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# The role of GATOR1 complex, upstream regulator of mTORC1 pathway, in mitochondrial function

Yahir Alberto Loissell Baltazar

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# The role of GATOR1 complex, upstream regulator of mTORC1 pathway, in mitochondrial function

*Le rôle du complexe GATOR1, régulateur en amont de la voie mTORC1, dans la fonction mitochondriale*

## Thèse de doctorat de l'université Paris-Saclay

École doctorale n° 582, cancérologie : biologie – médecine – santé (CBMS)  
Spécialité de doctorat : Aspects Moléculaires et Cellulaires de la Biologie  
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Thèse soutenue à Villejuif, le 13 décembre 2022, par

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## Résumé

Le *mammalian target of rapamycin* (mTOR) ou la cible de la rapamycin chez les mammifères, est une protéine hautement conservée au cours de l'évolution. mTOR est une enzyme avec une activité serine-thréonine kinase qui est la sous-unité catalytique de deux différents complexes, mTOR complexe 1 (mTORC1) et mTORC2.

La voie de mTORC1 est une des plateformes centrales de la signalisation cellulaire. Ce complexe répond à divers signaux, notamment les niveaux de nutriments, d'énergie et d'oxygène ainsi que des facteurs de croissance. D'autre part, mTORC1 répond aussi aux différents types de stress comme les dommages à l'ADN et les espèces réactives de l'oxygène (EROs). Le complexe mTORC1 concentre l'information pour pouvoir diriger la fonction cellulaire vers deux chemins différents. Avec une abondance de nutriments et en absence de stress, mTORC1 promeut l'activation des voies anaboliques qui conduisent vers une augmentation de la prolifération et de la croissance cellulaire. D'autre part, lorsque les nutriments sont peu abondants, ou en présence de stress, mTORC1 inhibe les voies anaboliques et conduit la cellule vers un état catabolique, en promouvant l'activation des voies de dégradation, en particulier le système d'ubiquitine-proteasome et l'autophagie.

Les données actuelles montrent que cette voie régule de façon directe plus de 65 protéines cibles, néanmoins les effets régulateurs de cette voie peuvent être perçus aussi au niveau d'organites tels que les mitochondries. De ce fait, la voie mTORC1 est fortement liée à la régulation de la fonction mitochondriale. La littérature existante fait preuve que mTORC1 participe à la régulation de la biogenèse ainsi qu'à la dégradation sélective des mitochondries, connue aussi comme mitophagie. De plus, mTORC1 participe à la maintenance de l'équilibre entre les événements de fission et de fusion des mitochondries, aussi appelé dynamique mitochondriale, et joue un rôle important dans la régulation du métabolisme oxydatif

et par conséquence de la production des EROs. Malgré ces liens, les mécanismes moléculaires à travers lesquels mTORC1 et la mitochondrie communiquent, restent peu décrits.

Un des principaux régulateurs de la voie mTORC1 en fonction des acides aminés est le complexe *GAP-activity towards RAGA 1* (GATOR1). Ce complexe multi-protéique, hautement conservé au cours de l'évolution, est constitué des trois différentes parties nommées *Nitrogen permease regulator like 2* (NPRL2), *Nitrogen permease regulator 3* (NPRL3) et *DEP-domain containing protein 5* (DEPDC5). De manière remarquable, les trois composants de GATOR1 ont d'abord été identifiés comme des suppresseurs des tumeurs. Chez la levure, il est connu que les protéines homologues du GATOR1 montrent une interaction avec la mitochondrie, et leur délétion impacte le métabolisme oxydatif et provoque des altérations dans la mitophagie. Chez les mammifères, l'interactome du GATOR1 a mis en évidence une relation avec la mitochondrie, et la sous-unité NPRL2 a été retrouvée dans des fractions mitochondriales. Néanmoins, le rôle de GATOR1 dans la fonction mitochondriale est peu connu.

Dans ce travail de thèse nous présentons des résultats qui montrent un lien important entre GATOR1 et la mitochondrie. Nous avons décrit pour la première fois que les membres de ce complexe peuvent être localisés dans la mitochondrie. Ainsi, nous avons utilisé le système CRISPR-Cas9 pour créer des lignées avec des délétions génétiques pour chacun des membres du complexe GATOR1 chez des cellules HEK293. Après la délétion des membres du complexe, nous avons trouvé des indicateurs du dysfonctionnement mitochondrial, tels que des altérations dans l'ultrastructure, notamment dans les crêtes. Nous avons aussi remarqué des changements importants de la morphologie mitochondriale, ainsi qu'une augmentation de la fragmentation des mitochondries dans des conditions basales, mais aussi lors d'un manque d'acides aminés. Ces altérations peuvent être partiellement expliquées par une dérégulation de l'expression des protéines clés de

la dynamique mitochondriale mitofusine 1 et 2, et *dynamamin-related protein 1* (DRP1) dans les lignées avec les délétions. D'autre part, nous avons observé une dérégulation dans la dégradation sélective des mitochondries au niveau basale, sans différences significatives lors de l'induction de la mitophagie avec le protonophore carbonylcyanure m-chlorophénylhydrazone (CCCP). Finalement, nous avons aussi observé des changements dans le taux de consommation d'oxygène, qui indiquent une altération du métabolisme oxydatif, sans changements significatifs de l'expression des protéines de la chaîne de transport des électrons.

L'ensemble de nos résultats montre une interaction importante entre le complexe GATOR1 et la mitochondrie. Ces résultats prouvent que la régulation de la fonction mitochondriale par mTORC1 peut partiellement être modulée par des membres de GATOR1.

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## **ABSTRACT**

The mammalian Target of Rapamycin (mTOR) is a serine-threonine kinase and the core subunit of two complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 pathway is one of the central hubs of cellular signaling. It responds to a variety of signals, such as energy, oxygen and nutrient levels, growth factors, DNA damage, etc. To maintain cellular homeostasis, mTORC1 keeps an equilibrium between activation of biosynthetic pathways and suppression of catabolic processes, such as autophagy. mTORC1 pathway has a key role in regulation of mitochondrial function and controls mitochondrial biogenesis, dynamics, and selective degradation of damaged or non-functional mitochondria. Many details of this regulation remain unclear.

One of the main upstream regulators of mTORC1 in response to amino acid availability is the GAP-activity towards Rags (GATOR1) complex composed of DEPDC5, NPRL2 and NPRL3. All three components of GATOR1 are tumor suppressors. The inactivation of NPRL2 is also associated to the resistance to the anticancer drug cisplatin. An interaction between mitochondria and GATOR1 homologue (SEACIT complex) was demonstrated in yeast, which have impaired oxidative metabolism and mitochondria degradation upon deletion of SEACIT proteins. Whether these functions are conserved in mammals is unknown.

In this work, we demonstrate that mammalian GATOR1 components can be localized at the mitochondria. Knockdown of GATOR1 proteins in HEK 293 cells trigger a significant alteration of mitochondrial morphology, and mitochondrial fusion and fission events, impacts oxidative metabolism, and triggers dysfunctions in mitochondrial membrane polarization. Taken together, our results show that in human cells mTORC1 pathway modulates mitochondrial function in part via the GATOR1 complex.

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## ABBREVIATIONS

AKT	AKT serine/threonine kinase 1
AMPK	5'Adenosin Monophosphate-Activated Protein Kinase
ATG	Autophagy Related Protein
ATM	Ataxia Telangiectasia Mutated
BNIP3	BCL2 Interacting protein 3
BNIP3L	BCL/adenovirus E1B-interactin Protein 3-Like
CASTOR1	Cellular Arginine Sensor for mTORC1
DDR	DNA Damage Response
DEPDC5	DEP Domain Containing 5
DRP1	Dynamin Related Protein 1
ETC	Electron Transport Chain
FUNDC1	FUN12 domain containing protein 1
GAP	GTPase-Activating Protein
GATOR	GAP Activity Towards RAGA
GEF	Guanine Nucleotide Exchange Factor
GTP	Guanosine Diphosphate
GTPase	Guanosine Triphosphate Hydrolase
LC3	Microtubule Associated Protein 1 Light Chain 3 Alpha
MFN1	Mitofusin 1
MFN2	Mitofusin 2

MIOS	Meiosis Regulator for Oocyte development
mTORC1	Mammalian Target of Rapamycin complex 1
NPRL2	Nitrogen Permease regulator like 2
NPRL3	Nitrogen Permease Regulator like 3
OCR	Oxygen Consumption Rate
OPA1	Optic Atrophy 1
OXPPOS	Oxidative Phosphorylation
PI3K	Phosphatidylinositol 3-Kinase
PINK1	Phosphatase and Tensin Homologue-Induced kinase 1
ROS	Reactive Oxygen Species
S6K1	Ribosomal Protein S6 Kinase Beta-1, p70s6 Kinase
SEA	Seh1-Associated
SEACAT	SEA Complex Activating TORC1
SEACIT	SEA Complex Inhibiting TORC1
SEH1L	Seh1-Like 1 Nucleoporin
TCA	Tricarboxylic Acids Cycle
TFEB	Transcription Factor EB
ULK1	Unc-51 Like Autophagy Activating Kinase 1
UPS	Ubiquitin Proteasome system
WDR24	WD-Repeat Domain 24
WDR59	WD-Repeat Domain 59



# **1.INTRODUCTION**

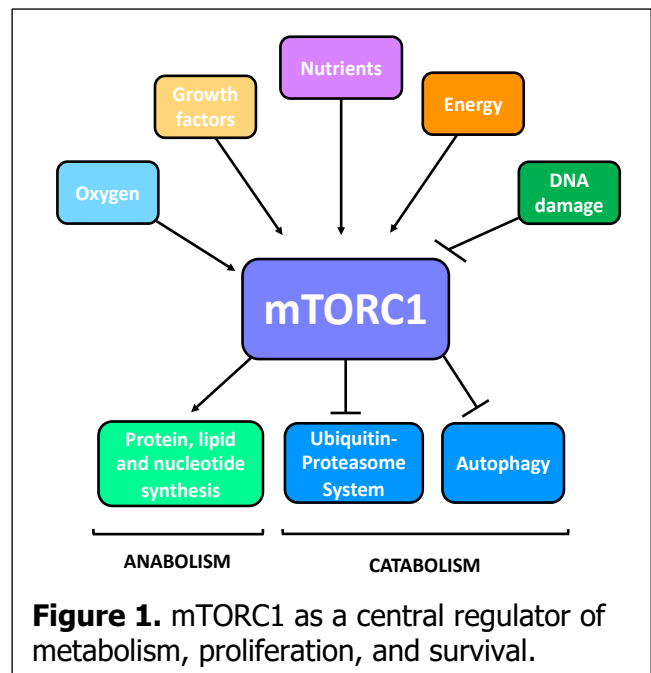
# 1. INTRODUCTION

## 1.1. mTOR complexes composition and structure

The highly conserved mechanistic (or mammalian) target of rapamycin (mTOR) plays a key role in cellular homeostasis. mTOR responds to a variety of intra- and extra cellular signals and stresses in order to coordinate cellular feedback to different cues. Therefore mTOR is considered as the master regulator of proliferation and survival (González and Hall, 2017; Kim and Guan, 2019; Saxton and Sabatini, 2017) (Figure 1).

mTOR is a 289-kDa, serine/threonine protein kinase that belongs to the phosphatidylinositol 3-kinase PI3K-related family (PIKK). Budding yeast, *Saccharomyces cerevisiae*, encodes for two different TOR proteins, TOR1 and TOR2, which form two structurally and functionally different complexes, TOR complex 1 (TORC1) and 2 (TORC2) (Heitman et al., 1991; Kunz et al., 1993).

Although mammals also have two different mTOR complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2), they encode only for one mTOR protein present in both complexes. These complexes differ in composition, functions and sensitivity to macrolide drug rapamycin, with mTORC1 being much more sensitive to rapamycin than mTORC2 (Liu and Sabatini, 2020).



mTOR protein contains multiple Hungtingtin, EF3A, ATM, TOR (HEAT) repeats at its N-terminal region, followed by a FRAP, ATM, TRRAP (FAT) domain, the FKBP12-rapamicyn-binding (FBR) domain, the catalytic kinase domain and the FATC domain at its C-terminal (Figure 1). Despite the fact that mTOR contains so many different domains, other enzymatic functions beyond its kinase activity were not reported (Yang et al., 2016; Yip et al., 2010).

**Table 1.** Composition of mTORC1 and mTORC2 and their yeast orthologues.

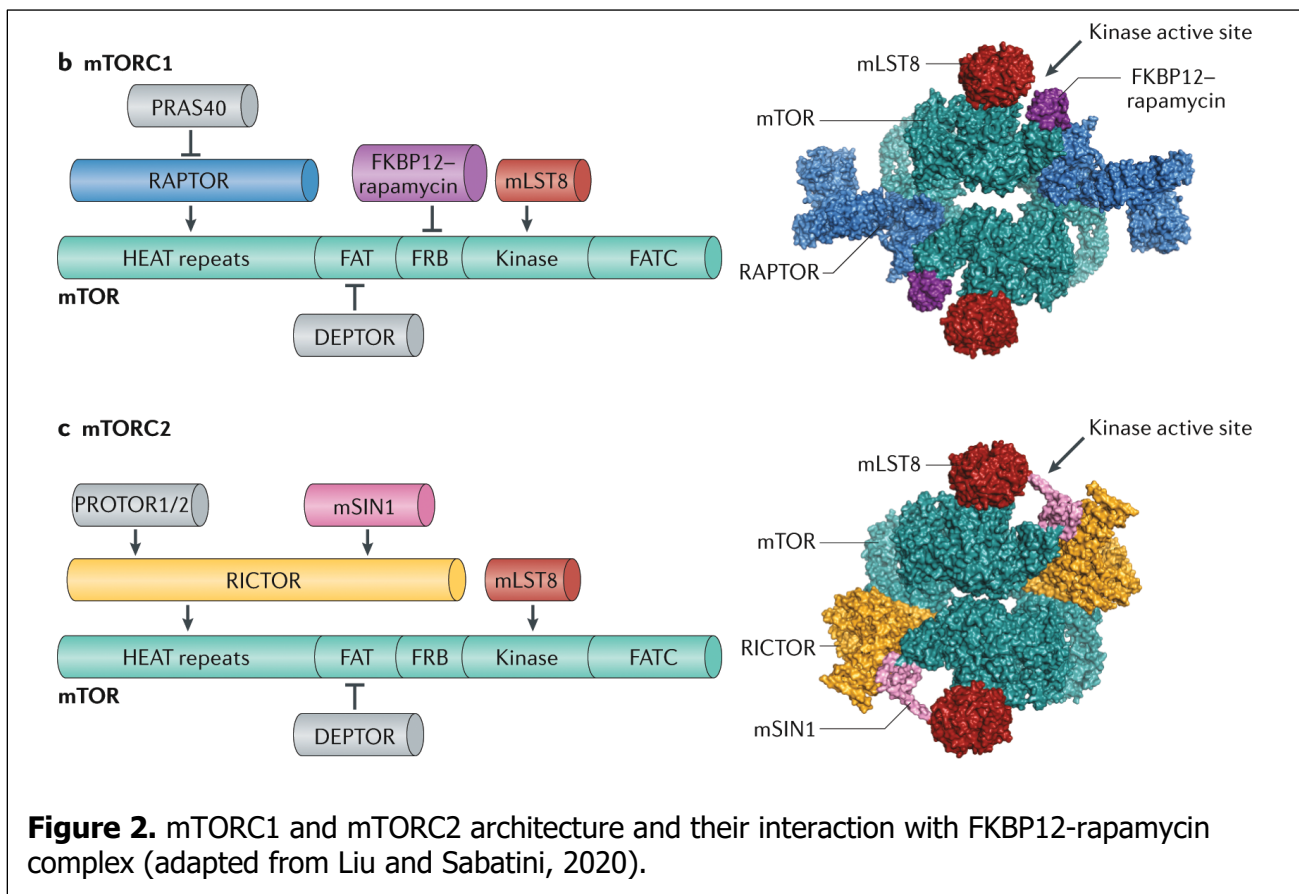
<b>Complex</b>	<b>Mammalian protein</b>	<b>Yeast orthologue</b>
<b>mTORC1</b>	mTOR	TOR1/2
	mLST8	LST8
	RAPTOR	KOG1
	*	Tc89
<b>mTORC2</b>	mTOR	TOR1/2
	mLST8	LST8
	RICTOR	AVO3
	mSin1	AVO1
	Protor-1/2	Bit6, Bit2
	*	AVO2

\*Unidentified orthologues.

mTORC1 is composed of the kinase mTOR, mammalian lethal with SEC13 protein 8 (mLST8), which interacts with the kinase domain of mTOR, and the regulatory associated protein of mTOR (RAPTOR) (Aylett et al., 2016; Yang et al., 2016). Additionally, the subunit RAPTOR, which acts as a scaffold for mTORC1 subcellular localization, interacts with the

mTORC1 accessory factor the proline-rich AKT substrate of 40 kDa (PRAS40) (Sancak et al., 2007), which along with the DEP-domain containing mTOR interacting protein (DEPTOR), act as endogenous inhibitors of mTORC1 pathway (Caron et al., 2018; Peterson et al., 2009) (Table 1). Cryo-electron microscopy structure of mTORC1 showed a dimer of mTOR proteins at its core, whilst the whole complex has a rhomboid-shape and it is formed by dimerization of the heterotrimer composed of mTOR, mLST8 and RAPTOR (Yang et al., 2016).

mTORC2 shares with mTORC1 both mTOR and mLST8, whereas RAPTOR is unique for mTORC1 (Chen et al., 2018a; Stutfeld et al., 2018) (Figure 2). It also contains the mTORC2-specific components Rapamycin insensitive companion of mTOR (RICTOR) and mammalian



stress-activated map kinase-interacting protein 1 (mSIN1). Additionally, the PROTOR-1/2 and DEPTOR can bind to mTORC2 (Gaubitz et al., 2016) (Table 1) (Figure 2).

mTORC1 and mTORC2 also differ in their regulation and the targets they activate. The most notable difference between these two complexes is their sensitivity to rapamycin. Rapamycin forms a gain-of-function complex with its intracellular receptor FK506-binding protein of 12 kDa (FKBP12) which binds to the FBR domain of mTOR in mTORC1 (Yip et al., 2010), but not in mTORC2 (Gaubitz et al., 2015). In yeast TORC2, the FBR domain of TOR interacts with AVO3, the yeast orthologue of RICTOR, which inhibits the binding with FKBP12-Rapamycin complex by steric hindrance. (Chen et al., 2018a; Gaubitz et al., 2016; Stutfeld et al., 2018). This structural feature was later confirmed in mTORC2, hence the architecture that confers mTORC2 rapamycin insensitivity is conserved from yeast to mammals (Scaiola et al., 2020; Stutfeld et al., 2018). In contrast to mTORC1, mTORC2 can be fully activated by growth factors and insulin through phosphoinositide 3-kinase (PI3K) signaling (Liu and Sabatini, 2020), it regulates cell survival and proliferation by activating AGC family kinases: Akt, PKC and SGK (Liu et al., 2015a), it is also involved in the upregulation of lipid biosynthesis (Kazyken et al., 2019; Masui et al., 2014). mTORC1 signaling pathway is closely related to mTORC2 signaling pathway. Long term mTORC1 inhibition will feed back to mTORC2 through signaling network, which is the reason why prolonged rapamycin treatment will eventually inhibit mTORC2, although it is not intrinsically sensitive to rapamycin. Since, the main scope of this work regards mTORC1 regulation, we will focus on the pathways that modulate upstream and downstream mTORC1 signaling.

## **1.2. mTORC1 upstream regulation**

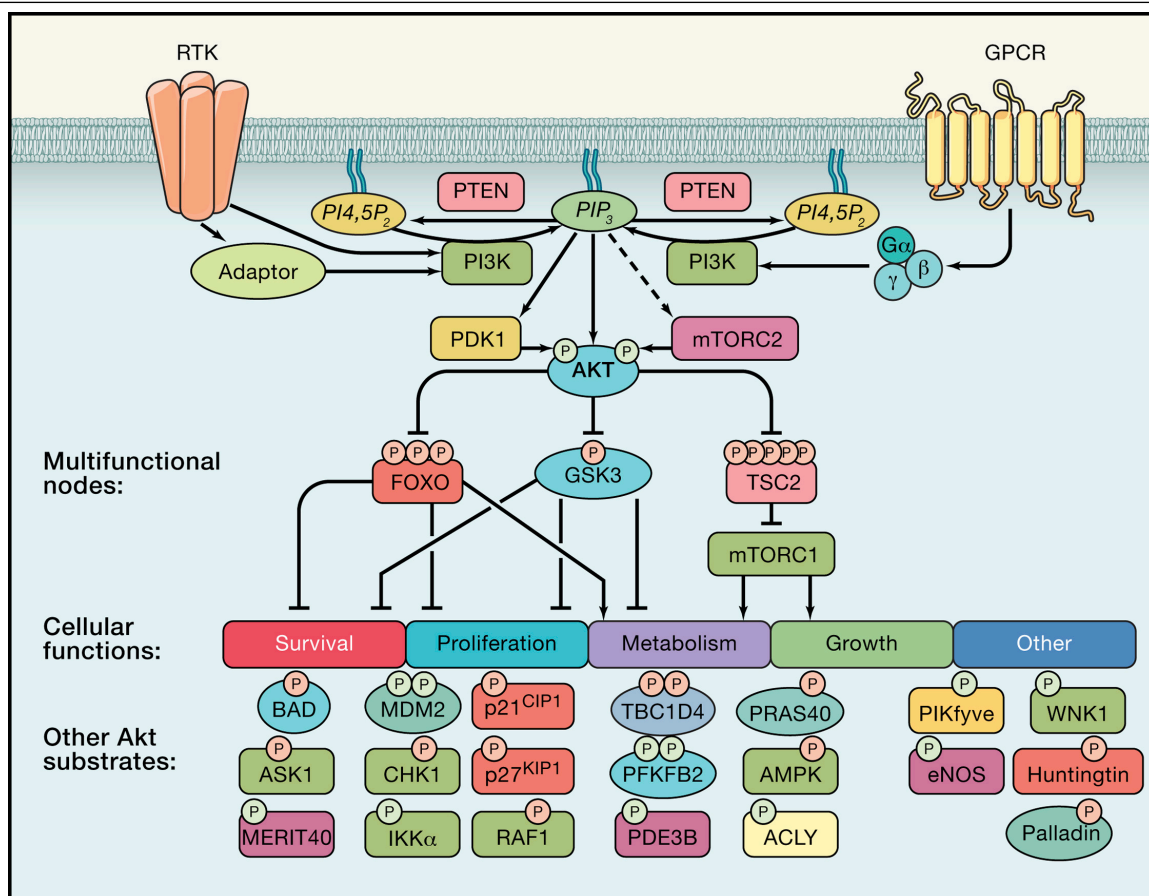
The mTORC1 pathway responds to a variety of signals and regulate the metabolic activity of cells to meet energetic demands and to respond to intra and extracellular stresses. mTORC1 pathway is subjected to a tight regulation allowing its activation, when there is sufficiency of growth factors, energy and nutrients. (Ben-Sahra and Manning, 2017; Liu and Sabatini, 2020; Saxton and Sabatini, 2017). In order to have an adequate and timely response to extra and intracellular inputs, mTORC1 responds to upstream signals through two different members of the small GTPases – Ras homologue enriched in brain (RHEB) and Ras-Related GTP-binding proteins (RAGs). GTP loading state of these small GTPases modulates mTOR kinase activity (Carroll, 2020; Kim et al., 2008; Sancak et al., 2008). When growth factors and energy are abundant, RHEB is loaded with GTP, in the lysosomal surface, which promotes mTORC1 activation. However, mTORC1 can only colocalize with RHEB when amino acids, glucose and other nutriments are plentiful (Liu and Sabatini, 2020). This mechanism of double activation ensures that cells only potentiate anabolism, when all growth factors, energy and nutriments are available.

### **1.2.1. Growth factors and energy signaling in mTORC1 regulation**

The ubiquitous pathway phosphoinositide 3-kinase (PI3K)/AKT is a key regulator of metabolism that responds to three main inputs: growth factors, hormones, chemokines and cytokines (Dibble and Cantley, 2015). To support cellular homeostasis, this pathway regulates glucose metabolism, cellular redox balance, and biosynthesis of macromolecules. The PI3/AKT

pathway can modulate cell metabolism by two different mechanisms: either directly through phosphorylation of key metabolic enzymes or nutrient transporters, or indirectly by control of transcription factors. It can also activate downstream effectors that play a role in metabolic reprogramming, such as mTORC1 (Dibble and Cantley, 2015; Fruman et al., 2017; Hoxhaj and Manning, 2020).

Growth factors can signal to PI3K/AKT pathway through binding to cell surface receptors, such as, cytokine receptors, receptor tyrosine kinase (RTKs), G-protein-coupled receptors (GPCRs), or adaptor molecules (Hoxhaj and Manning, 2020) (Figure 3). This interaction activates the lipid kinase activity of PI3K, which catalyzes the phosphorylation of



**Figure 3.** PI3K activation and downstream AKT activation (adapted from (Manning and Toker, 2017)).

phosphatidyl-inositol-3,4-bisphosphate (PtdIns-3,4-P<sub>2</sub>) to phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P<sub>3</sub> or PIP3) in the cytoplasmic side of the plasma membrane (Thorpe et al., 2015). PIP3 acts a second messenger for the activation of AKT, which is recruited to the plasma membrane or to endosomal membrane locations in a PIP3 dependent manner (Ebner et al., 2017). The PI3K/AKT signaling can be terminated by the phosphatase and tensin homologue deleted on chromosome 10 (PTEN), which exerts its phosphatase activity towards PIP3 (Vanhaesebroeck et al., 2012).

After PIP3-mediated membrane recruitment, the AGC serine/threonine kinase AKT is activated by phosphorylation in Thr308 by phosphoinositide dependent protein kinase 1 (PDK1) (Manning and Toker, 2017). Although phosphorylation on Thr308 is enough to promote downstream signaling, AKT is also phosphorylated by mTORC2 at Ser473 in a growth factor-dependent manner, it has been demonstrated that this modification enhances AKT downstream activity (Liu et al., 2015a; Sarbassov, 2005).

As mentioned before, mTORC1 is a well-recognized downstream effector of PI3K/AKT signaling pathway (Figure 3). This pathway funnels a variety of signals important for mTORC1 modulation of metabolism through a heterotrimeric complex: the Tuberous sclerosis complex (TSC), formed by Tuberous sclerosis complex 1 (TSC1), TSC2, and Tre2-Bub2-Cdc16-1 domain family member 7 (TBC1D7) (Dibble et al., 2012). From which, TSC2 exerts a GTPase activating protein (GAP) activity towards RHEB GTPase, an important upstream regulator of mTORC1 activation at the lysosomal membrane (Inoki, 2003).



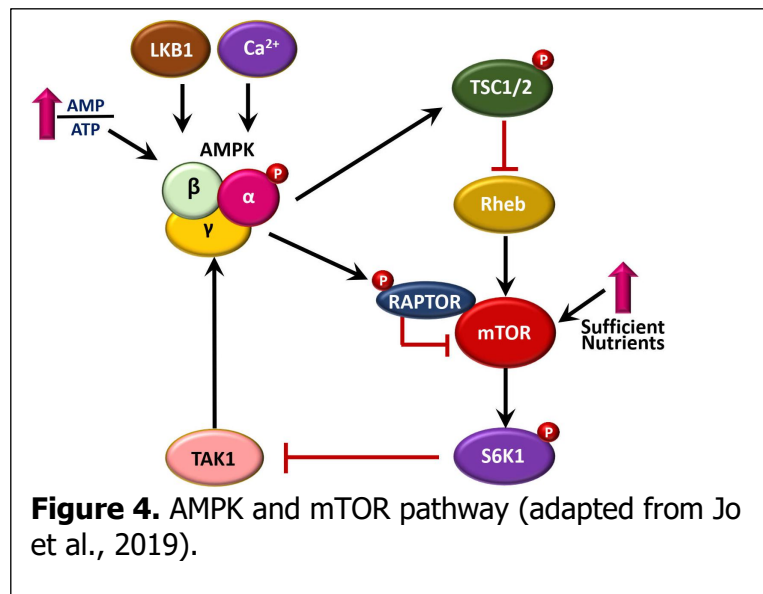
The primary mechanism through which PI3K/AKT pathway activates mTORC1 is through the dissociation of TSC complex by the phosphorylation of three amino acid residues adjacent to the GAP domain of TSC2: Ser 939, Ser1086 and Thr1462 (Inoki et al., 2002; Manning and Toker, 2017; Manning et al., 2002). Active AKT catalyzes the multisite phosphorylation of TSC2, which triggers a conformational change that promotes its dissociation from the lysosomal membrane, hence promoting the GTP-loaded state of RHEB on the lysosomal surface (Menon et al., 2014) (Figure 3).

The PI3K/AKT pathway can also modulate mTORC1 activity in an insulin dependent manner, through the phosphorylation of its subunit PRAS40, an endogenous inhibitor of mTORC1 activity (Sancak et al., 2007). Upon AKT mediated phosphorylation, PRAS40 dissociates from the RAPTOR subunit, allowing further RAPTOR interaction with other activators, such as RAGs. However, the details of this communication remain elusive.

Active mTORC1 can also act as an upstream negative regulator of PI3K/AKT signaling, since inactivation of the insulin receptor substrate 1 (IRS1) is a an event mediated by S6K1, a well-defined mTORC1 downstream target (O'Reilly et al., 2006). In addition, another negative feedback regulation from mTORC1 towards PI3K/AKT is via the inhibition of another adaptor protein, the growth factor receptor bound protein 10 (GRB19), this dampens the activation of the pathway (Manning and Toker, 2017) (Figure 3).

In contrast to growth factors sufficiency, energy depletion triggers the inhibition of mTORC1. Macromolecules synthesis are major energy consuming processes, which use adenosine triphosphate (ATP) as energetic currency, therefore its regulation must be tightly controlled in order to maintain cell homeostasis (Carroll and Dunlop, 2017; Herzig and Shaw, 2018). ATP and ADP are the major components of the “rechargeable battery” used by all organisms. When catabolism is active this battery is replenished by converting ADP into ATP, whereas anabolism requires energy investment, which triggers the conversion of ATP into ADP (Hardie et al., 2016). In addition, eukaryotic cells can interconvert adenine nucleotides through specific kinases that catalyze the reversible reaction  $2ADP \Leftrightarrow ATP + AMP$ , this led to the conclusion that cells rely on AMP levels for being the ultimate residue of ATP use (Gowans et al., 2013; Lin and Hardie, 2018).

Intense metabolic activity, glucose, or oxygen depletion can exhaust the cellular stores of ATP, hence leading to the accumulation of AMP. When the ratio AMP/ATP increases, cells can adapt their metabolism through the activation of the 5'-AMP-activated protein kinase



(AMPK), with mTORC1 as one of its main effectors (Hardie et al., 2016; Herzig and Shaw, 2018) (Figure 4). When ATP is scarce, the liver kinase B (LKB) 1 activates AMPK by

phosphorylation at Thr172 (Shaw et al., 2004). AMPK can also be activated by  $\text{Ca}^{2+}$  signaling and by growth factors through TGF-activated kinase 1 (TAK1) (Hardie et al., 2016; Herzig and Shaw, 2018). Once in active form, AMPK triggers the phosphorylation of TSC2 which enhances its GAP activity towards RHEB and thus inhibits mTORC1 (Inoki et al., 2003). In addition, AMPK can also inactivate mTORC1 by binding to its Raptor subunit, this event hinders the interaction between Raptor and the RAGs promoting mTORC1 release from lysosomal surface (Gwinn et al., 2008). This regulation appears to be bi-directional as very recent findings in fission yeast revealed, that under nutrient stress mTORC1 can directly phosphorylate and inhibit AMPK (Ling et al., 2020). In addition, mTOR signaling suppresses the activation of AMPK through S6K1-mediated inhibition of TAK1 (Xu et al., 2017) Also, by reprogramming the metabolism towards catabolic pathways, AMPK reduces the oxidative phosphorylation (OXPHOS) in mitochondria, hence reduces the production of reactive oxygen species (ROS) (Herzig and Shaw, 2018). Taken together, AMPK-mediated modulation of metabolism reduces ATP consumption under energy stress.

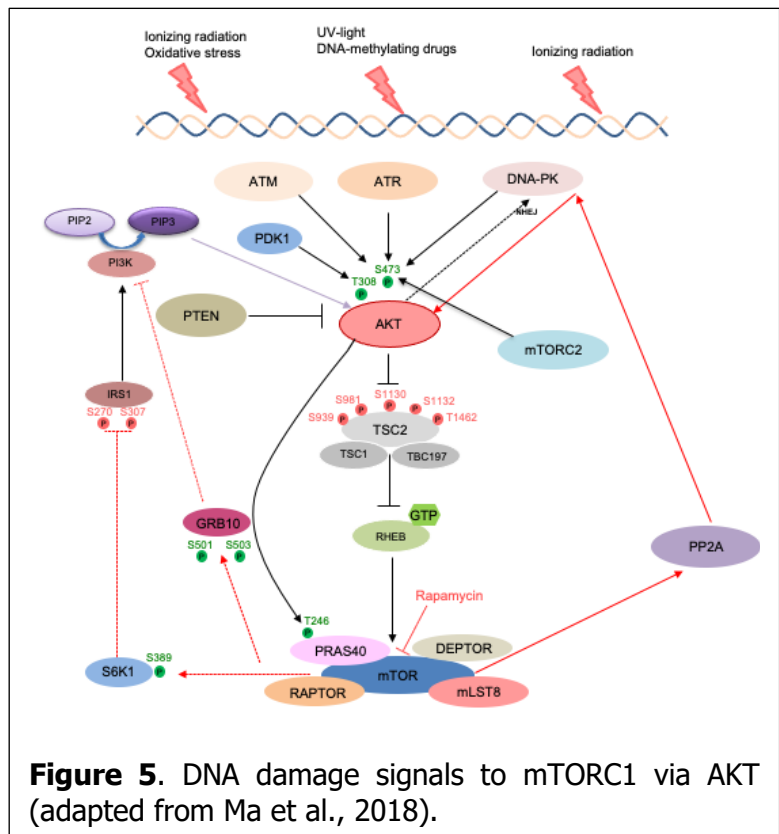
### **1.2.2. DNA damage response in mTORC1 regulation**

mTORC1 signaling is closely related to another cellular protection mechanism – DNA damage response (Ma et al., 2018).

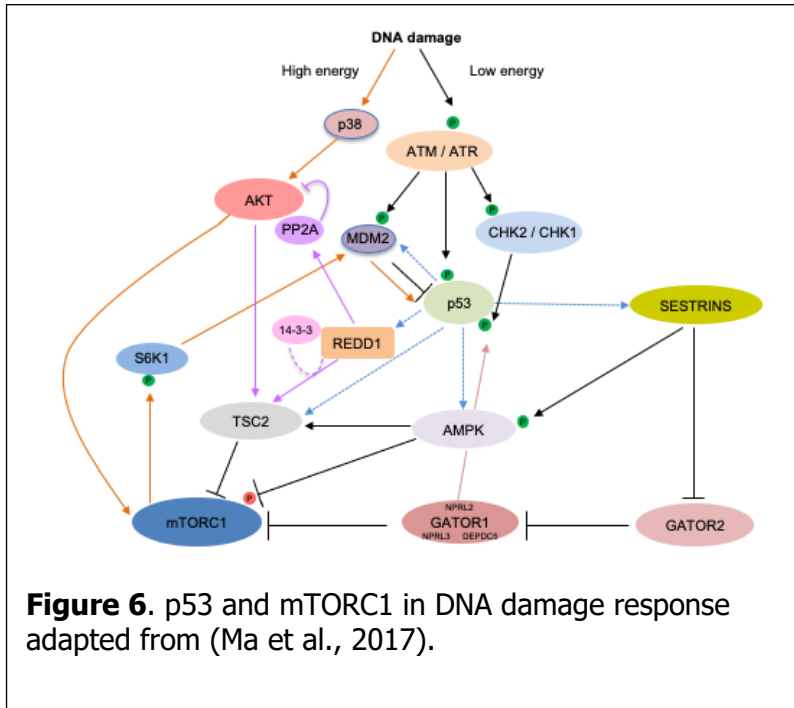
The maintenance of genome integrity is crucial for cell homeostasis and survival, however extra and intracellular insults can alter its integrity, such as ionizing radiation (IR), ultraviolet (UV) light and DNA damaging drugs (Figure 5). Additionally, by-products of metabolic reactions such as ROS can alter DNA integrity (Roos et al., 2016). When DNA damage

is detected, cells can respond to insults, either by activating DNA repair mechanism, if damage is mild, or by triggering apoptosis, when the damage is unreparable.

A group of effectors of the DNA damage response (DDR) from the PIKK family can signal to mTORC1 through the PI3K/AKT axis: ataxia telangiectasia mutated (ATM), DNA protein kinase (DNA-PK) and ataxia telangiectasia and Rag3 related (ATR). For example, exposure to  $\alpha$ -irradiation or oxidative stress activates ATM, which in turn triggers mTORC1 inhibition (Alexander et al.,



2010) (Figure 5). Upon DNA damage, these three proteins can phosphorylate histone H2AX at Ser139, producing  $\gamma$ -H2AX, a well-recognized marker of DNA damage (Blackford and Jackson, 2017). Remarkably, mTOR also belongs to PIKK family, underlying an evolutionary connection between the components of DDR and metabolic pathways (Imseng et al., 2018). Whereas, mTORC1 inhibition by rapamycin can induce AKT phosphorylation through protein phosphatase 2A (PP2A) and DNA-PK (Li et al., 2013).



mTORC1 response upon DNA damage is also regulated by p53 (Figure 6). This transcription factor is considered one of the main responders for DNA damage, its activity can trigger either pro-life or pro-survival, depending on the amount of damage in the DNA (Joerger and Fersht, 2016). p53 can signal upstream of mTORC1

through activation of the PI3K/AKT pathway, in fact several upstream regulators of mTORC1 that impact this pathway are targets of p53, i.e. AMPK, PTEN, TSC2 and SESTRIN2 (Feng et al., 2007; Hasty et al., 2013). Nevertheless, mTORC1 can also counteract in a negative feedback loop by downregulating p53 levels upon energy abundance (Shen and Houghton, 2013).

Interestingly, two proteins involved in amino acid sensing (reviewed in detail below) are also participating in mTORC1 modulation by DDR: SESTRIN2 and NPRL2. SESTRINS are a group of stress response proteins, activated by DNA damage, hypoxia, or ROS. SESTRIN 1 and SESTRIN 2 are targets of p53, and they can activate AMPK (Budanov and Karin, 2008; Sanli et al., 2012). Nitrogen permease regulator like 2 (NPRL2) is a member of the heterotrimeric complex GAP-activity towards RAGA 1 (GATOR1) (see below), (Loissell-Baltazar and

Dokudovskaya, 2021) Besides its role as mTORC1 regulator, it has been demonstrated that overexpression of NPRL2 leads to its nuclear accumulation and subsequently phosphorylation of p53 by interacting with Apoptosis-inducing factor (AIF), which induces cell cycle arrest and apoptosis. It also promotes the synthesis of mitochondrial ROS via activation of the ROS-producing enzyme NAPDH oxidase 2 (NOX2) and impairs mitochondrial function (Ma et al., 2017). In addition, NPRL2 overexpression overcomes cisplatin resistance in tumor cells, through phosphorylation of ATM with eventual apoptosis (Jayachandran et al., 2010). Taken together, it is evident, that communication between the DNA damage response and the mTORC1 pathway is one of the main strategies employed by the cell to face genotoxic and metabolic stresses.

