technique, the normalization of gene expression to multiple control genes is mandatory (for example *pmp-3*, *cdc-42*) [56,57]. RT-qPCR is very sensitive, and can be performed on many genes in the same experiment even with small samples, so it represents an interesting complementary tool.

2.3. Modifying Autophagy

In order to study the links between autophagy and cellular or developmental processes, it is essential to analyze the effects of a modification in the autophagic flux. Through using either genetic approaches or drug treatments, *C. elegans* allows the study of the consequences of an induction or a blockage of autophagy throughout a whole organism.

2.3.1. Genetic Approaches

Yoshinori Ohsumi identified *Atg* genes by genetic screens in *Saccharomyces cerevisiae*, for which he was awarded the 2016 Nobel Prize in Physiology or Medicine. The large majority of those genes have a single ortholog in *C. elegans*, and are involved in autophagy process (Table 1). For example, one of the first autophagy genes characterized in *C. elegans* is *bec-1* [31]. The usage of RNAi to deplete BEC-1 causes defects in dauer formation and extension of the lifespan [31]. Thanks to the international

C. elegans Gene Knockout Consortium and the Japanese National BioResource Project, numerous knocked-out mutants in autophagy genes are available. Noticeably, additional autophagy genes have been discovered by screening in *C. elegans*. Zhang et al. performed a non-lethal genetic screen to identify mutants for deficiency in the degradation of autophagy aggregates during embryonic development [6,9]. The newly identified genes involved in this autophagy process have been named *epg* (ectopic PGL granules). The *epg* genes are either distantly related to yeast *ATG* genes or have no yeast counterparts [12,14,15]. Numerous new tools such as mutants and fluorescent reporters have been generated by these studies. More recent RNAi screenings allowed the identification of signaling pathways involved in the control of autophagy. The results of the different screenings and the numerous genetic tools available have made *C. elegans* a powerful model to study the genetics behind autophagy process and its modulation. Indeed, the mutants of diverse autophagy genes involved in different steps of autophagy process, such as impairing or decreasing autophagy, are widely used. Additionally, mutants in the regulation of autophagy (*hlh-30*, *TOR*) and adaptors (*sepa*, *sqst-1*) also have been described [9,12,56,58]. The depletion of some autophagy proteins, such as BEC-1 and LGG-1, can be linked to sterility or lethality occurring during development, which can complicate their study at later stages. Nevertheless, RNAi in *C. elegans* can be performed by feeding and has been successfully used to provoke depletions in larva and adults. In addition, RNAi depletion can be performed in a tissue-specific manner, thanks to the development of a genetic tool [59]. Since *C. elegans* presents highly specialized tissues, tissue-specific autophagy genetic tools can be interesting for studying the involvement of autophagy in very specific processes. While tissue-specific knockout are not possible in classical mutants, a tissue-specific RNAi approach has been developed in *C. elegans*. It consists of using a strain impaired for RNAi processing machinery, then transfected with construct with a tissue-specific promoter, which restores the RNAi process. Intestinal-specific depletion of BEC-1 revealed that autophagy activity in the intestine is essential against *S. typhimurium* infection [33]. The main limit of the RNAi approach could be its efficiency, with some RNAi failing to reliably deplete the target protein. So, it is essential to check whether a specific RNAi has an effect on the amount of protein or mRNA. Temperature-sensitive mutants are generally a good tool to overcome lethality in *C. elegans*, but have not been yet reported for *atg* genes. Particular genetic tricks can be exploited for resolving lethality issues. For example, the *lgg-1(tm3489 maternal)* strain was obtained by the transgenesis of *Plgg-1::gfp::lgg-1*, which is expressed in somatic cells but not in germline cells. Indeed, the expression of GFP::LGG-1 starts when embryos reach 20-cell stage, rescuing the adult sterility and allowing the characterization of the phenotype of early embryos depleted for LGG-1 [23,25]. Altogether, the numerous autophagy-related genetic tools available in *C. elegans* allow an in-depth genetic analysis of pathways and epistasis.

2.3.2. Pharmacological Treatments

In mammalian cells, drugs are commonly used for modifying and also analyzing the autophagic flux. In regards to the links between pathologies (neurodegenerative diseases, cancer) and autophagy, drugs that modulate autophagy activity have a strong potential. Those molecules have various effects, and can be sorted into two main categories: autophagy activators and autophagy inhibitors. Drugs causing starvation or ER stress also increase the induction steps of autophagy, whereas some activators have an effect on later steps of the autophagic process, during the autophagosome maturation. Several inhibitors are effective for the blockage of the induction steps (class III PI3P inhibitors), while others impair the autophagosome's degradation.

In *C. elegans*, several constraints made the pharmacological approaches less preponderant for modulating autophagy. Indeed, worms present a protective cuticle that reduces the penetration and diffusion of drugs. Additionally, it has been suggested that *C. elegans* metabolism reduces the efficiency of drugs, leading to the usage of higher concentrations of drugs in this model [60]. Lastly, numerous studies on autophagy have been performed on the embryos, whose eggshell is impermeable to most molecules, which complicates pharmacological approaches. Nevertheless, several drug screenings

and pharmacological approaches have been validated in *C. elegans* for both inducing and inhibiting autophagy. A drug screen on *C. elegans* showed that fluphenazine, a potent autophagy enhancer, was efficient at reducing the proteotoxicity of ATZ (alpha-1-antitrypsin Z) in both *C. elegans* and a mouse model. In mammals, the accumulation of ATZ is linked to liver disease, as well as associated with hepatic fibrosis and hepatocellular carcinoma [48]. In regard to longevity, both resveratrol and spermidine increase the lifespan of the nematode in an autophagy-dependent way [61,62]. On the other hand, Bafilomycin A1 can be used to block the late steps of autophagy and avoid autophagosome maturation, thus delaying the degradation of autophagy substrates, and simplifying their study. It can be administered by feeding or injection; both approaches showed an effect on autophagy [63,64]. The inactivation of autophagy using inhibitors of type III PI3K, Wortmannin, and 3-methyladenin, causes an hypoxic lethality in the worms [21].

Alternatively, exposure to stress such as starvation or heat-stress are also an option that can be exploited to induce autophagy. Starvation strongly increases the volume of autophagosomes [34], and thermotolerance affects the transcription of autophagic genes, autophagosome numbers, and mitochondrial degradation [20,35,65,66]. The main limitation of using either pharmacological or physical treatments is the potential secondary effects on various other cellular processes. Additionally, autophagy induction or inhibition caused by treatments could be a secondary effect of the potential disruption of an unrelated process. In summary, this approach is powerful for performing mechanistic analyses, but should be used cautiously for interpreting physiological processes.

3. Tools to Monitor Selective Autophagy

Depending on the cargoes that are sequestered and degraded, autophagy has been qualified as bulk or selective. Selective autophagy specifically recognizes and engulfs proteins or organelles through autophagy receptors that mediate the interaction with ATG8/LC3 proteins. Autophagy adaptors could have a function during selective autophagy, but have no roles on cargo recognition, and are not degraded during the process [67]. It can be sometimes difficult to distinguish between bulk and selective autophagy because they mainly share the same autophagy machinery and can contain similar organelles, such as mitochondria or ER. This part concentrates on two selective processes: the aggrephagy and the mitophagy, for which *C. elegans* has been efficiently used for in vivo analyses.

3.1. Aggrephagy

Autophagy is an efficient mechanism for the degradation of proteins, and in particular of aggregates, through a selective process called aggrephagy. During *C. elegans* embryogenesis, several aggregate-forming proteins are selectively removed by aggrephagy [53].

3.1.1. P Granules Degradation through Aggrephagy

P granules are ribonucleoprotein aggregates synthesised in germline and transferred to offspring through oocyte. P granules were proved to have a function in germ cell determination during embryogenesis, and are restricted to germline precursor cells, while the P granules components in somatic cells are quickly degraded [7,68]. Two types of P granules components, the proteins PGL-1 and PGL-3, are selectively degraded through aggrephagy, and in autophagy mutants, P granules-like (PGL) structures accumulate until the larva stage. By a genetic screening, the SEPA-1 protein was identified as an important factor for P granules degradation. SEPA-1 binds with both PGL-3 and LGG-1 for cargo recognition in this process, and SEPA-1 itself is also degraded by autophagy [7]. Western blot analysis showed that the *sepa-1* mutant caused the accumulation of PGL granules. Immunostaining assay revealed the co-localization of SEPA-1 with both PGL granules and LGG-1 dots, which indicated that the SEPA-1 protein may be a bridge molecule in mediating P granule selective autophagy. Then, the co-immunoprecipitation confirmed the interaction of SEPA-1 with PGL granules and LGG-1. These data demonstrated that SEPA-1 is a receptor for selective autophagy in P granules degradation.

Several tools have been generated to allow the analysis of autophagy in the embryo. P granules can be analyzed by EM, immunofluorescence or Western blot on embryo extract, and transgenic worms expressing GFP::PGL-3 or GFP::PGL-1 in germline allow in vivo observation. In wild-type embryos, GFP::PGL-1 granules are detected only in germ cell precursors, while in autophagy mutant embryos (*lgg-1*, *atg-3*, *atg-4.1*, *atg-7*, *unc-51*), GFP::PGL-1 granules accumulate in the whole embryo. Using this selective autophagy process, Zhang et al. performed very powerful genetic screens that permitted the identification of the EPG proteins [6,9] (see also Section 2.3.1).

3.1.2. Poly-Q Aggregates

Due to overexpression or misfolding, some proteins form aggregates within the cell that must be then degraded. In mammalian cells, proteins containing polyglutamine (polyQ) are prone to form aggregates when the number of glutamine residues is above the normal length, which leads to a cellular toxicity. It has been reported that the formation of some polyQ aggregates can be responsible for neurodegenerative diseases [69], and that autophagy is a major pathway to degrade polyQ aggregates within the cell [70,71]. Thus, polyQ reporter proteins have been used for the study of autophagy in *C. elegans* as a substrate to reflect the autophagy activity. A series of transgenic strains have been generated that express a reporter GFP protein fused to different length polyQ tracts in various tissues [72–75]. Diffuse GFP fluorescence indicates that the polyQ constructs are not aggregated, while GFP clusters correspond to polyQ aggregates, allowing the tracing of polyQ aggregates in vivo by time-lapse microscopy. Interestingly, it has been shown that huntingtin-like polyQ aggregates can be extruded out of neuron cells, and that the extrusion is increased when autophagy is blocked [76]. Moreover, polyQ aggregates remain insoluble after worm lysis, and can be detected by WB [72]. The capacity of polyQ to form aggregates is correlated with the number of glutamines, although it is also affected by aging or stress. The inactivation of autophagy genes affects the amount of polyQ aggregates and increases their toxicity [77,78]. The polyQ aggregates can also be directly detected and quantified by electron microscopy. In *C. elegans*, it has been shown that polyQ aggregates formed during aging were associated with ubiquitination. In mammalian cells, p62 functions as an autophagy receptor for recognizing ubiquitinated protein aggregates, and SQST-1, the homologue of p62 in *C. elegans*, mediates autophagy during embryogenesis [9]. Similarly to p62 in mammalian cells, SQST-1 aggregates also accumulate in autophagy deficiency contexts in both embryo and larva stages. However, whether SQST-1 is involved in the degradation of polyQ aggregates has not been studied in *C. elegans*.

3.2. Selective Degradation of Mitochondria by Autophagy

Mitophagy is one of the most studied of the selective autophagy processes. Studies in yeast and mammalian cells have revealed that mitophagy is an important mechanism for mitochondrial quality control, and its impairment has been linked with pathologies such as cancers as well as neurodegenerative and mitochondrial diseases. In *C. elegans*, the study of mitophagy was mainly focused on its role in eliminating paternal mitochondria [19,24]. More recently, some studies have started to exploit the advantages of *C. elegans* to explore the roles of mitophagy in the context of aging or other stress conditions [65,66]. Since mitophagy and bulk autophagy share a common machinery, the tools described previously (see Section 2) are useful to study mitophagy in *C. elegans*. However, it is becoming crucial to develop a series of methods that are able to specifically discriminate mitophagy from bulk autophagy. We briefly describe here the recent tools that allow the analysis of the mitophagy flux.

3.2.1. Mitophagy of Paternal Mitochondria

In most metazoans, mitochondrial DNA (mtDNA) is maternally inherited [79], and paternal mitochondria are eliminated by diverse mechanisms that have been studied in several model animals. These degradative mechanisms can occur before or after fertilization, and result in the clearance of the whole paternal mitochondria, including its mtDNA. The presence of autophagy markers around

sperm material in the fertilized oocyte suggested that autophagy may be involved in this process. Indeed, studies in *C. elegans* have shown that upon fertilization, the paternal mitochondria and other sperm specific membranous organelles (MOs) are selectively removed by autophagy during a process called allophagy [19,24]. In vivo imaging and immunofluorescence are the most widely used approaches to analyze allophagy. The main substrates of allophagy are paternal mitochondria and MOs, which can be labelled by the antibodies 1CB and SP56 [80,81]. In the absence of a specific antibody for paternal mitochondria, sperm mitochondria are labelled with a mitochondrial-targeted GFP or through using a dye. For instance, applying mitoTracker or tetramethylrhodamine, ethyl ester (TMRE) to males before mating allows researchers to stain and trace paternal mitochondria in the embryo [19,24]. Tracing mtDNA has been also used for monitoring paternal mitophagy through the labelling of males with the nucleic acid dye SYTO11 [82]. Alternatively, a specific genetic strain can be used to detect paternal mtDNA by PCR. In this particular heteroplasmic strain, a part of mtDNA harbors the uaDf5 deletion that is easily discriminated from wild-type mtDNA [83]. Sperm mitochondria carrying the uaDf5 mtDNA are normally quickly degraded upon fertilization, but persist in the progeny when autophagy is blocked in the oocyte [19,24]. Autophagosomes are analyzed with tools described above (see Section 2.1) using LGG-1 and LGG-2 antibodies or fluorescent reporters as well as EM. In early embryos, the presence of double-membrane vesicles engulfing paternal mitochondria was revealed by EM analysis, further demonstrating mitophagy [24]. Immunostaining analyses demonstrated the co-localization of paternal mitochondria and MOs with LGG-1 and LGG-2 soon after fertilization until 8-cell stage. In *unc-51*, *atg-5*, *atg-7*, and *atg-18* autophagy mutants, the number of LGG-1/2 dots strongly decreased and paternal mitochondria persisted. A recent study has identified the prohibitin PHB-2 as a novel mitophagy receptor involved in the degradation of paternal mitochondria in *C. elegans* [84]. Interestingly, analysis of allophagy revealed that despite the formation of LGG-1 and LGG-2 double-positive autophagosomes, LGG-1 and LGG-2 participate differently in the process. Time-lapse assay with a tandem fusion protein GFP::mCherry::LGG-1, EM, and Western blot experiments were used to characterize the autophagic flux. In the *lgg-1* null mutant, the formation of autophagosomes is blocked, whereas the *lgg-2* null mutant only delays the formation of autolysosomes during allophagy. Further studies using a yeast two-hybrid screen and co-localization in vivo revealed that LGG-2 interacts with the HOPS protein VPS-39 to facilitate the fusion between autophagosomes and lysosomes [25].

Allophagy is essential for the elimination of sperm materials, but it is probable that other degradative mechanisms involving proteasome or other proteins could also be involved in this process [85]. A recent study reported that the mitochondrial endonuclease G, encoded by the gene *cps-6*, is involved in the breakdown and aggregation of sperm mtDNA within the mitochondrial matrix after fertilization, but before autophagosome engulfment. The mutant of paternal *cps-6* slows down the internal breakdown of paternal mitochondria, and in turns leads to a delay of paternal mitochondria degradation [82].