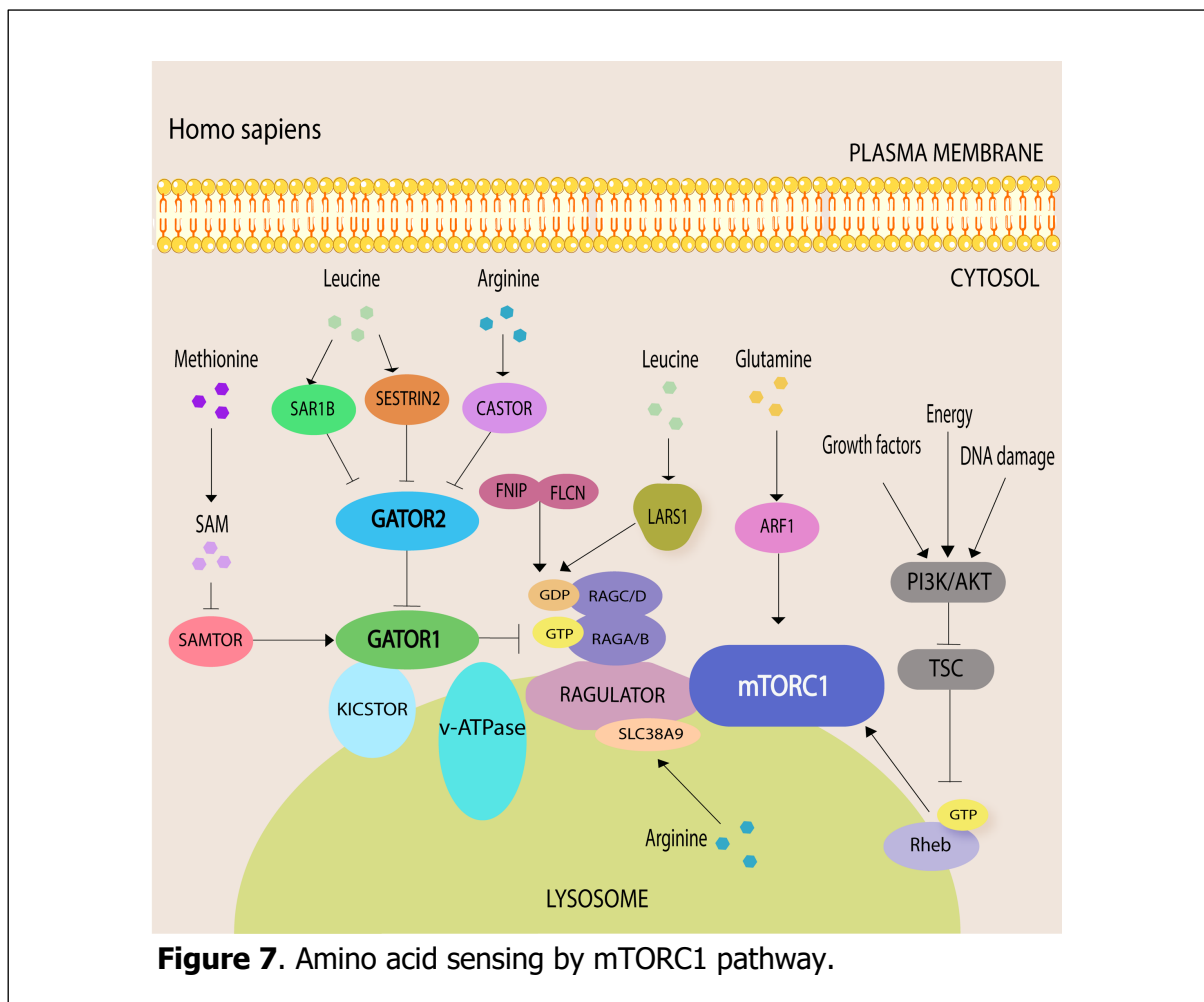
### **1.3. Amino acid signaling to mTORC1**

Amino acids are the monomer units for protein synthesis, precursors of hormones, and small nitrogen-based signaling molecules. Besides, they have been identified as regulators in a variety of metabolic pathways (Wu, 2009). Together, these characteristics give amino acids a strong biological importance and it is hence not surprising that mTORC1 has evolved a fine amino acid sensitivity. Amino acid signaling to mTORC1 was extensively studied during the last decade, which allowed to obtain many mechanistic details of this regulation (Figure 7).

#### **1.3.1. Lysosomal sensing of amino acids**

The lysosome has been long recognized as a central organelle for metabolism in which the degradation of proteins, polysaccharides and complex lipids takes place. The lysosomal

lumen contains more than 60 different hydrolases that breakdown these polymers to replenish the pool of cellular building blocks (Rabanal-Ruiz and Korolchuk, 2018). Extra and intracellular cargo can be delivered into the lysosome for degradation; therefore, it is considered a key organelle for catabolic processes and maintenance of metabolic homeostasis (Rabanal-Ruiz et al., 2017).



Since lysosomes provide a read-out of nutritional status of the cell, it is logical that they are fully involved in the mTORC1 activation. When nutrients are abundant, mTORC1 preferentially localizes to lysosomes. Amino acid deprivation promotes the mTORC1 diffusion

throughout the cytoplasm (Kim et al., 2008; Sancak et al., 2008). Effective functioning of mTORC1 pathway in respect to cellular amino acid levels requires coordinated action of RAGs and their effectors, such as GTPase-activating proteins (GAPs) and guanine-nucleotide-exchange factors (GEFs), which promote an inactive and active state, respectively. (Wolfson and Sabatini, 2017).

The major site of mTORC1 activation is the vacuole/lysosomal surface. When amino acids are abundant, mTORC1 is recruited to it, and further activated in a RAGs-dependent manner (Kim et al., 2008; Sancak et al., 2008). Mammals express four members of small Rag GTPase family which function as obligate heterodimers, RAGA and RAGB (RAGA/B); RAGC and RAGD (RAGC/D), which are structurally highly similar, but not completely functionally redundant (Bar-Peled and Sabatini, 2014) (Anandapadamanaban et al., 2019). In the presence of amino acids, RAGs are active when RAGA/B is loaded with GTP, and RAGC/D is bound to GDP. Reversely, when amino acids are low, RAGs are inactive, and RAGA/B is loaded with GDP and RAGC/D is bound to GTP (Figure 7). mTORC1 lysosomal translocation relies on the binding of activated RAGs to the RAPTOR subunit of mTORC1. RAPTOR-RAGs interact with the pentameric complex RAGULATOR, comprising of p18, p14, C7orf59 and HBXIP, which forms a scaffold that anchors the RAGs-mTORC1 to the lysosomal surface (Sancak et al., 2010) (Park et al., 2020; Rogala et al., 2019).

When amino acids are scarce, some amino acid sensors interact with and inhibit the GATOR2 complex, thus preventing inhibition of the GATOR1 by GATOR2. A mammalian-specific



KICSTOR complex tethers GATOR1 to the lysosomal surface (Peng et al., 2017; Wolfson et al., 2017) where GATOR1 acts as a GAP for RAGA (Bar-Peled et al., 2013), thereby transforming RAGA to its inactive, GDP bound form, which leads to mTORC1 suppression.

In the presence of amino acids, RAGULATOR and v-ATPase undergo a conformational change that results in RAGULATOR exerting GEF activity towards RAGA or RAGB (Sancak et al., 2010). RAGULATOR also trigger GTP release from RAGC (Shen and Sabatini, 2018). In parallel, upon arginine binding, lysosomal arginine sensor human member 9 of the solute carrier family 38 (SLC38A9) stimulates GDP release from RAGA (Shen and Sabatini, 2018). A complex between folliculin (FLCN) and folliculin-interacting protein (FNIP) 1 and/or 2 is a GAP for RAGC/D (Tsun et al., 2013). In addition, leucyl-tRNA synthetase (LeuRS or LARS1 or LRS) also has GAP activity towards RAGD (Han et al., 2012a).

Active RAGULATOR-RAG stimulates recruitment of mTORC1 to the lysosomal membrane where it is fully activated by small GTPase, RHEB, loaded with GTP (Long et al., 2005). RHEB is under the control of another signaling node - the TSC complex, where TSC2 acts as a GAP to inhibit RHEB. TSC is a nexus of multiple physiological stimuli (e.g. energy status, growth factors, DNA damage) that signal to mTORC1 through PI3K-AKT network (Hoxhaj and Manning, 2020). RAGs regulate the recruitment of TSC to the lysosome in response to amino acid starvation, growth factors removal and to other stresses that inhibit mTORC1 (Demetriades et al., 2014; Demetriades et al., 2016; Yang et al., 2020). Both RAGs and RHEB are necessary for mTORC1 activation at the lysosome, as the lone presence of either one is

not sufficient for full mTORC1 activation. Accordingly, only when both the RAGs and RHEB are inactive, mTORC1 can be released from the lysosome (Demetriades et al., 2014).

The lysosomal lumen was first proposed as the main site for amino acid sensing, particularly through the vacuolar H<sup>+</sup>-ATPase (v-ATPase). This proton pump is responsible for the maintenance of acidic conditions inside the lysosome and communicates lysosomal amino acid levels to the RAGULATOR complex. The underlying mechanism of this communication is not well understood (Zoncu et al., 2011a). Hence, the RAGULATOR complex provides a physical a functional link between v-ATPase and RAGs. When amino acids are available RAGULATOR affects the GTP loading state of RAGs, acting as a GTPase-activating protein (GAP) for RAGC, which promotes mTORC1 docking to the lysosomal membrane (Shen and Sabatini, 2018). However, whether v-ATPase is the sensor of a specific amino acid remains an open question.

The growing evidence of the lysosome as a hub for amino acid signaling upstream of mTORC1 led to the identification of SLC38A9 as another member of the amino acid sensing machinery. The SLC38A9 has homology with amino acid transporters and interacts with the RAGULATOR-RAGs (Rebsamen et al., 2015). SLC38A9 has a high degree of affinity for arginine transport, therefore, it was proposed as a specific arginine sensor. SLC38A9 can communicate arginine levels to mTORC1 in a RAG-RAGULATOR-v-ATPase mediated mechanism, however whether it is specific for arginine or it is a general amino acid sensor remains controversial (Jung et al., 2015; Wang et al., 2015).

### **1.3.2. Cytosolic sensing of amino acids**

Although the role of lysosome in the amino acid sensing upstream of mTORC1 has been described as an essential mechanism for cellular homeostasis. In recent years, several cytosolic sensors have been described, particularly for leucine, arginine, and methionine.

Cytosolic leucine can be sensed by the proteins from the SESTRIN family (SESTRIN 1-3) (Saxton et al., 2016a; Wolfson et al., 2016; Xu et al., 2019), by small GTPase SAR1B (Chen et al., 2021) and by leucyl-tRNA synthetase (Han et al., 2012b; Kim et al., 2021; Lee et al., 2018).

SESTRINS (1-3) are a group of stress response proteins, their genes were originally recognized as p53 targets (Budanov and Karin, 2008). SESTRINS also act as mTORC1 negative regulators through the AMPK stress-pathway (Ben-Sahra et al., 2013b; Budanov, 2011). SESTRIN2 interacts with GATOR2 protein upon amino acid sufficiency, which triggers mTORC1 activation (Chantranupong et al., 2014). This interaction inhibits mTORC1 activity by preventing its localization to the lysosomal membrane in a RAGs dependent manner (Parmigiani et al., 2014).

SESTRIN2 was the first specific cytosolic amino acid sensor identified (Wolfson et al., 2016). At physiological concentrations, leucine binds to SESTRIN2, disrupting the GATOR2-SESTRIN2 interaction, which releases GATOR2 from GATOR1, therefore promoting mTORC1 activity. SESTRIN2 has a highly conserved GATOR2 binding site near the leucine pocket. Upon

leucine binding, SESTRIN2 undergoes a conformational change that prevents its interaction with GATOR2, an event that can be reversed upon leucine starvation (Saxton et al., 2016b).

During leucine starvation, the GATOR2 complex interacts with another cytosolic leucine sensor, the SAR1B (Chen et al., 2021). Importantly, SAR1B and SESTRIN2 interacts with different subunits of GATOR2 (see below).

Leucyl-tRNA synthetase (LRS) catalyzes the ligation of leucine and tRNA to form leucyl-tRNA<sup>Leu</sup> (Park et al., 2005), and has also been identified as a cytoplasmic leucine sensor. In mammalian cells LRS has a GAP activity towards RAGD, which triggers mTORC1 activation (Han et al., 2012a). However in yeast cells, upon leucine sufficiency, LRS interacts with Gtr1 (the yeast homologue for RAGA/B) but not with Gtr2 (yeast RAGC/D) (Bonfils et al., 2012). Although these data are controversial, they suggest a conserved role for LRS in mTORC1 regulation through a leucine-dependending mechanism.

Two putative GATOR2 interactors GATS protein-like 2 (GATS2) and 3 (GATS3) were identified as cytosolic arginine sensors for mTORC1 and renamed Cellular Arginine Sensor for mTORC1 1 (CASTOR1) and 2 (CASTOR2) (Chantranupong et al., 2016). They can form homo- or heterodimers, (CASTOR1/1 or CASTOR1/2), that specifically communicate cytosolic arginine levels to mTORC1 again via interaction with GATOR2 complex (Chantranupong, et al., 2016).

While arginine and leucine bind directly to their sensors, methionine can be sensed

through its intermediate metabolite S-adenosyl methionine (SAM) by the S-adenosylmethionine (SAM) sensor to mTORC1 (SAMTOR). Under methionine deprivation, SAMTOR binds to GATOR1-KICKSTOR complex. This interaction anchors the GATOR1 to the lysosome where it can exert its GAP activity towards RAGA (Wolfson et al., 2017), and thus, prevents mTORC1 translocation to the lysosome (Gu et al., 2017).

RAGs-independent induction of mTORC1 by amino acids both at the vacuole/lysosome and Golgi has also been described in yeast and human (Jewell et al., 2015; Meng et al., 2020; Thomas et al., 2014; Ukai et al., 2018). In addition, both in yeast and in mammals, amino acids can activate TORC1 on Golgi membrane. A small GTPase Ypt1 in yeast (Rab1A in mammals) recruits TORC1 to this organelle. In mammals, mTORC1 is subsequently activated by RHEB, located at the Golgi (Thomas et al., 2014).

In RAGA/B deficient cells mTORC1 can be stimulated by glutamine (but not leucine) and this activation is dependent on v-ATPase (Jewell et al., 2015). Moreover, a pool of ten amino acids (alanine, arginine, asparagine, glutamine, histidine, leucine, methionine, serine, threonine, and valine), referred together as AA<sup>mTORC1</sup> are essential for mTORC1 lysosomal localization. However, whereas eight amino acids signal to mTORC1 through the RAGs, asparagine and glutamine can still activate mTORC1 in KO-RAGA/B MEFs and HEK293 cells via ADP-ribosylation factor 1 (ARF1). Thus AA<sup>mTORC1</sup> can regulate mTORC1 activity, at least, through two different pathways (Meng et al., 2020).

Specific amino acid sensors identified so far are not conserved among eukaryotes. That is probably because the mammals need to take essential amino acids with food while yeast can synthesize all amino acids if nitrogen is available.

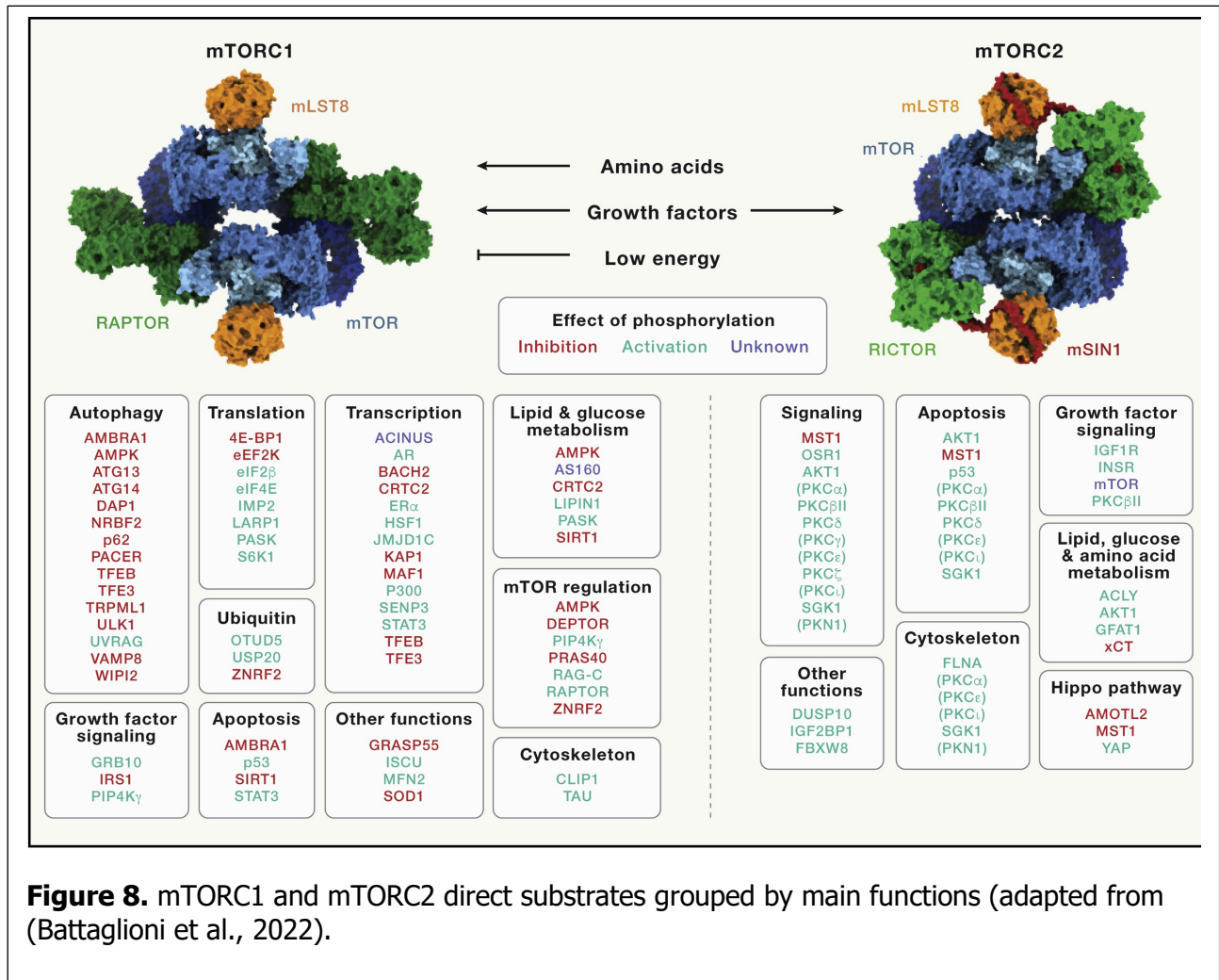
#### **1.4. mTORC1 downstream regulation**

For sustaining growth and division cells require a balance between biosynthesis of proteins, nucleotides and lipids and repression of catabolic pathways, such as autophagy. mTORC1 has a crucial role in the regulation of the equilibrium of the afore-mentioned processes, therefore it maintains the balance between anabolism and catabolism in response to nutritional or environmental conditions. mTORC1 controls synthesis of three major cell constituents: proteins, nucleotides, and lipids *via* phosphorylation of its multiple substrates (Figure 8).

##### **1.4.1. Anabolic pathways: protein, lipid, and nucleotide synthesis**

In active state, mTORC1 stimulates protein synthesis through the phosphorylation of two key effectors, p70S6 Kinase 1 (S6K1) and members of the eIF4E Binding Protein Family (4E-BPs). mTORC1 phosphorylation of S6K1 at Thr389 allows its interaction with PDK1. Once activated by PDK1 phospho-S6K1 exerts its kinase activity over several substrates. Such as eIF4B, a factor related to increased mRNA translation (Hannan et al., 2003; Holz et al., 2005). The phosphorylation of 4EBPs by mTORC1 pathway, promotes their dissociation from eIF4E, hence mRNA translation can occur (Gingras et al., 1999). Although studies are mainly focused

of 4E-BP1, other members of 4E-BP family share the conserved phosphorylation sites for mTORC1 activation (Thoreen et al., 2012).



**Figure 8.** mTORC1 and mTORC2 direct substrates grouped by main functions (adapted from (Battaglioni et al., 2022)).

Lipids are important for new membrane synthesis; therefore, they are crucial for cell growth and development. Sterol responsive element binding protein transcription factor 1 (SREBP1) and 2 (SREBP2) are canonically activated upon low sterol levels and control the expression of genes involved in fatty acid and cholesterol biosynthesis. mTORC1 can also regulate the lipid synthesis both through SREBPs activation or indirectly through an S6K1-

dependent mechanism (Laplante and Sabatini, 2010; Porstmann et al., 2008). mTORC1 signaling can also regulate lipid synthesis upstream SREBPs by phosphorylation of Lipin1, which in the absence of mTORC1 signaling sequesters SREBPs (Peterson et al., 2011).

mTORC1 promotes nucleotide synthesis through the phosphorylation of its target activating transcription factor 4 (ATF4) which promotes the expression of methylenetetrahydrofolate dehydrogenase 2 (MTFHD2), a supplier of carbon units for purine synthesis (Ben-Sahra et al., 2016). mTORC1 can also increase cell nucleotide pool by S6K1 dependent activation of carbamoyl-phosphate synthetase (CAD), an essential enzyme for the *de novo* pyrimidine synthesis pathway (Ben-Sahra et al., 2013a).

#### **1.4.2. Catabolic pathways**

Intra- and extracellular availability of nutrients, such as amino acids and glucose, as well as energy levels and hormonal signals, are dynamic. Therefore, a tight cellular response to these signals is required for maintenance of healthy cellular function. The aim of such response mechanisms are ensuring that intracellular levels of free biosynthetic precursors are in the appropriate concentration for promoting anabolic processes, including protein translation, energy production and driving cellular growth and proliferation (Carroll et al., 2015). As one of the central modulators of metabolism, the spatial and temporal regulation between nutrients availability, mTORC1 pathway regulation and catabolic process is fundamental for cell homeostasis.



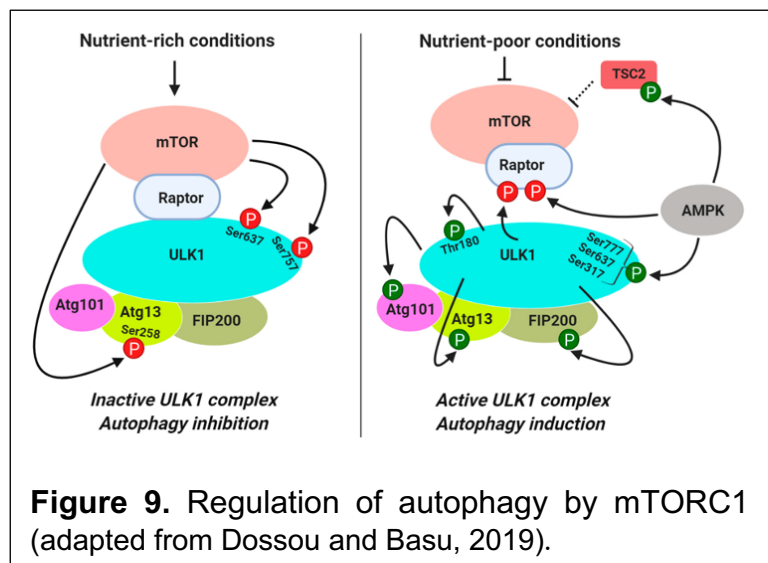
Under amino acid and energy deprivation conditions, mTORC1 is inactive. This leads to the activation of ubiquitin proteasome system (UPS) and autophagy to release free amino acids for replenishment of their pools.

The proteasome is a multi-protease complex, which catalyzes the ATP-dependent degradation of proteins into oligopeptides and/or amino acids for their further release in the cytoplasm. The UPS is responsible for the proteasome-mediated degradation of short-life or misfolded soluble proteins and depends on the addition of ubiquitin chains to their targets and proteasomal chaperones for their delivery (Ciechanover et al., 1980; Ciechanover et al., 1978; Hershko et al., 1980; Hershko et al., 1982). It has been proposed that mTORC1 can regulate this proteasome-dependent proteolysis through either, a general increase in protein ubiquitination rate (Zhao et al., 2015), or an upregulation of proteasomal chaperones via the inhibition of ERK5 pathway (Rousseau and Bertolotti, 2016). Although normal UPS function is needed for cellular homeostasis, the major axis through which mTORC1 controls cell catabolism relies on autophagy (Korolchuk et al., 2010).

The term autophagy derives from the Greek words "*authos*" and "*phagos*" which translates as "self-eating". Autophagy, also referred to as macroautophagy, is a tightly regulated multistep process in which cytosolic components and macromolecules, are first engulfed by specialized double-membrane structures known as autophagosomes. Then, delivery of cargo is carried out through autophagosome-lysosome fusion, further cargo degradation is completed by lysosomal hydrolases (Levine and Kroemer, 2019; Mizushima et

al., 1998; Takeshige et al., 1992). The molecular pathway of autophagic degradation involves a set of autophagy related proteins (ATGs), which coordinate distinct autophagic events, such as autophagosome formation, elongation of autophagosomal membrane, and lysosomal maturation and fusion. Basal levels of autophagy are needed in order to maintain metabolic homeostasis (Kawabata and Yoshimori, 2020; Sancak et al., 2008; Zoncu et al., 2011b). Deprivation of oxygen, energy and nutrients can trigger autophagy initiation through mTORC1 inactivation (Rabanal-Ruiz et al., 2017). mTORC1 can directly regulate autophagy by exerting its kinase activity on the autophagy-initiating Unc51-like autophagy activating kinase (ULK) 1 complex, which consists of ULK1, the FAK family kinase interacting protein of 200 kDa scaffold (FIP200), ATG13 and ATG101 proteins (Ganley et al., 2009). When mTORC1 is active, interaction of RAPTOR subunit with ULK1 leads to autophagy inhibition through mTORC1-mediated phosphorylation of ULK1 at Ser 637 and Ser 757, as well as, ATG13 at Ser 258 (Dunlop et al., 2011) (Figure 9).

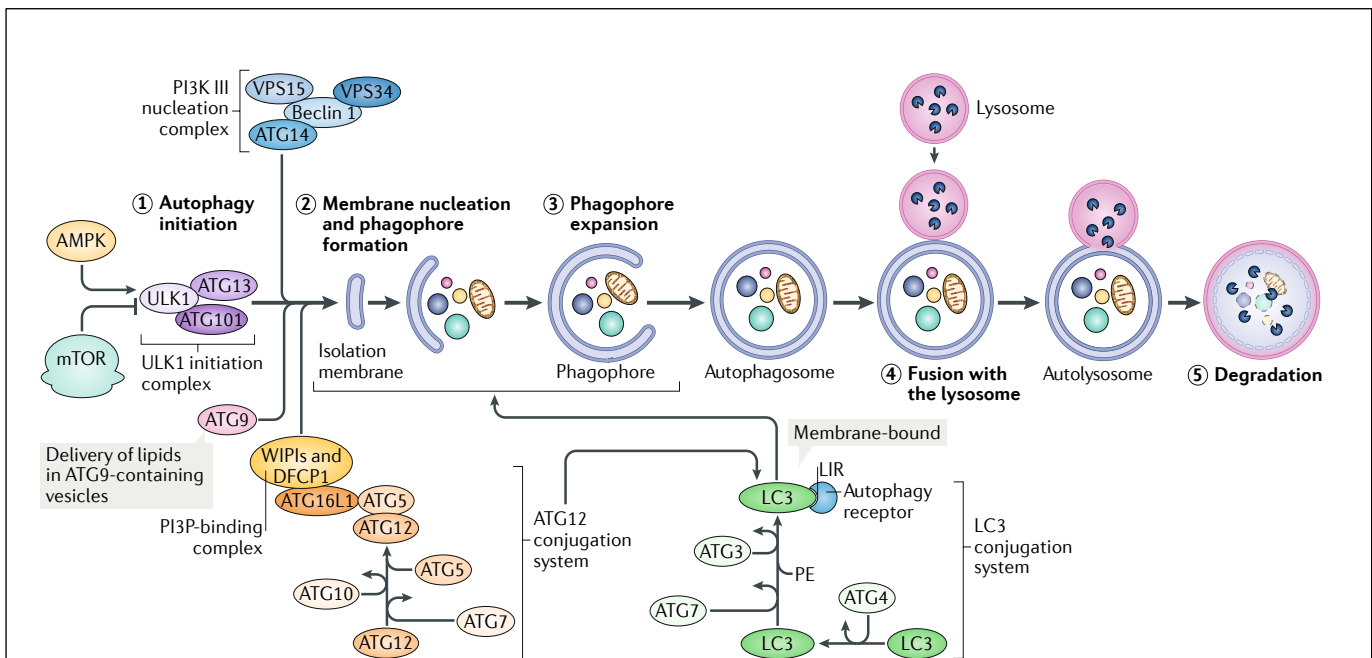
Under starvation conditions, cellular stress or rapamycin treatment, mTORC1 dissociates from the ULK1 complex (Lin and Hurley, 2016). This event triggers ULK1 protein dephosphorylation by protein phosphatase 2A (PP2A) and protein phosphatase 1D magnesium-



dependent (PPM1D) (Torii et al., 2016; Wong et al., 2015). Dephosphorylated ULK1 protein auto-activates by phosphorylation at Thr180, then active ULK1 phosphorylates ATG101 at Ser 11 and Ser103 and at ATG13, FIP200 at multiple sites (Egan et al., 2015). In addition, ULK1 mediates the phosphorylation of mTORC1 component RAPTOR at multiple sites, which promotes a negative-feedback to sustain mTORC1 suppression and provides a feed forward loop for autophagy (Dunlop et al., 2011). Once it is completely activated, the ULK1 complex initiates the autophagosome formation through a mechanism that involves class III PI3K complex I, which comprises vacuolar protein sorting 34 (VPS34), BECLIN1 and VPS15 in complex with either UV radiation resistance-associated gene protein (UVRAG) or ATG14 (Fogel et al., 2013; Russell et al., 2013). mTORC1 can inhibit Class III PI3K complex via the phosphorylation of ATG14 in an amino acid dependent manner (Yuan et al., 2013). In addition,

under energy deficiency AMPK promotes autophagy by ULK1 phosphorylation at Ser 555, Ser 317 and Ser 777, which leads to ULK1 complex activation (Bach et al., 2011). However, under nutrient sufficiency, mTORC1 mediated phosphorylation of ULK1 disrupts the interaction between ULK1 and AMPK, which indicates a tight crosstalk between nutrient and energy sensing pathways upstream of autophagy initiation (Carroll and Dunlop, 2017).

The ULK1 complex orchestrates autophagosome nucleation by phosphorylation of a series of downstream effectors (Ganley et al., 2009; Levine and Kroemer, 2019). Furthermore, ATG proteins, ATG5, ATG12, ATG16, are recruited to the autophagosome (Abada and Elazar, 2014) (Figure 10). Then the microtubule-associated protein 1 light chain 3 (LC3), which is



**Figure 10.** Molecular mechanisms of autophagy activation (adapted from Hansen et al., 2018). mTORC1 and AMPK are main regulators of autophagy with mTORC1 acting as an inhibitor and AMPK as an activator. When autophagy is induced, cytoplasmic material (the autophagic cargo) is engulfed by double membranes, starting from the formation of a cup-shaped structure called the phagophore to the sequestration into double-membrane vesicles, called autophagosomes, which subsequently fuse with acidic lysosomes and form autolysosomes, where cargo is degraded.

initially synthesized as a cytosolic protein, is recruited to the autophagosome membrane where it is cleaved at its C-terminus by the cysteine protease ATG4. After cleavage it is identified as LC3-I, which becomes rapidly conjugated with phosphatidylethanolamines (PE) by ATG3-ATG7, and thus referred to, as LC3-II (Kabeya, 2000). The expanded membrane retains LC3-II on the surface. The elongation of autophagosome membrane involves the sequestration of lipid membranes where the ER, Golgi, mitochondria or plasma membrane have all been studied as main membrane suppliers (Axe et al., 2008; Ge et al., 2013; Hailey et al., 2010; Ravikumar et al., 2010). Mature autophagosomes can recognize their cargo by binding to adaptor proteins such as sequestosome-1 (SQST1) also known as p62, or neighbor of BRCA1 gene protein 1 (NBR1) (Carroll et al., 2015). Once sequestered, the cargo is transported to the lysosome and upon autophagosome-lysosome fusion, lysosomal acid hydrolases degrade its content, a function tightly regulated by v-ATPase (Carroll and Dunlop, 2017).

Whereas non-selective autophagy, also known as “bulk autophagy” is a mechanism that responds to environmental cues status in order to meet cell energetic demands for maintaining homeostasis in a non-specific manner, other forms of autophagy recognize specific cargo, such as damage organelles, i.e. mitochondria or ER, or pathogens and deliver them for degradation (Johansen and Lamark, 2020).

### **1.5. Multifunctional mitochondria**

Mitochondria are essential double-membrane organelles that originated from the integration of an endosymbiotic  $\alpha$ -proteobacterium into a host proto-eukaryotic cell, most

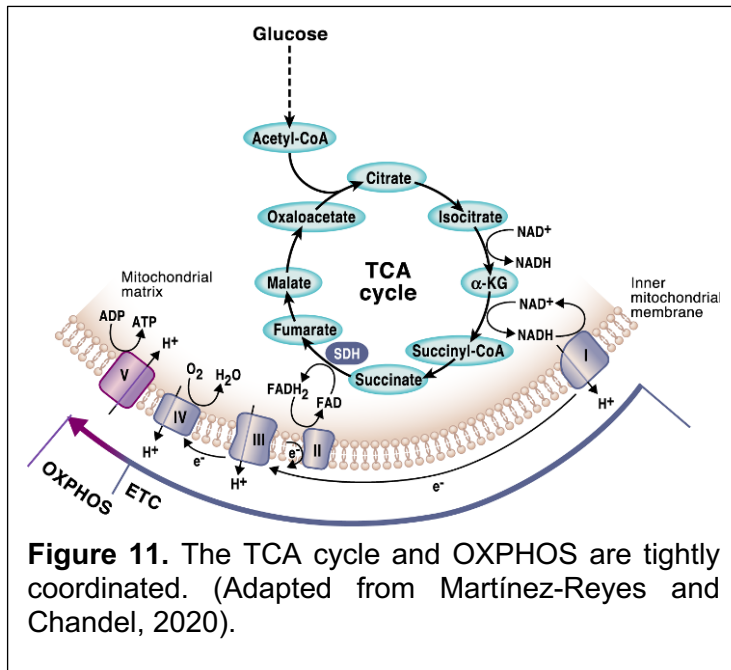
likely, related to Asgard taxa in Archea (Spang et al., 2015). It is suggested that direct mitochondrial ancestors were aerobic bacterium with the ability to reduce O<sub>2</sub> into H<sub>2</sub>O, which conferred a selective advantage for the host proto-eukaryotic cell in aerobic environments (Wang and Wu, 2014). The transition from  $\alpha$ -proteobacterium into mitochondria involved several changes throughout evolution where genome reduction and migration of prokaryotic genes into the nucleus stand as the most notorious (Dacks et al., 2016; Roger et al., 2017).

Modern mitochondria, like their bacterial ancestors, have two structurally and functionally different membranes, the outer membrane (OM) and the inner membrane (IM), with an interspace between them and a matrix compartment (Nunnari and Suomalainen, 2012). Another evidence of their bacterial origin is that mitochondria possess their own circular genomes, mitochondrial DNA (mtDNA) (Lecrenier et al., 1997; Saccone, 1994). Proteomic and bioinformatic analysis have shown that mammalian mitochondria contain ~1000 proteins (Gaston et al., 2009). However mtDNA only encodes for 13 proteins, all of them involved in OXPHOS (Mootha et al., 2003). In addition, proteins and lipids from the cytosol are imported and sorted into their corresponding mitochondrial compartment (Neupert and Herrmann, 2007; Schmidt et al., 2010). Taken together, this is evidence of the mitochondrial dependence on nucleus, cytosolic ribosomes, and other organelles in order to sustain cellular homeostasis.

Mitochondria host oxidative metabolism and most of ATP production, thus they are considered as the “power house” of the cells (Vakifahmetoglu-Norberg et al., 2017). Also, mitochondria have a critical role in apoptosis by regulating the release of proapoptotic factors

into the cytosol (Liu et al., 1996). In addition, metabolism of amino acids, lipids and nucleotides, biosynthesis of iron-sulfur (Fe/S clusters and cofactors), import and processing of precursor proteins that are synthesized on cytosolic ribosomes, gene expression, epigenetic regulation,  $\text{Ca}^{2+}$  homeostasis, inflammation and antiviral immune response have also been related to mitochondrial function (Baughman et al., 2011; Mills et al., 2017; Nunnari and Suomalainen, 2012). Mitochondrial matrix is also the site of ROS production, which are necessary for cellular homeostasis. A tight regulation of ROS generation is important, since ROS overproduction can have deleterious effect on the cells, such as DNA damage or lipid and protein oxidation (Lee and Wei, 2005). Hence, it is not surprising that mitochondrial function must be tightly regulated.

### 1.5.1. Mitochondria and oxidative metabolism



**Figure 11.** The TCA cycle and OXPHOS are tightly coordinated. (Adapted from Martínez-Reyes and Chandel, 2020).

Mitochondria are the providers of most of the ATP consumed by the cell through OXPHOS coupled to the electron transport chain (ETC) (Figure 11). In aerobic conditions, fatty acids, carbohydrates, and proteins are degraded into smaller units and metabolites that converge at the level of the tricarboxylic acid (TCA) cycle.

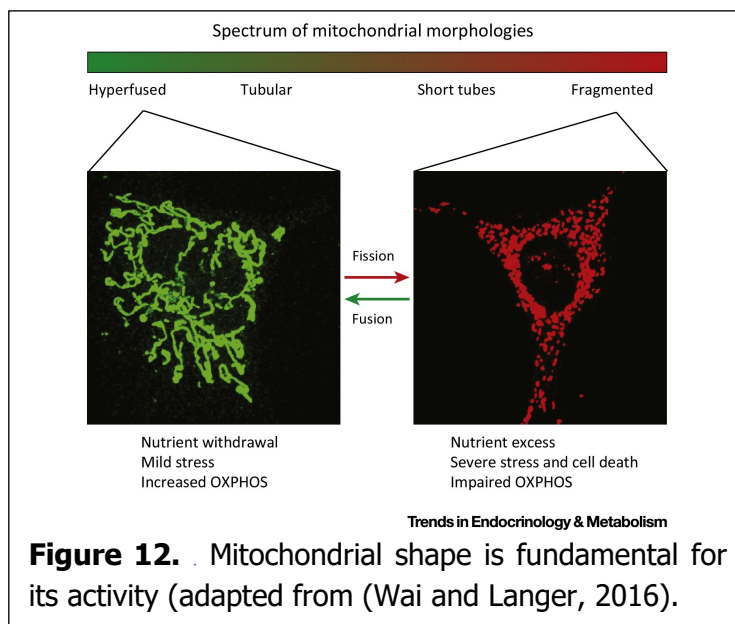
While there are many pathways to integrate catabolites into the TCA cycle, most of them converge in the formation of acetyl-CoA, which is considered the crucial fuel for TCA cycle (Martínez-Reyes and Chandel, 2020; Martínez-Reyes et al., 2016). This metabolic pathway is the final step for the oxidation of carbon molecules for generation of energy. The oxidative reactions in this cycle use two electron acceptors to store energy: nicotinamide adenine nucleotide (NAD<sup>+</sup>) and flavin adenine dinucleotide (FAD<sup>+</sup>). When loaded with electrons, NADH and FADH<sub>2</sub>, can donate their electrons to the ETC for further ATP synthesis. The enzymes in charge of TCA cycle reactions reside in the mitochondrial matrix, therefore the import of TCA cycle intermediates is crucial for maintaining cell homeostasis. Several intermediates of TCA cycle can serve as precursors of many biosynthetic pathways (Nelson and Cox, 2001). Therefore, TCA cycle activity can influence on the levels of amino acids, lipids and carbohydrates (Owen et al., 2002).



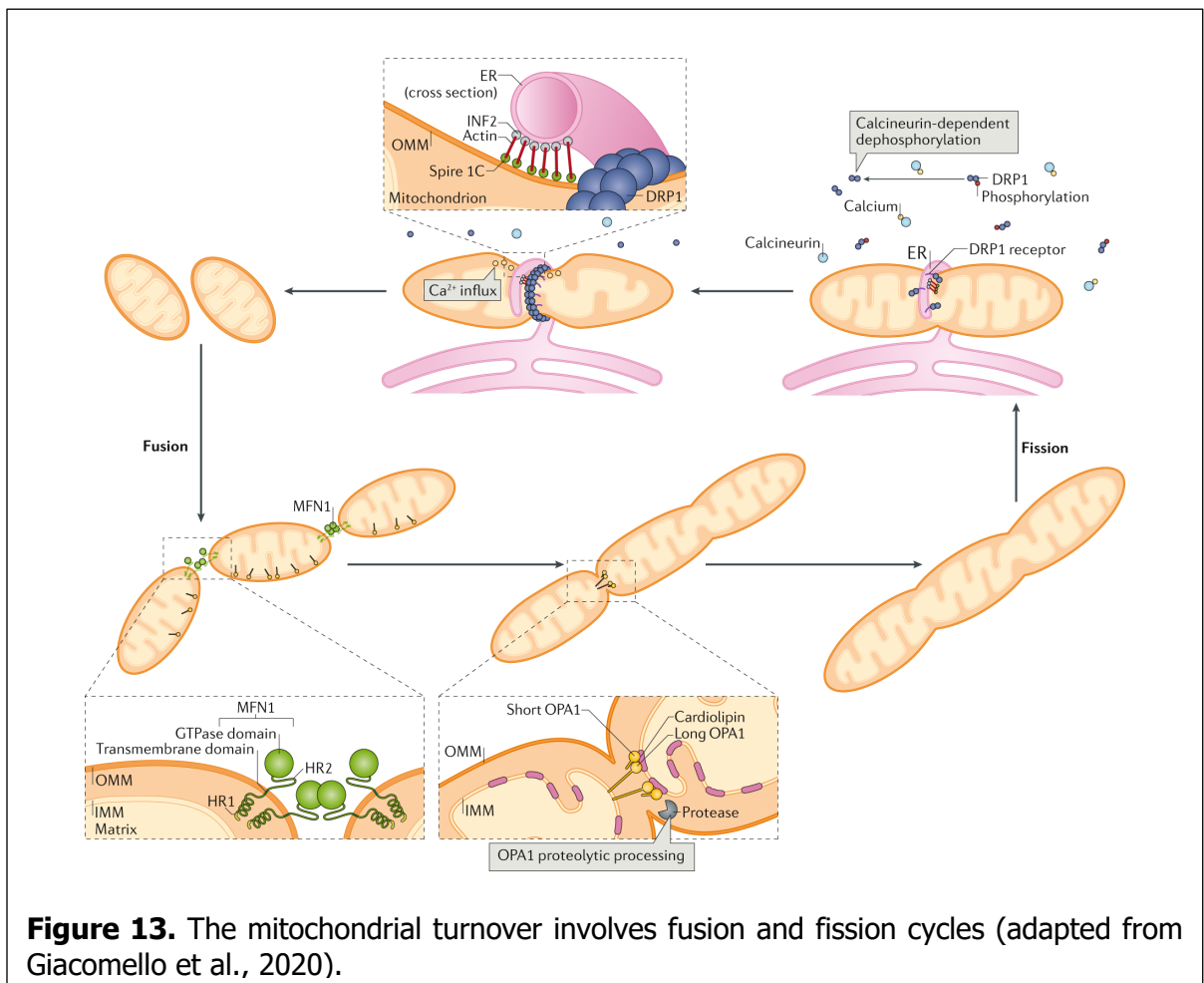
The final part of OXPHOS is ATP synthesis, achieved through the formation of a proton gradient ( $\Delta\text{pH}$ ) between the intermembrane space and the mitochondrial matrix, which creates the mitochondrial membrane potential ( $\Delta\psi\text{m}$ ) (Zorova et al., 2018). Through  $\Delta\text{pH}$ , ETC drives the formation of a proton driving force, which in turn, sustains the activity of ATP-synthetase, also known as complex V. This process allows ATP-synthetase to phosphorylate ADP to produce ATP (Cheng and Ristow, 2013). ETC is also the main source of ROS. ROS are important signal transducers; however their imbalance can trigger lipid, DNA and protein damage, in a process known as oxidative stress (Schieber and Chandel, 2014). Thus, mitochondria are constantly communicating with their surroundings, through changes in the  $\Delta\psi\text{m}$ , release of specific ETC factors or TCA intermediates into the cytoplasm, and also through the generation of ROS (Gaude and Frezza, 2014; Nunnari and Suomalainen, 2012; Venditti et al., 2013).

### 1.5.2. Mitochondrial dynamics

Mitochondria are highly mobile and interconnected in a complex network (Ni et al., 2015). Mitochondrial dynamics is defined as the capacity of these organelles to change its size, shape and distribution by modulating fission and fusion events in response to metabolic demands (Figure 12) (Romanello and Sandri, 2016).



Mitochondrial turnover through balance of fission and fusion, allow them to share membranes, metabolites, solutes, proteins and maintain their electrochemical gradient, while isolating aged, damaged or non-functional mitochondria (Figure 13) (Tilokani et al., 2018). Therefore appropriated balance between fission and fussion events is critical for sustaining mitochondrial function (Ploumi et al., 2017). Besides, disruption of mitochondrial fusion affects mtDNA quality and thus affects OXPHOS, probably due to accumulation of mutations and uneven mtDNA distribution between organelles (Chen et al., 2007) . Mitochondrial network also responds to cell cycle stage, typically mitochondria show a tubular-like elongated morphology during G1-S phase (Mitra et al., 2009). On the other during mitosis, a fragmented



**Figure 13.** The mitochondrial turnover involves fusion and fission cycles (adapted from Giacomello et al., 2020).

network composed of smaller mitochondria results in a more even distribution of these organelles among daughter cells (Figure 13) (Taguchi et al., 2007).

A more fused mitochondrial network is correlated with enhanced OXPHOS activity, thus an increase in ATP production (Schrepfer and Scorrano, 2016). On the other hand, inhibition of fusion triggers impaired OXPHOS, loss of  $\Delta\psi_m$ , accumulation of mtDNA mutations and oxidative stress (Liesa and Shirihai, 2013). Fusion events promote content exchange and dampen the deleterious effect of mtDNA mutations. Interestingly, genetic inhibition of fusion promotes individual heterogenic properties among mitochondrial population, such as differences in  $\Delta\psi_m$  level (Chen et al., 2010). Therefore, it can be assumed that fusion is important for a homogeneous distribution of mitochondrial contents to reduce variability from one single mitochondria to another.

Nutrient availability drives mitochondria fragmentation, whereas starvation results in the formation of an elongated, tubular-like mitochondrial network. Fragmented mitochondrial networks are associated with higher mitochondrial uncoupling, an event that triggers mitophagy (Gilkerson et al., 2012; Twig et al., 2008) (discussed in detail below), in order to avoid energy waste and damage resulted from non-functional mitochondria (Smirnova et al., 2001).

Taken together, mitochondria are highly dynamic organelles that can take different sizes and shapes. Thus, the balance between events of fission and fusion are critical to sustain proper mitochondrial function.

#### **1.5.2.1. Mitochondrial fusion**

Mitochondrial fusion is the merge of two mitochondria into one through a series of tightly regulated events starting with the close physical interaction of two mitochondria. Then, fusion of the OMM and subsequent fusion of IMM (Chan, 2020).

Fusion events are under the control of 3 large GTPases from the dynamin superfamily, the OMM residing: Mitofusin1 (MFN1) and MFN2, and the IMM specific Optic Atrophy 1 (OPA1). MFN1 and MFN2 are both anchored to the OMM and required for tethering two subjacent mitochondria either by forming homo or heterodimers (Mishra and Chan, 2014). MFN1 and MFN2 seem redundant, normal mitochondrial morphology can be rescued by overexpression of either MFN1 or MFN2 in MFN1/2 *KO* in MEF cells (Chen et al., 2003). Although MFN2 is also implicated in mitochondria-endoplasmic reticulum contact sites, a site for mitochondrial fusion (de Brito and Scorrano, 2008). Interestingly, MFN2 can also be a substrate of PARKIN for UPS degradation during mitophagy (Chen and Dorn, 2013). However, many of the details of their redundancy and other functions beyond mitochondrial morphology regulation remain controversial.

The IMM-residing protein OPA1 orchestrates the fusion of the IMM, and its loss triggers mitochondrial fragmentation, whereas its overexpression induces mitochondrial elongation

(Olichon et al., 2002). Unlike MFNs it is needed in only one side of the IMM to exert its GTPase activity. In humans, at least eight splicing variants of OPA1 have been described (Delettre et al., 2001). In addition, OPA1 can suffer proteolytic cleavage by two different IMM-residing peptidases: OMA1 and YMEIL, which originates Long-OPA1 (L-OPA1) isoform anchored to the IMM, and soluble short-OPA1 (s-OPA1), respectively. L-OPA1 is correlated with maintenance of mitochondrial fusion (Head et al., 2009), whereas S-OPA1 is associated with uncoupled mitochondria and enhanced fragmentation. However, their exact roles and their distribution among different tissues remain an active area of study.

#### **1.5.2.2. Mitochondrial fission**

Mitochondria cannot generate *de novo*, therefore all mitochondria come from preexisting organelles through processes of fission from a bigger mitochondrial network. One of the most extensively studied and central players of mitochondrial fission is the highly conserved GTPase Dynamin related protein 1 (DRP1)(Smirnova et al., 2001).

In contrast to proteins of the fusion machinery, DRP1 lacks mitochondrial targeting sequences (MTS) and is thus mostly found in the cytosol (Serasinghe and Chipuk, 2016). Therefore, the initial step for mitochondrial fission is the recruitment of DRP1 to the OMM where it oligomerizes in a ring-like structure specialized to constrict mitochondria for further scission(Tilokani et al., 2018).

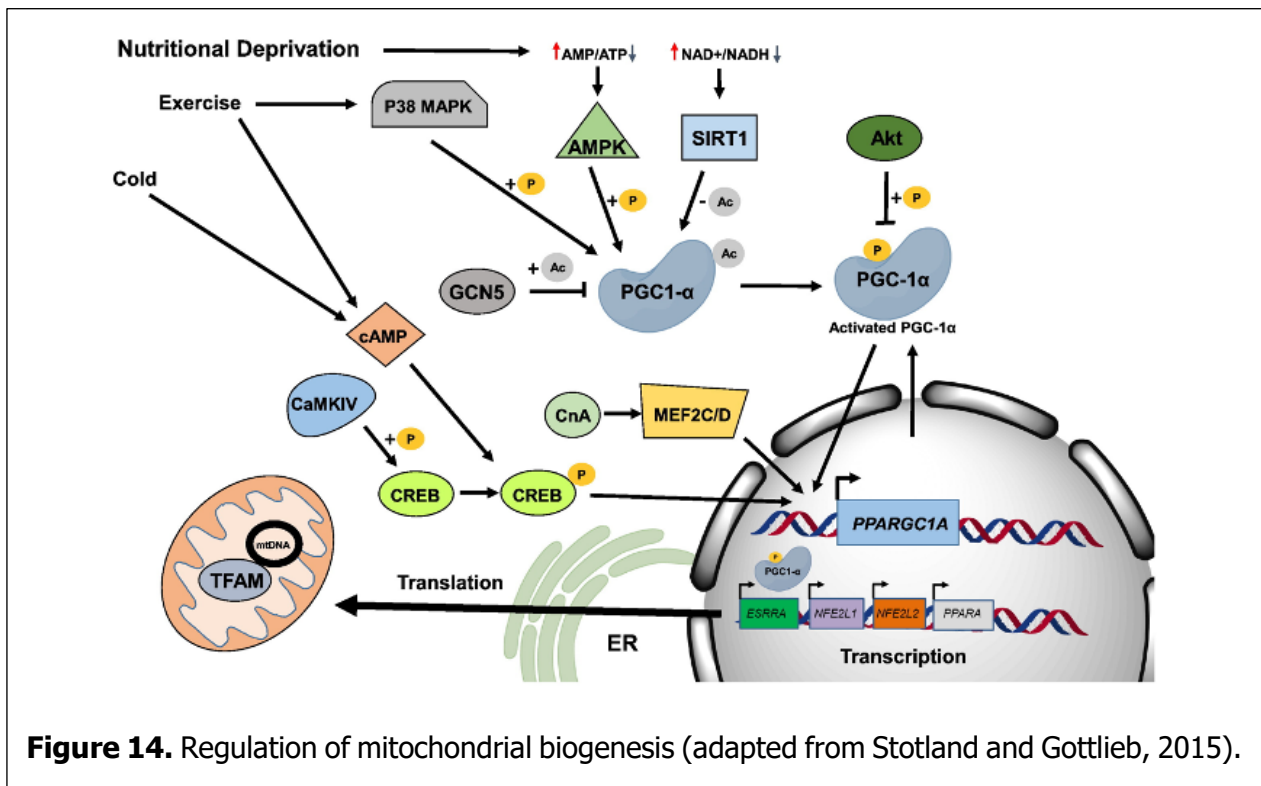
In yeast, Dnm1 (yeast homologue of mammalian DRP1) recruitment to the OMM depends on the Fis1 protein, an OMM-anchored protein adaptor, besides it also needs Mdv1 and Caf4 as receptors. However, there are no known mammalian orthologues for Mdv1 and Caf4. Recently, it was reported that mammalian Fis1 is not directly involved in mitochondrial dynamics, however it may have a role in mitophagy, although more details of this processes remain to be elucidated (Pryde et al., 2016). For mammals, DRP1 interact with the OMM-anchored proteins mitochondrial fission factor (MFF) and mitochondrial dynamics protein 49 (MiD49) and MiD51, which act as a receptor for DRP1 in the OMM (Osellame et al., 2016).

DRP1 modulation of mitochondrial morphology is tightly controlled by upstream pathways that integrate upstream signals to react accordingly. DRP1 activity is under the control of posttranslational modifications, particularly the phosphorylation at Ser616 and Ser637 (Kashatus et al., 2015), which are correlated with enhancement of fission and fusion, respectively. In accordance phosphorylation of DRP1 at Ser616 is correlated with more fragmented mitochondria, whereas Ser637 correlates to an increase in mitochondrial fusion (Cereghetti et al., 2008; Cribbs and Strack, 2007; Han et al., 2008).

### **1.5.3. Mitochondrial biogenesis**

Healthy mitochondria rely on mechanisms that regulate the equilibrium between mitochondrial biogenesis and degradation. Since mitochondria cannot be synthesized *de novo*, new organelles are formed from preexisting ones through a interaction with the already existing network, *via* both fusion and fission events (Ni et al., 2015). Mitochondrial biogenesis

also needs the replication of mtDNA, transcription and translation of mtDNA encoded genes, import of lipids and nuclear-encoded proteins (Popov, 2020; Scarpulla, 2008; Scarpulla, 2011). Therefore, the addition of new mitochondrial biomass requires the tight coordination of two separated genomes (Pickles et al., 2018). After transcription and translation, nuclear-encoded mitochondrial proteins are targeted to the mitochondria and imported to different mitochondrial sub-compartments (Priesnitz and Becker, 2018; Schulz and Rehling, 2014).



The peroxisome proliferation-activated receptor gamma coactivator 1 $\alpha$  (PGC-1  $\alpha$ ) is one of the master regulators of mitochondrial biogenesis. (Shao et al., 2010) The nuclear respiratory factor 1 (NRF1) and NRF2 and nuclear factor erythroid 2 related factor 2 (NF2L2) are the key effectors of mitochondrial biogenesis and both are targets of PGC-1  $\alpha$ . They trigger

the transcription of a variety of nuclear genes involved in mitochondrial functions, such as cytochrome C, involved in the ETC; the translocase of outer mitochondrial membrane (TOM) 34, a part of protein import machinery; superoxide dismutase 1 (SOD), necessary for ROS scavenging (Dhar et al., 2008; Ongwijitwat et al., 2006). NRF1/2 and NF2L2 can also activate mtDNA transcription factors (Ploumi et al., 2017). NRF1/2 and NF2L2 are targets of master regulator of mitochondrial biogenesis, (Figure 14). The overexpression of PGC-1  $\alpha$  can increase the number and function of mitochondria, and its loss impairs mitochondrial function (Lehman et al., 2000). In addition, PGC-1  $\alpha$  also enhances the expression of the transcription factor A, mitochondrial (TFAM), which in turn promotes the transcription and repair of mtDNA (Kang et al., 2007). Overexpression of PGC-1  $\alpha$  leads to the upregulation of more than 150 genes that encode mitochondrial proteins involved in fatty acid oxidation, TCA cycle, OXPHOS, mitochondrial ribosomal machinery and membrane transport proteins. mTORC1 can also activate mitochondrial biogenesis through phosphorylation of 4E-BP1 and S6K1, which leads to stimulation of translation of nuclear encoded mitochondrial proteins and promotes the transcription of microRNAs related to mitochondrial functions (Morita et al., 2013). Another direct target of mTORC1, the TFEB transcriptional factor, which is involved in lysosomal biogenesis and autophagy, also binds the PGC1- $\alpha$  promoter and trigger its activation (Settembre et al., 2013).

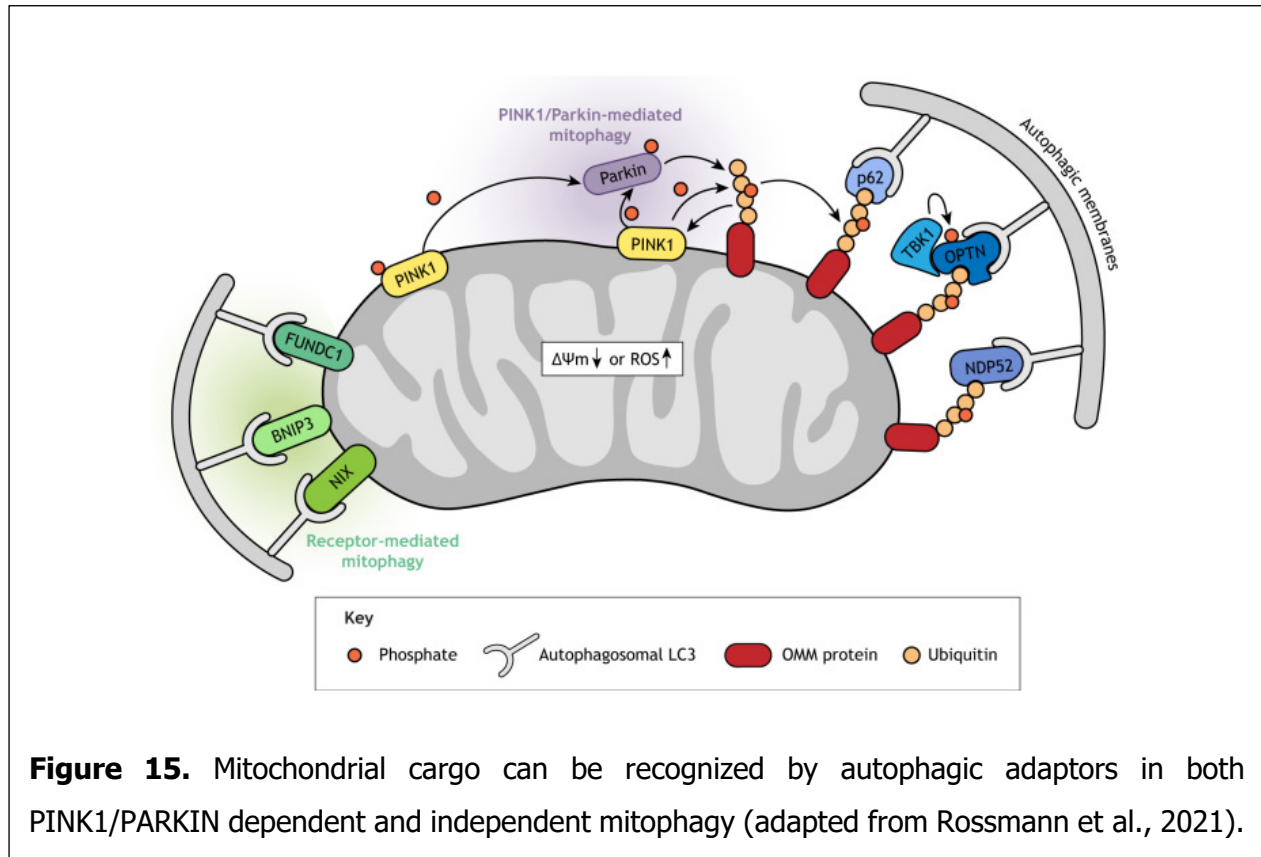
Mitochondrial biogenesis responds to starvation, hypoxia, or intense physical activity. AMPK and SIRT1, are activated when energy levels are low (Cantó et al., 2010; Herzig and Shaw, 2018). AMPK and SIRT1 can modulate mitochondrial mass, OXPHOS rate and ATP



production via PGC1-  $\alpha$  (Jeninga et al., 2010). The human PGC-1  $\alpha$  promoter contains binding sites for ROS-sensitive transcription factors, including nuclear factor-kB, and p53 (Hood et al., 2015).

### 1.5.4. Selective degradation of mitochondria

Two opposing processes are crucial for mitochondrial quality control: biogenesis and the selective removal of damaged mitochondria, also known as mitophagy. The adequate interrelation of such processes is known as mitochondrial turnover which is influenced by stress, metabolic changes, nutriment scarcity or developmental processes (Youle and Narendra, 2011). Depending on the physiological context, mitophagy can be classified as basal, programmed or stress induced. Basal mitophagy, refers to the normal mitochondrial turnover



that recycles old and damage organelles to ensure continuous function of mitochondrial network. When extracellular stress signals compromise mitochondrial function cells can activate stress-induced mitophagy pathways (Ni et al., 2015; Ploumi et al., 2017; Romanello and Sandri, 2016).

In metazoans and mammals targeting of damaged mitochondria for specific autophagic degradation involves two proteins related to the onset of recessive Parkinson disease: the PTEN-induced putative kinase 1 (PINK1), a mitochondrial localized kinase and PARKIN - a cytosolic E3 ubiquitin ligase (Palikaras et al., 2017; Pickles et al., 2018). Mitochondrial dynamics, biogenesis and transport rely on the PINK1/PARKIN pathway to ensure degradation of damaged organelles (Figure 15). Under normal conditions, PINK1 is imported into IMM through the TOM and TIM complexes (Sekine and Youle, 2018), where it is cleaved by matrix processing peptidase (MPP) and the inner membrane protease PINK1/PGAM5-associated rhomboid-like protease (PARL) (Deas et al., 2011). After cleavage, the truncated form of PINK1 is degraded by the UPS (Yamano and Youle, 2013).

When mitochondrial protein import is affected, *i.e.* during mitochondrial membrane potential dissipation, PINK1 is stabilized on the OMM in a complex that requires TOM7, 20, 22, 40 and 70 (Sekine and Youle, 2018). Upon homodimerization on the mitochondrial surface, PINK1 activates itself by autophosphorylation, which triggers the recruitment of PARKIN to the OMM (Narendra et al., 2008; Okatsu et al., 2013). Phosphorylation of PARKIN at Ser65 in by PINK1 leads to the activation of its ubiquitin ligase E3 activity (Kondapalli et al., 2012). The

kinase activity of PINK1 is essential for PARKIN translocation to mitochondria (Park et al., 2006). Besides PARKIN activation, PINK1 also exerts its kinase activity on preexisting Ub molecules on the mitochondrial surface (Okatsu et al., 2015). Inactive PARKIN binds phospho-Ub which then facilitates its activation by PINK1. Activated PARKIN creates a feed-back loop in order to create poly-Ub chains in other substrates of PINK1 (Ordureau et al., 2014).

Five adaptors have been linked to mitophagy in mammalian cells: the sequestosome 1 (SQSTM1) or p62, NBR1, NDP52, optineurin (OPTN) and TAX1BP1. Cells lacking all five receptors were unable to activate the PINK1/PARKIN pathway upon mitochondrial damage but were rescued by overexpression of NDP52 and OPTN. NDP52 and OPTN, which binds to the phospho-Ub chains on the OMM of depolarized mitochondria are essential receptors for autophagic machinery (Lazarou et al., 2015). Mitochondrial uncoupling promotes the PINK1/PARKIN-mediated activation of TANK-binding kinase 1 (TBK1), which phosphorylates OPTN at Ser177 resulting in increasing affinity towards LC3 (Heo et al., 2015), thus creating a feedback loop between PINK1/PARKIN activation and the capacity of autophagic receptors for recognition of damaged mitochondria. PINK1 and PARKIN contribute to creation an amplification loop of mitophagy signals.

The PINK1/PARKIN pathway is involved in other mitochondrial quality control mechanisms - mitochondrial dynamics and motility. After mitochondrial uncoupling, MFN2 is phosphorylated in PINK1-dependent manner, and serves as PARKIN receptor in the OMM of damaged mitochondria (Chen and Dorn, 2013). PARKIN can exert its E3 ubiquitin ligase activity

towards MFNs, which triggers their degradation via the UPS (Tanaka et al., 2010). PINK1/PARKIN mediated clearance of MFNs triggers the disruption of ER-mitochondria contact sites, thereby promoting isolation of damaged organelles for degradation (McLelland et al., 2018). PINK1 also triggers the indirect activation of DRP1 which promotes fission of dysfunctional mitochondria (Pryde et al., 2016). DRP1 may serve as an insulator for damaged mitochondria, which enhances their autophagic degradation (Burman et al., 2017).

Hundreds of proteins can be ubiquitinated via PARKIN, with most of them located at the OMM (Sarraf et al., 2013). Thus, ubiquitinated proteins create feedback loops to amplify mitophagy signals, which serve both as substrates for PARKIN and autophagy receptor proteins.

Mitochondrial proteins can act as mitophagy receptors by direct binding to autophagosomes for degradation through their LC3-interactin regions (LIR) motifs in PARKIN/PINK1 independent fashion (Gatica et al., 2018) (Figure 15). Among OMM proteins identified as mitophagy receptors, BCL/adenovirus E1B-interactin protein 3-like (BNIP3L) also known as NIX (NIP3-like-protein X), BNIP3 (BCL2 interacting protein 3) and FUNDC1 (FUN12 domain containing protein 1) (Pickles et al., 2018).

Under hypoxic conditions BNIP3 is upregulated and stabilized in the OMM where it promotes mitochondrial degradation by interaction with LC3 through its LIR domain. Upon impaired oxidative phosphorylation or oxidative stress, the RHEB GTPase, a critical regulator of mTORC1 pathway, is translocated to the mitochondria to form a complex with BNIP3L/NIX

and LC3 and hence promotes mitophagy (Melser et al., 2013) (Bartolomé et al., 2017). This points out to a crosstalk between mitochondrial quality control and mTORC1 pathway regulation. To activate mitophagy, both BNIP3 and BNIP3L/NIX require a dimerization upon stabilization on the OMM. Although this event is independent of PINK1-PARKIN, it has been shown that BNIP3L/NIX can be ubiquitinated by PARKIN (Gao et al., 2015). BNIP3 also interacts with PINK1, which enhances PINK1 accumulation on the OMM and activates the PINK1-PARKIN mediated mitophagy (Zhang et al., 2016).

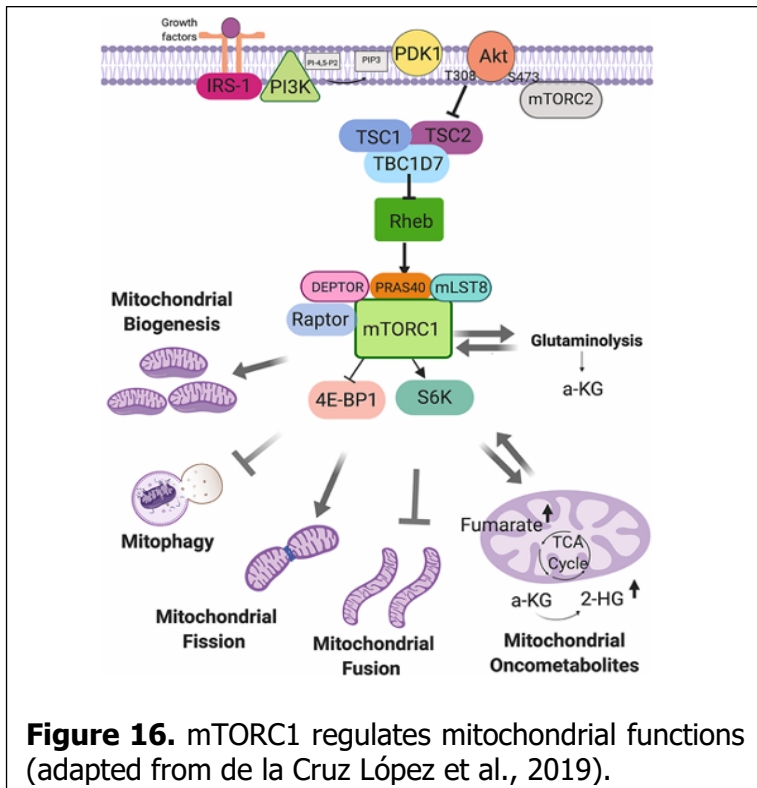
FUNDC1 is an OMM-residing protein that responds to hypoxic stress and triggers mitophagy through its LIR domain (Liu et al., 2012). FUNDC1 acts as a receptor of ULK1 complex to target mitochondria for degradation during hypoxia (Wu et al., 2014). In addition, FUNDC1 regulates mitochondrial dynamics: through an interaction with OPA1 in normoxic conditions, but not during hypoxia, which enhances interaction of FUNDC1 LIR domain with LC3 (Chen et al., 2016).

In addition to receptor proteins for mitophagy, lipids can also play a significant role in mitochondrial fate. Upon mitochondrial uncoupling, cardiolipin, an IMM phospholipid, is externalized to the OMM as an “eat me” sign, which allows its interaction with LC3 and hence promotion of engulfment of damaged mitochondria by the autophagosome (Chu et al., 2013). In addition, prohibitin 2 (PHB2) has been described as an IMM mitophagy receptor, since it is found to bind to LC3 through its LIR domains upon breaks in mitochondrial membranes due to mitochondrial damage (Wei et al., 2017).

Taken together, the complex interplay of signals and effectors reveal the existence of compensatory mechanisms for effective mitochondrial clearance. Thus, maintenance of mitochondrial function, through a balance between biosynthesis and clearance, is essential for cell survival.

#### **1.5.5. mTORC1 and mitochondrial homeostasis regulation**

Because functional mitochondria are required for survival, the cells have developed elaborated mechanisms for responding to stress and different metabolic demands. Since mTORC1 is one of the central hubs for metabolic modulation, mitochondria and mTORC1 tightly communicate (Albert and Hall, 2015).



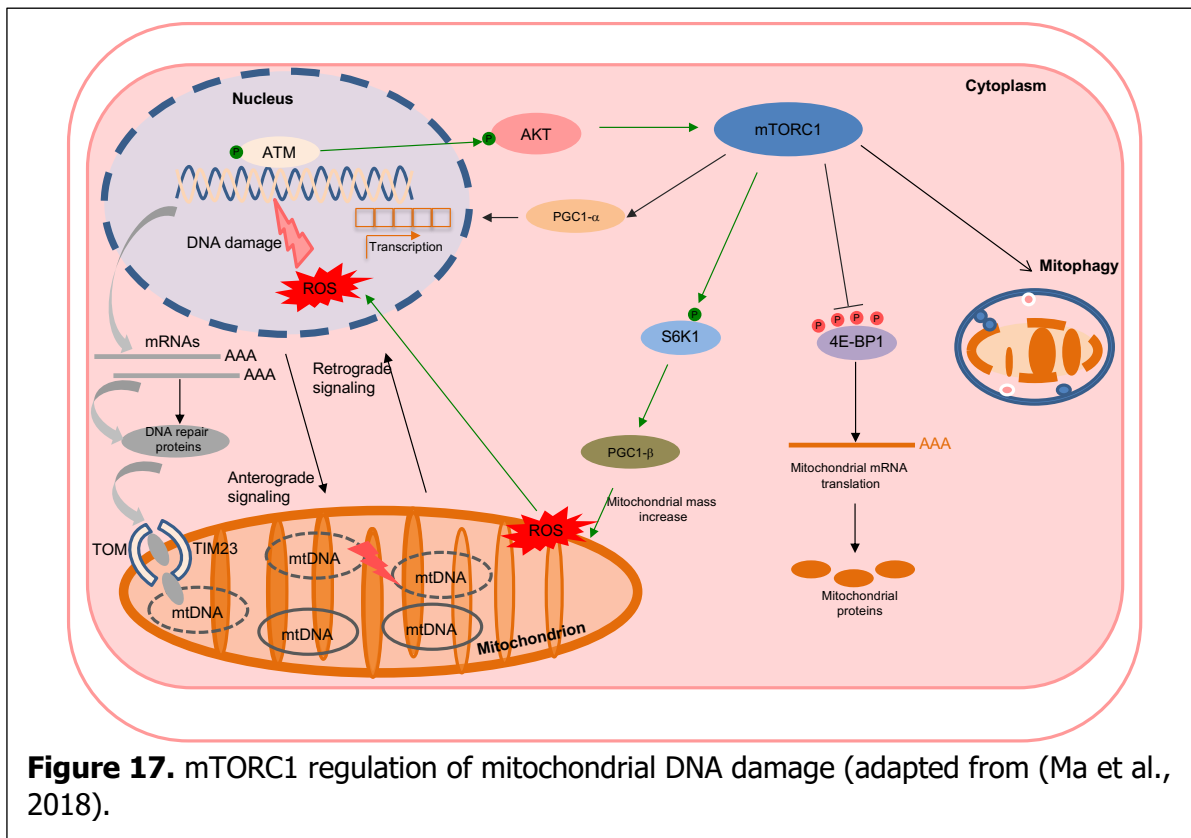
mTORC1 is essential for mitochondrial biogenesis, dynamics, phosphorylation of mitochondrial proteins, and regulation of mitophagy. In addition, the mTORC1 pathway is involved in the mitochondrial genome repair, synthesis of the proteins required for this process, and in the cellular response to mitochondrial ROS (Figure 16).

mTORC1 regulates transcription of mitochondrial genes *via* a master regulator of mitochondrial biogenesis, PGC-1- $\alpha$  transcriptional coactivator (Cunningham et al., 2007), and translation of nucleus-encoded mitochondria-related mRNAs *via* inhibition of 4E-BP1 (Figures 14 and 16, see above) (Morita et al., 2013). Mitochondrial dysfunctions that impair ATP production stimulate the activation of PGC1 $\alpha$  in an AMPK-dependent manner (Cantó et al., 2010). When energy levels are low, mTORC1 can promote mitochondrial biogenesis through the activation of ying-yang 1 (YY1) transcription factor, which interacts with PGC-1 $\alpha$  to stimulate transcription of mitochondrial genes (Cunningham et al., 2007). Translational regulation can be considered a feed-forward mechanism whereby translation of nucleus-encoded mitochondria-related mRNAs is modulated by the mTORC1/4E-BP1 pathway to induce

mitochondrial ATP production and thus provide sufficient energy for protein synthesis (Morita et al., 2015).

Although mitochondria have their own genome, most of mitochondrial proteome is encoded by nuclear DNA, therefore a tight communication with nucleus is needed in order to maintain mitochondrial quality control and supply of enzymes needed to sustain oxidative metabolism (Quirós et al., 2016). The mitochondria and the nucleus communicate through retrograde (mitochondria to nucleus) and anterograde (nucleus to mitochondria) signaling (Guaragnella et al., 2018) (Figure 17). Yeast *S. cerevisiae* grown in optimal conditions show an enhanced TORC1 activity, which is correlated to a decrease in the expression of genes of yeast electron transport chain. On the other hand, mitochondrial dysfunctions triggers mitochondria-to-nucleus communication, by enhancing the expression of mitochondrial genes to sustain metabolic activity. Both, anterograde and retrograde pathway are conserved among eukaryotes and are under the control of mTORC1.





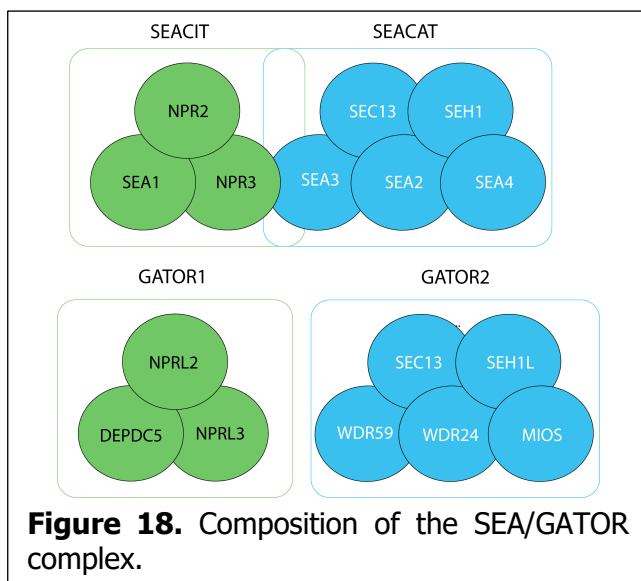
Mitochondria are particularly sensitive to ROS-mediated DNA damage (Venditti et al., 2013), especially because mtDNA lacks histones, that confer protection from DNA damage insults. DNA damage is signaled to mitochondria *via* ATM, AKT and mTORC1-mediated dependent phosphorylation cascades that promote mitochondrial mass increase through PGC-1b-driven mitochondrial biogenesis (Ma et al., 2018). When the damage is too strong mitophagy is activated (Babbar et al., 2020). ROS can modulate mTORC1 activity via oxidation of cysteine groups of mTORC1 or upstream regulators such as TSC2 (Sarbasov and Sabatini, 2005; Yoshida et al., 2011).

To sustain cell growth, mTORC1 can affect mitochondrial function through the regulation of glucose uptake. The PI3K/AKT branch of mTORC1 upstream signaling can increase glucose uptake by enhancing the expression of the glucose transporter 1 (GLUT1) on the plasma membrane (Düvel et al., 2010; Wieman et al., 2007). In certain pathological states such as cancer, mTORC1 can drive the switch to OXPHOS under hypoxic conditions, which contributes to the tumor microenvironment (Saxton and Sabatini, 2017). Moreover, the abnormal accumulation of several TCA intermediates including, fumarate, succinate or 2-hydroxyglutarate have a role in carcinogenic events and highlight the role of mTORC1 in mitochondrial events related to cancer (Gaude and Frezza, 2014).

mTORC1 is also involved in regulation of mitochondrial dynamics. The mitochondrial fission process protein 1 (MTPP1), also known as mitochondrial fission process 18 (MTPP18), is IMM located protein whose loss is related to enhanced mitochondrial fusion. In contrast, its overexpression leads to mitochondrial fragmentation (Tondera, 2005). mTORC1 modulates mitochondrial fission via 4EBP1 through the translational regulation of MTPP1. Interestingly, treatment with active-site mTOR inhibitors and simultaneous overexpression of MTPP1, leads to cell death, switching mTOR inhibition from cytostatic to cytotoxic (Morita et al., 2017).

mTORC1 hyperactivation not only impairs general autophagic initiation but also hinders selective degradation of uncoupled mitochondria by altering the expression of PINK1/PARKIN (Bartolomé et al., 2017).

Proteins, involved in upstream regulation of mTORC1 pathway have also been described as mitochondrial function modulators. For example, in yeast, deletions of members of the (Seh1-Associated) SEA complex impair development of selective mitochondrial degradation under respiratory conditions, whereas other forms of autophagy remained unaffected (Liu and Okamoto, 2018) (Ma et al., 2019). SESTRIN2, a leucine sensor upstream of mTORC1, directly interacts with mitochondria under glucose deprivation condition and has a role in PARKIN recruitment to the surface of damaged mitochondria through interaction with ULK1. (Kovaleva et al., 2020; Kumar and Shaha, 2018). As mentioned above SESTRIN2 interacts with GATOR2 complex (Parmigiani et al., 2014), whose yeast homologue, the SEA complex, has an active role in mitophagy regulation and maintenance of mitochondria and vacuole contact sites (Ma et al., 2019). Whether these or other mTORC1 pathway proteins are able regulate mitochondrial function either in a mTORC1-dependent or independent way awaits to be investigated.



## 1.6. SEA/GATOR complex

In order to coordinate its vast network, mTORC1 relies on many upstream modulators and downstream effectors. In this network the SEA/GATOR complex is the major upstream regulator of the mTORC1 pathway (Dokudovskaya et al., 2011).

The SEA complex was initially identified in yeast *Saccharomyces cerevisiae* (Alber et al., 2007; Algret and Dokudovskaya, 2012; Dokudovskaya and Rout, 2011; Dokudovskaya et al., 2011). The immunopurification of one of the components of the nuclear pore complex (NPC), nucleoporin Seh1, revealed that this protein did not only co-purify with proteins of the NPC scaffold, but also with the following four high-molecular-weight proteins with completely unknown functions at the time: Yjr138p (Iml1), Yol138p (Rtc1), Ydr128p (Mtc5) and Ybl104p (Alber et al., 2007). Four years later, in 2011, a paper that described the full SEA complex for the first time was published (Dokudovskaya et al., 2011). The four proteins, which were first observed in Seh1 pullouts in 2007, were given a common name, Sea (for Seh1-associated) and named Sea1 through Sea4, respectively. The following three other protein components completed the full SEA eight-protein complex: Sec13, Npr2 and Npr3. The proteins of the SEA complex appeared to be dynamically associated with the vacuole membrane and have a role in autophagy. The function of Iml1-Npr2-Npr3 in autophagy was also described by the Tu group that same year. Meanwhile, in 2009, Npr2 and Npr3 were shown to form an evolutionary conserved heterodimer, involved in the upstream regulation of TORC1 in response to amino acid starvation in *S. cerevisiae* (Wu and Tu, 2011). This fundamental function of the SEA complex was further confirmed both in yeast and humans by de Virgilio and Sabatini laboratories in 2013 (Bar-Peled et al., 2013; Panchaud et al., 2013a).

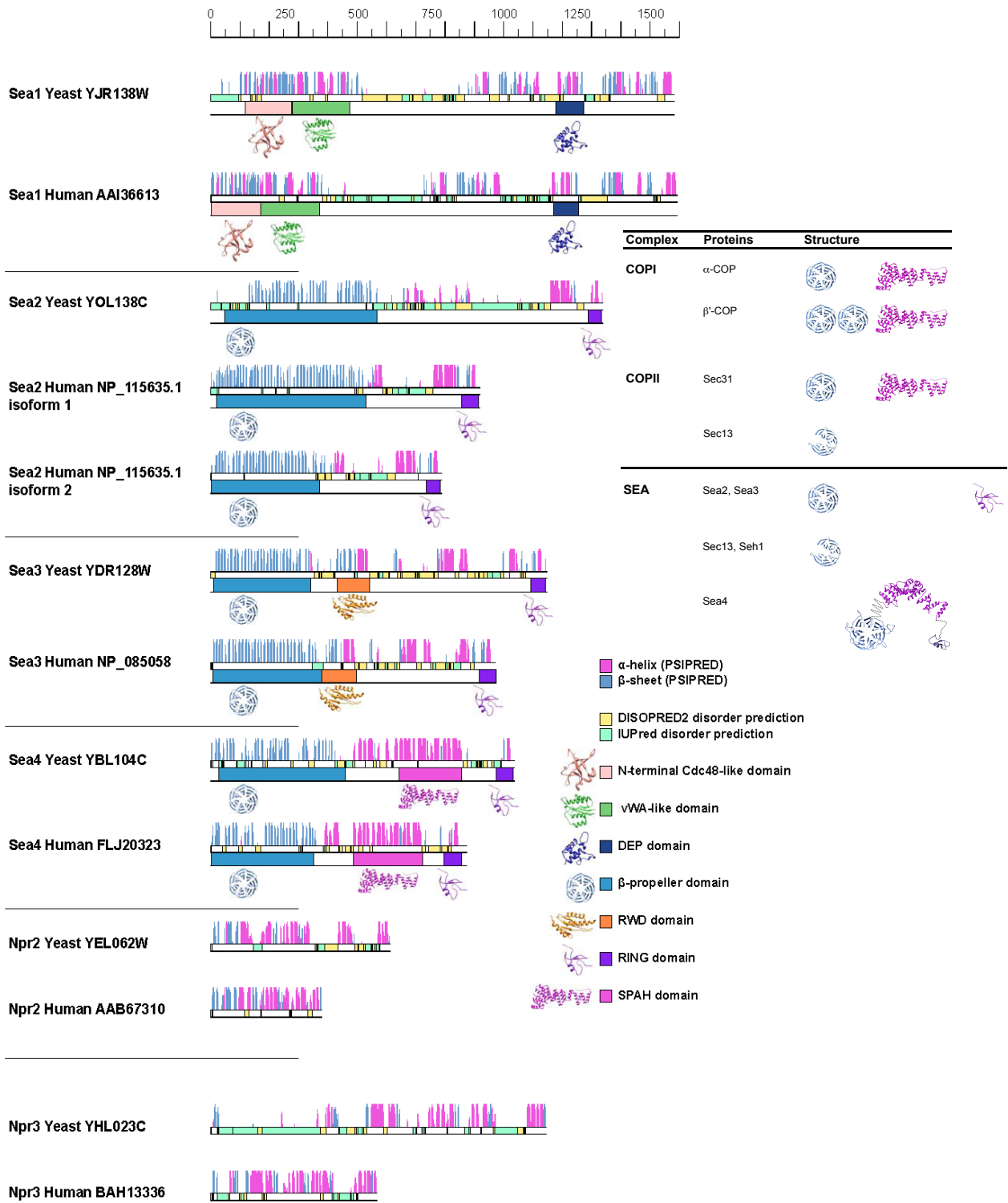
The SEA complex in *S. cerevisiae* consists of two subcomplexes, named SEACIT (SEA subcomplex inhibiting TORC1) and SEACAT (SEA subcomplex activating TORC1) (see below) (Panchaud et al., 2013a; Algret et al., 2014; Panchaud et al., 2013b). In 2013, these complexes

were characterized for the first time in humans and were re-named to GATOR1 (GTPase activating protein activity toward RAGA, see below) and GATOR2, respectively. SEACIT is composed of Iml1/Sea1, Npr2 and Npr3 (DEPDC5, NPRL2 and NPRL3 in GATOR1), and SEACAT contains Sea2, Sea3, Sea4, Seh1 and Sec13 (WDR24, WDR59, MIOS, SEH1L, SEC13 in GATOR2) (Figure 18).