3.2.2. Mitophagy in Stress Conditions

In normal conditions, basal mitophagy in *C. elegans* is kept at a low level. Many stresses can cause mitochondrial damages, and the dysfunction of mitochondria could result in cell toxicity linked to the increase of ROS level or the triggering of the apoptotic cascade. However, if a severe mitochondrial stress leads to mitochondrial dysfunctions and cellular toxicity, a mild mitochondrial stress could activate a beneficial adaptive response and extend lifespan in *C. elegans* [35,86]. The induction of mitophagy is essential to remove dysfunctional mitochondria, maintain cellular homeostasis, and play a protective role in stress conditions. As a model animal, *C. elegans* has been used to analyze the effect of various mitochondrial stresses, paving the way to study the regulation and the roles of mitophagy on stress tolerance. This part briefly presents the tools that are used to monitor and trigger mitophagy.

The original method used to identify mitophagy was EM, which has been widely used in yeast, mammalian cells, and *C. elegans*. The anatomy of mitochondria is very characteristic, and EM analysis

is a powerful approach for the identification of phagophores and autophagosomes that are sequestering fragmented mitochondria [87]. However, EM has not been extensively used in *C. elegans* for characterizing mitophagy in stress conditions, probably because it is time-consuming and costly. As a result, in vivo approaches that are easy to carry out in *C. elegans* and enable the analysis of numerous samples have been preferred, such as monitoring the co-localization of fragmented mitochondria with autophagosomes marked by fluorescence (Figure 2). Thanks to the genetic tools developed in *C. elegans*, researchers have generated transgenic worms expressing fluorescence proteins for observation in living animals. For instance, transgenic worms expressing mitochondria-targeted GFP and DsRed::LGG-1 have been used to detect the co-localization of mitochondria and autophagosomes indicating mitophagy events (Figure 2A) [66]. However, the overexpression of DsRed::LGG-1 may form aggregates, and this transgenic strain should be used carefully and with rigorous controls. Alternatively, the tandem fluorescence protein mitoRosella has been developed to monitor in vivo the mitophagic flux (Figure 2B). During the last step of autophagy, the autophagosomes fuse with lysosomes, which allows for the formation of an acidic compartment, the autolysosome, favorable for the degradation of cargoes. MitoRosella is a biosensor containing a fast maturing pH-insensitive DsRed and a pH-sensitive GFP addressed to the mitochondria. Thus, measuring the ratio between GFP and DsRed fluorescences allows researchers to monitor the fusion of mitochondria-containing autophagosomes with lysosomes [66]. Although mitoRosella has been efficiently developed for monitoring the mitophagic flux in the body wall muscle cells (Figure 2C), its heterogeneous level of expression between individual muscle cells may affect the morphology and the homeostasis of the mitochondrial network.

Upon stress conditions, mitophagy is generally considered to be a degradative process that enables researchers to recognize and selectively remove fragmented and dysfunctional mitochondria. When mitophagy is deficient, damaged mitochondria accumulate in the cells, which results in major consequences on the homeostasis of the cell. So, it can be useful to measure the reactive oxygen species (ROS), ATP, and cytoplasmic Ca^{2+} levels, as well as the oxygen consumption, which are all indicators of the mitochondrial status and could reveal an induction or a defect of mitophagy. Similarly, the analysis of the mitochondrial morphology is informative because mitophagy is often accompanied by mitochondrial fission [88,89]. Transgenic worms expressing mitoGFP or GFP fused with mitochondrial protein such as DCT-1 or a fragment of the yeast TOM70 have been efficiently used to detect mitochondrial morphology in specific tissues [66,90]. During mitophagy in *C. elegans*, the fragmentation of mitochondria can be visualized using $P_{myo-3}::mtGFP$ worms in body wall muscle cells, or MitoTracker in numerous tissues. The quantification of mitochondrial fluorescence is also informative, because a reduced amount of mitochondria is one of the features of active mitophagy [66,91].

Since the basal level of mitophagy is very low in *C. elegans*, the use of mitochondrial stress often has been a necessity to better understand the mechanism and physiological function of mitophagy. Drugs that cause the loss of mitochondrial potential (CCCP) or produce oxidative stress in mitochondria (paraquat) are also powerful mitophagy inducers in *C. elegans* [66]. Recent studies revealed that urolithin A, a natural compound belonging to ellagitannins, could induce mitophagy and autophagy in both mammalian cells and *C. elegans*, and prolong the nematode lifespan [91]. Heat stress has been efficiently used to induce mitophagy in *C. elegans*, but other types of autophagy have not been excluded. Finally, the facility to carry out genetic approaches in *C. elegans* could be a powerful means of inducing mitophagy. For example, in *C. elegans*, a partial depletion of frataxin, a main protein of the Fe-S-cluster-containing complex, causes an iron-depletion stress in mitochondria that induces mitophagy [65]. Alternatively, the blockage of mitophagy can be achieved by knocking down either the autophagy general machinery or mitophagy-specific genes. For instance, the mutated form of BEC-1, which belongs to nucleation PI3K complex, blocked mitophagy in *C. elegans* [66], while other studies in HeLa cells indicated that BECN1 is dispensable for CCCP-induced mitophagy [92]. Recent studies confirmed that the homologs of Parkin and PINK1 (PDR-1/PINK-1), two well-studied proteins critical and specific for mitophagy in mammalian cells, are also involved in mitophagy in *C. elegans* [66,93].

The deficiency of *pdr-1/pink-1* specifically blocked CCCP, paraquat, or heat stress-induced mitophagy in *C. elegans*, and also reduced the lifespan of long-lived animals. Recently, DCT-1 protein, which contains a classic LC3-interacting region (LIR) motif and can interact with LGG-1, has been identified as a receptor for mitophagy in *C. elegans* [66].