### **1.6.1. Structural features of the SEA and GATOR complexes**

The overall architecture of SEA/GATOR proteins is evolutionary conserved (Dokudovskaya et al., 2011). DEPDC5 is only 10 amino acid residues longer than Iml1/Sea1, but both have an identical fold arrangement. The human orthologs of Sea2-Sea4, Npr2 and Npr3 are smaller than yeast proteins, mainly because of the deletion of protein regions, predicted to be disordered in yeast. It is quite reasonable to expect that the mammalian GATOR components repertoire would be larger compared to yeast due to the expression of alternative splicing products. For example, bioinformatical predictions revealed that WDR24 has at least two isoforms, one of which is missing about 130 amino acid residues in the N-terminal part

(Dokudovskaya et al., 2011). One of the NPRL3 isoforms that lacks the N-terminal part and is highly expressed in red blood cells has just recently been characterized (Bertuzzi et al., 2020). A splicing variant that led to exon 3 skipping in *NPRL2* was detected in an individual with familial focal epilepsy (see below) (Zhang et al., 2022).



**Figure 19.** Domain organization of SEA/GATOR components adapted from (Dokudovskaya et al., 2011). A panel on the right shows a similar domain organization of SEACAT proteins and components of coated vesicles.

SEACIT/GATOR1 members have domains, found in proteins that control the functions of small GTPases. SEACAT/GATOR2 components are enriched with domains found in coating assemblies. Seh1, Sec13 and the N-termini of Sea4 and Sea2 in SEACAT appears to form a large cluster of  $\beta$ -propeller domains. Similar arrangements of  $\beta$ -propeller domains have been described at the vertex of the evolutionarily related complexes COPI and COPII (C. Lee and Goldberg, 2010).

In yeast, SEACAT and SEACIT interact to form the full SEA complex (Algret et al., 2014). A 3D map of the *S. cerevisiae* SEA complex, suggests that SEACAT and SEACIT are connected by interactions between the N-termini of Sea3 from SEACAT and both Npr3 and Iml1/Sea1 from SEACIT (Algret et al., 2014). Similar observations have recently been made in *S. pombe*, where Sea3 anchors other GATOR2 components to GATOR1, although as expected, Sea3 was not required for the assembly of GATOR1 components (Fukuda et al., 2021). In humans, GATOR1 and GATOR2 do not form a stable GATOR complex (Bar-Peled et al., 2013), yet NPRL3 is necessary and sufficient for the interaction with GATOR2 (Shen et al., 2018).

#### **1.6.1.1. SEACAT/GATOR2**

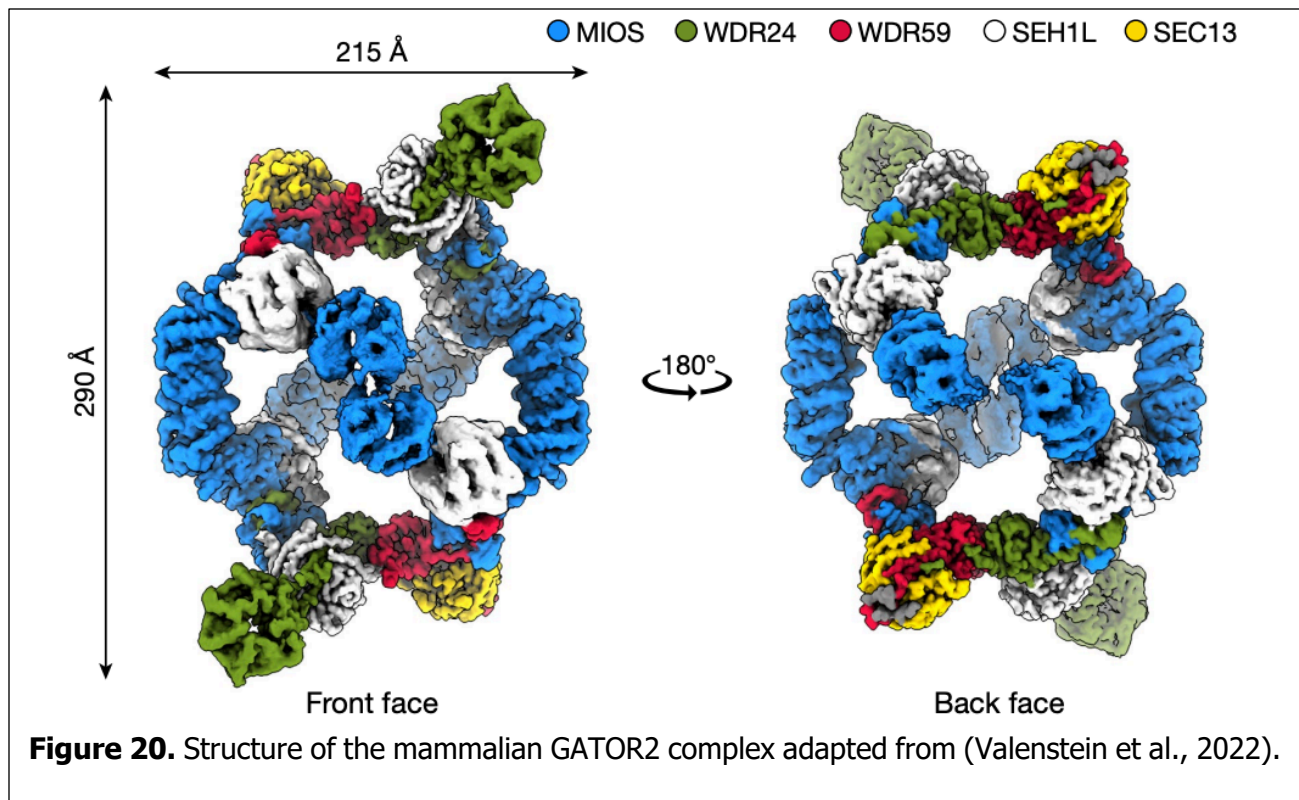
SEACAT and GATOR2 have components that moonlight between functionally unrelated complexes and are structurally connected with vesicle-coating scaffolds. The SEACAT/GATOR2 complex closely resembles the membrane coating assemblies, such as COPII vesicles and nuclear pore complexes (Alber et al., 2007; Balderhaar and Ungermann, 2013; Devos et al., 2004; Rout and Field, 2017a). It also shares common subunits with both COPII (Sec13/SEC13)

and nuclear pore complex (Sec13/SEC13 and Seh1/SEH1L). Sea4/MIOS contains N-terminal WD40 repeats arranged into a  $\beta$ -propeller structure followed by an  $\alpha$ -solenoid stretch, which is a structure that is characteristic for proteins that form oligomeric coats (*e.g.* clathrin and Sec31) in vesicle-coating complexes (Figure 19). Furthermore, every protein in SEACAT contains a  $\beta$ -propeller (and Sea3 probably has two  $\beta$ -propellers), a domain common in coating assemblies (Field et al., 2011). Lastly, there are two dimers, Seh1-Sea4 and Sec13-Sea3 (Algret et al., 2014; Dokudovskaya et al., 2011), that could be analogues to the Sec13-Sec31 dimer, which forms the structural unit of the COPII complex. These dimeric interactions in the SEACAT are most probably conserved, because it was found that the Seh1 in *Drosophila* also directly interacts with Sea4/Mio (Senger et al., 2011).

Sea4 also contains a C-terminal RING domain, which together with its  $\beta$ -propeller and  $\alpha$ -solenoid motifs, makes it closely resemble to several protein subunits of the homotypic fusion and protein sorting (HOPS) and class C core vacuole/endosome tethering (CORVET) complexes, which have been implicated in tethering of membranes prior to their fusion. HOPS and CORVET are associated with the vacuoles/lysosomes and endosomes, respectively, and play a role in endosomal and vacuolar assembly and trafficking, as well as in nutrient transport and autophagy (Balderhaar and Ungermann, 2013; Hesketh et al., 2020). Sea2/WDR24 and Sea3/WDR59 also have a C-terminal RING domain. Clusters of RING domains are associated with E3 ubiquitin ligase activity, suggesting SEACAT might have such a role. In *S. cerevisiae* the RING domains appear to be crucial for maintaining the interactions between Sea2, Sea3, Sea4 and the rest of the complex. For example, Sea4 that lacks the RING domain can only



interact with Seh1, whereas Sea2 or Sea3 without the RING domain are no longer able to interact with any of the SEACAT complex components (Algret et al., 2014). In addition, Sea3 contains an RWD domain that is enriched in  $\beta$ -sheets and common in proteins that also contain a RING motif and a  $\beta$ -propeller (Doerks et al., 2002). The RWD domain of Sea3 significantly resembles the RWD domain of the GCN2 protein, which is involved in general amino acid sensing and that of ubiquitin-conjugating E2 enzymes. In 2022 the structure of the mammalian GATOR2 complex with overall resolution of 3.7 Å has been solved by cryo-electron microscopy (Valenstein et al., 2022). To determine this structure all five components of the complex were transiently co-expressed and purified by affinity chromatography, followed by size exclusion chromatography. A 1.1 MDa GATOR2 has a two-fold symmetric cage-like architecture of approximately 290 x 215 x 160 Å and consists of octagonal scaffold formed by the following dimers: two WDR24-SEH1, two WRD59-SEC13 and four MIOS-SEH1 (Figure 20). Integration of SEH1 and SEC13 into the scaffold is made through  $\beta$ -propeller blade donation as in membrane coating complexes. The eight GATOR2 RING domains assemble into four heterodimeric C-terminal domain (CTD) junctions that hold the structure together. CTD dimers link MIOS to WDR24 and WDR59. GATOR2 that lacked the MIOS CTD failed to incorporate either WDR24, WDR59 or SEC13. RING domains of WDR24, WDR59 and MIOS form portions of larger zinc-binding domains that dimerize to construct the scaffold, impairing any potential role of the RING domains in ubiquitin ligase activity.



The presence of the same folds and fold arrangements in the SEACAT/GATOR2 complex and in coating and tethering assemblies, and the fact that they contain the same “moonlighting” components, are suggestive that these complexes share a common evolutionary origin. The majority of intracellular membranes are likely a result of evolutionary expansion of an ancestral membrane-curving module – termed the “protocoatomer” complex (Devos et al., 2004; Field et al., 2011). The SEACAT/GATOR2 complex is a member of the coatomer group, and its existence thus provides further evidence that an expansion of the protocoatomer family underpins much of the functional diversity of the endomembrane system.

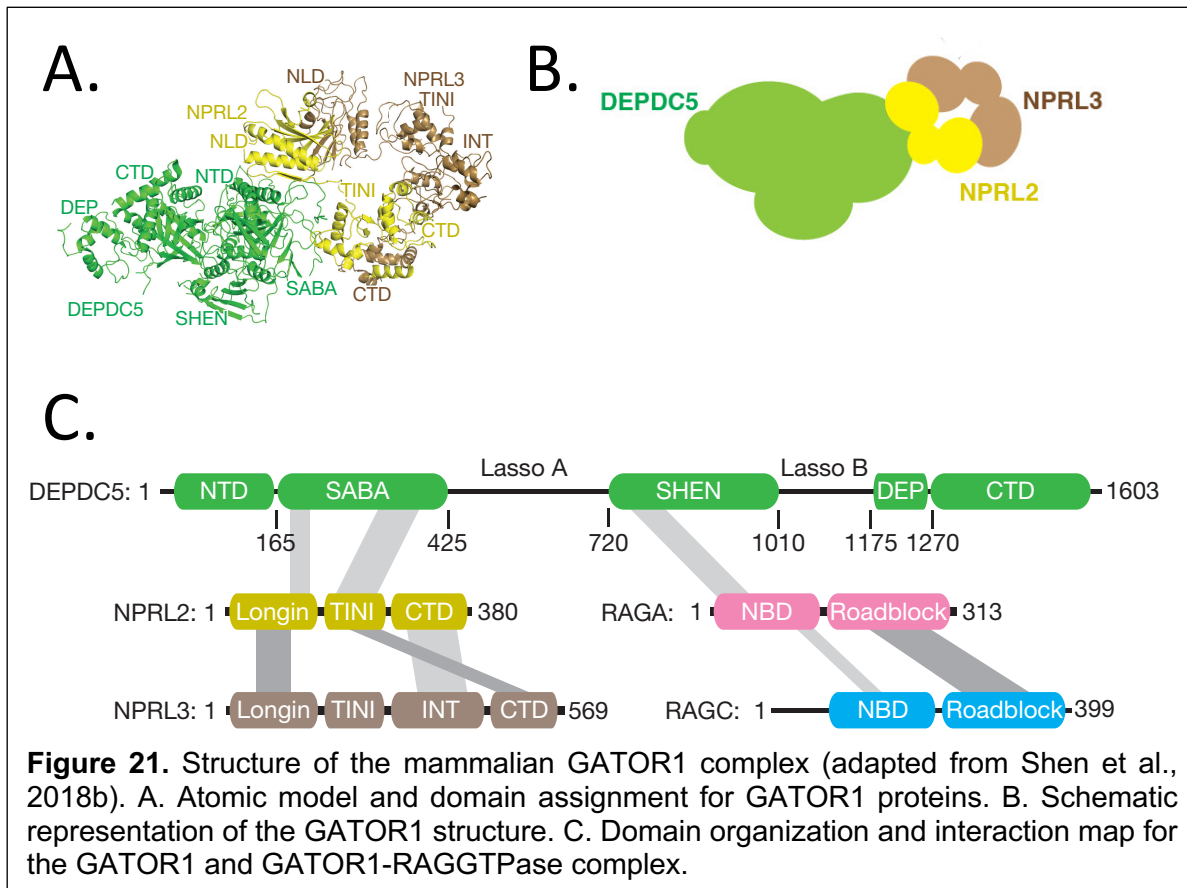
#### 1.6.1.2. SEACIT/GATOR1

The structural profile of the SEACIT/GATOR1 subunits is completely different (Figure 21). Npr2/NPRL2 is a paralog of Npr3/NPRL3 (Kowalczyk et al., 2012; Neklesa and Davis, 2009)

and both proteins possess N-terminal longin domains (Levine et al., 2013; Nookala et al., 2012). Iml1/Sea1 and its human homologue DEPDC5 contain a unique composition of domains that are not found in any other proteins. SEACIT components also have PEST motifs that are often exist in rapidly degraded protein (Dokudovskaya et al., 2011). However, PEST motifs are not well preserved in mammalian orthologues and thus could be a specific feature of the yeast SEA complex. The structure of GATOR1, resolved recently by cryo-EM revealed the architecture of each GATOR1 component (Shen et al., 2018) (Figure 21). DEPDC5 has five defined domains: N-terminal domain (NTD), followed by SABA (structural axis for binding arrangement), SHEN (steric hinderance for enhancement of nucleotidase activity), DEP (Dishevelled, Egl-10 and Plekstrin) and C-terminal (CTD) domains. Interestingly, NTD, SABA and DEP domains can be found in membrane-associated proteins. For example, a domain similar to NTD exists in the SNARE chaperone Sec18/NSF, the SABA domain - in Sec23 of COPII vesicles (again returning to the theme of coating complexes). The DEP domain, which has diverse functions in signal transduction, is involved in the interactions between regulator of G protein signaling (RGS) proteins and their membrane-bound receptors, the GPCRs (Consonni et al., 2014). The DEP domain is also found in a DEPTOR subunit of mTORC1 (Caron et al., 2018).

NPRL2 and NPRL3 have similar structure with N-terminal longin domains that heterodimerize (Figure 21). C-terminal domains of NPRL2 and NPRL3 also form large contact surface. SABA domain in DEPDC5 interacts with NPRL2 TINI domain (tiny intermediary of NPRL2 that interacts (with DEPDC5)). By the way, the domain nomenclature within the GATOR1 complex created a doubtful precedent, where protein domains are named after the

first (SHEN) and the last (SABA-TINI) authors of the article that reported the structure (Shen et al., 2018).



### 1.7. Posttranslational modifications of SEA/GATOR

All the SEA and GATOR members are heavily phosphorylated and ubiquitinated (Albuquerque et al., 2008; Bretkreutz et al., 2010; Hu et al., 2019; Iesmantavicius et al., 2014; Martínez-Montañés et al., 2020). Yet, there are few studies, which explore the functional role of PTMs. Several papers, which describe the effect of ubiquitination are mainly focused on the role of this modification on protein stability. Thus, Npr2 in yeast interacts with Grr1, the F-box component of the SCF<sup>Grr1</sup> E3 ubiquitin ligase (Spielewoy et al., 2010). Moderately unstable Npr2

is stabilized in *gtr1* $\Delta$  mutants. In response to amino acids, CUL3-KLH22 E3 ubiquitin ligase induces K48 poly-Ub on multiple DEPDC5 sites leading to its degradation (Chen et al., 2018b). Accordingly, DEPDC5 levels are increased during amino acid starvation. In the rich media, NPRL3 is more resistant to proteasome degradation than NPRL2 (Ma et al., 2017). It is reasonable to expect in the following years that we will gain more information about the role of posttranslational modifications not only on the stability of SEA/GATOR members, but also on their function.

## **1.8. Function of the SEA and GATOR in nutrient sensing and responding**

One of the principal roles of SEA and GATOR as upstream regulators of mTORC1 is responding to amino acid availability (Bar-Peled et al., 2013; Panchaud et al., 2013a), although the role of both GATOR subcomplexes in glucose sensing has also been reported recently (Orozco et al., 2020).

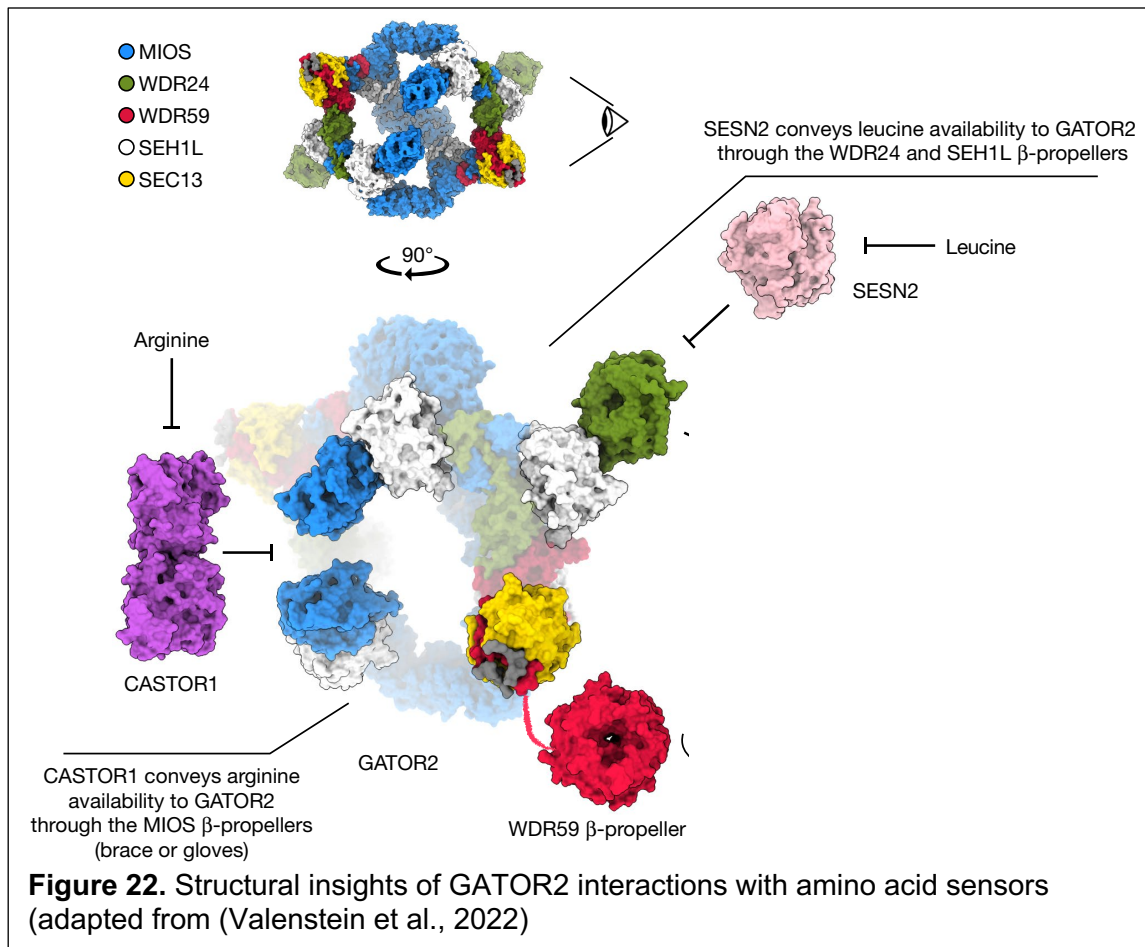
### **1.8.1. GATOR2 interactions with leucine sensors SESTRINs and SAR1B and arginine sensor CASTOR1**

Cytosolic leucine can be sensed by the proteins from the SESTRIN family (SESTRIN 1-3) (Saxton et al., 2016a; Wolfson et al., 2016; Xu et al., 2019), by small GTPase SAR1B (Chen et al., 2021) and by leucyl-tRNA synthetase (Han et al., 2012b; Kim et al., 2021; Lee et al., 2018). Arginine is sensed by CASTOR1 protein homodimer in the cytoplasm (Chantranupong et al., 2016; Saxton et al., 2016d) and by SLC38A9 together with TM4SF5 protein at the lysosomal membrane (Jung et al., 2015; Rebsamen et al., 2015; Wang et al., 2015).

During leucine or arginine starvation SESTRIN2 (Wolfson et al., 2016), SAR1B (Chen et al., 2021) or CASTOR1 respectively (Chantranupong et al., 2016; Saxton et al., 2016d) interact with and inhibit the GATOR2 complex (Figure 22). WDR24 and SEH1L are essential for interaction with SESTRIN2, but it is not known which component of GATOR2 interacts with SESTRIN2 directly (Kowalsky et al., 2020; Parmigiani et al., 2014). SAR1B directly binds MIOS, but not other GATOR2 subunits (Chen et al., 2021). WDR24, SEH1L and MIOS were sufficient for interaction with CASTOR1 (Chantranupong et al., 2016), CASTOR1 N-terminal domain is involved into direct interaction with MIOS (Gai et al., 2016). Binding sites for SESTRIN2 and CASTOR1 are located at different parts of GATOR2 (Chantranupong et al., 2016). These interactions prevent inhibition of the GATOR1 by GATOR2 (Kim et al., 2015) and as a consequence, lead to mTORC1 inhibition. Neither SESTRIN2 nor CASTOR1 interact with GATOR1 (Chantranupong et al., 2014; Chantranupong et al., 2016; Parmigiani et al., 2014).

In the presence of amino acids, interaction of leucine to the defined binding pocket in monomeric SESTRIN2 (Saxton et al., 2016a) or arginine with its binding pocket at the homodimeric CASTOR1 (Gai et al., 2016; Saxton et al., 2016d; Xia et al., 2016) results in dissociations of these sensors from GATOR2 and relieves mTORC1 inhibition (Figure 22). It is important to note, however, that SESTRIN2-GATOR2 interactions were initially observed in the cell-lines cultured in leucine-rich conditions (Chantranupong et al., 2014; Parmigiani et al., 2014), even if amino acid starvation enhanced this interaction. *In vitro* addition of leucine reduces the SESTRIN1-GATOR2 or SESTRIN2-GATOR2 interactions, but it does not affect

SESTRIN3-GATOR2 interaction (Wolfson et al., 2016; Xu et al., 2019). Interestingly, SESTRIN2 and SAR1B detect different part of leucine; SAR1B recognizes the amino group and side chain of leucine (Chen et al., 2021), while SESTRIN2 interacts with leucine’s amino and carboxyl groups (Saxton et al., 2016a).



Interactions of SESTRINs to GATOR2 depends on a cell type and physiological conditions. Thus, in skeletal muscle of rats, SESTRIN1 is the most abundant isoform, and SESTRIN2 expression is much lower relative to either SESTRIN1 or SESTRIN3. Accordingly, oral administration of leucine to fasted rats induced SESTRIN1-GATOR2 disassembly, but did not affect the interaction of other SESTRIN isoforms with GATOR2 (Xu et al., 2019). This

suggests that in rat skeletal muscle it is probably SESTRIN1 which has a primary role as a leucine sensor and leucine-induced activation of mTORC1 in skeletal muscle happens via SESTRIN1 release from GATOR2. SESTRINs-GATOR2 interactions can also be age dependent. Thus, in skeletal muscle of young pigs, SESTRIN2 is more abundant than SESTRIN1 but GATOR2 amounts are the same. Accordingly, during amino acid starvation the abundance of SESTRIN2-GATOR2 complex reduced more in younger pigs (Suryawan and Davis, 2018).

Recently GATOR2 was reported to be required for SESTRIN2-induced AKT activation and AKT translocation to plasma membrane (Kowalsky et al., 2020). GATOR2 physically bridges SESTRIN2 with mTORC2 where WDR59 interaction with mTORC2 component RICTOR is essential for the communication between GATOR2 and mTORC2, and WDR24 crucial for GATOR2-SESTRIN2 interaction. In HeLa cells GATOR2 promotes AKT activation and facilitates AKT-dependent inhibitory phosphorylation of TSC2 (Yang et al., 2020).

A recent GATOR2 structure (Valenstein et al., 2022) revealed that MIOS  $\beta$  -propeller is specifically required for GATOR2 to associate with CASTOR1, and the WDR24  $\beta$  -propeller is essential for GATOR2 to co-immunoprecipitate SESN2. Interestingly, it was not possible to generate a WRD24 variant that restores mTORC1 activity to WDR24-knockout cells and also cannot bind to SESN2. This suggests that the WDR24  $\beta$  -propeller mediates an essential GATOR2 function and that SESN2 inhibits it, probably by directly interfering with the same surface of the propeller which is crucial for the essential functions. Thus, GATOR2 uses MIOS



and WDR24  $\beta$ -propellers to receive inputs from the cytosolic amino acid sensors while WDR24 and WDR59 propellers – to transduce amino acid availability to mTORC1.

### **1.8.2. GATOR1 interaction with SAM sensor SAMTOR**

SAM sensor, SAMTOR, binds to GATOR1 during SAM or methionine deprivation, and negatively regulates mTORC1 activity (Gu et al., 2017). Which component of GATOR1 interacts with SAMTOR is currently unknown. In the presence of SAM, this metabolite occupies its binding pocket in SAMTOR, which disrupts the interaction of SAMTOR with GATOR1 promoting mTORC1 activity. SAMTOR and GATOR1 interactions are dependent on KICSTOR (see below). When SAMTOR is bound to SAM it dissociates from GATOR1-KICSTOR, inhibiting GATOR1 and promoting mTORC1 activation (Rathore et al., 2021). Methionine starvation promotes interaction between SAMTOR and GATOR1-KICKSTOR complex, but weakened the interaction between GATOR1 and GATOR2, thus leading to mTORC1 suppression (Gu et al., 2017). SAM levels can be affected by the availability of vitamin B12. Mice NPRL2 KO embryos have significantly reduced methionine levels and demonstrate phenotypes reminiscent of B12 deficiency (Dutchak et al., 2015). It is unknown if methionine can be sensed directly. Interestingly, leucine can also signal to mTORC1 through its metabolite, acetyl-coenzyme A, but in a RAG-independent and cell-specific manner (Son et al., 2019).

In the recent study Jewell laboratory investigated the potency of each amino acids to stimulate mTORC1 in MEF or HEK293 cells (Meng et al., 2020). Ten amino acids were able to re-stimulate mTORC1 and promote its lysosomal localization. Glutamine and asparagine signal

to mTORC1 through a RAG-independent mechanism via ADP-ribosylation factor ARF1. Eight amino acids (alanine, arginine, histidine, leucine, methionine, serine, threonine, and valine) filter through RAGs. While three cytoplasmic sensors for leucine, arginine, and methionine (SAM) have been identified, it is not known whether other five amino acids also have their specific sensors and whether they will interact with GATORs.

### **1.8.3. SEACIT/GATOR1 as GAP for EGO/RAG**

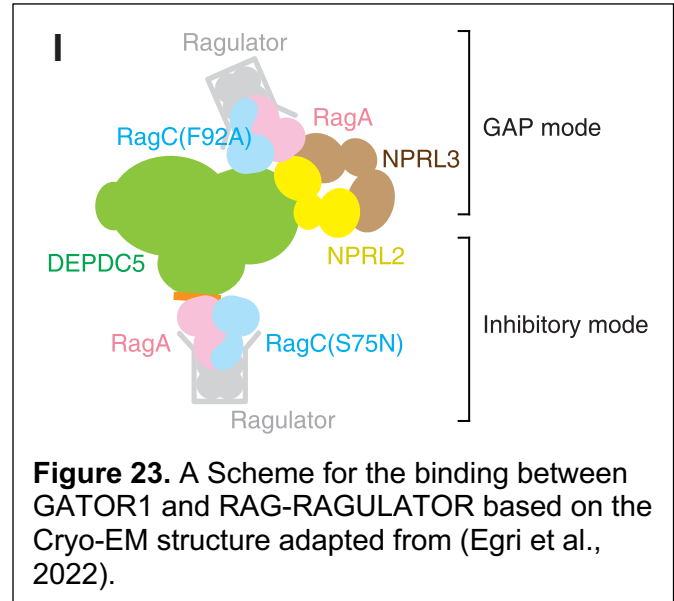
Two papers published simultaneously in 2013 reported the results that have dramatically increased the significance of SEA/GATOR complex in the regulation of mTORC1 pathway. The laboratory of Claudio de Virgilio found that in *S. cerevisiae*, the SEA subcomplex, which was subsequently named SEACIT (SEAC subcomplex inhibiting TORC1 signaling (Panchaud et al., 2013a), acts as a GAP for Gtr1 and thus inhibits TORC1 (Panchaud et al., 2013b). In the parallel study David Sabatini's laboratory characterized for the first time the human homologue of the SEA complex, and also found the GAP activity of the SEACIT analogue, which received the GATOR1 name (GTPase activating protein activity towards RAGA) (Bar-Peled et al., 2013). In both yeast and human SEACAT/GATOR2 acts upstream of SEACIT/GATOR1 suppressing its GAP activity, thus being "an inhibitor of an inhibitor", although how exactly this suppression is achieved is completely unknown.

A molecular mechanism of how SEACIT/GATOR1 acts as a GAP has been addressed in several functional and structural studies, but complete consensus of how exactly the GAP function is exerted has not yet been achieved. Indeed, in the initial study by the de Virgilio

group it was demonstrated that in *S. cerevisiae*, Iml1/Sea1 co-precipitate with Gtr1 in the presence but substantially less in the absence of other SEACIT subunits. In the *in vitro* binding and GAP assays, Iml1/Sea1 directly binds to Gtr1 and promote GTP hydrolysis in the absence of Npr2 and Npr3. GAPs often supply a catalytic amino acid residue (Arg, Asp, or Gln) in their active sites, thus forming an “arginine finger” or “Gln/Asn thumb” which can be inserted into nucleotide-binding pocket of a GTPase (Wittinghofer and Vetter, 2011). In the conserved Iml1/Sea1 domain, essential for its GAP activity (aa 929-952), a conserved Arg<sup>943</sup> was critical for GAP activity. Human DEPDC5 could partially complement TORC1 inhibition defect in *iml1Δ* cells, suggesting a conserved role of Iml1/Sea1 and DEPDC5 across the species. Therefore, when the cryo-EM structure of GATOR1 (Figure 23) and GATOR1 in the complex with RAG GTPases was solved, it came as a surprise because it revealed a very unexpected mode of interaction between GTPases and GAPs (Shen et al., 2018).

For the structural studies, GATOR1 was copurified with RAG GTPase heterodimer, containing wild type RAGA and mutant RAGC, which can bind GTP, but not GDP. In addition, this heterodimer was loaded with GDP and non-hydrolysable GTP analogue (GppNHp) to create the most favorable nucleotide-binding configuration for interaction with GATOR1. The structure demonstrated that the overall conformation of the GATOR1 in a complex with RAG GTPases is similar to a free GATOR1 (see above). SHEN domain of DEPDC5 can contact directly with a site proximal to nucleotide binding pocket of GTP analogue-bound RAGA. However, quite surprisingly, this interaction did not appear to be responsible for stimulation of GTP hydrolysis. Kinetic analysis of GTP hydrolysis of DEPDC5 alone with RAGA or NPRL2/NPRL3 dimer with

RAGA revealed that it is rather NPRL2/NPRL3, which has GAP activity. Moreover, a conserved Arg<sup>78</sup> localized on the loop of NPRL2 longin domain is the “arginine finger”, responsible for GAP activity (Shen et al., 2019a). However, this Arg<sup>78</sup> located far away and is opposite to the RAGs binding interface of DEPDC5. Moreover an earlier study from



Wang laboratory showed that amino acid stimulation enhances the interaction of RAGA with both endogenous DEPDC5 and NPRL3 (Deng et al., 2015). To explain these rather contradictory observations, a two-state model of GATOR1 interaction with RAG GTPases was proposed: in the inhibitory mode DEPDC5 SHEN domain interacts strongly with RAGs and GAP activity of GATOR1 is weak; alternatively, a low affinity interaction dependent on NPRL2/NPRL3 stimulates GAP activity. Such bi-modal activity has not been previously observed between a GAP and a GTPase. Moreover, before this study longin domains were found to be highly represented in many GEFs, where they would serve as adaptable platforms for GTPases (Levine et al., 2013). In addition, in a structure of *Chaetomium thermophilum* Mon1-Ccz1-Ypt7 complex, Mon1-Ccz1 GEF contacts its cognate GTPase Ypt7 through a face of a conserved longin domain heterodimer (Kiontke et al., 2017). NPRL2 and NPRL3 also form a heterodimer using their longin interaction domains, therefore it is quite intriguing why in case of Mon1-Ccz1 longin heterodimer supports a GEF activity, while NPRL2/NPRL3 longin domains assist to GAP function. One of the plausible explanations might involve a possibility that NPRL2-NPRL3

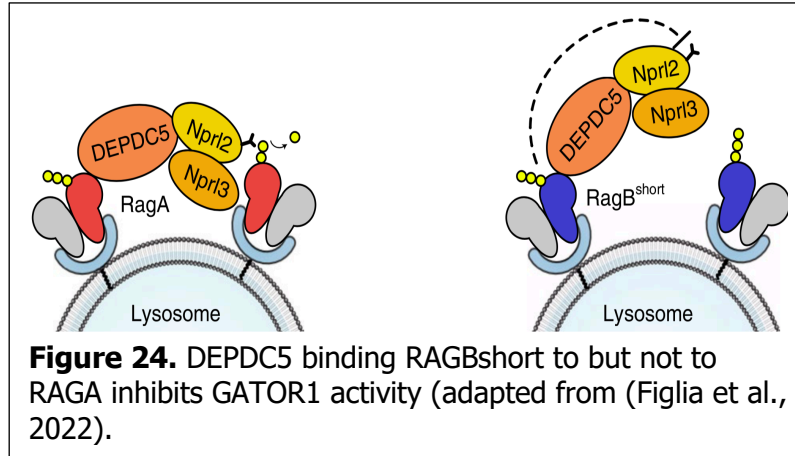
interaction with RAGs can be sterically compromised by GATOR2, because it is NPRL3, which is necessary and sufficient for interaction with GATOR2. Finally, to add even more complexity, one (and the only) study reported that NPRL2 interacts with RAGD in amino acid scarcity, and with Raptor during amino acid sufficiency to activate mTORC1 (Kwak et al., 2016). Although the authors explain this behavior by suggesting that NPRL2 may not solely exist as a part of GATOR1, these findings require more clarifications.

Recent cryo-EM structures of the human GATOR1-RAG-RAGULATOR complex with 4Å resolution provided additional elements to understand the function of GATOR1 as a GAP (Egri et al., 2022). In this work three structures have been solved: one – with the RAG-RAGULATOR occupying the inhibitory state (I mode), another – the GAP state (G mode), and the third one – both binding sites simultaneously (D mode). In all three complexes RAGA/RAGC GTPase heterodimer directly contacts GATOR1 and RAGULATOR binds to the RAGs. For the I-mode RAGA binds to the DEPDC5, for the G-mode RAGA/RAGC is located near the Longin domain heterodimer of NPRL2-NPRL3. For the D-mode two copies of RAG-RAGULATOR sandwich the GATOR1 in the middle. Thus NPRL2/NPRL3 interacts with RAGA to execute the catalytic reaction, while DEPDC5 interacts with RAGC to secure the nucleotide loading state of the GAP product. Two binding sites on GATOR1 may be mutually exclusive to one another for the RAG-RAGULATOR complexes that localize on the lysosomal membrane. GATOR1 may bind to the RAG-RAGULATOR either in the inhibitory mode or in the GAP mode, but not simultaneously. Using the auxiliary interaction formed between DEPDC5 and RAGC, GATOR1 Promotes RAGC into its GTP-bound state while stimulating GTP hydrolysis on RAGA with the arginine finger on

NPRL2. These coordinated actions ensure that only RAGA<sup>GDP</sup>-RAGC<sup>GTP</sup> is released to inactivate mTORC1.

In most studies the RAGA/RAGB and RAGC/RAGD paralogues are referred to as functionally redundant. Yet, a number of the studies indicate that it is not the case. For example, LARS GTPase binds and regulate RAGD, but not RAGC. While NPRL2 and NPRL3 preferentially bind to RAGD over RAGC in an amino acid and GTP/GDP-loading dependent manner (Kwak et al., 2016). RAGD is responsible for the regulation of mTORC1 on lysosomes, where it phosphorylates TFEB/TFE3, whereas RAGC is more loosely connected to the lysosome and more relevant for the phosphorylation of non-lysosomal mTORC1 substrate S6K (Gollwitzer et al., 2022).

RAGB isoforms RAGB<sup>short</sup> and RAGB<sup>long</sup>, which are highly expressed in neurons, change the dynamics of mTORC1 activity, causing it to persist despite low amino acid levels (Figlia et al., 2022). RAGB<sup>short</sup> inhibits GATOR1 by binding it in the inhibitory mode via DEPDC5, while RAGB<sup>long</sup> acts as a RAG isoform with low affinity for GTP and high affinity for NPRL2/NPRL3, this titrating away the GAP activity of GATOR1 (Figure 24). This creates a gradient of mTORC1 activity that could be further modulated by the relative coexpression of RAGA, RAGB<sup>short</sup> and/or RAGB<sup>long</sup>. The long RAGB isoform in the brain may allow neurons to continue critical mTORC1 activities during starvation. Interestingly, some forms of cancer seem to preferentially express RAGB rather than RAGA, which may provide advantages during nutrient deprivation.



#### 1.8.4. SEA/GATOR recruitment to the vacuolar/lysosomal membrane

In yeast both TORC1 and SEA complex localize at the vacuole membrane regardless presence or absence of amino acids (Binda et al., 2009; Dokudovskaya et al., 2011; Sturgill et al., 2008; Urban et al., 2007). Iml1/Sea1 did not require other SEA components to localize to the vacuole membrane in both budding and fission yeast (Fukuda et al., 2021; Panchaud et al., 2013a). Npr2 and Npr3 mutually depend on each other and on Iml1/Sea1 for vacuolar localization (Fukuda et al., 2021; Panchaud et al., 2013a). Deletion of any of the SEACIT components during nitrogen starvation caused re-localization of Tor1 to the cytoplasm (Algret et al., 2014).

In mammalian cells, mTORC1 is recruited to the lysosome in the presence of the amino acids, where it is fully activated by RHEB (Betz and Hall, 2013). The activation of mTORC1 by RHEB can happen at the surface of other organelles, because both RHEB and mTORC1 have been detected at the Golgi apparatus, the peroxisome, the plasma membrane and ER (Hao et al., 2017; Thomas et al., 2014; Zhang et al., 2013). Stably expressed GFP-tagged components

of GATOR1 (NPRL2 and DEPDC5) and GATOR2 (MIOS and WDR24) localizes to the lysosome regardless of amino acid levels (Bar-Peled et al., 2013; Wolfson et al., 2017). During amino acid starvation WDR24, MIOS and mTOR can be found at rough ER membrane (Zhang et al., 2020). Similarly, *Drosophila* GATOR2 components Mio and Seh1 localize to lysosomes in both fed and starved flies. Mammals, however, developed additional mechanisms to maintain GATORs at the lysosomal membrane, which include an interaction with protein complex KICSTOR, that is not present in non-vertebrates and regulation of GATOR1-RAGA interaction via ubiquitination.