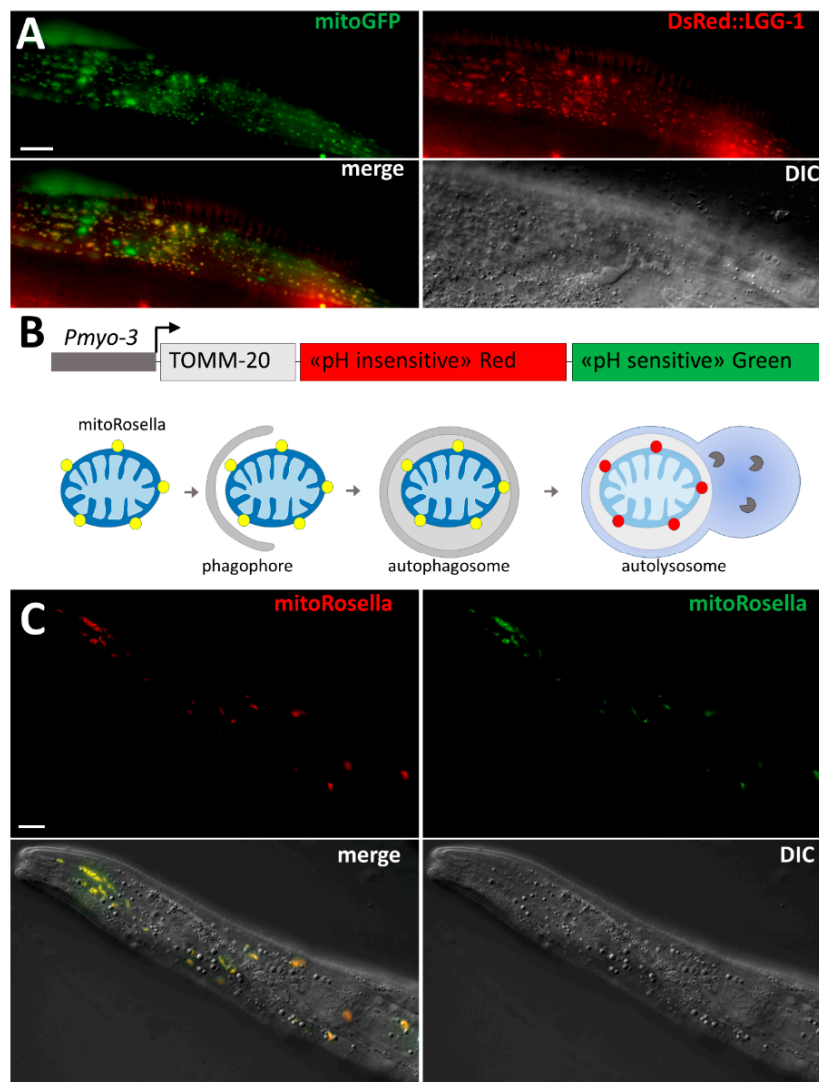


Figure 2. Tools for the study of mitophagy in *C. elegans*. (A) In vivo confocal images of mitophagy in the body wall muscle cells of an adult worm. After heat stress (37 °C for 2 h), mitophagy is visualized by co-localization between fragmented mitochondria (mitoGFP in green) and autophagic structures (DsRed::LGG-1); (B) Schematic diagram of mitoRosella fluorescent protein biosensor under the control of the muscle-specific promoter *myo-3*. MitoRosella is a chimeric protein-containing fragment of TOMM20, which leads to mitochondrial localization, the pH-stable DsRed fluorescent protein, and the pH-sensitive GFP. During mitophagic flux, mitochondria turn from yellow (DsRed and GFP) to red in autolysosome, due to the quenching of the GFP signal; (C) Confocal images of mitoRosella in body wall muscle cells of an adult worm in standard condition (20 °C). All mitochondria are fluorescent for GFP and DsRed, indicating that the basal level of mitophagy in muscle is very low. Scale bar = 10 µm.

4. Conclusions

Although this review mainly focuses on macroautophagy, other autophagy pathways, such as chaperone-mediated autophagy and microautophagy, should not be ignored. However,

their implications in the physiology of *C. elegans*, has not been yet analyzed. Since macroautophagy is a complex and dynamic process, each method for studying autophagy presents some limitations. In fact, the combination of different approaches is essential when analyzing autophagy to minimize the drawbacks. In regards to recent publications, it is clear that new methods for monitoring autophagy will be rapidly developed to improve and optimize the currently available assays.

If autophagy is a key mediator for cell metabolism, it is now clear that it is acting together with the other degradative mechanisms, and particularly the ubiquitin-proteasome system. As mentioned previously, proteasome may contribute to paternal organelles degradation together with autophagy. Moreover, a recent study identified a complementary way to analyze autophagy for the elimination of protein aggregates and organelles [67]. Under stress conditions, adult *C. elegans* neurons could extrude vesicles, called exophers, which contain protein aggregates and mitochondria [76]. Both mitophagy and exopher-genesis are involved in proteostasis and mitochondria quality control, and may contribute to relieve neurodegeneration.

Acknowledgments: The authors would like to thank their colleagues for helpful discussion and particularly Céline Jenzer and Céline Largeau for providing pictures. We are grateful to Nektarios Tavernarakis for sending the mitoRosella and the DsRed::LGG-1; mtGFP strains. We are also grateful to the *C. elegans* autophagy community for sharing informations and reagents and apologize if some data could not be mentioned in this review due to size limitations. The Legouis' group is supported by the Agence National de la Recherche (project EAT, ANR-12-BSV2-018) and the Association pour la Recherche contre le Cancer (SFI20111203826). Y.C. is a recipient of a fellowship from the China Scholarship Council and VS received a fellowship from of the Ligue Nationale contre le Cancer.

Conflicts of Interest: The authors declare no conflict of interest.

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FRENCH SUMMARY

Mécanismes et fonction de la mitophagie dans l'adaptation au stress thermique pendant le développement de *C. elegans*

Mots clés : stress thermique, mitochondries, autophagique, mitophagie, *Caenorhabditis elegans*

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1. Comment *C. elegans* réagit-il au stress thermique aigu?

C. elegans, un animal modèle largement utilisé pour l'étude de l'autophagie, les vers multiplient les stress au cours de leur cycle de vie. Les vers ont également développé de nombreux mécanismes pour ajuster et surmonter les conditions de stress. Par exemple, le stress de famine déclenche la formation de dauer à partir du stade L1; Les dauers sont plus résistants au stress de l'environnement et peuvent vivre plusieurs semaines sans nourriture. Plus important encore, les dauers peuvent devenir des larves L4 une fois le stress éliminé (Golden et Riddle 1984, Hu 2007). L'autophagie est importante pour la formation de dauer et la résistance au stress dans des conditions de famine (Melendez, Talloczy et al. 2003). En fait, non seulement en état de famine, d'autres stress, comme une température élevée (ou un stress thermique), semblent également nécessiter la fonction d'autophagie pour la résistance au stress et la survie des vers. Chez l'adulte *C. elegans*, il a été démontré que l'autophagie induite par le stress thermique pourrait être bénéfique pour la survie et la longévité des vers. Le groupe Tavernarakis a démontré que le blocage de la mitophagie lors d'un stress thermique sévère (37 ° C, 7h) compromet la survie des personnes âgées (Palikaras, Lionaki et al. 2015). Le groupe Hansen a également montré qu'un stress thermique hormétique (36 ° C, 1h) appliqué sur les jeunes adultes est bénéfique pour la longévité des vers (Kumsta et al, 2017). Cependant, quelles sont les influences du stress thermique sur la larve? Nous essayons d'introduire un stress thermique aigu qui nuirait aux vers mais ne les tuerait pas, et nous nous concentrons sur ses effets sur le développement des larves. De plus, quel est le rôle de l'autophagie dans l'ajustement du ver à un stress thermique aigu? Les résultats ci-dessus sont

principalement présentés dans le manuscrit suivant, mais j'ai également ajouté une partie supplémentaire pour les données étendues afin de mieux illustrer notre travail. Les principaux résultats de notre projet sont énumérés ici:

- **Un stress thermique aigu (aHS) induit des modifications des organites et un retard de développement.**

Nous avons remarqué qu'un stress thermique aigu (aHS) de 37 ° C sur une larve de L4, à 37 ° C pendant 1 heure, pourrait fortement empêcher le ver de devenir un adulte sans tuer de ver. Après 24 heures de récupération à 20 ° C, les vers traités avec l'aHS sont beaucoup plus petits et retardent la ponte des œufs par rapport aux vers témoins. Les images de la CID ont montré que les vers stressés étaient également plus transparents et présentaient divers degrés de défaut de développement tissulaire, par exemple la fusion bloquée de cellules de couture dans l'épiderme, la disposition désordonnée dans les lignées germinales et les grandes cavernes dans l'intestin (Figure 16). Ces défauts de développement évidents ont été observés après une récupération après 24 heures sur aHS, alors que les images de la CID suggéraient qu'il n'y avait pas de dégradation évidente des tissus des vers versés après une aHS (récupération de 1 à 3 heures). Mais les données de microscopie électronique à transmission (TEM) ont révélé que, par rapport aux vers témoins, les organites des vers stressés, récupérés à 20 ° C pendant 1 heure, avaient déjà été modifiés. Les images EM nous ont montré que la aHS tant chez les larves L4 que chez les jeunes adultes induisait l'inflation du réticulum endoplasmique et la fragmentation des mitochondries, les taches denses aux électrons dans la matrice mitochondriale indiquant un endommagement grave des mitochondries (Figure 1E-L, E'-L').