Mammalian specific KICSTOR complex plays a key role in the localization of GATOR1 to its GTPase substrates (Peng et al., 2017; Wolfson et al., 2017). KICSTOR consist of KPTN, ITFG2, C12orf66 and STZ2, whose initial letters gave to the complex its name. *C.elegans* only encode a homologue of SZT2, while yeasts and *Drosophila* lack entire KICKSTOR (Wolfson and Sabatini, 2017). Both GATOR1 and GATOR2 associate with KICKSTOR in amino-acid insensitive manner. STZ2 is responsible for the interaction of KICKSTOR with GATOR1. SZT2 is also necessary for the coordinated GATOR1 and GATOR2 binding and for GATOR1-dependent inactivation of mTORC1 at the lysosome. SZT2 contains several regions that allow interaction with GATOR1 and GATOR2 (Peng et al., 2017). SZT2-DEPDC5 interactions can occur in the absence of other GATOR components (Shen et al., 2018). SZT2 does not bind GATOR2 in the absence of NPRL3, once again underlining a crucial role of this protein in GATOR1-GATOR2 interactions. In addition, lysosomal localization of WDR59 is abolished in the absence of SZT2.



Thus, KICKSTOR, and especially its largest component 380 kDa SZT2, may facilitate interaction between GATOR1 and GATOR2.

Recent structure of GATOR2 complex revealed that the WDR59  $\beta$ -propeller is necessary to bind to GATOR1 and KICSTOR (Valenstein et al., 2022). Expression of GATOR2 lacking the WDR59  $\beta$ -propeller failed to rescue mTORC1 signaling in *WDR59* knockout cells. The CTDs of MIOS WDR24 and WDR59 were all required for GATOR2 to interact with the GATOR1 and KICSTOR complexes and to complement the mTORC1 signaling defect of the respective knockout cells.

GATOR1 is also implicated to the recruitment to the lysosomal surface of another GAP – FLCN/FNIP. GATOR1-dependent control of the RAGA nucleotide state drives FLCN recruitment to lysosomes when amino acids are scarce (Meng and Ferguson, 2018). In this case the GAP activity of GATOR1 promotes the GDP-RAGA/B conformation and FLCN/FNIP is recruited to the lysosome to act as a GAP towards RAGC/D. These findings help to resolve the apparent contradiction reported earlier, that FLCN-FNIP heterodimer binds to RAGA/B, but acts as a GAP for RAGC/D (Petit et al., 2013; Tsun et al., 2013). Cryo-EM structures of the human FLCN-FNIP-RAG-RAGULATOR complex containing inactive form of the RAG heterodimer confirmed that the FLCN-FNIP2 heterodimer binds to the GTPase domains of both RAGA and RAGC (Lawrence et al., 2019; Shen et al., 2019b)

GATOR1-RAGA interactions are controlled by several kinases and E3 ubiquitin ligases, which are not present in lower eukaryotes. For example, an oncogenic non-receptor tyrosine kinase SRC, disrupts GATOR1-RAGs interactions, promoting mTORC1 recruitment and activation at the lysosomal surface (Pal et al., 2018). Currently it is not known what are the mechanisms that activate SRC in response to amino acids and whether GATOR1 subunits or RAGs can be phosphorylated by SRC. On the other hand DEPDC5 can be phosphorylated by Pim1 kinase at S1002 and S1530, and by AKT also at S1530 (Padi et al., 2019). This phosphorylation seems not affect the ability of DEPDC5 to interact with neither NPRL2 nor SZT2.

Two lysosome localized E3 ligases, RNF152 and SKP2, mediate K63-linked polyubiquitination of RAGA at different sites, which promote GATOR1 recruitment to RAGA and consequent inactivation of mTORC1 (Deng et al., 2015; Jin et al., 2015). SKP2 ubiquitinates RAGA at K15 during prolonged amino acid stimulation (Jin et al., 2015), while, quite opposite, RNF152 ubiquitinates RAGA at different set of lysines (K142, 220, 230, 244) during amino acid starvation (Deng et al., 2015). SKP2 provides a negative feedback loop, where RAGA ubiquitination and GATOR1 recruitment restrict mTORC1 activation upon sustained amino acid stimulation. Inversely, during amino acid starvation, it is RNF152-dependent RAGA ubiquitination, which enhances GATOR1-RAGA interaction. Interestingly, RNF152 can also ubiquitinate RHEB, sequestering RHEB in its inactive RHEB-GDP form and promoting its interaction with TSC2, which leads to mTORC1 inactivation (Deng et al., 2019). Thus, RNF152

acts as a negative mTORC1 regulator in both amino acid and growth factor branches of mTORC1 signaling.

#### **1.8.5. SEA/GATOR in autophagy**

One of the major functions of mTORC1 is in the regulation of autophagy, which is induced when mTORC1 is inhibited. Thus, it is not surprising that deletions of SEACIT/GATOR1 components suppresses autophagy in yeast (Algret et al., 2014; Dokudovskaya et al., 2011; Graef and Nunnari, 2011; Kira et al., 2014; Laxman et al., 2014; Sutter et al., 2013; Wu and Tu, 2011), *Drosophila* (Wei et al., 2016), *C. elegans* (Qi et al., 2017) and mammals (Kira et al., 2014; Luo et al., 2020). Just as opposite, mutations in GATOR2 may promote autophagy, which can happen even in the absence of nutrient starvation, as it is a case of *wdr24* mutants in *Drosophila* (Cai et al., 2016). In contrast, deletions of SEACAT members in yeast seems not to have a drastic effect on autophagy initiation and flux (Dokudovskaya et al., 2011). Interestingly, upon nitrogen starvation deletion of *SEA1* or double deletion of *NPR2* and *NPR3* resulted in inhibition of vacuolar fusion (Algret et al., 2014). Because inactivation of TORC1 during nitrogen deprivation promotes vacuole coalescence (Michaillat et al., 2012), deletions of any of the SEACIT members increase TORC1 activity during starvation, and therefore induce vacuolar fragmentation and defects in autophagy. Recently a bi-directional feedback loop, which regulates autophagy and involves SEACAT, has been described (Hu et al., 2019). In order to control autophagy TORC1 phosphorylates and inhibits Atg1 kinase essential for autophagy initiation, but Atg1 in turn can phosphorylate SEACAT components. This finding uncovers the important node of convergence between TORC1 and Atg1, with the SEACAT

being both regulator and effector of autophagy.

SEA complex is also important for specific types of autophagy. Thus, yeast with deletions of SEACIT complex members failed to activate selective degradation of mitochondria via mitophagy (Liu and Okamoto, 2018; Ma et al., 2019). Given the conservation of the SEA/GATOR function, it is reasonable to assume the similar role of GATOR in mammals, although the involvement of GATOR in specific types of autophagy in mammals has not yet been described.

### **1.9. SEA and GATOR functions in mitochondrial quality control**

As a central controller of mTORC1 pathway, SEA/GATOR is also involved in the regulation of mitochondria function and quality control. The analysis of synthetic genetic interactions in *S.cerevisiae* revealed already in 2011 that SEA genes interact with many mitochondrial genes, with Npr2 located close to the mitochondrial gene cluster (Costanzo et al., 2010; Costanzo et al., 2016; Dokudovskaya and Rout, 2015). About 20% of proteins that co-precipitate with SEA components are mitochondrial proteins (Algret et al., 2014; Ma et al., 2019) and, inversely, enriched mitochondrial fractions contain SEA proteins (Elbaz-Alon et al., 2014). Both C-terminal GFP tagged Iml1/Sea1 and Sea4 can be localized to the mitochondria (Ma et al., 2019). Moreover, treatment with rapamycin significantly increases the amount of cells with cytoplasmic and mitochondrial localizations of Iml1/Sea1, although a fraction of Iml1/Sea1 can still be observed at the vacuole (Chong et al., 2015). Similarly, in HEK 293T cells NPRL2 can be localized to the mitochondria and many mitochondrial proteins can be found

in proteome of NPRL2 and NPRL3 (Ma et al., 2017). Recently SESTRIN2, which interacts with GATOR2 during leucine starvation (see above), was also found to be localized to mitochondria and silencing of GATOR2 genes considerably reduced the mitochondrial pool of SESTRIN2 (Kovaleva et al., 2020). Finally, Sec13 was shown to be interacting with mitochondrial antiviral signal protein (MAVS, also known as VISA) (Chen et al., 2018d; De Falco et al., 2021). MAVS is localized on the outer membrane of mitochondria, with the small proportion present at mitochondria associated membranes (MAMs). Sec13 overexpression increases MAVS aggregation and facilitates interferon- $\beta$  production, while low levels of Sec13 results in weaker host antiviral immune response. Currently it is not clear whether other proteins from nuclear pore complex or COPII or GATOR2 are also involved in these interactions.

Deletion of SEA/GATOR components affects mitochondria functions. Total abundance of SEA proteins is increased during respiratory growth and decreased upon nitrogen starvation, *sea2* deletion impairs respiration capacity in *S. cerevisiae* (Perrone et al., 2005). *npr2* $\Delta$  cells have defective mitochondrial-housed metabolic pathways, such as synthesis of amino acids, and an impaired tricarboxylic acid (TCA) cycle activity. *npr2*-deficient cells showed decreased pools of nitrogen-containing intermediates of TCA cycle and nucleotides. Yet *npr2*  $\Delta$  yeast use TCA cycle intermediates for replenishment of biosynthetic pathways to sustain the hypermetabolic state due to mTORC1 constant activation, suggesting a role of SEACIT in the regulation of cataplerotic reactions of the TCA cycle depending on amino acid and nitrogen status of the cell (Chen et al., 2017). This was later supported by another study which demonstrated that skeletal-muscle-specific NPRL2 loss in mice promoted aerobic glycolysis by

altering the tuning between the amino acid sensing pathway and TCA cycle function. NPRL2-mKO mice also had less oxidative muscle fibers and more glycolytic muscle fibers, a hallmark of aerobic glycolysis, which highlights the functional role of NPRL2 *in vivo* in the regulation of glucose entry into the TCA cycle (Dutchak et al., 2018).

The function of GATOR1 proteins in mitochondrial health seems not to be limited to NPRL2. A heterozygous mutation in the CTD domain of DEPDC5 gene found in autistic child was correlated with a significant decrease in mitochondrial complex IV activity and decrease of overall oxygen consumption rate in peripheral blood mononuclear cells. Therefore, this variant of DEPDC5 can be directly related to an altered mitochondrial function in autistic disease (Burger et al., 2017). Mice with skeletal-muscle specific deletion of DEPDC5 showed increased mitochondrial respiratory capacity and TCA cycle activity (Graber et al., 2019).

SEACIT is also involved in the communication of the mitochondria with other organelles. The mitochondria-to-nucleus communication pathway, known as the retrograde signaling, is triggered by mitochondrial dysfunctions in order to alter the expression of nucleus-encoded mitochondrial genes to effect metabolic reprogramming and to restore cellular fitness (Guaragnella et al., 2018; Quirós et al., 2016). *npr2Δ* and *npr3Δ* yeast strains failed to activate the retrograde signaling pathway when grown in media containing ammonia as nitrogen source (Chen et al., 2017; Neklesa and Davis, 2009). In order to recruit the substrates for biochemical reactions and export resulting products mitochondria rely on direct transport with organelles through contact sites (Zung and Schuldiner, 2020). The vacuole and mitochondria contact

sites, vCLAMPs, are important for lipid exchange (Hönscher et al., 2014) and may also serve for the sensing of the integrity and functionality of mitochondria (Figure 4 ) (Ma et al., 2019). Importantly, SEACIT is required for the maintenance of vCLAMPs and deletion of any SEACIT members drastically reduce the amount of vCLAMPs in yeast cells (Ma et al., 2019). Whether GATOR1 have the same functions in mammalian cells remain to be discovered.

### **1.10. Deletion phenotypes of the SEA/GATOR components**

In *S. cerevisiae* SEA genes (apart from Sec13) are non-essential and in rich media SEA deletion mutants grow practically with the same rate as wild type yeast (Dokudovskaya and Rout, 2011). In *S. pombe* deletion of any GATOR1 and GATOR2 component Sea3 results in severe growth defect (Chia et al., 2017; Fukuda et al., 2021). Homozygous deletions of *nprl2* and *nprl3* in *Drosophila* are semilethal and deletions of *iml* are lethal, with GATOR1 activity required for animals to transit the last stage of pupal development (Wei et al., 2016). In addition, *nprl2* null flies have significantly reduced lifespan (Xi et al., 2019). Similarly, *depdc5* knockout in zebrafish resulted in premature death at 2-3 weeks post-fertilization (Swaminathan et al., 2018). In mice homozygous knockouts of Seh1 (Liu et al., 2019a), Wdr59 and Wdr24 are embryonically lethal (The International Mouse Phenotyping Consortium et al., 2016). Constitutive knockout homozygous and heterozygous GATOR1 rodent models differs significantly. Thus, GATOR1 homozygous animals *Nprl2*<sup>-/-</sup> mice (Dutchak et al., 2015), *Nprl3*<sup>-/-</sup> mice (Kowalczyk et al., 2012b), *Depdc5*<sup>-/-</sup> rats (Marsan et al., 2016) and *Depdc5*<sup>-/-</sup> mice (Hughes et al., 2017) are lethal for embryos. Mice embryos deficient for NPRL2 expression show a compromise liver hematopoiesis, which has negative impact on embryonic viability

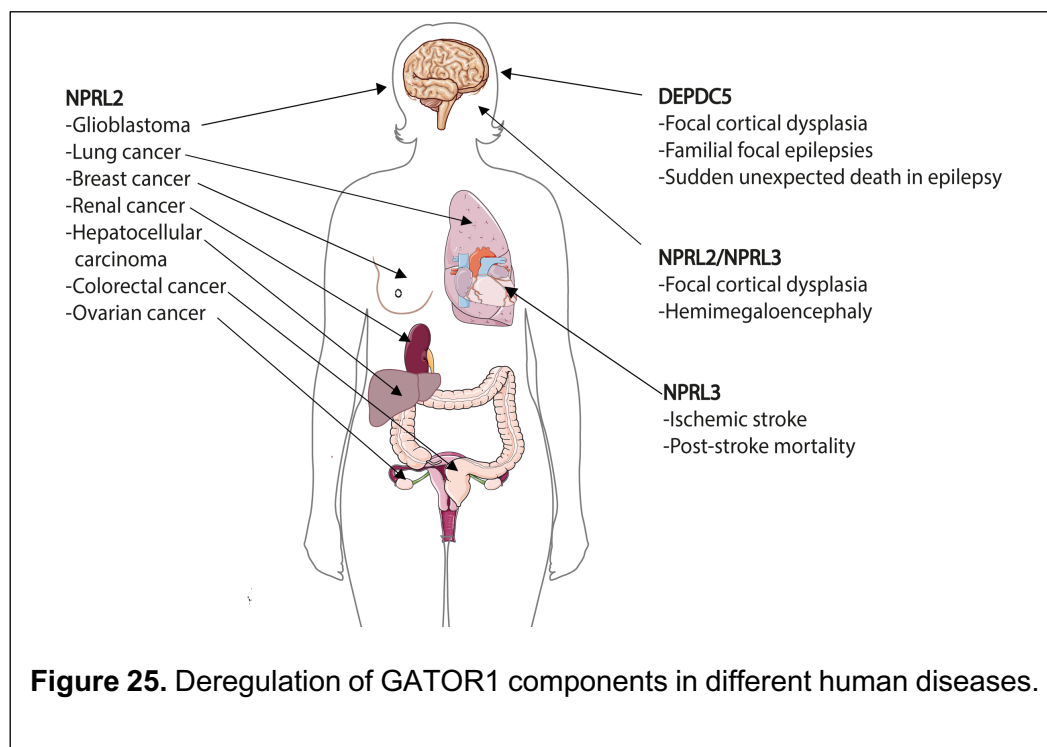
(Dutchak et al., 2015). Although mutations in GATOR1 genes are associated with epileptic disorders and brain malformations, heterozygous *Depdc5*<sup>+/-</sup> rats and mice did not present spontaneous epileptic seizures, but *Depdc5*<sup>+/-</sup> rats have subtle cortical malformations (Hughes et al., 2017; Marsan et al., 2016). Several tissue specific knockouts have also been investigated. A neuron-specific conditional homozygous *Depdc5* knockout mice lived till adulthood, but had larger brains and exhibited a decreased survival (Yuskaitis et al., 2018). Hepatic deletion of *Depdc5* in mice resulted in mild liver inflammation and decreased fat level (Cho et al., 2019). Skeletal muscle-specific *Depdc5* depletion in mice resulted in muscle hypertrophy, but neither physical nor contractile muscle function of these mice improved (Graber et al., 2019). Similarly, mice with *Npr12* deletion in skeletal muscles had larger muscle fibers and exhibited altered running behavior (Dutchak et al., 2018). In conclusion, deletions of SEA/GATOR components in every organism studied so far provoke severe defects on growth and viability.

### **1.11. GATOR in human diseases**

During last decade it became increasingly evident that alternations in expression of GATOR genes can cause various human diseases (Figure 25). Mutations of GATOR2 components can be found in various cancers according to The Cancer Genome Atlas (TCGA) and Cancer Cell Line Encyclopedia (CCLE), COSMIC and cBioPortal databases, although their recurrent mutation frequency is very low (Grabiner et al., 2014). None of the GATOR2 mutations in these cancers were studied on the molecular level and currently there is no data about the involvement of GATOR2 components in other human pathologies (Weckhuysen et al., 2016). One of the reasons of the low pathogenicity of GATOR2 mutations could be that



they would cause an increased, but most probably not complete suppression of the mTORC1 pathway, which can rather be associated with more healthy conditions. In striking contrast to GATOR2, many pathological mutations in GATOR1 genes have been reported. These mutations are mainly related with two main types of human diseases - cancer and epilepsy. Although alternations in sequence and gene expression associated with these pathologies have been reported for all three GATOR1 genes there are striking differences that mark some kind of "preferences" of a gene for a pathology (Figure 25). Thus, DEPDC5 mutations are more frequent in epilepsies in comparison with mutations in other GATOR1 members. NPRL2 mutations can be found more often in different types of cancers and are associated with resistance to anticancer drugs cisplatin and doxorubicin. Even though NPRL3 is a paralogue of NPRL2, its alternations in cancer are less recurrent. Instead NPRL3 is appeared to be required for the normal development of cardiovascular system.



### 1.11.1. Epilepsies and brain malformations

DEPDC5 was reported as the first gene implicated in familial focal epilepsies by Baulac and Scheffer groups (Dibbens et al., 2013; Ishida et al., 2013). Mutations in DEPDC5 are also related with brain malformations, notably with focal cortical dysplasia (FCD), which is a major cause of drug-resistant epilepsy (Scheffer et al., 2014) and can be associated with sudden unexpected death in epilepsy (SUDEP) (Nascimento et al., 2015). Mutations related with focal epilepsies, familial cortical dysplasia and SUDEP were also reported for Nprl2 and Nprl3 (Ricos et al., 2016; Sim et al., 2016; Weckhuysen et al., 2016). More than 140 variants of GATOR1 genes have been found in up to 37% of patients with familial focal and in other forms of epilepsies (Baldassari et al., 2019). These variants include loss-of-function mutations (67%), missense mutations (27%), splice site changes (4%), frameshifts and copy number variants (~1%). Interestingly, distribution of mutations in epilepsy cohort differs drastically from overall distribution of GATOR1 mutations listed in gnomAD database, were loss-of-function represent only 4% of variants with the majority (88%) being missense mutations. Histopathological analysis of brain tissues from individuals with GATOR1 gene mutations demonstrate the hyperactivation of mTORC1 pathway, suggesting that mTORC1 signaling plays an important role in brain development (Ricos et al., 2016; Sim et al., 2016; Weckhuysen et al., 2016).

Nearly 85% of GATOR1 mutations in epilepsies accounts for changes in DEPDC5 with both somatic and germline mutations detected all through the gene without clustering. Initially, it was not clear how germline *Depdc5* mutations can cause FCD, especially taking into account that these mutations are often dominantly inherited from an asymptomatic carrier parent

(Baldassari et al., 2019) and that in rodent models *Depdc5<sup>+/-</sup>* constitutive heterozygous mutations do not exhibit epileptic phenotype (Hughes et al., 2017; Marsan et al., 2016). The discovery of second hit somatic mutations *in trans* which lead to a biallelic inactivation in a subset of brain cells explained this phenomenon (Lee et al., 2019; Ribierre et al., 2018).

*Nprl2* and *Nprl3* mutation are less frequent (6% and 9% respectively), which might be partially related with the fact that their involvement in epilepsies and brain malformations has been tested in a low number of people (Baldassari et al., 2019; Zhang et al., 2022). Cases with simultaneous mutations in different GATOR1 genes have not been described so far. Several *Nprl2* or *Nprl3* variants found in individuals with FCD or hemimegalencephaly (HME) have been reported recently (Chandrasekar et al., 2021; D’Gama et al., 2017). Interestingly, *NPRL3* single nucleotide polymorphism has been associated with ischemic stroke susceptibility and post-stroke mortality (Ryu et al., 2020), which can be related with increased mTOR activity, that is known to accelerate brain recovery after stroke. The role of *NPRL3* in this disease is most probably related with its function in focal epilepsies that might occur in ischemic cerebrovascular disorders (Cocito and Loeb, 1989). Finally, genetic alternations of KICSTOR complex, required for GATOR1-mediated repression of mTORC1 signaling (see above) have also been linked to epilepsies and brain malformations (Baple et al., 2014; Basel-Vanagaite et al., 2013; Trivisano et al., 2020).

GATOR1 plays an essential role in the formation and cortical development. Mutations of GATOR1 components became important features of “mTORopathies” – a set of pathological

conditions characterized by brain malformations, neurological disorders and mTORC1 hyperactivity due to either gain-of-function mutations in a pathway activators (e.g. *AKT*, *RHEB*, *MTOR* itself) or loss-of-function mutations of inhibitors (e.g. *TSC1*, *TSC2*) (Crino, 2011; Lim and Crino, 2013). However, mutations of GATOR1 genes seems to result in a broader spectrum of neurological disorders than other “mTORopathic” genes. Not only these mutations are related with medically epilepsies, and, especially SUDEP, but they are also observed in autism spectrum disorders (Burger et al., 2017) and could be implicated in Parkinson disease (Fang et al., 2021). Therefore, it was recently proposed to name GATOR1-related neurological disorders as GATORopathies (Iffland et al., 2019).

### **1.11.2. Cancer and anticancer drug resistance**

Among GATOR1 components, NPRL2 was the first that was suggested to be a tumor suppressor (Lerman and Minna, 2000) almost a decade before the GATOR1 complex was described for the first time. NPRL2 has the higher cancer associated recurrent mutational frequency out of all GATOR1 genes (Grabiner et al., 2014). For example, missense mutations in metastatic breast cancers are twice more frequent in Nprl2 (1,55%), than in Nprl3 or Depdc5 (0.78%) (Bertucci et al., 2019). Low levels of NPRL2 expression have been detected mostly in solid tumors, including hepatocellular carcinoma (Otani et al., 2009), glioblastoma (Bar-Peled et al., 2013), as well as in renal (Li et al., 2004; Tang et al., 2014) ovarian (Bar-Peled et al., 2013; Li et al., 2004), colorectal (Li et al., 2004; Liu et al., 2019; Liu et al., 2015; Yogurtcu et al., 2012), breast (Li et al., 2004; Peng et al., 2015) and lung cancers (Anedchenko et al., 2008; Jayachandran et al., 2010; Ji et al., 2002; Ueda et al., 2006). Paradoxically, NPRL2 might

also have functions as an oncogene. Recent studies in castration resistant prostate cancer (CRPC), revealed that poor prognosis is associated with high expression of NPRL2 (Chen et al., 2018).

Alterations of NPRL2 expression is also related to the resistance to a number of anticancer drugs. The most recurrent cases are associated with the resistance to cisplatin and doxorubicin which has been initially observed in Npr2 deletion mutants in yeast (Schenk et al., 2003) and further confirmed in human lung cancer cell lines (Jayachandran et al., 2010; Ueda et al., 2006). The reason of this resistance is still not clear, but it could be related with a role of NPRL2 in DNA damage response (Jayachandran et al., 2010; Ma et al., 2017). Overexpression of NPRL2 in colon cancer cells increases the sensitivity to a topoisomerase I inhibitor irinotecan (CPT-11) by activation of the DNA damage checkpoints (Liu and Liu, 2018). Genomic alterations of all three GATOR1 components have recently been associated with the resistance to PIK3Ka inhibitors in primary and metastatic breast cancer (Cai et al., 2021). This resistance is explained by the sustained activation of mTORC1 pathway due to the loss of function mutations of GATOR1 components. In this case it is reasonable to expect that concomitant mTOR blockage by rapalogs or mTOR pan-inhibitors might overcome resistance. Inversely, CRPC cells, where NPRL2 expression is elevated, are resistant to everolimus (Chen et al., 2019).

Surprisingly, during last decade not a single article reported a study about involvement of NPRL3 in cancer and drug resistance, even if in the COSMIC database there are almost three times more somatic cancer mutations listed for NPRL3 than for its paralogue NPRL2.

Low frequency DEPDC5 inactivation mutation have been observed in glioblastoma and ovarian cancer (Bar-Peled et al., 2013). DEPDC5 downregulation was also detected in breast cancer patients (Chen et al., 2018b), where it is strongly correlated with upregulation of KLHL22 E3-ubiquitin ligase, responsible for DEPDC5 poly-Ub and degradation. DEPDC5 inactivation was discovered in gastrointestinal stromal tumors (GIST), one of common sarcomas. Chromosome 22q deletions are observed in ~50% of GIST and recurrent genomic inactivation of DEPDC5 (>16%) makes it the *bona-fide* tumor suppressor contributing to GIST progression via increased mTORC1 pathway signaling (Pang et al., 2019). This is in striking contrast with >250 non-GIST sarcomas where DEPDC5 aberrations are infrequent (~1%). Interestingly, cancer occurrence in epilepsy probands with germline GATOR1 variants is very low. At present it is considered that there is no link between epileptic germline GATOR1 variants and cancer (Baldassari et al., 2019).

Currently, >2000 somatic mutations in different tumors are listed for GATOR1 genes in COSMIC database, none of them has been studied in detail. It is reasonable to expect that in the following years we should gain more information about the molecular mechanisms associated with the tumorigenesis provoked by these mutations.

### **1.11.3. Cardiovascular diseases**

In striking contrast to other GATOR1 components, and especially to its paralogue NPRL2, NPRL3 seems to be less important for epilepsy and cancer. Rather it appears as an crucial gene, necessary for the normal development of the cardiovascular system(Kowalczyk et al., 2012b). Mice with the deletion of NPRL3 promoter often have severe embryonal cardiac defects and die in late gestations. Single nucleotide polymorphism of NPRL3 was reported in sickle cell anemia (Milton et al., 2013), a disease characterized by various hemoglobin abnormalities. These defects are explained by the fact that the introns of NPRL3 contain super-enhancers required for high level expression of the genes encoding the  $\alpha$ -globin subunits of hemoglobins in humans and mice (Hay et al., 2016; Kowalczyk et al., 2012a). These regulation elements appeared to be deeply preserved during evolution. Recent genomic study revealed that NPRL3 gene carrying strong regulatory element became linked to at least two different globin genes in ancestral vertebrate, just before the divergence between jawless and jawed vertebrates. Each of these ancestral globin genes evolved in the modern hemoglobin genes but kept their enhancers in NPRL3.

Therefore, the pathologies associated with NPRL3 mutations are related with the disturbances of the transcriptional elements in the Nprl3 gene rather than with the function of the protein product in the mTORC1 pathway. Similarly, the higher recurrence of NPRL2 mutations in cancers and DEPDC5 mutations in epilepsies could be related with specific moonlighting functions of these GATOR1 members beyond the regulation of mTORC1 pathway.

## **2. RESULTS AND DISCUSSION**



## 2. Results and discussion

The results obtained throughout the development of this thesis work are presented in the format of a manuscript for further publication entitled "*GATOR1 maintains mitochondrial functionality*".

The most studied role of the SEA/GATOR complex is its implication in the amino acid sensing network that signals to mTORC1. However, recent evidence suggests a role for SEA/GATOR in the regulation of mitochondrial function. Most of the currently existing evidence for the implication of the SEA/GATOR complex in mitochondrial function comes from studies in yeast. Since this complex has a high level of conservancy among eukaryotes, we considered reasonable to evaluate the participation of GATOR1 complex in maintenance of mitochondrial function in mammalian cells.

The first part of this study consisted in finding a physical interaction between GATOR1 proteins and mitochondria. Furthermore, we assessed the stability of GATOR1 proteins upon metabolic and mitochondrial stress conditions. Then, since these proteins are related to mitophagy regulation in *Saccharomyces cerevisiae*, we performed CCCP-induced mitophagy in 3 different HEK293 cells lines, each one deleted for one of the GATOR1 components. We also found out that GATOR1 proteins are important for maintaining normal mitochondrial morphology and ultrastructure, and alterations in normal expression of proteins involved in mitochondrial dynamics are found upon GATOR1 deletion. We finalized with functional assays for finding the role of GATOR1 in mitochondrial health. We evaluated mitochondrial membrane

potential, oxygen consumption level, and expression level of proteins of the oxidative phosphorylation. Whether such novel roles of GATOR1 in mitochondrial quality control is mTORC1-mediated remains an open question.

In summary, the data presented in this manuscript show for the first time a link between the GATOR1 complex and the maintenance mitochondrial morphology, ultrastructure, and function. Whereas it is widely accepted that mTORC1 pathway and mitochondria signal to each other, most of the details of such communication remain unclear. In conclusion, since GATOR1 are mTORC1 upstream regulators, the results shown in this manuscript open the door for new perspectives in mitochondrial research that will contribute to our understating of regulation of mitochondrial function through the GATOR1-mTORC1 axis.

## **GATOR1 maintains mitochondrial functionality**

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## **Abstract**

mTORC1 pathway has a key role in regulation of mitochondrial function and controls mitochondrial biogenesis, dynamics, and selective degradation of damaged or non-functional mitochondria. One of the main upstream regulators of mTORC1 in response to amino acid availability is GATOR1 complex composed of DEPDC5, NPRL2 and NPRL3. Yeast cells with deletions of GATOR1 homologue (SEACIT complex), have impaired oxidative metabolism and defects in mitophagy. Whether these functions are conserved in mammals is unknown. In this work, we demonstrate that mammalian GATOR1 components can be localized at the mitochondria. Knockdown of GATOR1 proteins in HEK 293 cells trigger an alteration of mitochondrial morphology due to impairment of fusion and fission events. In addition, GATOR1-depletion alters oxidative metabolism by enhancing oxygen consumption rate upon mitochondrial damage. Taken together, our results show that in human cells mTORC1 pathway modulates mitochondrial function in part via the GATOR1 complex.

## Introduction

The highly conserved mTORC1 pathway plays a key role in cellular homeostasis (Liu and Sabatini, 2020). In order to maintain optimal growth and metabolism, the mTORC1 pathway integrates signals from a wide variety of intracellular and extracellular cues, which include amino acids, growth factors, energy, oxygen and DNA damaging agents (Ma et al., 2018). Depending on the nature of the signal, mTORC1 will drive the cell either to the anabolic pathway, through the synthesis of proteins, nucleotides and lipids, promoting the proliferation and survival, or to the catabolic pathway by controlling autophagy or the ubiquitin-proteasome system (Liu and Sabatini, 2020).

Among many functions exerted by mTORC1, one of the most complex involves the regulation of mitochondrial homeostasis. mTORC1 is essential for mitochondrial biogenesis, phosphorylation of mitochondrial proteins, mitochondrial dynamics and regulation of mitophagy, the selective degradation of mitochondria by autophagy (de la Cruz López et al., 2019). Cells with constitutive mTORC1 activation accumulate mitochondria damaged after uncoupling of oxidative phosphorylation due to treatment with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and mTORC1 inhibition is necessary to restore mitophagy under this condition (Bartolomé et al., 2017). An information about amino acids abundance is transmitted to mTORC1 through Rag GTPases and their upstream regulator the SEA/GATOR complex, while growth factors, insulin and hypoxia signaling converges on TSC complex. Mammalian cells with TSC deletions exhibit impaired mitophagy and increased mitochondria

protein aging (Bartolomé et al., 2017). Yeast cells with deletions of the SEA complex also have defects in mitophagy and accumulate other mitochondria defects, such as impaired respiration capacity, disturbed communication between nucleus and mitochondria and poor maintenance of vacuole-mitochondria contact sites (Loissell-Baltazar and Dokudovskaya, 2021). Whether or not these functions preserved in mammalian cells is not well known.

In yeast the SEA complex is formed via interaction of two subcomplexes SEACAT and SEACIT. *S.cerevisiae* strains with deletion of SEACIT members demonstrate defects in mitophagy (Ma et al., 2019)(Liu and Okamoto, 2018), while deletions of SEACAT components affect contacts between vacuole and mitochondria (Ma et al., 2019). In human GATOR1 (SEACIT homologue) and GATOR2 (SEACAT homologue) do not form a stable complex. While nothing is known about the role of GATOR2 in mitochondrial function, some information is available for the components of the GATOR1 complex (DEPDC5, NPRL3, NPRL2). For example, mice with skeletal-muscle specific deletion of NPRL2 and DEPDC5 showed increased mitochondrial respiratory capacity and TCA cycle activity (Dutchak et al., 2018)(Graber et al., 2019). In addition, both NPRL2 and NPRL3 can be co-precipitated with a number of mitochondrial proteins (Ma et al., 2017).

Here we investigated the involvement of the GATOR1 components in various mitochondrial functions. We demonstrate that a fraction of the GATOR1 proteins is localized to the mitochondria. Deletion of these proteins disturb respiration capacity of cells, mitochondrial shape and dynamics.

## Materials and methods

### Cell culture, transfection, and treatments

HEK293 cells were cultured in DMEM + Glutamax (Gibco™, 31966-047), supplemented with 10% Fetal Bovine Serum (FBS) (Sigma, F7524) and 1% Penicillin-Streptomycin (P/S) (Gibco™, 15140122). Cells were maintained in a humidified incubator at 37 °C and 5% CO<sub>2</sub>. For maintenance cells were trypsinized by washing once with PBS and were incubated with Trypsin-EDTA, Phenol red (Sigma, 25300-054), centrifuged at 500g for 5 min before seeding in a new flask. Cells were regularly tested for mycoplasma contamination.

Cell lines stably expressing Flag-NPRL2-GFP and NPRL3-GFP under the control of EF1 promotor have been described previously (Ma et al., 2017). For transfection with pFLAG-DEPDC5 plasmid (Addgene 46340) cells were grown until 70% confluence and 1µg/ml of plasmid was transfected with Viafect Transfection Reagent (Promega, E4981) following the manufacturer instructions.

3 x 10<sup>5</sup> of HEK293 cells (wild type and deletions) were seeded in 6-well plate containing DMEM, 10%FBS, 1%P/S and were allowed to reach 70% confluence in a humidified atmosphere inside an incubator at 37 °C. Before treatments cells were washed with pre-warm DMEM, and all compounds were dissolved in DMSO, unless otherwise specified. Compounds were prepared in DMEM, 1%FBS, and used with following final concentrations: 10 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (C2759, Sigma), a mixture of 10 µM Oligomycin A

(HY-16589, MedChemExpress) and 2.5  $\mu$ M Antimycin A (A8674, Sigma), 1  $\mu$ M TORIN-1 (HY-13003, MedChemExpress), 500 nM Rapamycin (TO-ROO1, Euromedex), 20  $\mu$ M hydroxychloroquine sulfate HCQ (HY-B1370, MedChemExpress). For stability assay of GATOR1 proteins the proteasome inhibitor MG-132 (ST-SIH-326, Euromedex) was added at 20 $\mu$ M. All treatments were carried out for 4h, unless otherwise specified. For starvation experiments complete medium was replaced with Earle's balanced salts with sodium bicarbonate (EBSS) (E2888, Sigma) or with Hank's balanced salts solution (HBSS) (14025092, Gibco) and then cell were incubated with EBSS or HBSS for 4 h.

### **Subcellular fractionation**

Cells from five T-175cm<sup>2</sup> culture flasks were pooled, washed with pre-warmed PBS, trypsinized and centrifuged at 250g for 5 min at RT. Cell pellets were resuspended in PBS buffer, containing 1 mM HEPES, 1 mM EDTA, pH 7.4, incubated in ice for 10 min, homogenized for 2 min in ice with a dounce homogenizer and centrifuged at 700 g for 15 min at 4 °C. Supernatant was recovered and centrifuged at 10,000g for 20 min for isolation of cytosolic fraction. The pellet was washed with ice-cold PBS, centrifuged 10 min at 10,000 g and resuspended in Buffer 2 pH 7.2 (75 mM Sucrose, 20 mM HEPES, 225 mM Mannitol, 0.5 mM EDTA, 2  $\mu$ M NH<sub>4</sub>OH). Cell fraction was incubated 10 min in ice with B2, homogenized for 2 min with a dounce homogenizer, and centrifuged at 800g for 20 min. Supernatant with mitochondrial fraction was recovered in a new tube and centrifuged at 10,000g for 10 min at 4°C. Mitochondrial fraction was resuspended in NETN lysis buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris pH 7.5, 0.5% NP40) and incubated on ice for 30 min. The cellular suspension was



sonicated, spun at 10,000g, 10 min, 4°C. The LDS sample buffer was added to a supernatant, incubated at 75°C for 10 min and stored at -20°C.

### **Knock-down of GATOR1 components by CRISPR-Cas9**

For generating heterozygous knock-down cell lines, two pairs of single guide-RNAs (sgRNAs) targeting exogenic regions of either NPRL2 (5'-CAC CGC CGT GGG CTA GAT CGC CAT C-3', 5'-CAC CGA GCG TGT ACC ACG CCG TCG-3') or NPRL3 (5'-CAC CGA TGA ACA TGC CTC GGT TGC G-3', 5'-CAC CGC GAT TCT CCT CTA GGA TTA C-3') were annealed and cloned into the vector pHU6-gRNA (Addgene, 53188). HEK293 cells were co-transfected with pHU6-NPRL2 gRNAs or pHU6-NPRL3-gRNAs and EGFP-CRISPRCas9, after 24h GFP-positive cells were single-sorted in 96-well culture plates by flow cytometry in an ARIA FUSION-UV (BD Biosciences) and allowed to grow in DMEM, 10% FBS, 1%P/S. Total DNA of clones was isolated with NucleoSpin Tissue kit (Macherey-Nagel, 740952.50) according to the manufacturer instructions and clones were screened by end-point PCR by using primers designed to detect exon-exon junctions at the expected cutting-site (for NPRL2, 5'-GAC AGC CCG GAG CCT TAA AA-3', 5'-CAG CCG TGC TAG TGG TTG TA-3', for NPRL3: ( 5'-AAC TGG TGC CCT CAA TAG CC-3', 5'-CGT CCC TCT CGA TGT TGG TT-3')). Finally, end-point PCR positive clones for heterozygous deletions were confirmed by quantitative real-time PCR and western-blot analysis. HEK293 cell line with heterozygous deletion of Depdc5 was a kind gift of Stephanie Baulac laboratory (ICM, U1127, Paris, France).

## **Western blot analysis of GATOR1 proteins response to metabolic stress and stability assays**

Cells were washed with PBS and centrifuged at 500g for 5 min at room temperature, cell pellets were resuspended in 100  $\mu$ L of ice-cold NETN lysis buffer, supplemented with protease inhibitor cocktail (Roche, 11873520001) and phosphatase inhibitor (Roche, 4906845001)) and incubated in ice for 30 min. Cell suspensions were sonicated and centrifuged at 10,000g for 10 min at 4 °C. The total amount of protein in whole lysates were quantified using Pierce BCA Protein Assay Kit (Thermo, 23227). Cells were mixed with NuPAGE™ LDS sample buffer 4X (Life Technologies, NP0008) and 100 mM DTT, incubated at 90°C for 5 min and chilled in ice. Protein from lysates samples were resolved by SDS-PAGE electrophoresis using NuPAGE™ 4-12% Bis-Tris Protein Gels (Thermo, NP0323) in either NuPAGE™ MOPS (Thermo, NP0001) or MES running buffer (Thermo, NP0002). Proteins were transferred from gel to a PVDF membrane Immobilon-P (Sigma, IPVH00005) in a wet chamber system with transfer buffer (25 mM Tris, 192 mM Glycine, 20% Ethanol,) for 2h at 90V. Membranes were incubated for 1h at RT with 5% fat-free milk in Tris-Buffered Saline, 0.1% Tween (TBS-T) and washed 3 times with TBS-T and incubated with primary antibodies overnight at 4°C. The following primary antibodies were purchased at Cell Signaling Technology: pP70 S6K (Thr389) (9206S), p70 (9202S), pTFEB (Ser211) (37681), TFEB (37785), MFN1 (14739S), MFN2 (11925S), OPA1 (80471), DRP1 (8570), pDRP1 (Ser616) (3455), LC3B (2775), TOM20 (42406S), GAPDH (51332S). Total OXPHOS Human Wb Antibody cocktail (ab110411) and anti-DEPDC5 (ab213181) were from ABCAM. Anti-GFP is from Roche (11814460001). Anti-NPRL2 (SAB2501073) was from Sigma. Anti-mouse and anti-rabbit IgG-HRP secondary antibodies

were from Jackson ImmunoResearch (JIR315-035-003 or JIR111-035-144). Immunodetection by chemiluminescence was carried out with Immobilon Western Chemilum HRP Substrate (Sigma, WBKLS0500) ImageQuant 800 (Amersham).

### **Confocal microscopy for visualizing mitochondrial network**

$2 \times 10^5$  cells were seeded on coverslips pretreated with 0.1mg/ml poly-D-lysine (A-003-E, Sigma) inside a 12-well plate with DMEM, 10% FBS, 1% P/S. After 48h, the media was replaced with DMEM, 1%FBS containing 100 nM of MitoTracker Orange CMTMRos (M7510, ThermoFisher) and incubated in the dark for 45 min at 37 °C. After incubation, cells were washed 3 times with warm PBS for 5 min and once with H<sub>2</sub>O. Coverslips were mounted on a glass slide with Fluoroshield Mounting Media with DAPI (F6057, Sigma). For DAPI and MitoTracker, images were observed at 405nm and 561nm respectively. Images were acquired on a Confocal Leica SPE DM4000B microscope with 63x oil-immersion objective.

For quantification of mitochondrial length, three to five confocal images were acquired per coverslip. Mitochondrial length was measured by using the free-hand line tool in ImageJ software on a clearly visible individual mitochondrion. Mitochondria were chosen randomly with a distinguishable morphology as the only inclusion criteria in at least ten cells per image. Three intervals for mitochondrial length were defined as following: from 0.8-1.2  $\mu$ M, 1.2-2 $\mu$ M,  $\leq 2\mu$ M, were considered as fragmented, intermediate, and elongated, respectively. Mitochondrial lengths are expressed as percentages of the total amount of mitochondria (n=300), per condition in at least three different images.

## **Electron microscopy**

For ultrastructural studies, samples were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer for 1h at 4°C and were postfixed with 2% osmium tetroxide in 0.1 M phosphate buffer for 1h at room temperature. Following dehydration through a graded ethanol series, samples were embedded in Epon™ 812. Polymerization was completed after 48 h at 60 °C. Ultrathin sections were stained with standard uranyl acetate and lead citrate and observed with FEI Tecnai 12 electron microscope (FEI, Eindhoven, Netherlands). Digital images were taken with a SIS MegaviewIII CCD camera (Olympus, Tokyo, Japan).

To evaluate mitochondrial shape, the length and width of at least 100 mitochondria per condition was calculated using the segmented line tool of ImageJ software. The ratio length/width of mitochondria for each cell line was calculated and expressed the results as the mean. Ratios equal to  $1 \pm 20\%$  were considered representative of a round mitochondrion.

## **Mitochondrial membrane potential ( $\Delta\psi_m$ ) assay by flow cytometry**

For assessing the status of mitochondrial membrane potential ( $\Delta\psi_m$ )  $3 \times 10^5$  HEK293 of WT and *Npri2*, *Npri3*, *Depdc5* cells were seeded in a 6-well plate and allowed to grow until 70% confluence. The day of the experiment, cells were washed with warm DMEM without FBS, trypsinized, cells pellets were resuspended in DMEM 1% FBS, containing 100 nM of Tetramethylrhodamine methyl ester (TMRM) (Sigma, T5428) and were incubated for 10 min at 37 °C in the dark. Fluorescence intensity was acquired at the basal level by using the FL2

detector in a BD Accuri™ C6 Plus Flow Cytometer. After basal acquisition, each sample was treated for 10 min with 10  $\mu$ M Oligomycin A (HY-16589, MedChemExpress) and then 100  $\mu$ M CCCP (C2759, Sigma) as a positive and negative control for  $\Delta\psi_m$  status, respectively. 104 cells were acquired per condition and relative TMRM intensity was calculated by using the HEK WT basal level for normalization in the FlowJo X software.

### **Measurement of mitochondrial respiration by extracellular flux analysis**

Extracellular flux analysis was carried out in Seahorse XF96 extracellular flux analyzer (Agilent Technologies).  $0.25 \times 10^3$  cells were seeded in a Seahorse XF96 Cell Culture Microplate (Agilent Technologies, 101085-004) and allowed to grow in DMEM 10%FBS, 1%P/S at 37 °C in a humidified incubator until 70% confluence. The day of the experiment, cells were washed 3 times with XF DMEM medium pH 7.4 (Agilent Technologies, 103575-100) supplemented with 1mM Pyruvate (Agilent Technologies, 103578-100), 2 mM Glutamine (Agilent Technologies, 103579-100) and 10 mM glucose (Agilent Technologies, 103577-100) and incubated with supplemented XF DMEM medium in a CO<sub>2</sub>-free incubator for 1h. A 96-well plate for Seahorse XFe96 Extracellular Flux Assay Kits (Agilent Technologies, 102601-100), previously calibrated with 200  $\mu$ L of XF calibrant solution (Agilent Technologies, 100840-000) was loaded with Seahorse XF Cell Mito Stress Test Kit compounds (Agilent Technologies, 103015-100) according to the manufacturer instructions. Data was normalized by assessment of fluorescence intensity with Hoesch staining and quantification of total protein amount by BCA. All analyses were carried out using the Seahorse Wave software.

## **Statistical methods**

Data are presented as mean  $\pm$  SD from at least 3 independent experiments. Reported results were statistically evaluated using GraphPad Prism 9 (GraphPad Software Inc.) The normality of the data was tested using Shapiro Wilk normality test. Data that passed the normality test was analyzed using One-Way ANOVA or Unpaired t-test with Welch's correction. The remaining data was analyzed using Kruskal-Wallis test or Dunnett's Multiple Comparison test according to the case.

## Results

Our previous work demonstrated that both in yeast and in mammalian cells SEACIT/GATOR1 components co-precipitate with mitochondrial proteins (Algret et al., 2014)(Ma et al., 2017)(Ma et al., 2019). Moreover, in subcellular fractionation experiments ectopically expressed NPRL2 can be found in the mitochondrial fraction (Ma et al., 2017). Here, we checked if NPRL3 and DEPDC5 can also be associated with mitochondria in this type of experiments. Indeed, similar to NPRL2 (Figure 1A), in the HEK293 cells stably expressing NPRL3 this protein can be found in the mitochondria fraction (Figure 1B), as well as ectopically expressed DEPDC5 (Figure 1C).

We next verified whether stability of GATOR1 components can be affected during the stresses that change mitochondrial functionality. We have already shown that under normal conditions NPRL2 has a rapid turnover via proteasome mediated degradation while NPRL3 is quite stable (Ma et al., 2017). Here we treated cells with stable expression of GATOR1 components with CCCP and the mix of oligomycin and antimycin (OA). CCCP is a mitochondrial uncoupler, oligomycin inhibits mitochondrial ATP synthetase, while antimycin A is respiratory chain inhibitor that blocks the reduction of semi-ubiquinone by cytochrome b, resulting in the increased production of ROS. These treatments are known to induced mitochondrial degradation, followed by mitochondrial clearance via mitophagy. In addition, a stability of NPRL2 was also reduced when cells were maintained in the conditions that block mTORC1 (growth in poor in nutriments EBSS media, treatment with Torin1), Incubating cells with a

proteasome inhibitor MG132 partially rescued NPRL2 degradation (Figure 2A). In contrast, a stability of NPRL3 was only slightly reduced during CCCP treatment and starvation and was not affected during other stresses. DEPDC5 is a stable protein and was not degraded in all the condition tested (Figure 2B).

We and others have previously shown that homologues of GATOR1 from yeast SEACIT complex are implicated in mitophagy (Ma et al., 2019)(Liu and Okamoto, 2018). We wanted to verify if this function is conserved. We have created heterozygous deletions of all three GATOR1 components by CRISPR-Cas9 system and demonstrated that (as expected) an activity of mTORC1 complex in these cells is elevated as we can conclude because of increased phosphorylation of classical mTORC1 substrates p70 kinase and TFEB (Figure 2C). Accordingly, the basal autophagic activity is decreased in GATOR1 deletion cells (Figure 2D-F), as can be observed by reduced level of LC3 lipidation in the cells treated with hydroxychloroquine, a drug that blocks a fusion between lysosome and the autophagosome (Mauthe et al., 2018). Surprisingly though, we did not detect significant changes in mitophagy in the cells treated with CCCP (Figure 2 D-F). Currently we cannot exclude that GATOR1 can be important for mitophagy, triggered by other inducers.

mTORC1 inhibition leads to mitochondrial branching and hyperfusion (Morita et al., 2017). Because GATOR1 deletions increase mTORC1 activity, it is reasonable to expect that in these cells we should observe more fragmented mitochondria. To examine mitochondrial morphology, we stained cells with MitoTracker orange and performed quantitative analysis of



300 cells for each cell type to detect a percentage of fragmented, intermediate, and elongated mitochondria. In wild type cells more than 50% of mitochondria are elongated, while, quite opposite, 50% of mitochondria in *Depdc5* cells are fragmented (Figure 3A, B). The amount of fragmented and intermediate mitochondria was also elevated in *Npr12* and *Npr13* knockouts, although not as drastically as in *Depdc5* cells. We then observed the details of mitochondrial structure by electron microscopy (Figure 3 C, D). In the wild type cells, we can detect elongated mitochondria with very structured cristae, while in the deletion cells mitochondria are rounder, with less structured cristae, especially in the *Npr13* cells.

Fusion of mitochondrial outer membranes is mediated by dynamin related GTPases mitofusin 1 (MFN1) and mitofusin2 (MFN2), while fusion of mitochondrial inner membrane is under the control of dynamin-related proteins optic atrophy 1 (OPA1). Mitochondrial fission requires dynamin-related protein 1 (DRP1) which is recruited to the outer membrane and leads to the division of the organelle. During fission process is accompanied by the increased phosphorylation of DRP1 at Ser616. We checked the expression of all these fusion/fission players in GATOR1 deletions (Figure 4 A). We noticed while OPA1 expression was practically not affected, MFN1 and MFN2 expression was reduced in cells with GATOR1 deletions, indicating and decreased fusion (Figure 4 B upper panel). Accordingly, DRP1 phosphorylation at Ser616 was increased in *Depdc5* cells, pointing to the increased fission (Figure 4 B lower panel).

Finally, we checked how morphological changes can impact on mitochondrial functionality. We evaluated mitochondrial membrane potential ( $\Delta\Psi_m$ ) by observing an accumulation of TMRM, a cell-permeant dye, that can be stock in intact mitochondria (Figure 5A). We found that cells with GATOR1 deletions, and especially, *Depdc5*, have reduced  $\Delta\Psi_m$ . We then reasoned that this defect may reflect on basic mitochondrial functions in these cells. Accordingly, we evaluated basal and maximal respiration as well as the ATP production and proton leak in the Seahorse extracellular flux analyzer. Our data show that all these functions are significantly elevated in the *Npr12* and *Npr13* cells, but practically not changed in *Depdc5* (Figure 5 B). Finally, analysis of expression of proteins of OXPHOS system demonstrated an increase of complexes I and IV expressions (Figure 5 C, D).

Taken together, our data indicate, that GATOR1 proteins can be localized to the mitochondria, and appear to be important for mitochondrial dynamics and functionality.

## Discussion

The tripartite GATOR1 complex, a negative mTORC1 pathway regulator in response to amino acids availability, has been linked to other functions beyond mTORC1 modulation (Loissell-Baltazar and Dokudovskaya, 2021). Notably, the proteome of NPRL2 and NPRL3 proteins revealed their interactions with mitochondrial proteins (Ma et al., 2017). Strong overexpression of NPRL2 promotes its interaction with mitochondrial apoptosis-inducing-factor (AIF) (Ma et al., 2017). In order to know whether the mitochondrial related functions are exclusive of NPRL2, we performed mitochondrial isolation on HEK cells with stable and ectopic expression of tagged GATOR1 proteins and discovered that all three proteins can be localized to mitochondria. Recently, an upstream regulator of mTORC1 in response to leucine, SESN2 protein, was reported to be localized at the mitochondrial outer membrane or in the intermembrane space and thus can be directly involved in the regulation of mitochondrial functions (Kovaleva et al., 2020). SESN2 interacts with GATOR2, and one of the GATOR2 members were found to co-localize with SESN2 at the mitochondria (Kovaleva et al., 2020). Thus, both GATOR complexes can be found at the mitochondria.

A stability of GATOR1 may change as the response to different mitochondrial stressors (Figure 2A-B). Thus, upon mitochondrial membrane uncoupling, OXPHOS shut-down, amino acid starvation or mTOR inhibition by Torin1, NPRL2 protein levels decreased by 50% (Figure 2). Treatment with MG-132, a ubiquitin-proteasome system inhibitor, stabilized NPRL2. Because NPRL2 is heavily ubiquitinated it can be a target for UPS (Ma et al., 2017)(Loissell-

Baltazar and Dokudovskaya, 2021). NPRL2 localization at the mitochondria and its degradation upon mitochondrial damage, may suggest a potential role of NPRL2 as a target of the PINK1/PARKIN mitophagy pathway, which starts by the UPS-mediated degradation of OMM residing proteins, to enhance mitochondrial fission and further engulfment of mitochondria by autophagosome. However, when we induced mitophagy in *Nprl2*, *Nprl3* and *Depdc5* cells with the mitochondrial uncoupler CCCP we found no significant difference in the LC3-II accumulation levels in comparison with wild type cells even upon cotreatment with HCQ. These results are not in accordance with the role of GATOR1 homologue in yeast, the SEACIT complex (Ma et al., 2019)(Liu and Okamoto, 2018). This discrepancy can be partially explained by the fact that yeast do not have PINK1 and PARKIN homologues. Other mitophagy inducers or read-outs should be investigated to find out whether GATOR1 is important for mitophagy.

The lack of GATOR1 proteins disturb mitochondrial morphology, with a significant increase of fragmented mitochondria in *Nprl2*, *Nprl3* and *Depdc5* cells compared to the WT (Figure 3), which may be partially explained by a decrease in MFN1 and MFN2 expression in the GATOR1-depleted cells (Figure 5). Mitochondria in GATOR1-depleted cells are rounder with disorganized and irregularly spaced cristae (Figure 3).

Mitochondrial morphology affects its functionality. Accordingly, in *Depdc5* cells with increased phosphorylation level of DRP1 at Ser616 a sign of mitochondria fragmentation (Figure 4), we also observed decreased mitochondrial membrane polarization (Figure 5 A), while *Nprl2* and *Nprl3* cells, exhibit an enhanced OCR (Figure 5 B). In addition, a significant

increase of expression level of Complex I and IV, was observed in *Nprl2* cells (Figure 5). Since OXPHOS chain is the main production site of ROS in the cell, this might be related to NPRL2 role in ROS balance, as NPRL2 strong overexpression triggers ROS production (Ma et al., 2017).

A fragmented mitochondrial network is prone to be less efficient in oxidative metabolism and tends to accumulate mtDNA mutations, which can have a detrimental effect on cells (Wai and Langer, 2016). An increase in Drp1-dependent mitochondrial network fragmentation has been correlated with cisplatin-resistance in cancer cells (Xie et al., 2020). Impairment of mitochondria-nucleus communication could also be an anticancer drug-resistance mechanisms because of aberrant metabolic rewiring to sustain tumor cells proliferation (Cocetta et al., 2019). Interestingly, NPRL2 loss-of-function mutations have been reported to play a role in modulation of cisplatin resistance in lung cancer cells (Ueda et al., 2006). Whether there is a direct correlation between a role of GATOR1 members in the maintenance of mitochondrial morphology, metabolic reprogramming, and drug-resistance in cancer, remains to be discovered.

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## RESULTS

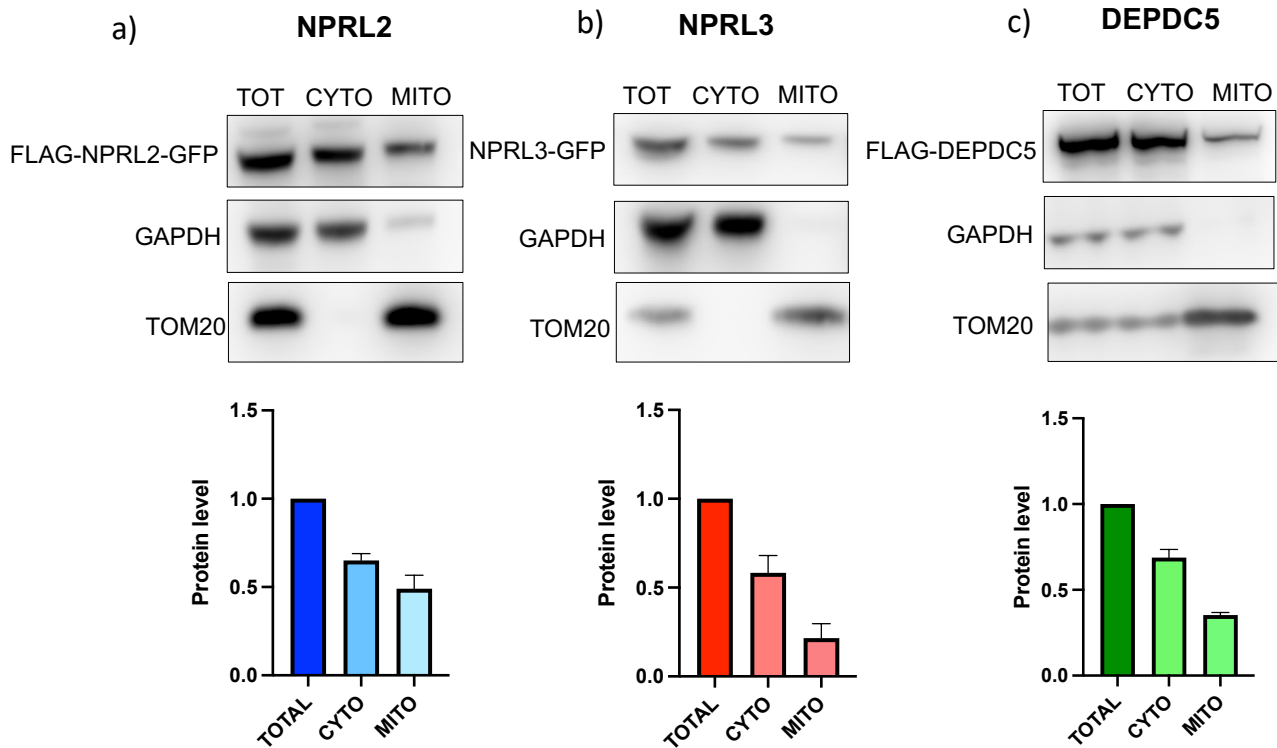
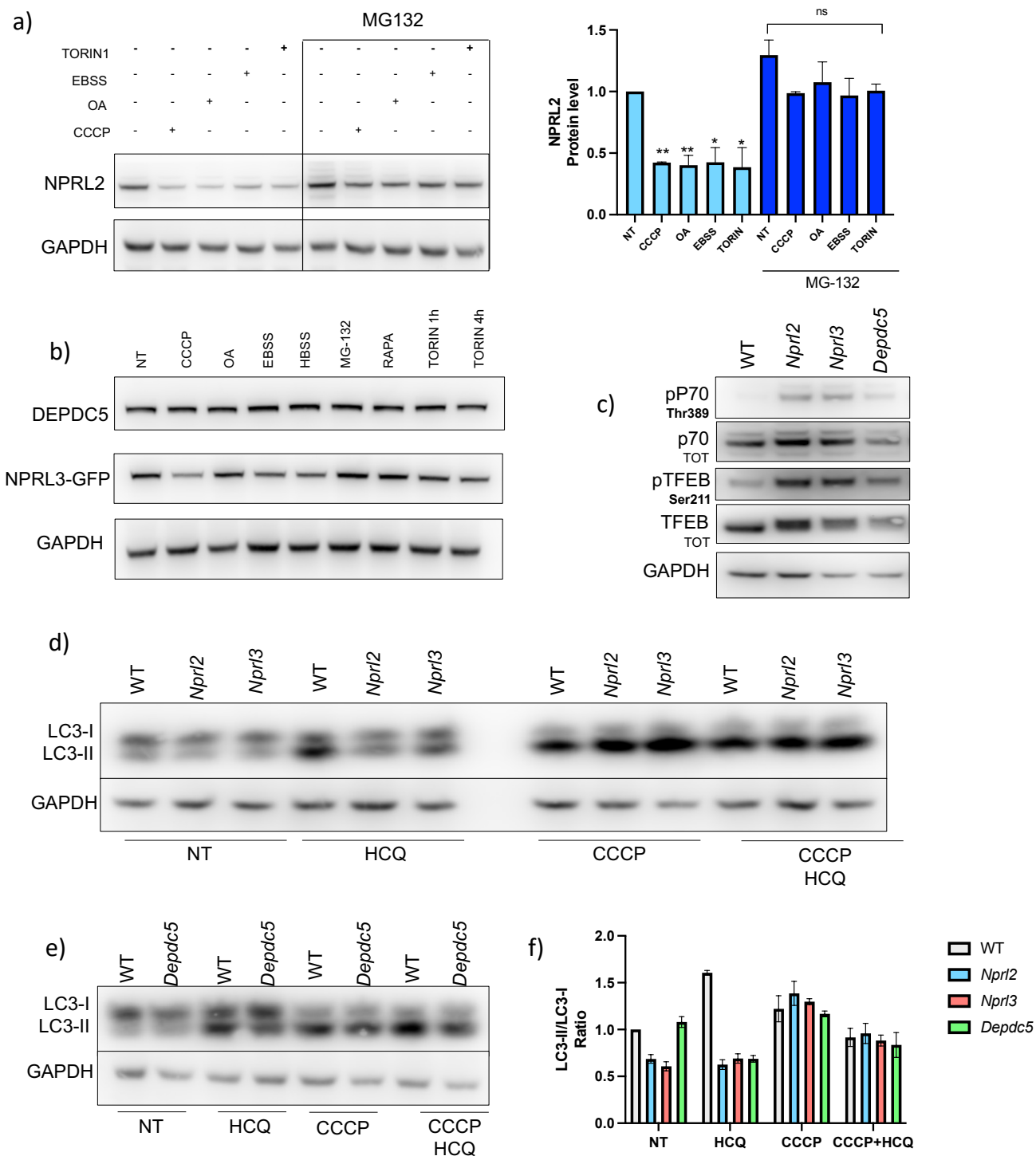
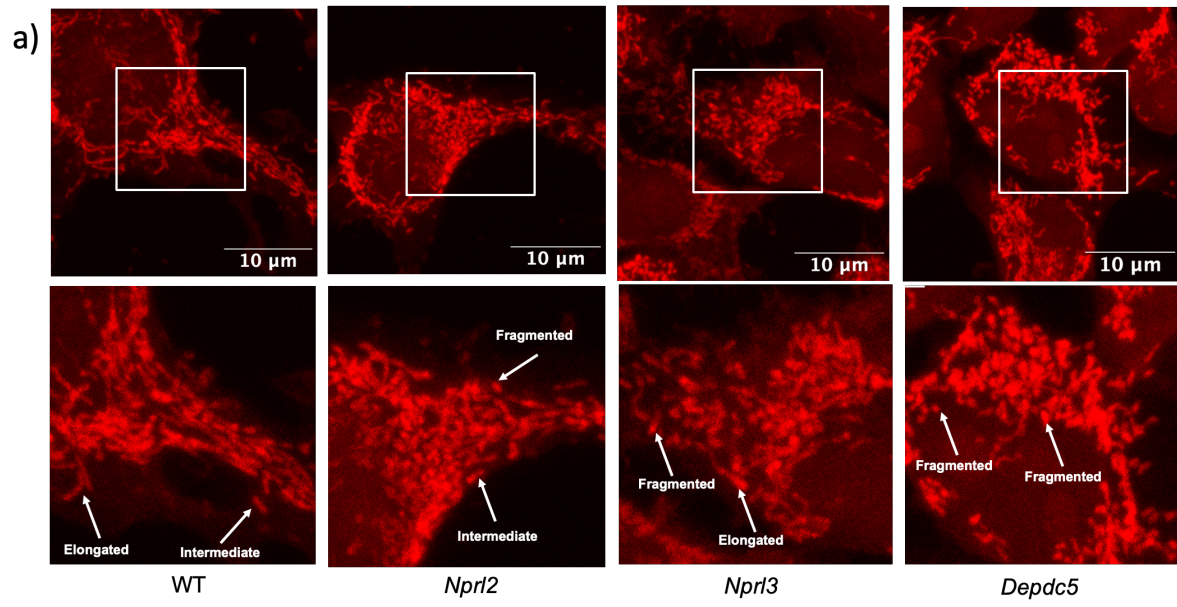


Figure 1. **GATOR1 proteins can be localized at mitochondria.** Cell fractionation of HEK293 cells expressing either a) FLAG-NPRL2-GFP, b) NPRL3-GFP or c) pFLAG-DEPDC5. TOT (whole cell lysate), CYTO (cytosolic fraction) and MITO (mitochondrial fraction) fractions were probed with anti-GAPDH and anti-TOM20 as a control of fractionation. Bar charts represent the mean of band intensity of at least 3 independent experiments.



**Figure 2. GATOR1 proteins involvement upon metabolic stress and CCCP-induced mitophagy.** GATOR1 proteins show different response to metabolic stressors, a) NPRL2 protein levels upon treatment with 10 $\mu$ M CCCP, a combination of Oligomycin-Antimycin (OA) 10  $\mu$ M-2.5  $\mu$ M, starvation with EBSS, and mTOR inhibitor, TORIN-1 1 $\mu$ M for 4h, NPRL2 protein levels upon inhibition of proteasome by treatment with 20  $\mu$ M MG-132. b) NPRL3 and DEPDC5 protein levels upon metabolic stress c) Assessment of phosphorylation levels of p70 kinase and TFEB as a read out of mTORC1 pathway activity, in GATOR1 depleted cells d,e) Cells were treated with hydroxychloroquine (HCQ) 20 $\mu$ M, CCCP 10  $\mu$ M, or a combination of both, for 1h, and LC3 was detected as an autophagy read-out. \* $p < 0,05$ , \*\* $p < 0,01$ .



b) **Mitochondrial Morphology**

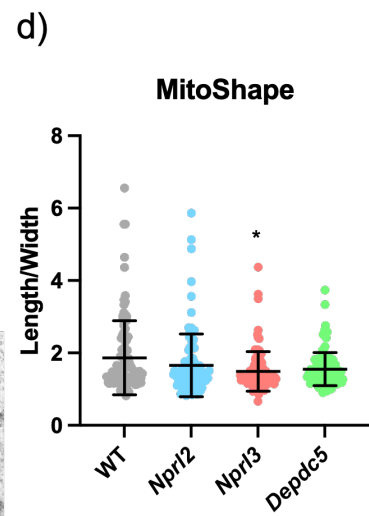
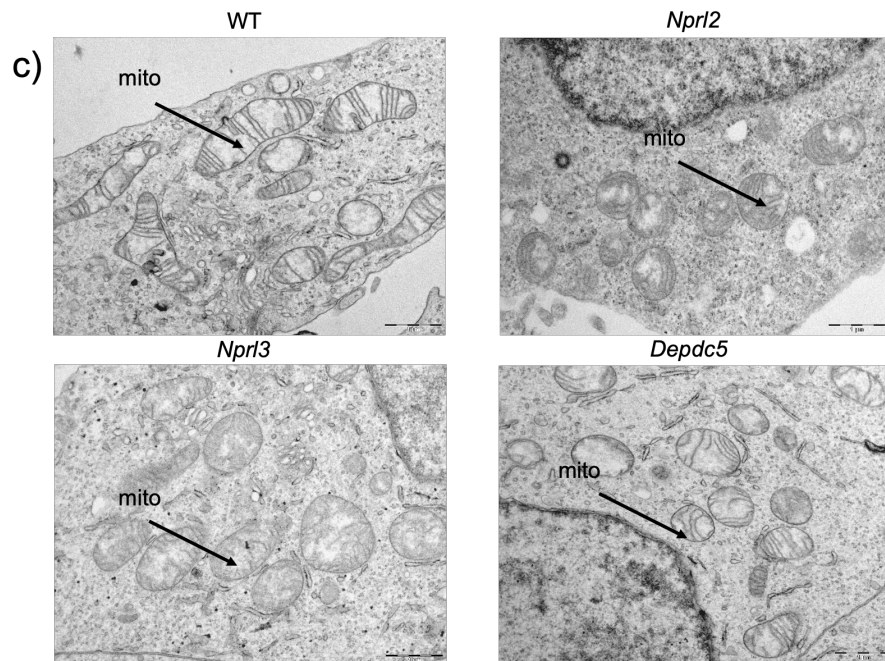
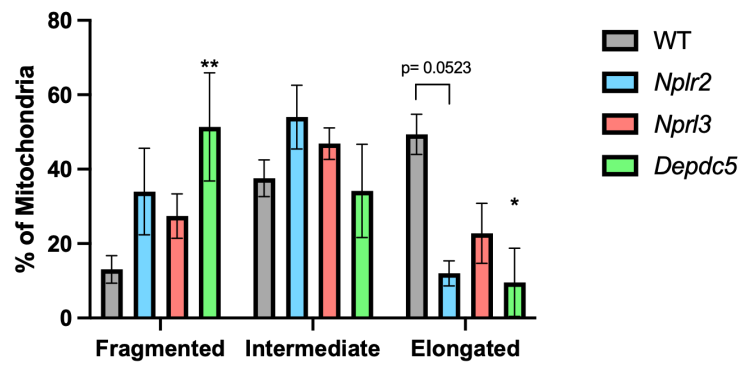


Figure 3. **GATOR1 proteins deletion impacts mitochondrial morphology.** a) Upper panel, Representative confocal images of mitochondrial morphology of HEK cells WT, *Nprl2*, *Nprl3*, *Depdc5* stained with 100 nM MitoTracker Orange CMTROS for 30 min. a) Lower panel, magnifications of mitochondrial network. b) Cells with GATOR1 deletions show an imbalance in mitochondrial dynamics with increased fragmented mitochondria as well as decrease in elongated mitochondrial morphology. c) Representative transmission electron microscopy (TEM) of HEK WT, *Nprl2*, *Nprl3*, *Depdc5*. WT cells show well-preserved branched and elongated mitochondria with well-defined cristae ultrastructure, whereas *Nprl3*, *Nprl3*, *Depdc5* show rounder, isolated and smaller mitochondria with alterations in cristae structure. e) Graphical representation of length/wide ratio of 100 individual mitochondria per condition, *Nprl2*, *Nprl3*, *Depdc5* show ratios  $\sim 1$ , an indicator of roundness. \* $p < 0,05$ .

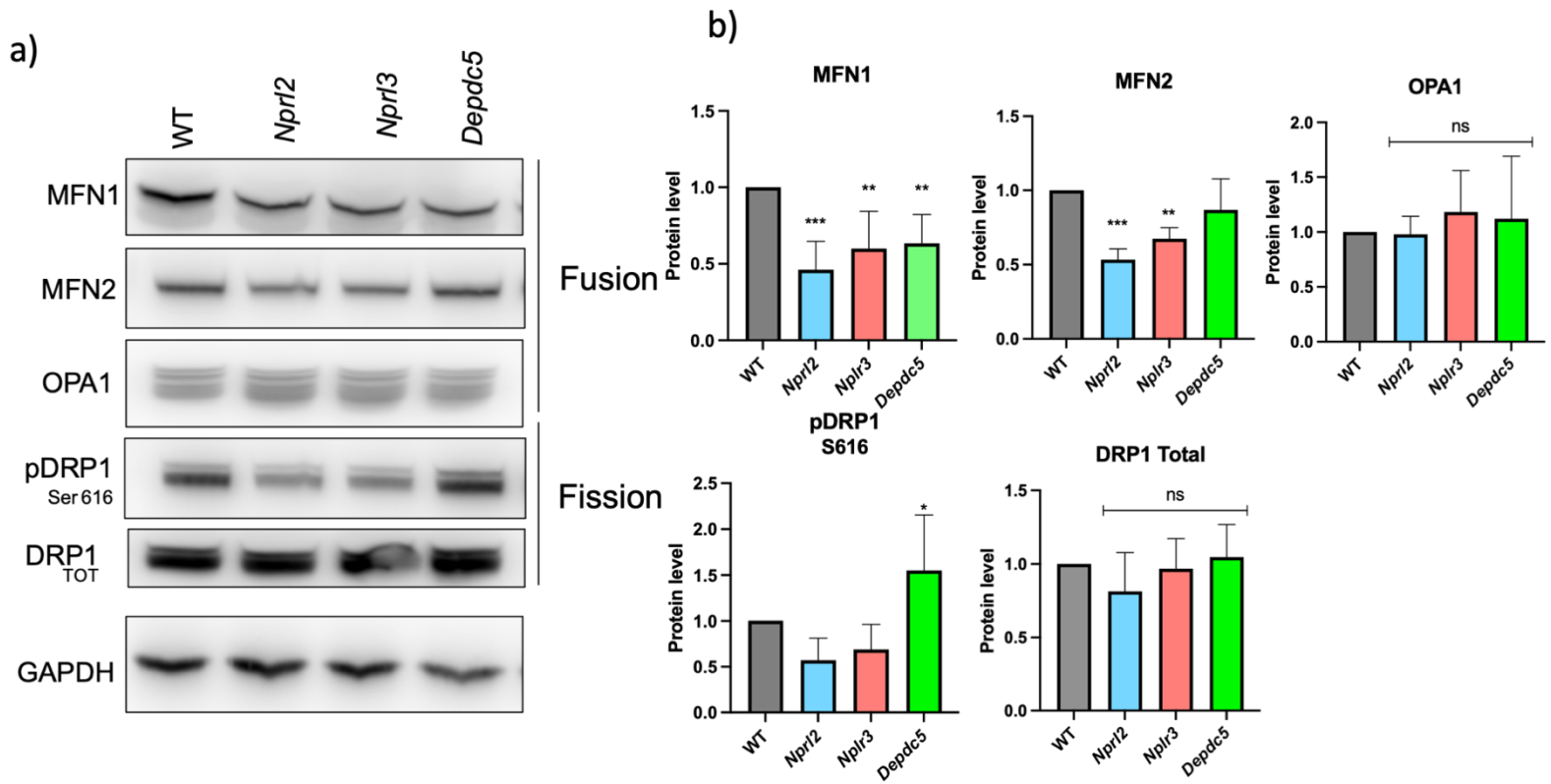


Figure 4. **Mitochondrial dynamic proteins expression is affected upon GATOR1 deletions.** a) Representative images of western blots for proteins involved in mitochondrial dynamics b) Analysis of protein level of MFN1, MFN2, OPA1, pDRP1 s616 and DRP1total of *Nprl2*, *Nprl3*, *Depdc5* compared to WT cells, a significant decrease was found in MFN1 for three deletions of GATOR1 members, MFN2 expression is decreased for *Nprl2*, *Nprl3*, while DRP1 phosphorylation at Ser 616 is enhanced in *Depdc5* cells. Bar charts represent the mean of pixel intensity for at least three independent experiments. \* $p < 0,05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

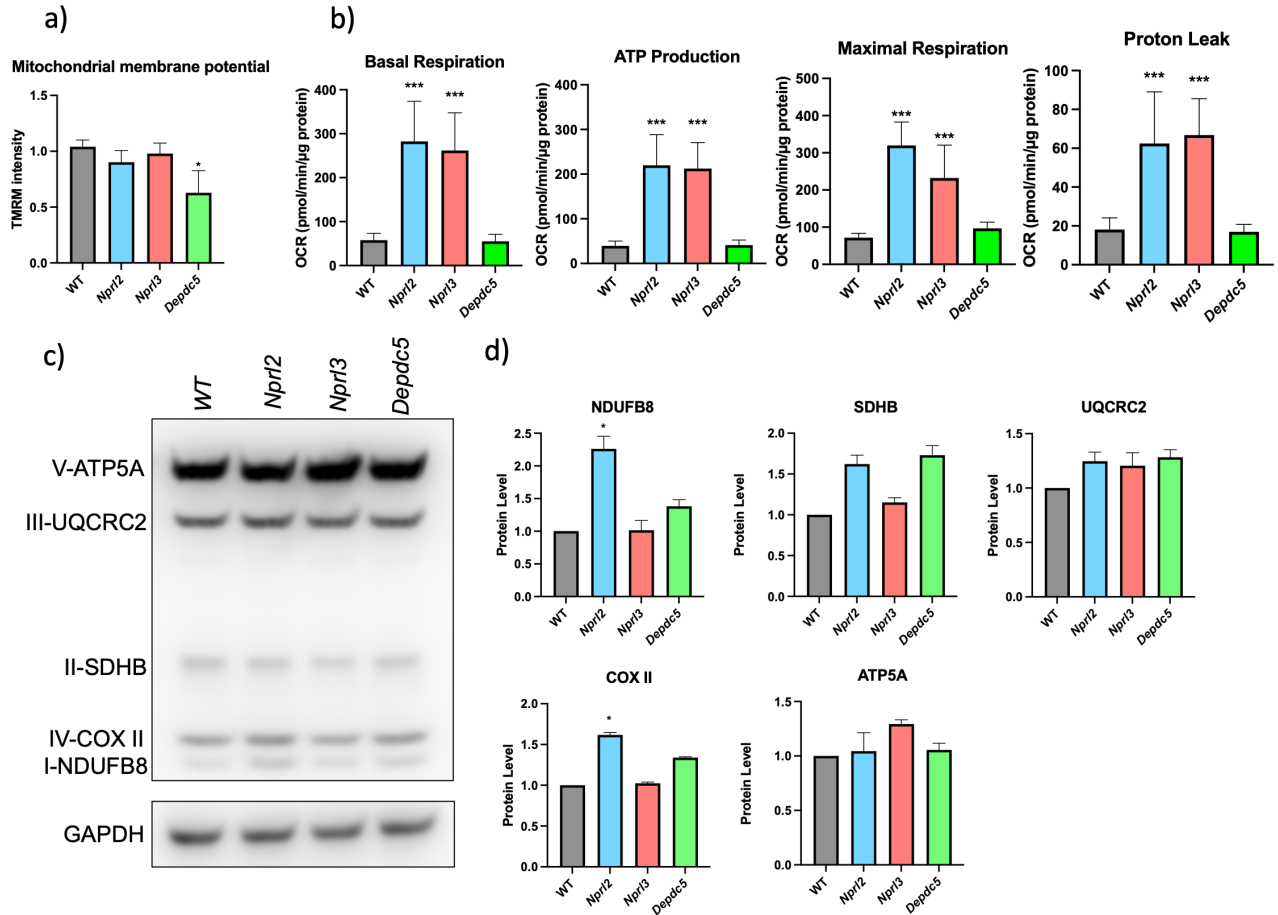


Figure 5. **GATOR1 deletions trigger mitochondrial dysfunctions.** a) Mitochondrial membrane potential ( $\Delta\Psi_m$ ) using  $\Delta\Psi_m$ -specific dye TMRM, *Depdc5* cells show a significant decrease in  $\Delta\Psi_m$ . b) Analysis of oxygen consumption rate (OCR) at basal level, after ATP synthesis inhibition (ATP production), maximal level and respiration non-coupled to ATP production (Proton Leak). c) Representative image of Western blot of 5 complexes of ETC in *Npr12*, *Npr13*, *Depdc5*. d) Bar charts represent the mean of band intensity of at least 3 independent experiments. \* $p < 0,05$ .

## **Identification of Small Molecules Inhibiting Cardiomyocyte Necrosis and Apoptosis by Autophagy Induction and Metabolism Reprogramming**

Original Article, Published: 29/01/2022, Cells, MDMPPI

During my thesis, I collaborated with the team of Dr. Brenner on a project which studied the damage caused by chemotherapeutic drugs to cardiac cells. The focus of this work was to identify new compounds that can potentially provide cardiotoxicity protection of chemotherapy-treated patients. My role in this project concerned the evaluation of mitochondrial function in the cells treated with drugs using Seahorse XFe96-Agilent analyzer, since I was actively participating in the activities of Real-Time Metabolism Evaluation Platform located at our unit UMR9018 in Gustave Roussy.







A high-throughput analysis of 1600 molecules was performed and identified six compounds capable to inhibit apoptosis and necrosis, upon camptothecin and H<sub>2</sub>O<sub>2</sub> treatment, in rat cardiomyoblast cell line and in rat neonatal ventricular myocytes. The cardioprotective effect of these compounds was diminished after downregulation of ATG5 and BECLIN-1, two key proteins for autophagy induction. Thus, the protective effect of these compounds depends on an intact autophagic machinery.

Cardiomyocytes rely on mitochondria for maintaining cell function. Therefore, the effect of the selected drugs on mitochondrial network structure and dynamics was assessed. Digitoxigenin, digoxin and SG6163 increased the total number of mitochondria per cell. Digitoxigenin and digoxin showed a decrease in the expression of the mitochondrial fusion machinery proteins MFN1 and MFN2. Digoxin and SG6163 enhanced phosphorylation levels of DRP1 at Ser 616, an indicator of mitochondrial fission. Finally, cells treated with these compounds exhibit enhanced oxidative phosphorylation. Taken together, our results show that cardioprotective mechanisms of six selected compounds depend on autophagy activation and promotes metabolism rewiring by modulating mitochondrial function.



## Article

# Identification of Small Molecules Inhibiting Cardiomyocyte Necrosis and Apoptosis by Autophagy Induction and Metabolism Reprogramming

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**Abstract:** Improvement of anticancer treatments is associated with increased survival of cancer patients at risk of cardiac disease. Therefore, there is an urgent need for new therapeutic molecules capable of preventing acute and long-term cardiotoxicity. Here, using commercial and home-made chemolibraries, we performed a robust phenotypic high-throughput screening in rat cardiomyoblast cell line H9c2, searching for small molecules capable of inhibiting cell death. A screen of 1600 compounds identified six molecules effective in preventing necrosis and apoptosis induced by H<sub>2</sub>O<sub>2</sub> and camptothecin in H9c2 cells and in rat neonatal ventricular myocytes. In cells treated with these molecules, we systematically evaluated the expression of BCL-2 family members, autophagy progression, mitochondrial network structure, regulation of mitochondrial fusion/fission, reactive oxygen species, and ATP production. We found that these compounds affect autophagy induction to prevent cardiac cell death and can be promising cardioprotective drugs during chemotherapy.