- **La fonction et la morphologie mitochondriales sont modifiées de façon transitoire lors d'une HS aiguë.**

Pour mieux visualiser la morphologie mitochondriale, nous avons effectué les marqueurs mitochondriaux in vivo: dosage du mitoGFP, du Tomm20 :: mKate2 ou de l'immunocoloration. Les deux ont prouvé que la aHS pouvait induire une fragmentation du réseau mitochondrial dans l'épiderme, le muscle et la lignée germinale (Figure 2B-E, G-J du manuscrit, quantifications en F, K). Non seulement la morphologie mitochondriale, mais aussi les fonctions mitochondriales ont été altérées par la aHS, confirmées par Seahorse et d'autres tests (Figure 2A et L manuscrites). Cependant, le potentiel de membrane mitochondriale ne semble pas être affecté par la aHS, car le MitoTracker™ Red CMXRos, qui tache les mitochondries en fonction du potentiel de la membrane mitochondriale, pourrait encore étiqueter les mitochondries après un traitement de HS (manuscrit, figures 2I et J). Plus important encore, le réseau mitochondrial fragmenté a commencé à se reconstituer après six heures de récupération, la majorité des vers stressés étant revenus au réseau tubulaire mitochondrial après 24 heures de récupération (Figure 2M-R du manuscrit).

- **L'induction d'un flux d'autophagie protège les larves du HS aigu.**

Les images EM et les images in vivo d'épifluorescences de marqueurs autophagiques ont montré une augmentation du nombre d'autophagosomes (Figure 1H-L, H'-L 'du manuscrit). Pour exclure la possibilité que l'augmentation des autophagosomes soit due au blocage de la fusion avec les lysosomes, nous avons détecté l'autophagie dans les ARNi de epg-5, dont le produit est requis pour la fusion entre autophagosomes et lysosomes (Wang, Miao et al. 2016) et ont découvert que les autophagosomes étaient encore accumulés par les ARNi d'epg-5 lors

d'un stress thermique (figures 3O et P du manuscrit, quantifications en L et Q). Ces données suggèrent que l'HS aigu induit un flux d'autophagie. Les deux homologues de la famille Atg8, LGG-1 et LGG-2, ont participé mais ont eu des rôles distingués au cours de l'autophagie induite par la HS (figure 3R-V du manuscrit). Les défauts d'autophagie n'avaient pas d'effet évident sur la survie du ver à la suite d'une aHS, alors qu'ils entraînaient un retard de développement plus important (Figure 3W et X du manuscrit). Pris ensemble, l'autophagie est un mécanisme de protection pour la récupération du ver après une HS aiguë.

- **La mitophagie favorise la reconstruction du réseau mitochondrial après une SH aiguë.**

Nous avons montré dans le manuscrit que les mitochondries sont l'une des cargaisons principales de l'autophagie induite par la aHS (figure 4B-E du manuscrit). L'autophagie fonctionnelle pour dégrader les mitochondries endommagées est importante pour la reconstruction du réseau mitochondrial après la aHS (Figure 4F-H du manuscrit, quantification en I). Cependant, l'ARNi ou la mutation des régulateurs bien connus de la mitophagie, PINK-1, PDR-1 et DCT-1, n'a eu aucun effet sur le processus d'autophagie ni sur la reconstruction mitochondriale après la SHA (Figure 4J-L manuscrite), ce qui suggère que la mitophagie au moins est partiellement indépendante de PINK-1, PDR-1 et DCT-1 (Palikaras, Lionaki et al. 2015).

- **Le DRP-1 est nécessaire à la fragmentation mitochondriale induite par la aHS.**

Il est largement admis que la fragmentation mitochondriale médiée par DRP-1 est une condition préalable à leur captage autophagique au cours de la mitophagie (Tatsuta et Langer 2008, Mao et Klionsky 2013), alors que des études démontrent également l'existence d'une fission mitochondriale indépendante du DRP-1. dans les systèmes de levure et de mammifères

(Mendl, Occhipinti et al. 2011) (Hamasaki, Furuta et al. 2013) (Yamashita, Jin et al. 2016). Chez *C. elegans*, de nombreux autres groupes ont montré que le DRP-1 est un facteur clé de la fission régulant la morphologie mitochondriale (Labrousse, Zappaterra et al. 1999), mais qu'il s'agit d'une fragmentation mitochondriale que nous avons observée lors d'une aHS dépendante également de DRP-1? Pour répondre à cette question, nous avons comparé la morphologie mitochondriale chez le mutant nul *drp-1* (Breckenridge, Kang et al. 2008) sous 20 ° C et l'aHS. Les résultats de la quantification nous ont montré que sans DRP-1, la morphologie des mitochondries était légèrement modifiée sous HS, mais que les mitochondries étaient incapables de terminer l'étape de fission (Figure 5 du manuscrit).

- **Le DRP-1 est nécessaire pour compléter l'autophagosome au cours d'une aHS.**

Bien que les régulateurs spécifiques de la mitophagie ne soient pas nécessaires pour l'autophagie / mitophagie induite par le aHS (Figure 4J-L manuscrite), une fission mitochondriale à médiation par DRP-1 est nécessaire pour la formation des autophagosomes. Dans les mutants *drp-1*, il y avait moins d'autophagosomes mais au lieu de cela, davantage de groupes autophagiques sous aHS, par rapport aux vers de type sauvage (figures 6A et B manuscrites, quantifications en C, H-J). Ces amas autophagiques ont été confirmés par plusieurs marqueurs d'autophagie, LGG-1, LGG-2 et ATG-18, indiquant une formation anormale d'autophagosomes plutôt que d'autolysosomes chez les vers mutants *drp-1*. De plus, les grappes autophages anormales dans l'appauvrissement de la *drp-1* étaient toujours en contact avec les mitochondries (Figure 6E, E' et G, G' du manuscrit). De plus, chez les vers de type sauvage, les autophagosomes étaient également souvent en contact avec les mitochondries, tant en condition basale qu'en aHS (Figure 6D et F manuscrites), ce qui suggère

que les mitochondries sont le site principal de formation des autophagosomes. Le réseau mitochondrial altéré dans les mutants drp-1 peut perturber la formation des autophagosomes et entraîner un regroupement anormal.

2. Une exploration préliminaire des candidats à la MAM chez *C. elegans*

Il a été démontré dans des cellules de mammifère que les sites de contact ER-mitochondries sont l'un des sites de biogenèse de l'autophagosome. Atg5, Atg14 et d'autres marqueurs précoces de l'autophagie sont présents sur les sites de contact ER-mitochondries pour l'assemblage de l'autophagosome (Hailey, Rambold et al. 2010, Hamasaki, Furuta et al. 2013). Chez la levure, cependant, il est considéré que les sites de contact ER-mitochondries sont nécessaires pour un processus spécifique de la mitophagie plutôt que pour l'autophagie générale (Bockler et Westermann 2014). Il est intéressant de noter que dans des conditions de contrôle ou induites par le stress, nous pourrions toujours détecter les autophagosomes à proximité des mitochondries. Il est intéressant de noter que la récente étude de Vincent SCARCELLI dans notre laboratoire a révélé que, dans l'embryon, les autophagosomes se localisent également près de l'ER (données non publiées). Tous ces résultats indiquent que des autophagosomes pourraient se former aux sites de contact ER-mitochondries chez *C. elegans*. En outre, une étude récente sur *C. elegans* a montré que la suppression de l'epg-3 bloque la libération de phagophore à partir de l'ER et, ultérieurement, de la maturation des autophagosomes (Zhao, Chen et al. 2017). À notre connaissance, il n'y a pas encore d'étude sur le rôle des sites de contact ER-mitochondries dans le processus d'autophagie chez *C. elegans*.

Comme le DRP-1 est recruté sur les sites de contact ER-mitochondries pour fission (voir Introduction 4.2 et 4.4.2), nous émettons l'hypothèse que les groupes autophagiques induits par l'ARN drp-1 ou une mutation nulle sont liés aux sites de contact ER-mitochondries, et l'appauvrissement de drp-1 peut entraîner une interaction anormale entre ER et les mitochondries, ce qui pourrait perturber davantage la biogenèse de l'autophagosome. Pour tester cette hypothèse, nous avons essayé d'interrompre les sites de contact ER-mitochondries par les candidats candidats à l'ARNi de MAM (membranes associées aux mitochondries) chez *C. elegans*, puis nous avons détecté leurs rôles dans la fission mitochondriale et l'autophagie induite par le stress thermique. Les résultats sont les suivants:

- **Les protéines potentielles de MAM chez *C. elegans*.**

Tout d'abord, sur la base des protéines MAM rapportées dans le système mammalien (van Vliet, Verfaillie et al. 2014, Lopez-Crisosto, Bravo-Sagua et al. 2015), par le test BLAST, nous avons répertorié les homologues de MAM dans *C. elegans* (Tableau 7). Ces candidats MAM, ainsi que la protéine MAM identifiée chez *C. elegans*, ARF-1.2 (Ackema, Hench et al. 2014), n'étaient pas tous impliqués dans la régulation de la morphologie de la mitochondrie. Nous avons constaté que seuls les ARNi contre arf-1.2, vdac-1 et miro-1 pouvaient altérer le réseau mitochondrial, suggérant que leurs produits codés sont les protéines potentielles de MAM.

- **L'effet de l'ARNi sur les candidats à la MAM dans les processus d'autophagie induite par le stress thermique de base et thermique.**

La question de savoir si les sites de contact ER-mitochondries sont nécessaires à la formation des autophagosomes a ensuite été testée à l'aide de l'ARNi des candidats au MAM, répertoriés dans le tableau 6. Malheureusement, aucun des ARNi ne présentait une

autophagie altérée de manière significative, tant dans les conditions de stress basal que thermique. Plus important encore, les groupes autophages dans l'ARNi drp-1 ou les vers mutants n'avaient jamais été découverts lorsque ces candidats à la MAM ont été renversés. Cela peut nous donner une idée du fait que le défaut d'autophagie induit par l'épuisement de drp-1 n'est pas dû au dérangement ER et à l'attachement des mitochondries.

- **L'autophagie / mitophagie est modifiée par l'ARNi de fzo-1.**

FZO-1 est l'homologue de Mfn2, un mammifère, qui joue un rôle clé dans l'attachement des récepteurs ER aux mitochondries au site de contact (de Brito et Scorrano 2008, Filadi, Greotti et al. 2015, Naon, Zaninello et al. 2016). L'ARNi de fzo-1, ainsi que d'autres candidats au MAM, n'a pas pu altérer la formation d'autophagosomes induite par le stress thermique. Cependant, dans des conditions basales (20 ° C), les vers fzo-1 ARNi présentaient plus de GFP :: LGG-puncta, qui étaient souvent colocalisés avec la mitochondrie de l'épiderme. L'hypothèse est que l'ARNi fzo-1 induit des mitochondries plus fragmentées, puis déclenche la dégradation de la mitophagie.

- **Nouveaux outils pour étudier les sites de contact ER-mitochondries.**

Finalement, nous avons essayé de développer de nouvelles approches pour détecter directement les sites de contact ER-mitochondries. Nous avons d'abord essayé de détecter les sites de contact ER-mitochondries par microscopie confocale. Pour ce faire, une protéine ER RET-1 a été marquée avec GFP et les mitochondries ont été colorées avec mitoTracker CMXRos. Cette approche a permis l'observation in vivo, mais les réseaux ER et mitochondriaux étaient très denses, ce qui a rendu difficile la détection des zones de contact. La deuxième

tentative consistait à construire une souche exprimant HRP-KDEL par approche EM (Schikorski, Young et al. 2007, Giordano, Saheki et al. 2013).

En résumé, nous avons exploré ici le rôle des protéines MAM dans notre autophagie / mitophagie induite par l'aHS, et ces données suggèrent que ces candidats MAM ne sont pas nécessaires pour la formation d'autophagosomes dans l'autophagie / mitophagie induite par le stress thermique. Afin d'étudier plus avant les protéines MAM ou les sites de contact ER-mitochondries chez *C. elegans*, nous avons développé la première souche HRP-KDEL chez *C. elegans* afin de détecter les sites de contact sous EM. Nous espérons que ce nouvel outil nous aidera à approfondir notre étude des relations entre les sites de contact des ER-mitochondries, la formation d'autophagosomes et la fission des mitochondries chez *C. elegans*.

3. Ether lipidique et autophagie (projet collaboratif)

Les phospholipides sont les composants principaux des membranes cellulaires et la plupart des phospholipides sont constitués des acides gras hydrophobes attachés à un groupe de tête hydrophile de phosphoglycérol par une liaison ester. Cependant, il existe un type de phospholipides dans lequel les alcools gras à longue chaîne sont reliés au phosphoglycérol par une liaison éther (Fahy, Subramaniam et al., 2005). Ce type de phospholipides est appelé éther lipides, constituant environ 20% du pool total de phospholipides chez les mammifères (Dean et Lodhi 2018). Les éthers lipidiques sont importants pour le maintien des propriétés physiques de la membrane, notamment la fluidité de la membrane, la promotion de la fusion membranaire et la contribution aux microdomaines lipidiques (Wallner et Schmitz, 2011). En outre, les lipides éthérés sont nécessaires au bon fonctionnement des protéines

membranaires intégrales, et le défaut de ces lipides est associé à de nombreuses maladies humaines (Braverman et Moser 2012).

Au cours de la synthèse des lipides d'éther, qui commence dans les peroxysomes et se termine dans ER (Figure 26A), trois gènes, *acl-7*, *ads-1* et *fard-1*, ont été identifiés comme essentiels chez *C. elegans*. La perte de fonction de l'un ou l'autre de ces gènes montre une diminution remarquable du taux de DMA 18:0 et un taux élevé de graisse saturée, ce qui suggère l'absence de capacité de biosynthèse des lipides d'éther. Les protéines codées de *acl-7*, *ads-1* et *fard-1* sont toutes requises dans l'étape initiale de la synthèse d'éther-lipide chez les peroxysomes, et leurs homologues correspondants chez les mammifères ont les mêmes fonctions que chez *C. elegans* (voir Figure 26A). . Alors que la déficience en éther lipides chez la souris était différente de la forte infertilité et d'autres troubles graves du développement, *C. elegans* est capable de se développer de la même manière que le type sauvage dans des conditions normales, malgré une durée de vie raccourcie et une taille de couvée réduite (Shi, Tarazona et al. 2016). Cet avantage fait de *C. elegans* un bon outil alternatif pour explorer le rôle des lipides d'éther dans le métabolisme cellulaire, en particulier dans des conditions de stress.

L'autophagie fournit une voie de dégradation pour la régulation du métabolisme lipidique, appelée lipophagie. D'autre part, les lipides pourraient en sens inverse réguler l'activité de l'autophagie, mais le mécanisme est complexe (Jaishy et Abel 2016). Comme nous avons déjà mis en place un bon outil pour induire l'autophagie par le stress thermique aigu, dans le cadre de la collaboration avec une équipe travaillant en autophagie à Dijon, nous détectons ensuite si le déficit en lipides d'éther modifie les réponses autophagie chez *C. elegans*. Nos résultats

indiquent que la biogénèse altérée des lipides d'éther a causé un défaut d'autophagie et une réduction de la survie du ver en état de famine.

III Discussion et perspectives

1. Le rôle de l'autophagie lors du stress thermique

1.1 Etudes de stress thermique

Le stress thermique est un stress courant chez les organismes, provenant de bactéries, de plantes et de mammifères. Lorsque les organismes sont exposés à un changement environnemental où la température est élevée, leurs métabolismes cellulaires changent rapidement pour s'adapter au stress thermique. Par exemple, le niveau d'expression des protéines de choc thermique (HSPs) est élevé pendant le choc thermique, afin d'éviter la formation d'agrégats de protéines et de maintenir la protéostase.