**Keywords:** apoptosis; autophagy; cardioprotection; cardiotoxicity; mitochondrion; screening

## 1. Introduction

One of the major problems in anticancer treatments is the management of toxicity that affects cardiac cells and leads to cardiac dysfunction and cardiomyopathy in many surviving patients. The number of patients at risk for cardiovascular diseases increases in correlation with the improvement of survival for most cancers resulting in higher cardiovascular morbidity and mortality [1,2].

Acute cardiac damages can be induced by tissue irradiation and chemotherapy, especially upon treatment with tyrosine kinase inhibitors and anthracyclines (doxorubicin and epirubicin), as often observed in childhood cancer survivors [1–6]. Cardiotoxicity can also develop in Her2-positive breast and stomach cancer patients treated with trastuzumab and other Her2-targeted drugs since Her2 is expressed not only in tumors but also in

cardiomyocytes. Depending on the anticancer agent and patient comorbidities, cardiotoxicity mechanisms can involve DNA damage, endoplasmic reticulum stress, mitochondrial dysfunction, reactive oxygen species (ROS) production, bioenergetic metabolism failure, apoptosis, and necrosis [6,7]. Of note, necrosis and apoptosis in the heart differ in terms of triggering stimuli, biochemical effectors, and sequence of the events leading to cell death [6,7]. For example, plasma membrane permeabilization occurs early in necrosis and later in apoptosis. Therefore, there is an urgent need to develop new cardioprotective molecules capable of preventing cardiotoxicity in cancer patients.

Here, in perspective to find novel cardioprotective drug candidates, we performed a phenotypic high-throughput screening using a rat cardiomyoblast cell line, H9c2, and tested commercial and home-made library of 1600 molecules searching for compounds capable of inhibiting both apoptosis and necrosis. We used camptothecin, a potent apoptosis inducer that acts both as the DNA-intercalating agent and topoisomerase I inhibitor [8] and H<sub>2</sub>O<sub>2</sub>, which causes oxidative damage and induces both necrosis and apoptosis [9]. We identified six molecules that could be used to maintain cardiomyocyte viability preventively during treatment with H<sub>2</sub>O<sub>2</sub> or camptothecin and further characterized their cellular and molecular effects in rat primary neonatal cardiomyocytes (RNVCs). To be effective, all molecules require autophagy regulators ATG5 and BECLIN-1 proteins but have differential abilities to regulate cell death, autophagy, and mitochondrial structure. Overall, these compounds are promising cardioprotective drugs to be used in the course of chemotherapy and should be further tested during preclinical studies.

## 2. Material and Methods

### 2.1. Phenotypic High Throughput Screening

#### 2.1.1. Chemical Libraries

Compounds obtained from Prestwick library (1200 molecules) and CEA SCBM library (400 molecules) were dissolved at 10 mM in 100% DMSO to prepare stock solutions. The distribution of compounds into 96 well plates was made with a Biomek Single Bridge 96 liquid handler (Beckman Coulter, Brea, CA, USA).

#### 2.1.2. Cellular Treatments

H9c2 cells (ATCC 30-2002™) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) complemented with Fetal Bovine Serum 10% (BioWhittaker, Walkersville, MD, USA) and penicillin-streptomycin mixture (Gibco, Waltham, MA, USA). H9c2 cells were seeded in 96 well plates (5,000 cells/well), let adhere for 48 h, and treated with compounds at 10 μM for 2 h at 37 °C. Compounds were removed and replaced with a culture medium containing either 10 μM camptothecin (Sigma, St. Louis, MO, USA) 24 h to induce apoptosis or 300 μM H<sub>2</sub>O<sub>2</sub> (Sigma, St. Louis, MO, USA) 2 h to induce necrosis, and 0.1% DMSO in culture medium was used as a negative control.

#### 2.1.3. Viability Measurement and Hit Selection

The percentage of viable cells was evaluated by methylene blue staining [10]. After treatment, cells were washed two times with PBS and fixed with ethanol for 30 min at room temperature. Ethanol was removed, and plates were left to dry overnight; cells were stained with 0.1 g/L methylene blue for 5 min, washed three times with water, and resuspended in 100 mM HCl. Absorbance was measured at 665 nm (Envision spectrofluorimeter, Perkin Elmer, Waltham, MA, USA). Results were normalized with negative control, and hits were selected if the absorbance value was higher than the mean cell death value plus 3 standard deviations (SD).

### 2.2. Neonatal Cardiomyocyte Isolation

Rat neonatal cardiomyocytes (RNVCs) were isolated as previously described [11]. Briefly, RNVCs were isolated from Wistar newborn rat hearts, and cells were cultured in Minimum Essential Medium (MEM) containing 1.2 mM Ca<sup>2+</sup>, 2.5% fetal bovine serum

(FBS), 1% penicillin-streptomycin, and 2% HEPES (pH 7.6) and plated on culture dishes, coated with 10 µg/mL laminin. RNVCs were left to adhere for 2 h in a 95% O<sub>2</sub>, 5% CO<sub>2</sub> at 37 °C before the medium change.

### 2.3. LDH Release Assay

A colorimetric assay was used to measure lactate dehydrogenase (LDH), a cytosolic enzyme released upon plasma membrane permeabilization, and to evaluate cell viability. Assay from Promega was performed using cell culture supernatants obtained from H9c2 cells or RNVCs, and LB was used as a positive control of total cell lysis. LDH release was measured at 490 nm (Infinite spectrofluorimeter, Tecan, Zurich, Switzerland).

### 2.4. Plasma Cell Membrane Permeabilization Assay

Propidium iodide (Sigma, St. Louis, MO, USA), a fluorescent impermeable DNA marker, was used to measure plasma membrane integrity. Propidium iodide at 10 µM was added in the culture medium, and fluorescence reading was performed (λ<sub>ex</sub>: 530 nm; λ<sub>em</sub>: 620 nm) using TECAN infinite spectrofluorimeter (Tecan, Zurich, Switzerland), commercial lysis buffer (LB) was used as a control.

### 2.5. Plasmid Transfection

Then, 4 × 10<sup>5</sup> neonatal cardiomyocytes were plated overnight on 35 mm culture dishes coated with 10 µg/mL laminin, and 24 h later, cells were transiently transfected with 1 µg plasmid coding for GFP-LC3 (generous gift from Dr. J.L. Perfettini, INSERM U1030, Gustave Roussy, Villejuif, France) by using 2.5 µL Lipofectamine<sup>®</sup> 2000 (Thermo Fisher, Waltham, MA, USA) for 48 h. Fluorescence was detected with a confocal microscope (SP5 Leica). Images were analyzed with Image J (Wayne Rasband, NIH, Bethesda, MD, USA).

### 2.6. Mitochondrial Network Analysis by Confocal Microscopy and Transmission Electron Microscopy

#### 2.6.1. Confocal Microscopy

4 × 10<sup>5</sup> RNVCs were plated overnight on 35 mm culture dishes coated with 10 µg/mL laminin, and 24 h later, cells were treated for 6 h with 1 µM or 10 µM of different compounds. Cells were incubated with Mitotracker Red 580 at 200 nM for 20 min at 37 °C, then with 4 µM calcein (Life Technologies, Carlsbad, CA, USA) for 10 min at 37 °C. Z stack images were acquired with a Leica (TCS SP8 gSTED) inverted confocal laser scanning microscope (Mannheim, Germany) equipped with a WLL Laser (495 nm excitation wavelength for calcein and 580 nm for Mitotracker Red 580). Green fluorescence emission was detected with 505–550 nm wide emission slits and 585–700 nm wide emission slits for the red signal under a sequential mode. The pinhole was set at 1.0 Airy unit, and 12-bit numerical images were done with the Leica Application Suite X software (Version 3.5.5; Leica, Wetzlar, Germany).

Mitochondrial network and cell volume 3D model were reconstructed by using the IMARIS software 9.7 version (Bitplane Company, Zurich, Switzerland); consequently, cell volume, mitochondria number, and volume were analyzed using the volume and surface rendering processes.

#### 2.6.2. Transmission Electron Microscopy

For ultrastructural analysis, cells were fixed in 1.6% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, washed in 0.1 M cacodylate buffer, fixed for 1 h in 1% osmium tetroxide, and 1% potassium ferrocyanide in 0.1 M cacodylate buffer to enhance the staining of membranes [12]. Cells were washed in distilled water, dehydrated in alcohol, and embedded in epoxy resin. Contrasted ultrathin sections (70 nm) were analyzed under a JEOL 1400 transmission electron microscope equipped with a Morada Olympus CCD camera.

### 2.7. ROS Detection in RNVCs

A total of 50 µg of MitoSOX mitochondrial superoxide indicator (MitoSOX™, Thermo Fisher, Waltham, MA, USA) was dissolved in 13 µL of DMSO to make 5 mM MitoSOX™ stock solution, which was further diluted in PBS to make a 5 µM MitoSOX working solution. RNVCs were treated with either 0.1% DMSO or 3 µM rapamycin, or 1 µM solutions of digitoxigenin, digoxin, SG6163F VP331, LOPA87, or minaprine in cell culture medium for 6 h. After treatments, cells were washed 2 times with PBS at 37 °C, incubated with 5 µM MitoSOX for 10 min at 37 °C, and gently washed three times with warm PBS. The nuclear fluorescence was deleted, and mitochondrial fluorescent intensity was measured by using ImageJ software.

### 2.8. Real-Time Bioenergetic Profile Analysis in H9c2 Cardiomyocytes

The XFe96 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA, USA) was used to measure cellular bioenergetic function. H9c2 cells were seeded at 20,000 cells per well in XFe96 cell culture microplates; all the pre-treatments were performed with a serum-free cell culture medium. The Agilent Seahorse XF Glycolysis Stress Test Kit (Agilent, Santa Clara, CA, USA) was used to measure glycolytic function by quantification of the extracellular acidification rate (ECAR) followed by 3 sequential injections of 10 mM glucose, 2 µM oligomycin, and 50 mM 2-deoxy-D-glucose. The oxygen consumption rate (OCR) was measured with Seahorse XF Cell Mito Stress Test Kit (Agilent, Santa Clara, CA, USA). The built-in injection ports on XF sensor cartridges were used to add modulators of respiration into cells during the assay to reveal the key parameters of mitochondrial function. Then, 2 µM oligomycin was injected first, followed by the addition of 1 µM carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP). Finally, 0.5 µM antimycin A was injected to stop mitochondrial respiration. The oxidation of exogenous fatty acids was measured using the XF Palmitate-BSA FAO Substrate kit (Agilent, Santa Clara, CA, USA) and the XF cell Mito Stress Test kit. Cells were grown in DMEM supplemented with 0.5 mM glucose, 1 mM GlutaMAX, 0.5 mM carnitine, and 1% fetal bovine serum. The FAO Assay Medium (111 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl<sub>2</sub>, 2 mM MgSO<sub>4</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, supplemented on the day of the assay with 2.5 mM glucose, 0.5 mM carnitine, and 5 mM HEPES pH 7.4), was kept at 37 °C. H9c2 cells were seeded at 20,000 cells per well in XFe96 cell culture microplates; all the pre-treatments were performed with a serum-free cell culture medium. A total of 24 h prior to the assay, the growth medium was replaced with the substrate-limited medium, and 45 min prior to the assay, cells were washed two times with FAO Assay Medium; 150 µL/well FAO Assay Medium was added to the cells and incubated in a non-CO<sub>2</sub> incubator for 30–45 min at 37 °C. The assay cartridge was loaded with XF Cell Mito Stress Test compounds (final concentrations: 2 µM oligomycin, 1 µM FCCP, and 0.5 µM antimycin A). Finally, 30 µL XF Palmitate-BSA FAO Substrate or BSA was added to the appropriate wells, then immediately inserted the XF Cell Culture Microplate into the XFe96 Analyzer for analysis.

### 2.9. SDS-PAGE and Western Blot

H9c2 cells and RNVCs were detached in LB containing 50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% Triton X 100, and 0.1% SDS. The cells were collected, placed on ice for 30 min and centrifuged at 2000× g for 20 min at 4 °C. The supernatant was transferred to a new tube and kept on ice. The protein concentration was determined by BCA assay. The protein samples were diluted with 2X Laemmli Sample Buffer (Sigma, St. Louis, MO, USA), incubated for 5 min at 95 °C, and loaded in 4–20% Tris-Glycine gel (Fisher Scientific, Waltham, MA, USA). Separated proteins were transferred onto PVDF membrane for 3 min at 2.5 V in Trans Blot Turbo System (BioRad, Hercules, CA, USA). The membrane was blocked with 5% milk in PBS/0.1% Tween and incubated overnight with an appropriate primary antibody in 5% milk in PBS/0.1% Tween at 4 °C. The membrane was washed 6 times × 5 min with PBS/0.1% Tween, incubated with a Horseradish Peroxidase-Conjugated secondary antibody for 1 h at room temperature, washed again with PBS/0.1%

Tween, incubated with an ultra-sensitive enhanced chemiluminescent substrate for 5 min, and visualized with a gel imaging system (BioRad, Hercules, CA, USA). The following antibodies were used: anti-Mitofusin 1 (ab126575, Abcam, Waltham, MA, USA), anti-Mitofusin 2 (ab124773, Abcam, Waltham, MA, USA), BCL-2 (C-2) (sc-7382, Santa Cruz Biotechnology, Dallas, TX, USA), BAX (B-9) (sc-7480, Santa Cruz Biotechnology, Dallas, TX, USA), BCL-XL (2764, Cell Signaling, Danvers, MA, USA), LC3B (D11) (3868, Cell Signaling, Danvers, MA, USA),  $\beta$ -actin (C4) (sc-47778, Santa Cruz Biotechnology, Dallas, TX, USA), phospho-DRP1 (Ser616) (D9A1) (4494, Cell Signaling, Danvers, MA, USA), and DRP1 (611112, BD Biosciences, San Jose, CA, USA).

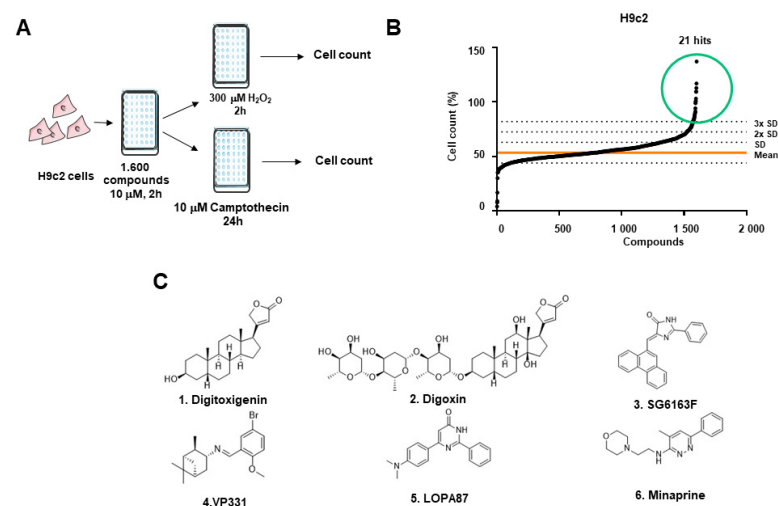
### 2.10. Statistical Analysis

Results are expressed as mean  $\pm$  standard error (SD) or standard error to the mean (SEM). The Origin software and Graphpad Prism 6 were used for statistical analysis. Differences between 2 groups were analyzed by one-way ANOVA, and differences between groups of two genotypes were analyzed by two-way ANOVA, Sidak's multiple comparisons. Statistical significance is indicated as follows: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , and \*\*\*\*  $p < 0.0001$ .

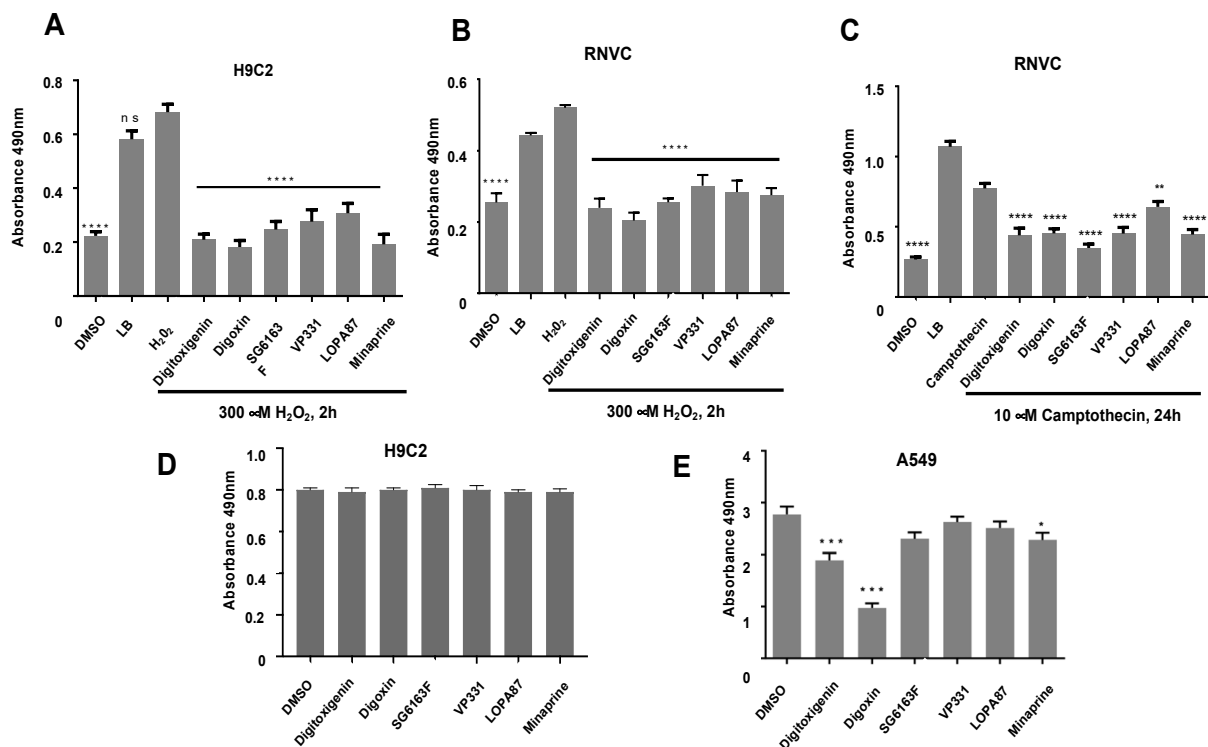
## 3. Results

### 3.1. Identification of Cardiomyocyte Apoptosis and Necrosis Inhibitors by High Throughput Screening

To identify inhibitors of  $H_2O_2$ -induced necrosis and camptothecin-induced apoptosis in rat cardiomyoblast H9c2 cell line, a phenotypic high-throughput screening was performed with 1200 molecules from the commercial library Prestwick and 400 molecules from the home-made chemical library (Figure 1A). Our screen revealed 21 statistically significant hits (Figure 1B), of which we chose to investigate further six compounds that were most potent during cell death inhibition. Three of these six molecules (digitoxigenin, digoxin, and minaprine) belong to the Prestwick library, and three others are new chemical entities named SG6163F, VP331, and LOPA87 [13,14] (Figure 1C). Among the selected compounds, digitoxigenin and digoxin exhibited the best protection from cell death inducers, while minaprine was less powerful. The effect of these components was further confirmed in LDH release assay (Figure 2A) and propidium iodide staining (Supplementary Figure S1).



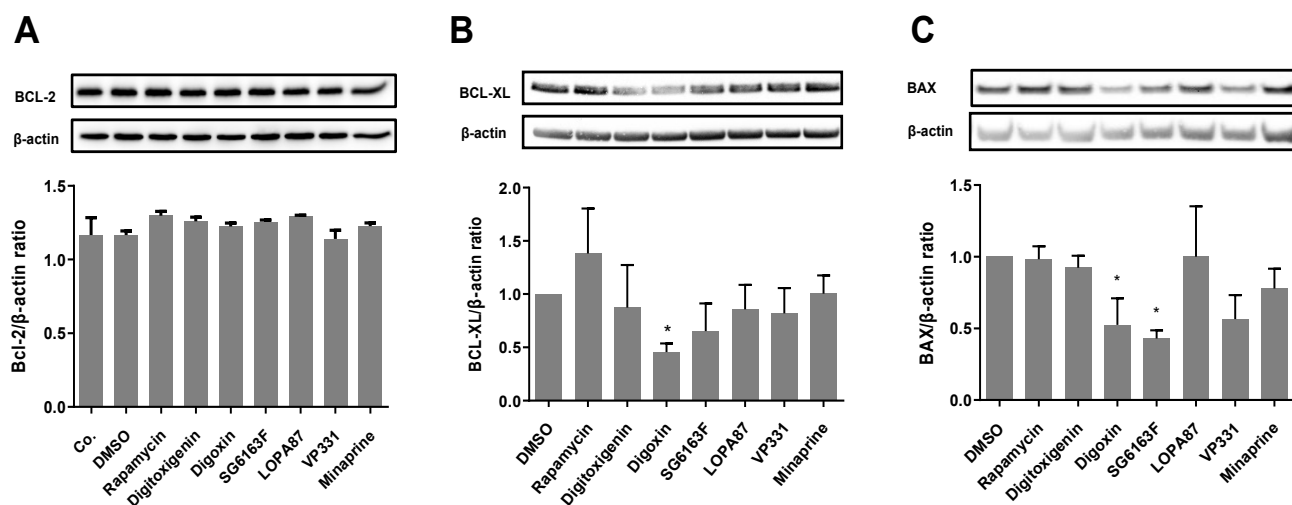
**Figure 1.** High-throughput screening for cardiac apoptosis and necrosis inhibitors. (A) Flow chart of the screening. Immortalized H9c2 cells were plated into 96-well plates, treated by 1600 compounds for 2 h, then by 10  $\mu$ M camptothecin for 24 h or 300  $\mu$ M  $H_2O_2$  for 2 h. (B) Cell survival was determined by methylene blue staining, percentage of survived cells was calculated in comparison to 0.1% DMSO as the vehicle and used to rank the compounds. (C) Ranked list and chemical formula of 6 best hits selected from Prestwick (hits 1, 2, and 6) and SBM CEA libraries (hits 3, 4, and 5).



**Figure 2.** Comparative analysis of cell viability after treatment of various cells with selected compounds. Cell viability was evaluated by LDH release assay. H9c2 cells (A) or RNVCs (B,C) were first cultured with indicated compounds followed by treatment with 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 2 h (A,B) or 10  $\mu\text{M}$  camptothecin for 24 h (C). Evaluation of compound's effect on cell growth in H9c2 cells (D) or lung carcinoma A549 cells (E). Cells were cultured in the presence of 10  $\mu\text{M}$  compounds for 48 h and lysed with lysis buffer (LB) before LDH assay. Experiments were repeated three times. Data are presented as mean  $\pm$  standard error to the mean (SEM) with one-way ANOVA, Sidak's multiple comparisons test. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ , \*\*\*\*,  $p < 0.0001$  vs. 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$  (A), 10  $\mu\text{M}$  Camptothecin (C), or 0.1% DMSO (vehicle) (A–E). ns, not significant.

The efficacy of selected compounds to inhibit cell death after treatment with  $\text{H}_2\text{O}_2$  or camptothecin was further confirmed on rat primary neonatal cardiomyocytes (RNVCs) using LDH assay and propidium iodide staining (Figures 2B,C and S1). All six compounds efficiently inhibit both necrosis and apoptosis in RNVCs. To evaluate the longer-term effect of each compound, we cultured H9c2 cells and lung cancer cells A549 with the compounds for 24 h and 48 h but did not see any additional differences in comparison with the 6 h treatment. Therefore, in all following experiments, we used 6 h treatment time. Every compound, used as single agent, did not interfere with cell proliferation of H9c2 or A549 cells (Figure 2D,E), except digitoxigenin, digoxin, and minaprine, which significantly induced cell death of A549 cells (Figure 2E).

To define cell death protective mechanisms of selected compounds, we first determined the protein expression level of anti-apoptotic B cell leukemia/lymphoma 2 family members BCL-2 and BCL-X and pro-apoptotic BAX (Figure 3) in RNVCs. The BCL-2 expression level was not changed after treatment with any compound (Figure 3A), whereas digoxin treatment decreased the expression of BCL-XL (Figure 3B). Digoxin and SG6163F decreased the expression of BAX (Figure 3C). Altogether, these results indicate the potential of the six compounds as necrosis and apoptosis inhibitors, with limited or no effect on BCL-2 family members' expression.



**Figure 3.** Effects of selected compounds on the expression of pro- and anti-apoptotic members of Bcl-2 family. Protein expression levels of BCL-2 (A), BXL-XL (B), and BAX (C) in RNVCs cultured for 6 h with indicated compounds.  $\beta$ -actin was used as a loading control. Co.—control of untreated cells. Representative Western blot images and quantification of three independent experiments are presented as mean  $\pm$  SEM with one-way ANOVA, Sidak's multiple comparisons test. \*,  $p < 0.05$  vs. DMSO.

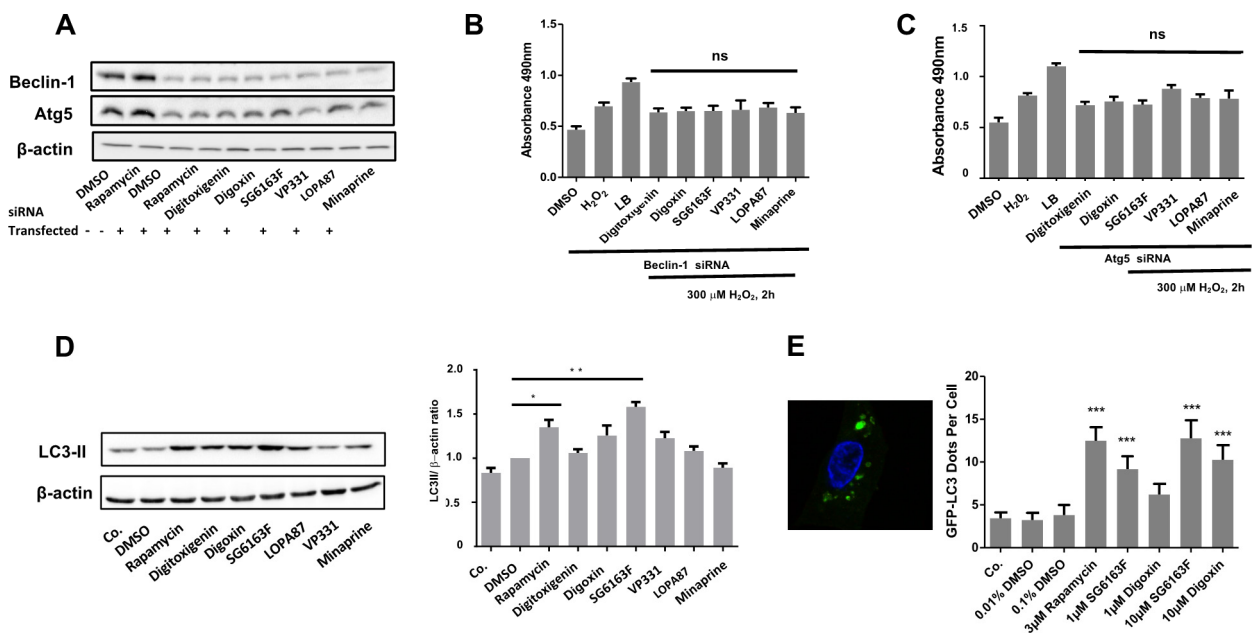
### 3.2. SG6163F Influences Autophagy Induction via ATG5 and BECLIN-1

We further hypothesized that the effect of compounds on cell viability might be through activation autophagy, an evolutionarily conserved catabolic process that removes damaged or unnecessary cellular components [15]. In order to induce autophagy, we treated cells with rapamycin, an inhibitor of mTORC1 [16], but we did not observe significant effects on the expression of BCL-2 family members (Figure 3A–C).

We further checked whether ATG5 and BECLIN-1, two proteins necessary for autophagy induction [17,18], can play a role during the inhibition of apoptosis and necrosis by selected drugs. ATG5 and BECLIN-1 expression were downregulated by siRNAs transitory transfection in RNVCs for 24 h (Figure 4A). Cells were subsequently treated with six selected chemicals, incubated with  $H_2O_2$  for 2 h, and cell death was analyzed by LDH assay (Figure 4B,C). In cells where ATG5 or BECLIN-1 expression was downregulated, all compounds lost their ability to protect RNVCs from  $H_2O_2$ -induced necrosis and camptothecin-induced apoptosis (data not shown), suggesting that selected compounds can induce autophagy as a cytoprotective mechanism. We next measured the capacity of compounds to activate autophagy following the conversion of cytosolic LC3 I to autophagosome associated LC3 II and found that LC3 II expression level was significantly increased by 1  $\mu$ M SG6163F (>1.5 fold) and 3  $\mu$ M rapamycin (>1.4 fold) treatment (Figure 4D). Next, RVNCs were transiently transfected with a GFP-LC3 plasmid, and localization of GFP-LC3 protein at autophagosomes was monitored by fluorescence 24 h post-transfection. Only treatments with 1  $\mu$ M and 10  $\mu$ M SG6163F and 10  $\mu$ M digoxin were able to induce autophagosome formation, as shown in Figure 4E.

We further measured the autophagic flux monitoring the accumulation of LC3 II and the ubiquitin-scaffold binding protein p62 after treatment with two autophagy inhibitors, 3-methyladenine (3MA) and chloroquine (CQ) (Figure 5). In the presence of CQ, but not 3MA, we observed an accumulation of LC3-II and p62 after cell treatment with SG6163F and rapamycin (Figure 5A) and an increase in GFP-LC3 puncta (Figure 5B).

Altogether, these results reveal that all compounds require ATG5 and BECLIN1 to exert their cell death inhibitory activity, but only SG6163F stimulates the autophagic flux.

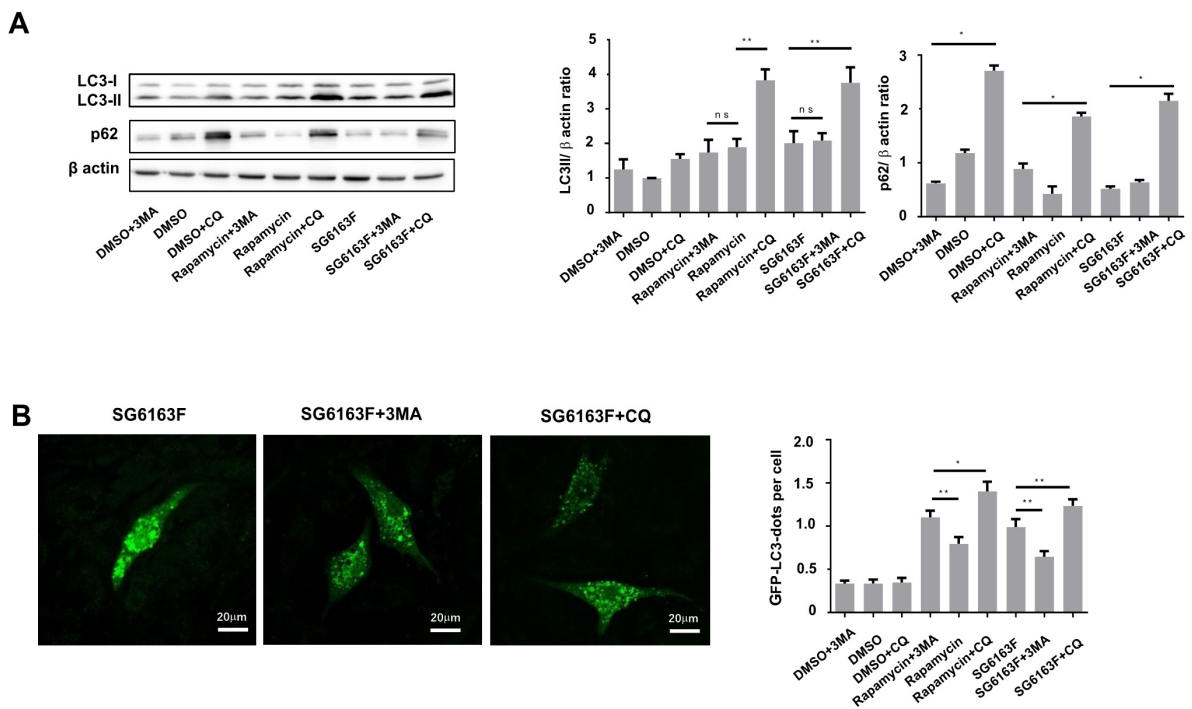


**Figure 4.** RNVCs cell death inhibition by compounds requires ATG5 and BECLIN-1. (A) RNVCs were transfected with pools of siRNAs targeting ATG5 or BECLIN-1, cultured for 48 h, and expression levels of both proteins were evaluated by Western blot. (B,C) Following BECLIN-1 (B) and ATG5 (C) siRNA transfection, LDH release was measured in RNVCs treated with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 2 h. Data are presented as mean  $\pm$  SEM with one-way ANOVA, Sidak’s multiple comparisons test. ns.—not significant vs. H<sub>2</sub>O<sub>2</sub> treated and siRNA transfected cells. (D) Protein level of LC3-II in RNVCs following treatment by compounds for 6 h was analyzed by Western blot, and the LC3II/b-actin ratio was determined in comparison to DMSO. Data are presented as mean  $\pm$  SEM with one-way ANOVA, Sidak’s multiple comparisons test. \*,  $p < 0.05$ , \*\*,  $p < 0.01$  vs. DMSO. (E) Redistribution of GFP-LC3. 24 h after transient transfection with a GFP-LC3 coding plasmid, cells were treated for 6 h with DMSO, rapamycin, and 1 and 10  $\mu$ M of SG6163F and Digoxin. A representative cell is shown (left). The frequency of dots per cell (right) was quantified for 150 cells for each condition. Dots correspond to clear vacuolar distribution of GFP-LC3. Nuclei were stained by 0.5  $\mu$ M Hoechst 33342. Data are presented as mean  $\pm$  SEM with one-way ANOVA, Sidak’s multiple comparisons test. \*\*\*,  $p < 0.001$  vs. DMSO. ns, not significant. +, transfection; -, no transfection.

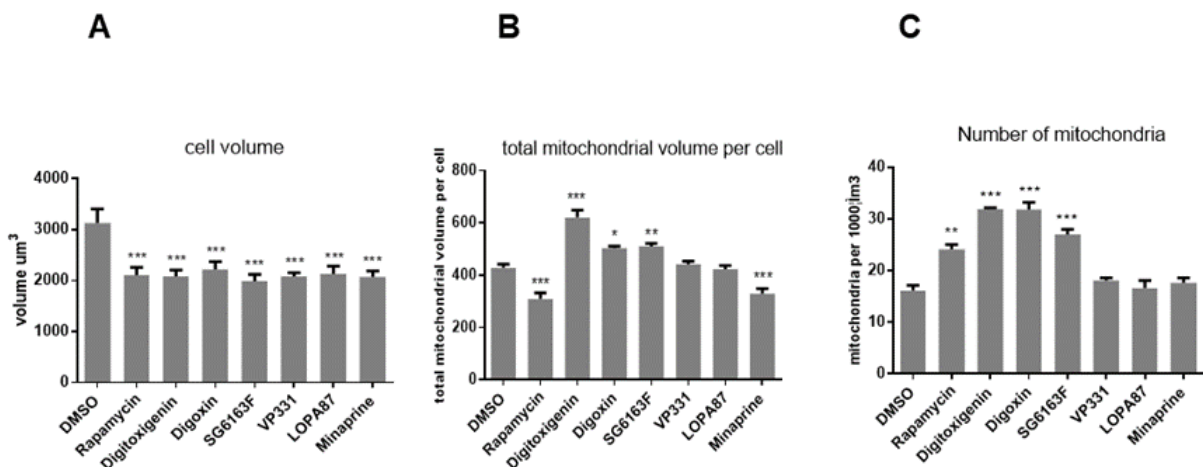
### 3.3. Compounds Impact on Mitochondrial Network Structure and Dynamics

We next verified the effects on mitochondria in cells treated with different compounds because these organelles play a major role in cardiomyocyte cell functioning [7,19,20]. Following RNVCs treatment by the compounds for 6 h, the mitochondria were labeled with 200 nM Mitotracker and the cells with 4  $\mu$ M calcein-AM. The mitochondrial network was visualized by confocal microscopy, and the numbers of individual mitochondria were analyzed using the software IMARIS. While all compounds significantly decreased the cell volume compared to the vehicle (Figure 6A), digitoxigenin, digoxin, and SG6163F increased the number of mitochondria and the total mitochondrial volume per cell (Figure 6B,C). In contrast, VP331, LOPA87, and minaprine had no effect on the number of mitochondria, whereas 10  $\mu$ M minaprine and 3  $\mu$ M rapamycin decreased the total mitochondrial network volume (Figure 6B,C). Moreover, digitoxigenin and digoxin decreased the expression of MFN1 and MFN2 proteins, essential for mitochondrial fusion (Figure 7A) and digoxin and SG6163F stimulated fission as detected by phosphorylation of Drp-1 at Ser616 (Figure 7B), which suggests that treatment with these compounds influences mitochondrial dynamics and induces mitochondrial fission.

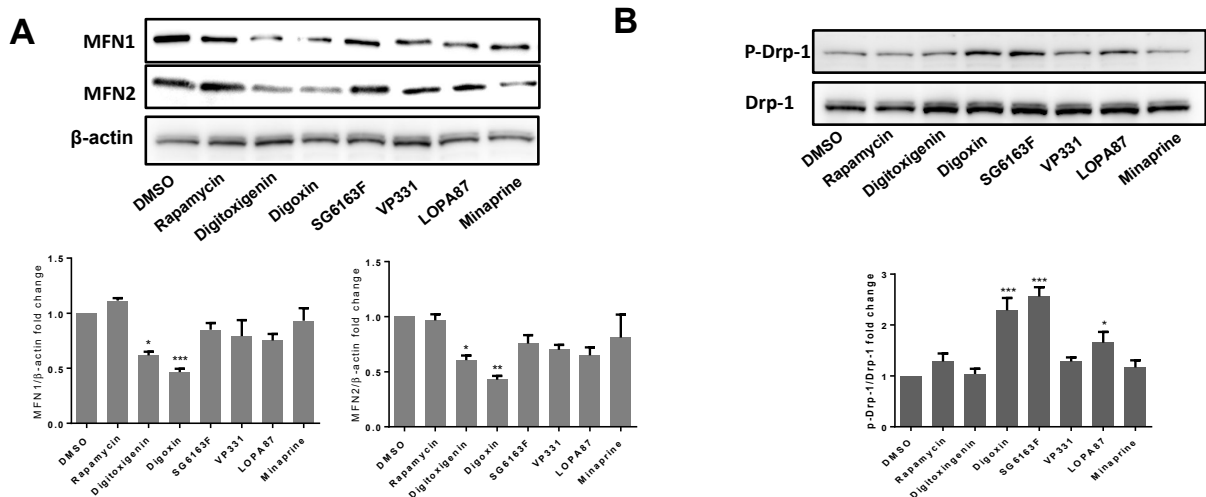




**Figure 5.** SG6163F stimulates autophagic flux in RNVCs. **(A)** Protein levels of LC3-I/II and p62 in RNVCs treated with 5 mM 3-methyladenine (3MA) and 20 μM chloroquine (CQ) and 10 μM SG6163F or 3 μM rapamycin for 6 h were analyzed by Western blot. LC3II/β-actin and p62/β-actin ratios were determined and presented as fold change in comparison to DMSO. Data are presented as mean ± SEM with one-way ANOVA, Sidak's multiple comparisons test. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ . **(B)** Upon cell transfection by GFP-LC3 for 24 h and 6 h of cell treatment by SG6163F, 3-methyl adenine (MA), chloroquine (CQ), and rapamycin (not shown as image), GFP-LC3 redistribution to vacuoles (dots) was visualized by fluorescence microscopy and quantified by Image J. Experiments were repeated three times. Data are presented as mean ± SEM with one-way ANOVA, Sidak's multiple comparisons test, \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , ns, no significant.