C. elegans est un animal modèle puissant qui explore les effets du stress thermique sur le développement et la longévité. *C. elegans* peut croître et se reproduire à des températures comprises entre 12 ° C et 26 ° C. Cependant, le taux de croissance est plus de 2 fois supérieur à 25 ° C par rapport à 16 ° C (Hirsh, Oppenheim et al. 1976). Une longue exposition à une température supérieure à 25 ° C provoque une stérilité qui constitue un obstacle à un entretien important (journal de travail), mais une exposition à température élevée pendant une courte période n'est pas toujours préjudiciable pour *C. elegans*. On sait que les effets du stress thermique varient en raison des différences de température, de durée et de stade des vers (Zevian et Yanowitz 2014). Il a été surprenant de découvrir que

les vers sont sensibles à un léger changement de température et qu'une augmentation de 26,0 ° C à 26,2 ° C pourrait être perçue par les vers pour ajuster leurs comportements (Dawe, Smith et al. 2006). Pour cette raison, le traitement de stress thermique doit être soigneusement choisi en fonction du but des expériences.

Ces dernières années, le stress thermique a été appliqué comme un simple outil d'induction de l'autophagie chez *C. elegans*, mais la méthodologie utilisée pour réaliser le stress thermique utilisé dans différentes études conduit à des effets très distincts. Dans notre étude, nous avons mis en place un stress thermique à 37 ° C pendant 1 heure sur les larves de L4. Nous qualifions le traitement de stress thermique aigu (aHS pour faire court) capable de déclencher l'autophagie mais sans létalité des vers. Dans la bibliographie, nous avons également noté d'autres traitements de stress thermique appliqués par d'autres groupes dans les études d'autophagie chez *C. elegans*. Par exemple, le groupe Tavernerakis a été le premier à révéler que le stress thermique pouvait induire la mitophagie (Palikaras, Lionaki et al. 2015), tandis que le groupe Hansen appliquait un stress thermique hormétique pour induire l'autophagie chez *C. elegans* (Kumsta, Chang et al. 2017).

La comparaison des trois études a montré que l'autophagie pouvait être induite par un stress thermique différent, mais différentes réactions et changements physiologiques de l'autophagie étaient déclenchés en fonction des traitements.

La première différence concerne les cargaisons autophages analysées et la question de l'autophagie sélective par rapport à l'autophagie en masse. La deuxième différence est la spécificité tissulaire de l'autophagie. Groupe Hansen a observé une autophagie accrue dans

plusieurs tissus, y compris l'épiderme, les muscles, l'anneau nerveux et l'intestin. La troisième différence observée entre les trois études concerne les effets du stress thermique sur les animaux. En résumé, nos données ont montré que le stress thermique est un outil puissant pour stimuler l'autophagie chez *C. elegans*, ce qui est cohérent avec les deux autres groupes. Nous proposons également une approche permettant de comprendre l'impact du stress thermique sur l'homéostasie cellulaire et le développement des vers.

1.2 Comment l'autophagie affecte la récupération du ver du stress thermique?

Il est intéressant de noter que, bien que les organites soient fortement altérées par l'aHS, la plupart des vers pourraient encore se remettre des dégâts et se développer en adultes. Nos résultats montrent que l'autophagie est un facteur bénéfique pour la récupération du ver. Nous offrons une possibilité que l'autophagie aide à éliminer les matériaux endommagés et les organites toxiques pour l'homéostasie cellulaire. Dans les mitochondries, par exemple, en cas de stress thermique, la morphologie et les fonctions des mitochondries sont toutes deux endommagées; l'autophagie est donc stimulée pour nettoyer ces mitochondries endommagées. Cependant, l'inhibition de la mitophagie par les ARNi de *lgg-1* ou *atg-7* nuit davantage à la reconstruction du réseau mitochondrial lors de la récupération du ver. Étant donné que les mitochondries sont les principaux organites fournissant de l'énergie et associées à de nombreux processus physiologiques, la reconstruction correcte du réseau mitochondrial est essentielle pour la récupération des vers à partir des dommages causés par le stress thermique.

La réponse au stress thermique la plus étudiée est via la régulation transcriptionnelle. L'analyse de l'expression génique à l'échelle du génome chez *C. elegans* montre qu'un stress

thermique à 35 ° C pendant 2h affecte l'expression de nombreux gènes impliqués dans le développement, la reproduction et le métabolisme (Jovic, Sterken et al. 2017). Par exemple, les gènes impliqués dans la synthèse de la cuticule et les processus métaboliques ont été atténués par le stress thermique, ce qui peut expliquer en partie le retard de développement (Jovic, Sterken et al. 2017). De plus, l'expression de ces gènes dépend du HSF-1 (facteur de stress thermique 1) (Brunquell, Morris et al. 2016). Ici, nous avons énuméré plusieurs mécanismes de la façon dont l'autophagie protège le ver du stress thermique. La réaction au choc thermique semble être l'un des principaux facteurs de ce processus. Cependant, étant donné que le stress thermique entraîne de nombreux changements cellulaires, d'autres voies peuvent également être importantes pour la résistance des vers de terre dans des conditions de stress thermique.

2. Sites de contact ER-mitochondries chez *C. elegans*

À notre connaissance, il existe peu d'études sur le rôle des sites de contact ER-mitochondries chez *C. elegans*. En fait, jusqu'à présent, il n'y avait pas de protéines MAM validées chez *C. elegans*. Comment détecter le contact ER-mitochondries et identifier les fractions de MAM n'a pas encore été étudié chez *C. elegans*. En 2014, un document combinant les outils de détection des sites de contact ER-mitochondries chez la levure et identifiant l'ARF-1.2 en tant que protéine MAM potentielle chez *C. elegans* (Ackema, Hench et al. 2014).

Chez *C. elegans*, il n'existe pas encore d'outil d'étude des sites de contact ER-mitochondries et il n'existe aucune preuve permettant de savoir si l'attachement ER-mitochondrie est similaire à un système de levure ou de mammifère. Les nouveaux vers transgéniques exprimant HRP-KDEL, qui permettent de mesurer les sites de contact ER-mitochondries sous

EM, pourraient être un outil utile pour promouvoir la compréhension de l'interaction des ER avec les mitochondries.

3. Le défaut d'autophagie chez les vers mutants drp-1

Nous avons montré des amas autophages dans le mutant drp-1 lors d'un stress thermique. Les preuves suggèrent que ces grappes sont dues au défaut lors de l'élongation de la membrane d'isolation. L'appauvrissement de la drp-1 sur la formation des autophagosomes nécessite des études complémentaires, mais j'ai proposé ici plusieurs hypothèses pour l'expliquer.

Hypothèse 1: Les amas autophages dans le mutant drp-1 résultent d'un engouffement incorrect pendant la mitophagie (un effet secondaire du défaut de fission de drp-1)

Notre manuscrit démontre que le DRP-1 est nécessaire pour la fission mitochondriale dans des conditions de stress thermique. En l'absence de DRP-1, les mitochondries sont encore saignées et entretenues en connexion chez *C. elegans* (Figure 6 du manuscrit). Dans les levures et les mammifères, DRP-1 est recruté sur les sites de contact ER-mitochondries pour s'assembler autour des mitochondries. Plus important encore, les RE pré-contractent les mitochondries sur ces sites pour permettre l'assemblage du DRP-1. Il est généralement admis que la fission mitochondriale induite par le DRP-1 est une condition préalable à l'enfouissement autophagique au cours de la mitophagie (Tatsuta et Langer 2008, Mao et Klionsky 2013). Pour cette raison, les énormes mitochondries du mutant drp-1 pourraient constituer un obstacle à la formation de mitophagosomes et conduire à la formation de grappes de membranes isolantes. Les autophagosomes observés atteignent normalement un

diamètre compris entre 300 et 900 nm dans les cellules en culture, ce qui suggère que la taille des autophagosomes est contrôlée par un mécanisme permettant d'éviter la prolifération. Des images confocales vivantes montrent que les structures GFP :: LGG-1 / -2 s'entremêlent autour des mitochondries dans le mutant *drp-1*, alors que les piles montrent clairement que le vésicule autophagique autour des mitochondries est incomplet. Ces données corroborent notre hypothèse selon laquelle lors de la mitophagie induite par le stress thermique, les membranes d'isolation sont allongées autour d'une partie des mitochondries, alors qu'en l'absence d'autorachidies *drp-1*, qui ne parviennent pas à enfermer les énormes mitochondries en croissance excessive. Une autre étude a également révélé qu'au cours de l'allophagie, la morphologie des mitochondries paternelles affecte leur dégradation ((Wang, Zhang et al. 2016). Un défaut de fission dans les mitochondries paternelles retarde leur élimination, alors que le défaut de fusion a un effet facilitant. Il en va de même que dans les cellules de mammifère, la dynamique mitochondriale est un facteur clé de la mitophagie.

Hypothèse 2: Les amas autophages dans le mutant *drp-1* résultent des sites de contact perturbés de l'ER-mitochondrie où l'autophagosome se forme

Il est intéressant de noter que la fission mitochondriale induite par DRP-1 et l'initiation de l'autophagosome peuvent avoir lieu aux sites de contact ER-mitochondries. Les protéines Mfn2 et Drp1, deux facteurs clés de la fusion et de la fission mitochondriales, sont des protéines MAM enrichies en co-fractions MAM. Le DRP-1 pourrait peut-être fonctionner comme une protéine MAM régulant l'interaction entre l'ER et les mitochondries et, par conséquent, la biogenèse de l'autophagosome.

Des études chez les mammifères ont montré que plusieurs marqueurs autophages, y compris les marqueurs phagophores, apparaissent dans la zone de contact ER-mitochondries et sont enrichis en MAM lorsque l'autophagie est stimulée. Le découplage des mitochondries des urgences par suppression de PACS-2 ou de MFN2 conduit à une autophagie atténuée, notamment une diminution de la lipidation de la LC3 et une altération du mécanisme d'autophagie sur les sites de contact (Hamasaki, Furuta et al. 2013). Dans notre étude, le mutant *drp-1* entraîne également une réduction des autophagosomes LGG-1, similaire au défaut d'autophagie dans les cellules non couplées ER-mitochondries. De plus, les autophagosomes (étiquetés par GFP :: LGG-1 ou GFP :: LGG-2) apparaissent souvent à proximité de la mitochondrie lors de l'autophagie basale et stimulée. Il n'y a pratiquement aucune étude sur l'origine des autophagosomes chez *C. elegans*, sauf qu'une suggère que ER pourrait être associé à la membrane de l'isolat pendant l'embryogenèse. Ils ont découvert que la protéine EPG-3 localisée dans l'ER (homologue de VMP1 chez les mammifères) régule la formation d'autophagosome au niveau des sites de contact de la membrane d'isolation de l'ER (ER-IM) et que la perte d'EPG-3 inhibe la libération de l'IM lors de la fermeture de l'autophagosome et conduit à un regroupement autophagique (Tian, Li et al. 2010, Zhao, Chen et al. 2017). Considérant que l'implication des mitochondries dans ce processus n'est pas claire. Pour vérifier si ces autophagosomes sont proches des zones de contact ER-mitochondries, nous prévoyons de réaliser des analyses de colocalisation entre ER, mitochondries et autophagosomes chez les vers.

Une autre question est de savoir si le groupe autophagique dans le mutant *drp-1* est spécifique de la mitophagie ou non. Des études chez la levure indiquent qu'ERMES est

spécifiquement requis pour la mitophagie sélective, mais pas pour l'autophagie en masse, qui est différente de celle des mammifères. Mais chez *C. elegans*, nous n'avons pas encore de réponse privilégiée à cette question.

Titre : Mécanismes et fonction de la mitophagie dans l'adaptation au stress thermique pendant le développement de *C. elegans*

Mots clés : stress thermique, mitochondries, autophagique, mitophagie, *Caenorhabditis elegans*

Résumé : Le stress thermique résulte d'une exposition à une température située au-delà de la plage optimale pour un organisme. L'impact du stress thermique est variable selon son intensité, allant d'un effet bénéfique à la mort de l'organisme. Mon travail de thèse a établi un modèle de stress thermique aigu (aHS pour acute Heat Stress) chez *C. elegans* et a étudié ses effets sur l'homéostasie cellulaire, le développement des vers et la réponse autophagique. Un aHS au cours du 4ème stade larvaire induit un retard de développement, mais aucune létalité ni stérilité. Ce stress de développement entraîne la fragmentation massive mais transitoire des mitochondries, la formation d'agrégats dans la matrice et la diminution de la respiration mitochondriale. En outre, l'aHS déclenche un flux autophagique associé à des événements de mitophagie dans de nombreux tissus et en particulier dans l'épiderme. Nous avons montré que la réponse autophagique à l'aHS était protectrice pour les animaux.

De plus, nous avons découvert que dans l'épiderme, les mitochondries sont les principaux sites de biogenèse des autophagosomes, en conditions physiologique et en aHS. Nous avons également constaté que la protéine DRP-1 (dynamamin related protein 1) est impliquée dans le processus de mitophagie induite par l'aHS. Chez les animaux mutants *drp-1* soumis au aHS, la fission mitochondriale est impossible, l'autophagie est induite mais les autophagosomes sont anormaux et agrégés sur la mitochondrie. À partir de ces données, nous proposons que DRP-1 participe au contrôle de la qualité des mitochondries stressées en coordonnant la fission mitochondriale et la biogenèse des autophagosomes. J'ai également étudié plusieurs protéines pouvant être impliquées dans les zones de contact entre le réticulum endoplasmique et les mitochondries, ainsi que leurs rôles sur la morphologie mitochondriale et l'autophagie, dans des conditions physiologiques ou d'aHS. De plus, nous avons développé de nouveaux outils pour analyser les sites de contact ER-mitochondries.

Title : Mechanisms and function of mitophagy in adaptation to heat stress during development of *C. elegans*

Keywords : heat stress, mitochondria, autophagy, mitophagy, *Caenorhabditis elegans*

Abstract : Heat stress results from an exposure to a temperature beyond the optimum range of an organism. The impact of heat stress can range from beneficial to lethal due to the severity of stress. My thesis work established an acute heat stress (aHS) model in *C. elegans* and studied its effects on cell homeostasis, worm development and autophagy response. aHS during the 4th larval stage induces a developmental delay but no lethality or sterility. This developmental stress results in the massive but transitory fragmentation of mitochondria, the formation of aggregates in the matrix and the decrease of mitochondrial respiration. In addition, aHS triggers an active autophagy flux associated to mitophagy events in many tissues and particularly in epidermis. We showed that the autophagy response upon aHS is protective for the animals.

Moreover, we discovered that in the epidermis, the mitochondria are the major sites for autophagosome biogenesis in both standard and aHS. We also found that the dynamamin related protein DRP-1 is involved in aHS-induced mitophagy process. In *drp-1* animals submitted to aHS, mitochondrial fission is unable to achieve, and despite autophagy induction the autophagosomes cluster and elongate abnormally on mitochondria. From these data, we propose that DRP-1 is involved in the quality control of stressed mitochondria by coordinating mitochondrial fission and autophagosomes biogenesis. I also studied several proteins which may be involved in contact zones between endoplasmic reticulum and mitochondria, and their roles on mitochondrial morphology and autophagy, in physiological or aHS conditions. Furthermore, we have developed new tools for further studying the ER-mitochondria contact sites.