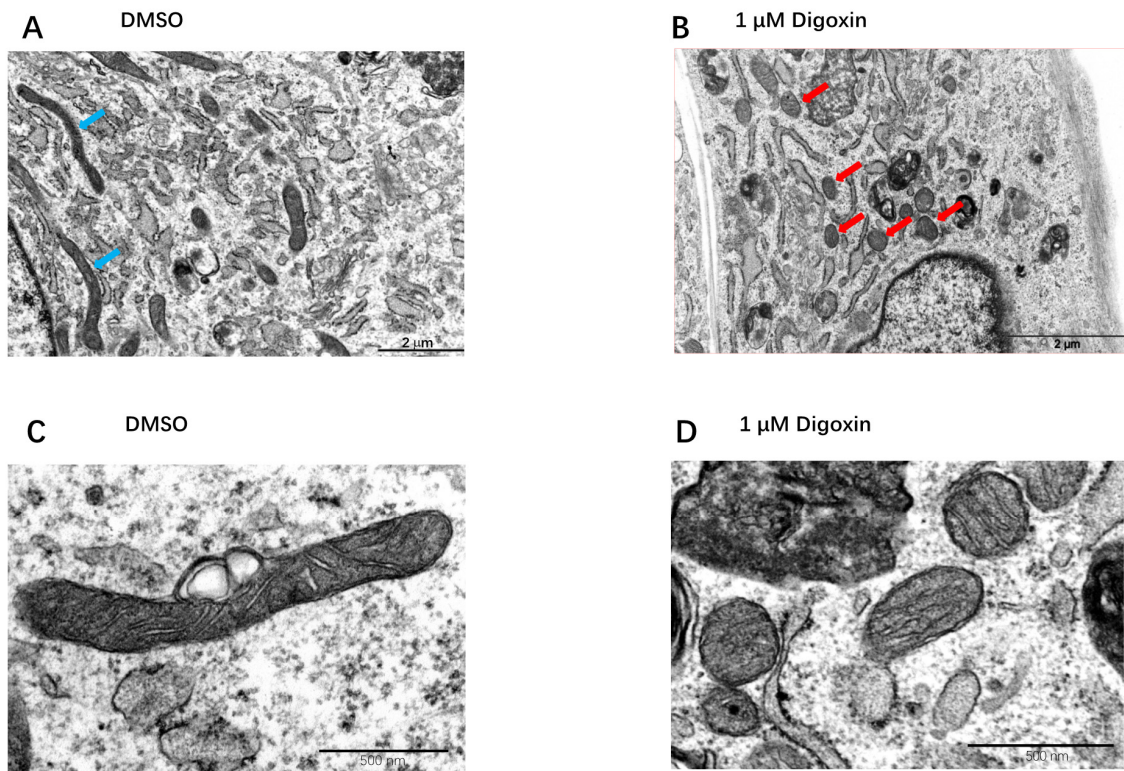


**Figure 6.** Effect of selected compounds on mitochondrial volume. RNVCs were treated with 1 μM compounds for 6 h and labeled with 4 μM calcein to determine the effect of compounds on the mitochondrial volume **(A)**. Mitochondria were labeled with 200 nM Mitotracker to evaluate the total volume of mitochondria per cell **(B)** and quantify the number of individual mitochondria per cell **(C)**. At least 150 cells were analyzed using a Leica confocal microscope and IMARIS software. Data are presented as mean ± SEM with one-way ANOVA, Sidak's multiple comparisons test. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  vs. DMSO. Experiments were repeated three times.

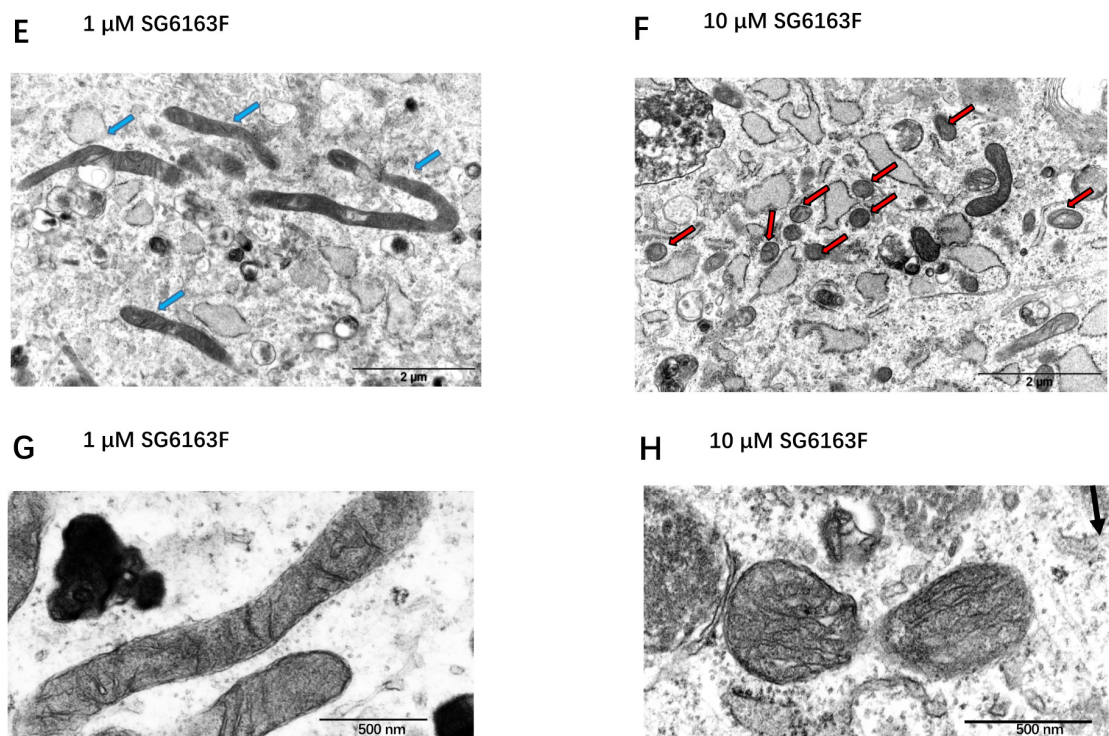


**Figure 7.** Effects of compounds on the expression of proteins of the mitochondria fusion/fission machinery. (A) Following RNVCs incubation with 1  $\mu$ M of indicated compounds, expression was analyzed by Western blot. The intensities of MFN1 and MFN2 bands were normalized to  $\beta$ -actin. (B) Drp-1 and p-Drp-1 expressions in treated RNVCs were analyzed by Western blot and their ratio quantified. Experiments were repeated 3 times. Representative Western blot images and quantification of three independent experiments are presented as mean  $\pm$  SEM with one-way ANOVA, Sidak’s multiple comparisons test. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  vs. DMSO.

In digoxin and SG6163F- treated cells, numerous short and round mitochondria can be observed compared to 0.1% DMSO-treated cells (controls), which have long and thin mitochondria (Figure 8). Thus, transmission electron microscopy confirms that mitochondria are smaller in H9c2 cells treated with 10  $\mu$ M SG6163F and 1  $\mu$ M digoxin in comparison to cells treated with DMSO or 1  $\mu$ M SG6163F.



**Figure 8.** Cont.



**Figure 8.** Mitochondrial morphology analysis in cells treated with SG6163F and digoxin by transmission electron microscopy (TEM). Cells were treated by 0.1% DMSO (A,C), 1  $\mu$ M digoxin (B,D), 1  $\mu$ M SG6163F (E,G), and 10  $\mu$ M SG6163F (F,H), fixed by glutaraldehyde and analyzed by TEM. Blue arrows in (A,E) indicate the long and thin mitochondria, and the red arrows in (B,F) indicate short round mitochondria which suggest fission events.

### 3.4. Metabolic Reprogramming in Cells Treated with Selected Compounds

To dissect the metabolic effects of compounds, we analyzed the energy metabolism of H9c2 cells in real time. We found that all compounds except rapamycin increased extracellular acidification suggesting an increase of ATP productions by anaerobic glycolysis (Figure 9A). Digitoxigenin and minaprine improved ATP production by oxidative phosphorylation (OXPHOS) using glucose and pyruvate, but not fatty acids as substrates (Figure 9B,C). VP331 Digoxin improved OXPHOS using fatty acids as substrate (Figure 9C) but not glucose (Figure 9B). SG6163F boosted OXPHOS, but rapamycin decreased it [21] (Figure 9B,C).

Finally, we evaluated ROS production by detecting anion superoxide in RVNCs by MitoSOX fluorescent probe following cell treatment by the compounds during 6 h. We found that rapamycin, digitoxigenin, VP331, LOPA87, and minaprine but not digoxin or SG6163F induced a local mitochondrial anion superoxide production in line with the observed activation of OXPHOS (Supplementary Figure S2).

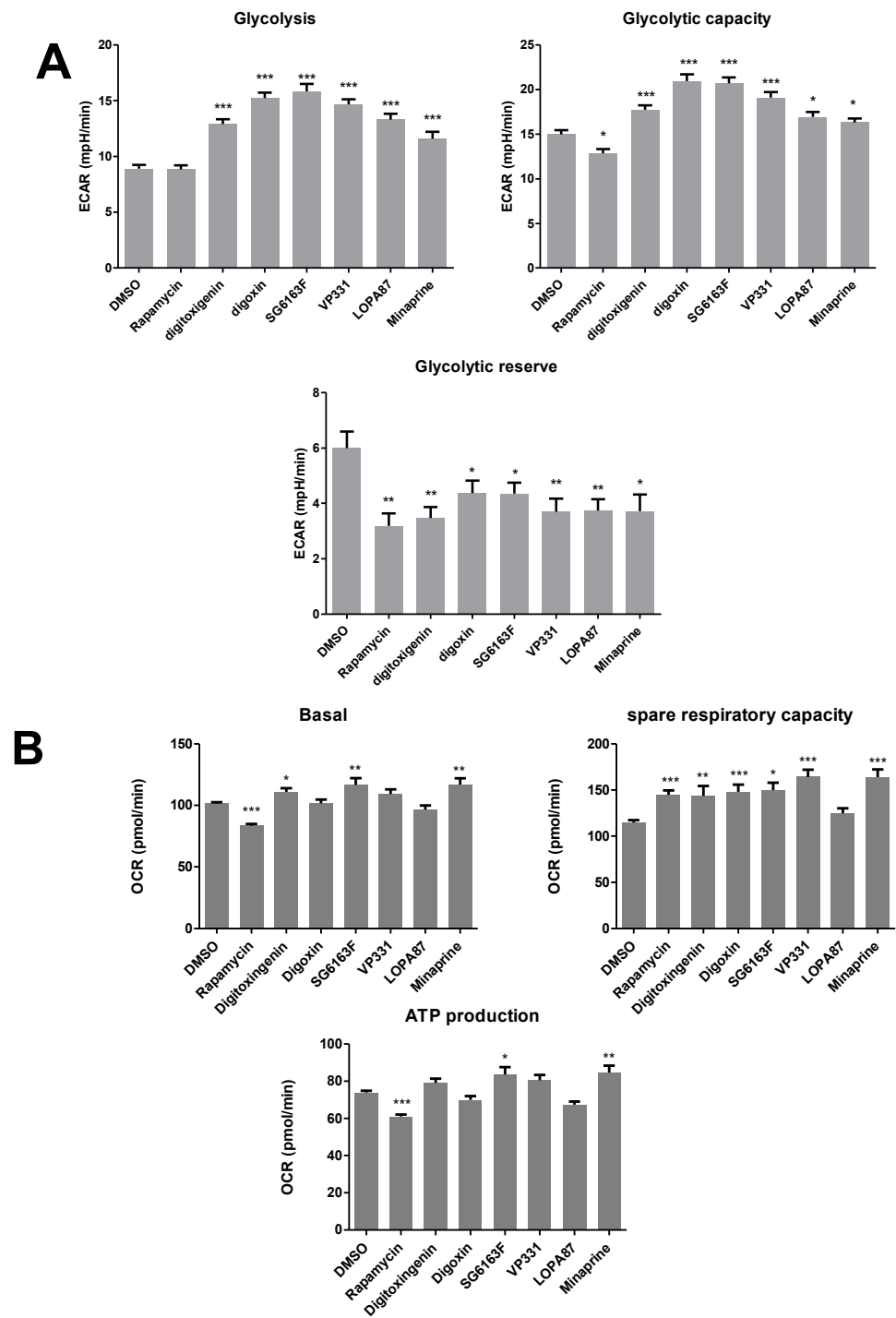
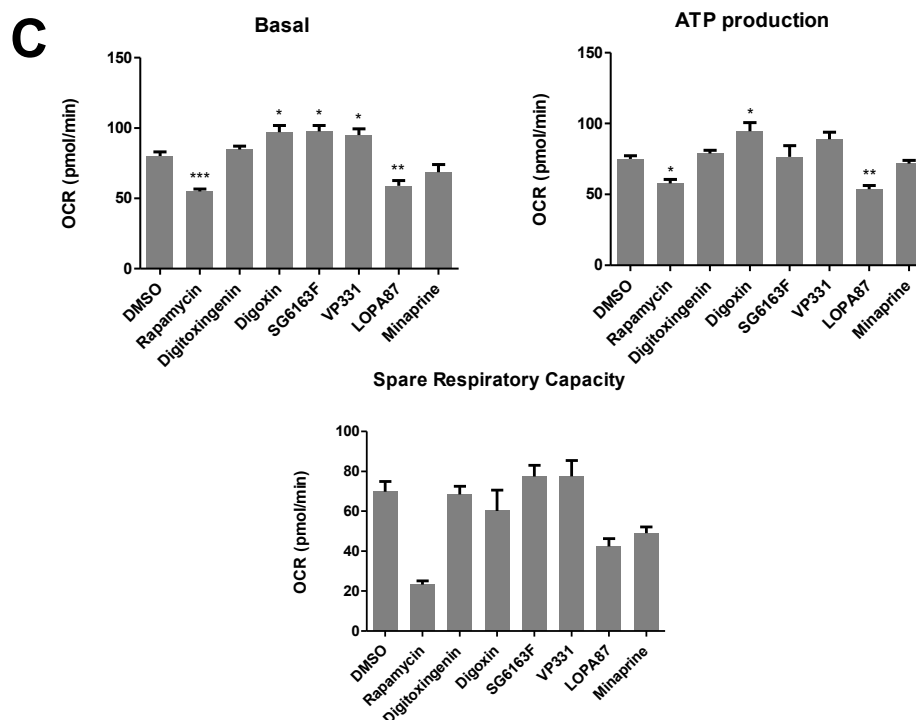


Figure 9. Cont.



**Figure 9.** Metabolic reprogramming effects. (A) H9c2 cells were treated with indicated compounds for 6 h, and glycolytic function (A) and mitochondrial respiration (B) were measured by XFe96 Extracellular Flux Analyzer. (C) The oxidation of exogenous fatty acids was measured using the XF Palmitate-BSA FAO Substrate kit. OCR rates are expressed for 20,000 cells per well. Experiments were repeated three times. Data are presented as mean  $\pm$  SEM with one-way ANOVA, Sidak's multiple comparisons test. \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  vs. DMSO.

#### 4. Discussion

By high-throughput screening, we have identified new compounds capable of inhibiting cardiac apoptosis and necrosis and characterized their effects in H9c2 cells and in primary RNVCs. Among these compounds, digitoxigenin and digoxin (cardiac glycosides) and minaprine are molecules from Prestwick library, a commercial library of 1200 off-patent small molecules, 95% being approved drugs. Three other chemicals, SG6163F, VP331, and LOPA87, are new small molecules, which were synthesized in our laboratories [13,14].

Cardiac glycosides are natural molecules used in clinical medicine known for their antagonistic action on  $\text{Na}^+, \text{K}^+$ -ATPase. Cardiac glycosides have increased sensitivity in cancer cells [22] and have an ability to induce apoptosis [23], promote immunogenic cell death [24], and mediate autosis, a form of cell death resulting from excessive autophagy [25]. Here, we observed that digitoxigenin and digoxin promote cell death of A549 lung cancer cells (Figure 2) and have a pro-survival ATG5 and BECLIN-1-dependent autophagic activity in RNVCs (Figure 3). This is accompanied by a downregulation of BCL-XL and BAX, two members of the BCL-2 family, but no effect on the BCL-2 expression level was observed (Figures 3 and 4). Importantly, because the disruption of interaction of BECLIN-1 and BCL-XL induces autophagy [26], our results are in line with the major role of BECLIN-1 in the heart, where changes in its expression affect functions and survival of cardiomyocytes [27].

Our results show that pharmacological manipulation of autophagy can be instrumental for protection from  $\text{H}_2\text{O}_2$ -induced oxidative alterations and DNA-damage events induced by camptothecin. Indeed, all compounds required BECLIN-1 and ATG5 to protect primary cardiomyocytes from cell death, as shown by the downregulation of these autophagy activators upon siRNA treatment (Figure 4). However, only SG6163F and digoxin treatment were shown to involve autophagosome formation (Figure 4D,E).

Treatments of RNVCs by compounds revealed that digitoxigenin, digoxin, and SG6163F modulate mitochondrial dynamics and/or biogenesis by increasing mitochondrial mass and number of mitochondria accompanied by a decrease of cell volume (Figure 6). In addition, cardiac glycosides, but not SG6163F, decreased the expression level of mitofusins MFN1 and MFN2, while digoxin and SG6163F activated organelle fission as revealed by phosphorylation of DRP1 on Ser 616 (Figure 7). In addition, digitoxigenin, digoxin, and SG6163F stimulated ATP production by anaerobic glycolysis. Digoxin and SG6163F boosted glucose and pyruvate-fueled OXPHOS (Figure 8), while digitoxigenin stimulated fatty acid-fueled OXPHOS.

In summary, digitoxigenin, digoxin, and SG6163F protected cardiomyocytes by decreasing the expression of pro-apoptotic protein BAX, affecting autophagy, increasing mitochondrial mass, and boosting ATP production by improving aerobic and anaerobic metabolism. However, the three molecules effects differ in BCL-XL, MFN, DRP-1/DRP1-*p* expression regulation, ROS production, and toxicity for cancer cell line A549. In contrast, VP331, LOPA87, and minaprine had no effect on BCL-2 family expression (Figure 3) and required ATG5 and BECLIN-1 (Figure 4). Mitochondrial ROS increase was observed following RNVCs treatments by VP331, LOPA87, and minaprine, which could be due to OXPHOS stimulation (Supplementary Figure S2). These molecules also boosted ATP production by anaerobic glycolysis.

To conclude, we conducted a robust high-throughput screening to search for cell death inhibitors. These assays are complementary to the previous low-throughput screens [28–31]. Our screening identified six inhibitors of cardiac cell death, which act through autophagy and metabolism reprogramming. These compounds have promising cardioprotective activities and, thus, might be useful in clinics for repositioning purposes or as new drug candidates. Since there is a high interconnection between metabolism, cell death, and malignancy [32,33], it was important to check the effect of our compounds on cancer cell proliferation. None of the six compounds favored cell proliferation in A549 lung cancer cells or led to the overexpression of oncogenic proteins BCL-2 and BCL-XL (Figures 2 and 3). In addition, all molecules showed no cytotoxicity for RNVCs and H9c2 cells (Figure 2). These are particularly interesting results in the perspective of the use of compounds in anticancer combination therapy.

As anticipated from chemical structures, compounds were rapidly metabolized in vitro mouse microsomes or showed poor solubility (data not shown), which might hamper preclinical studies in animals [34]. Therefore, if digitoxigenin, digoxin, and minaprine as approved FDA molecules could be repositioned and enter rapidly in preclinical studies in combination with radiation or chemotherapeutic agents, the three new compounds, SG6163F, VP331, and LOPA87, may require chemical optimization for further therapeutic development.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells11030474/s1>, Figure S1: Selected compounds inhibition of H<sub>2</sub>O<sub>2</sub> induced-necrosis in RNVCs and H9c2; Figure S2: Effects of compounds on mitochondrial ROS production.

**Author Contributions:** Conceptualization, C.B., F.T. and J.-C.C.; methodology, C.B., D.C. and F.P.; investigation, D.L., F.P., D.C., V.N., S.L.-G., E.J. and Y.A.L.-B.; writing—original draft preparation, C.B., J.-C.C. and D.L.; writing—review and editing, C.B., Y.A.L.-B. and S.D.; funding acquisition, C.B. and J.-C.C. All authors have read and agreed to the published version of the manuscript.

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

### Abbreviations

LDH	Lactate dehydrogenase
SD	Standard deviation
3-MA	3-methyladenine
CQ	Chloroquine

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## **3. PERSPECTIVES**

### 3. Perspectives

The results obtained in this worked confirmed a conserved function of the GATOR1 complex in regulation of mitochondrial function. We found a physical interaction between GATOR1 proteins and mitochondria and confirmed that lack of GATOR1 proteins impacts mitochondrial morphology and ultrastructure.

We found that NPRL2 protein stability is dramatically affected upon metabolic or mitochondrial stress, however NPRL3 and DEPDC5 remain unaffected. The exact reason of this difference between GATOR1 members response to stress remains elusive. Previous results in our group have shown that strong overexpression of NPRL2 induces its accumulation in the nucleus, where it interacts with mitochondrial protein AIF which provokes p53 phosphorylation and further activation of DNA damage response. However, whether other members of GATOR1 are also participating in this process remains unclear. In contrast, it is known that GATOR1 and GATOR1 do not form a stable holo-complex like their yeast homologues, could it be also possible that its individual components are performing separated specific mechanistic functions? These results provide evidence of independent roles for GATOR1 proteins in other functions beyond mTORC1 regulation, therefore in the future it would be reasonable to treat GATOR1 proteins as independent entities for the study of mitochondrial function.

As mentioned above, NPRL2 and AIF can interact in the nucleus to induce apoptosis. According to our results NPRL2 can be found in the mitochondria, still whether such interaction

with AIF or other mitochondrial proteins can also occur in the mitochondria it is currently unknown. In addition, can NPRL3 and DEPDC5 also interact with other mitochondrial proteins at the mitochondrial level or elsewhere in the cell? If so, what is their exact mechanistic function? Are they working as part of the platform of mTORC1 signaling? Or by such interaction are they exerting mTORC1-independent functions?

We did not find any significant difference in accumulation of LC3-II upon CCCP-induced mitophagy. Since CCCP induces loss of mitochondrial membrane potential, a molecular event that can signal to mTORC1 to undescribed mechanisms. We consider that it will be reasonable to assess other milder mitophagy inducers to evaluate the impact of GATOR1 in selective mitochondrial degradation.

In this work we found that GATOR1 depletion disrupts normal mitochondrial morphology which points to a link of GATOR1 proteins and regulation of mitochondrial dynamics. However, it would be interesting to describe mitochondrial morphology in both depletion and overexpression conditions of GATOR1 proteins. Since mitochondrial network shows small, round and fragmented mitochondria, it would be interesting to assess the response of such mitochondria to metabolic stress to a condition that promotes mitochondrial fusion, such as amino acid starvation in both GATOR1 depletion and strong overexpression.

There are many reports that link mutations of NPRL2, NPRL3 and DEPDC5 members to several diseases, such as cancer, cardiovascular and neurodegenerative diseases, respectively.

In all the three listed diseases mitochondria and metabolic regulation can play a critical role, therefore it would be interesting to explore the role of GATOR1 proteins in a pathological model. In addition, NPRL2 mutations are highly correlated to cisplatin and doxorubicin resistance in lung cancer, both drugs are DNA-damaging agents. A link between cisplatin resistance and DRP1-dependent mitochondrial network remodeling has already been described. However, whether NPRL2 is an active player in the development of such molecular events is completely unknown, thus we consider that this research axis should be explored in the future. Such findings could provide further evidence of their exact mechanistic role in the regulation of mitochondrial function and their impact in metabolic regulation in health and disease.

## **4. SEA and GATOR 10 years later**

Review

# SEA and GATOR 10 Years Later

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**Abstract:** The SEA complex was described for the first time in yeast *Saccharomyces cerevisiae* ten years ago, and its human homologue GATOR complex two years later. During the past decade, many advances on the SEA/GATOR biology in different organisms have been made that allowed its role as an essential upstream regulator of the mTORC1 pathway to be defined. In this review, we describe these advances in relation to the identification of multiple functions of the SEA/GATOR complex in nutrient response and beyond and highlight the consequence of GATOR mutations in cancer and neurodegenerative diseases.

**Keywords:** SEA complex; GATOR complex; mTORC1 pathway; autophagy; amino acid signaling; cancer; epilepsy; neurological disorders

## 1. Introduction

The highly conserved mechanistic (or mammalian) target of rapamycin (mTOR) plays a key role in cellular homeostasis. mTOR kinase forms the following two different complexes: mTORC1 and mTORC2, which regulate cellular responses to many stresses [1,2]. In order to maintain optimal growth and metabolism, the mTORC1 pathway integrates signals from a wide variety of intracellular and extracellular cues, which include amino acids, growth factors, energy, oxygen, DNA damaging agents, etc. Depending on the nature of the signal, mTORC1 will drive the cell either to the anabolic pathway, promoting the proliferation and survival, or to the catabolic pathway by controlling autophagy or the ubiquitin-proteasome system. In order to coordinate this vast network, mTORC1 relies on many upstream modulators and downstream effectors. Ten years ago, one of the major upstream regulators of mTORC1 pathway, the SEA/GATOR complex, was identified [3]. Over these years, many advances have been made in our understanding of the SEA/GATOR complex functions and their consequences to the operation of the mTORC1 pathway; however, many questions are still unsolved [4]. Our comprehension of the SEA/GATOR complex regulation and function is particularly important because of the consequences of its dysfunction in diverse pathological settings, especially in cancer and neurodegenerative diseases. This review covers the most important findings about the SEA/GATOR complex that have been made during the last decade.

## 2. Discovery of the SEA Complex

The SEA complex was initially identified in yeast *Saccharomyces cerevisiae* through an atypical way [3,5–7]. Back in 2007, a multidisciplinary approach was undertaken to solve the structure of one of the largest macromolecular machines in the cell—the nuclear pore complex (NPC) [5,8]. Central to this approach were the collection of many kinds of biophysical and proteomic data, the translation of these data to spatial restraints and the calculation of a final architecture that satisfies all the restraints. This was how the immunopurification of one of the NPC components, nucleoporin Seh1, revealed that this protein did not only co-purify with the Nup84 subcomplex, the major constituent of the NPC scaffold, but also with the following four high-molecular-weight proteins with completely unknown functions at the time: Yjr138p (Iml1), Yol138p (Rtc1), Ydr128p (Mtc5)



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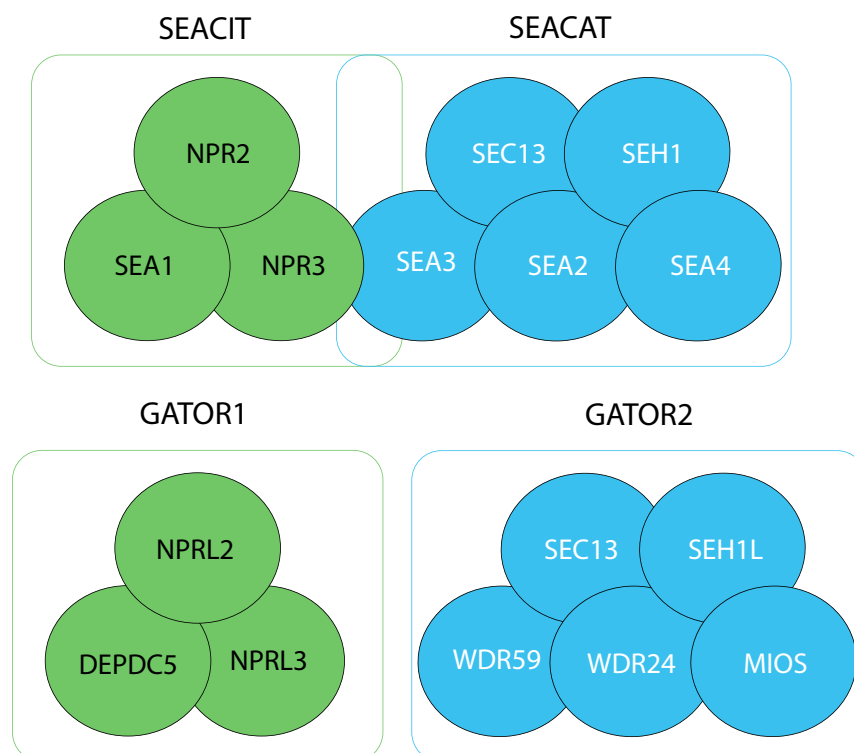
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and Ybl104p [5]. Four years later, in 2011, a paper that described the full SEA complex for the first time was published [3]. The four proteins, which were first observed in Seh1 pullouts in 2007, were given a common name, Sea (for Seh1-associated) and named Sea1 through Sea4, respectively. The following three other protein components completed the full SEA eight-protein complex: Sec13, Npr2 and Npr3. The proteins of the SEA complex appeared to be dynamically associated with the vacuole membrane and have a role in autophagy. The function of Iml1-Npr2-Npr3 in autophagy was also described by the Tu group that same year [9]. Meanwhile, in 2009, Npr2 and Npr3 were shown to form an evolutionary conserved heterodimer, involved in the upstream regulation of TORC1 in response to amino acid starvation in *S. cerevisiae* [10]. This fundamental function of the SEA complex was further confirmed both in yeast and humans by de Virgilio and Sabatini laboratories in 2013 [11,12].

The SEA complex in *S. cerevisiae* consists of two subcomplexes, named SEACIT (SEA subcomplex inhibiting TORC1) and SEACAT (SEA subcomplex activating TORC1) (see below) [11,13,14] (Figure 1). In 2013, these complexes were characterized for the first time in humans by Sabatini's laboratory and were re-named to GATOR1 (GTPase activating protein activity toward RAGA, see below) and GATOR2, respectively [12]. SEACIT is composed of Iml1/Sea1, Npr2 and Npr3 (DEPDC5, NPRL2 and NPRL3 in GATOR1), and SEACAT contains Sea2, Sea3, Sea4, Seh1 and Sec13 (WDR24, WDR59, MIOS, SEH1L, SEC13 in GATOR2) (Figure 1).



**Figure 1.** Composition of yeast SEA complex and mammalian GATOR complex. SEACIT subcomplex in yeast can tightly interact with SEACAT, most probably via Npr3/Sea3 connection. GATOR1 and GATOR2 do not form a stable full GATOR complex.

Phylogenetic analyses demonstrated that SEA/GATOR complex subunits are present across various eukaryotic kingdoms, suggesting an origin of these factors before the last common eukaryotic ancestor [3]. Homologs of all eight proteins could be clearly found in the genomes of fungi and metazoans, with some representation in protists, but cannot be identified in plants [3,15]. In 2021, the homologs of the SEA complex and its components were characterized in *Schizosaccharomyces pombe* [16], *Caenorhabditis elegans* [17], *Drosophila* [18], zebrafish [19], mice [20], rats [21] and humans [12]. The

majority of the structural and functional studies were usually performed in *S. cerevisiae* and in humans. *Drosophila* was very instrumental for the study of the SEA/GATOR role in development; while the zebrafish, mouse and rat models were used to study different human pathologies.

### 3. SEA/GATOR Nomenclature

The nomenclature of the SEA complex proteins and subcomplexes in different organisms is somewhat confusing. For example, in *S. pombe*, the complex is called GATOR, but the names of the constituent proteins are the same as in *S. cerevisiae* [22]. One of the *Drosophila* GATOR1 components is called Iml1 (impaired minichromosome loss), as its yeast homologue, but all other proteins are named after their human homologues [18]. Moreover, the yeast protein community has a tendency to drop the name Sea1 and call the protein with its initial name, Iml1. The SEA proteins Npr2 (nitrogen permease regulator 2) and Npr3 (nitrogen permease regulator 3) gave names to their human orthologues NPRL2 (Npr2-like) and NPRL3 (Npr3-like) [10,23]. GATOR2 component MIO obtained its name from its *Drosophila* orthologue Mio (missing oocyte) [24]. On the other hand, the two GATOR2 components, WDR24 and WDR59, still have their systematic names. In the future, it might be reasonable to revise their names so they reflect their function (currently, these functions are not yet defined). Alternatively, the proteins can be systematically named after their yeast homologues (as in the case of NPRL2, NPRL3), i.e., SEAL2 and SEAL3.

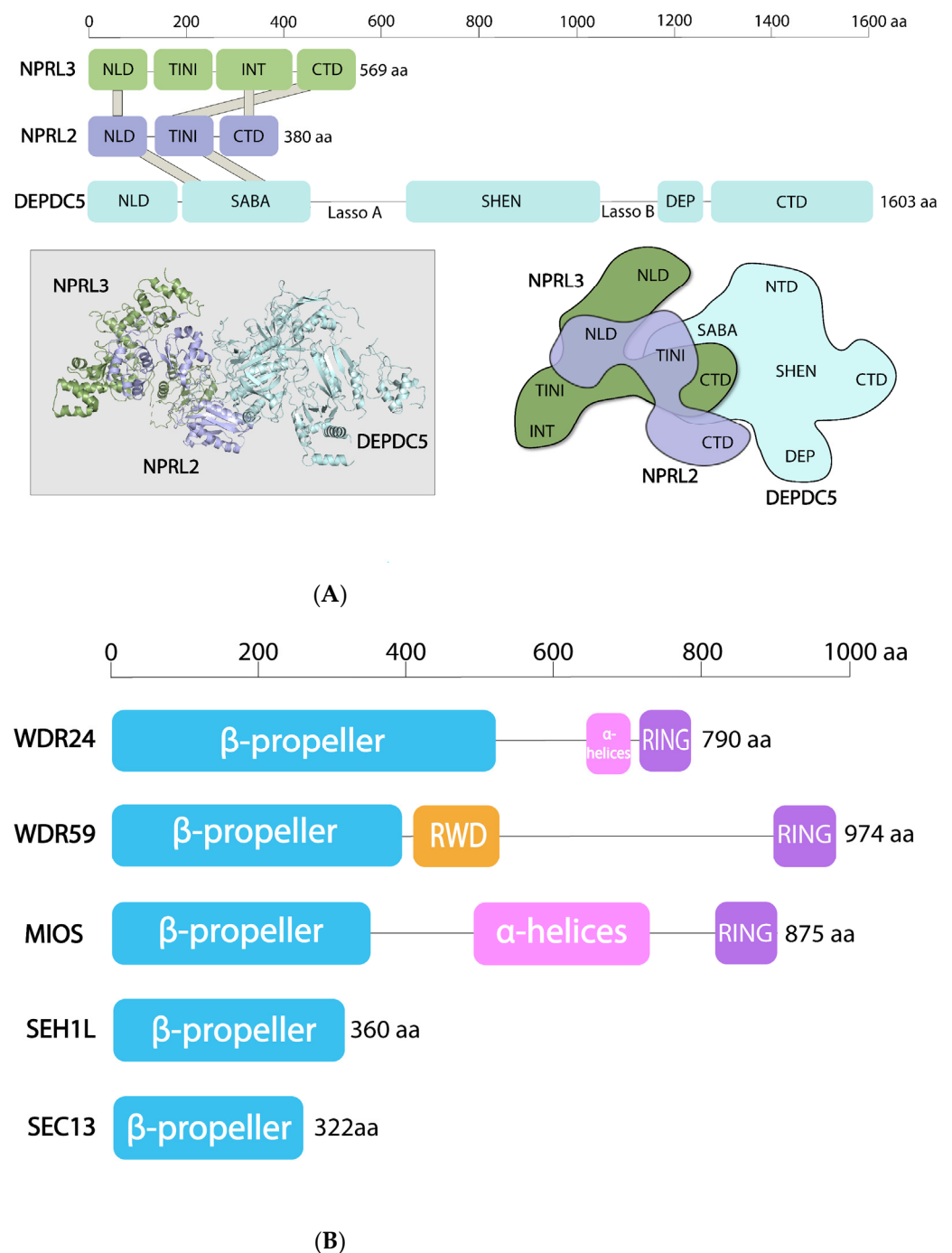
### 4. Structural Features of the SEA and GATOR Complexes

The overall architecture of SEA/GATOR proteins is evolutionary conserved [3]. DE-PDC5 is only 10 amino acid residues longer than Iml1/Sea1, but both have an identical fold arrangement. The human orthologs of Sea2-Sea4, Npr2 and Npr3 are smaller than yeast proteins, mainly because of the deletion of protein regions, predicted to be disordered in yeast. It is quite reasonable to expect that the mammalian GATOR components repertoire would be larger compared to yeast due to the expression of alternative splicing products. For example, bioinformatical predictions revealed that WDR24 has at least two isoforms, one of which is missing about 130 amino acid residues in the N-terminal part [3]. One of the NPRL3 isoforms that lacks the N-terminal part and is highly expressed in red blood cells has just recently been characterized [25]. A splicing variant that led to exon 3 skipping in NPRL2 was detected in an individual with familial focal epilepsy (see below) [26].

Two subcomplexes of the SEA/GATOR are very different structurally (Figure 2). SEACIT/GATOR1 members have domains, found in proteins that control the functions of small GTPases. SEACAT/GATOR2 components are enriched with domains found in coating assemblies (i.e., COPI and COPII coated vesicles, nuclear pore complex, etc.) (see below). Seh1, Sec13 and the N-termini of Sea4 and Sea2 in *S. cerevisiae* SEACAT appear to form a large cluster of  $\beta$ -propeller domains. Similar arrangements of  $\beta$ -propeller domains have been described at the vertex of the evolutionarily related complexes COPI and COPII [27].

In yeast, SEACAT and SEACIT interact to form the full SEA complex (Figure 1) [13]. A 3D map of the *S. cerevisiae* SEA complex, obtained by a combination of biochemical and computational approaches, suggests that SEACAT and SEACIT are connected by interactions between the N-termini of Sea3 from SEACAT and both Npr3 and Iml1/Sea1 from SEACIT [13]. Similar observations have recently been made in *S. pombe*, where Sea3 anchors other GATOR2 components to GATOR1, although, as expected, Sea3 was not required for the assembly of GATOR1 components [28]. In humans, GATOR1 and GATOR2 do not form a stable GATOR complex [12], yet, similar to yeast, NPRL3 is necessary and sufficient for the interaction with GATOR2 [29].





**Figure 2.** Domain organization of GATOR1 and GATOR2 proteins. **(A)** Domain structure and interaction of GATOR1 proteins (top); atomic model of GATOR1 complex (PDB:6CET), adapted from [29] and modified by PyMOL (bottom left) and cartoon representation of GATOR1 structure with domains indicated (bottom right). **(B)** Schematic representation of GATOR2 components, with domain boundaries according to secondary structure predictions from [3].

Despite the considerable progress in the structural determination of the constituents of the mTORC1 pathway that have been made in the last five years [30], only the structure of human GATOR1 has been solved (Figure 2A). All structural information that is currently available for GATOR2 or for the yeast SEA complex comes from bioinformatic predictions and interactivity assays [3,13]. The lack of high-resolution structures of the GATOR2 and of the entire complex both in yeast and humans are among the major reasons that prevent our full understanding of the SEA/GATOR functions at the present.

#### 4.1. SEACAT/GATOR2

SEACAT and GATOR2 have components that moonlight between functionally unrelated complexes and are structurally connected with vesicle-coating scaffolds. The SEACAT/GATOR2 complex closely resembles the membrane coating assemblies, such as COPII vesicles, nuclear pore complexes and HOPS/CORVET complexes [8,31–33]. It also shares common subunits with both COPII (Sec13/SEC13) and nuclear pore complex (Sec13/SEC13 and Seh1/SEH1L). Sea4/MIOS contains N-terminal WD40 repeats arranged into a  $\beta$ -propeller structure followed by an  $\alpha$ -solenoid stretch, which is a structure that is characteristic for proteins that form oligomeric coats (e.g., clathrin and Sec31) in vesicle-coating complexes [3,33] (Figure 2B). Furthermore, every protein in SEACAT contains a  $\beta$ -propeller (and Sea3 probably has two  $\beta$ -propellers), a domain common in coating assemblies [34]. Lastly, there are two dimers, Seh1-Sea4 and Sec13-Sea3 [3,13], that could be analogues to the Sec13-Sec31 dimer, which forms the structural unit of the COPII complex [35]. These dimeric interactions in the SEACAT are most probably conserved, because it was found that the Seh1 in *Drosophila* also directly interacts with Sea4/Mio [36].

Sea4 also contains a C-terminal RING domain, which, together with its  $\beta$ -propeller and  $\alpha$ -solenoid motifs, makes it closely resemble several protein subunits of the homotypic fusion and protein sorting (HOPS) and class C core vacuole/endosome tethering (CORVET) complexes, which have been implicated in the tethering of membranes prior to their fusion. HOPS and CORVET are associated with the vacuoles/lysosomes and endosomes, respectively, and play a role in endosomal and vacuolar assembly and trafficking, as well as in nutrient transport and autophagy [32,37]. Sea2/WDR24 and Sea3/WDR59 also have a C-terminal RING domain. Clusters of RING domains are associated with E3 ubiquitin ligase activity, suggesting SEACAT might have such a role. In *S. cerevisiae*, the RING domains appear to be crucial for maintaining the interactions between Sea2, Sea3, Sea4 and the rest of the complex. For example, Sea4 that lacks the RING domain can only interact with Seh1, whereas Sea2 or Sea3 without the RING domain are no longer able to interact with any of the SEACAT complex components [13]. In addition, Sea3 contains an RWD domain that is enriched in  $\beta$ -sheets and common in proteins that also contain a RING motif and a  $\beta$ -propeller [38]. The RWD domain of Sea3 significantly resembles the RWD domain of the GCN2 protein, which is involved in general amino acid sensing and that of ubiquitin-conjugating E2 enzymes [39]. Given that SEACAT contains three proteins with RING domains, as well as numerous  $\beta$ -propeller domains that can mediate the recognition of phospho-substrate within E3 ligase complexes [40], it will be very interesting to investigate whether SEACAT/GATOR2 can act as a E3 ubiquitin ligase, and if this is the case, what are its possible targets.

The presence of the same folds and fold arrangements in both the SEA complex and in coating and tethering assemblies, and the fact that they contain the same “moonlighting” components, are suggestive that these complexes share a common evolutionary origin (see below). The majority of intracellular membranes are likely a result of the evolutionary expansion of an ancestral membrane-curving module—termed the “protocoatome” complex [31,34]. The SEA complex is a member of the coatome group, and its existence, thus, provides further evidence that an expansion of the protocoatome family underpins much of the functional diversity of the endomembrane system.

#### 4.2. SEACIT/GATOR1

The structural profile of the SEACIT/GATOR1 subunits is completely different (Figure 2A). Npr2/NPRL2 is a paralog of Npr3/NPRL3 [10,41] and both proteins possess N-terminal longin domains [42,43]. Iml1/Sea1 and its human homologue DEPDC5 contain a unique composition of domains that are not found in any other proteins. SEACIT components also have PEST motifs that often exist in rapidly degraded proteins [3]. However, PEST motifs are not well preserved in mammalian orthologues and, thus, could be a specific feature of the yeast SEA complex.

The structure of GATOR1, resolved recently by cryo-EM, revealed the architecture of each GATOR1 component [29] (Figure 2A). DEPDC5 has the following five defined domains: N-terminal domain (NTD), followed by SABA (structural axis for binding arrangement), SHEN (steric hinderance for enhancement of nucleotidase activity), DEP (Dishevelled, Egl-10 and Plekstrin) and C-terminal (CTD) domains. Interestingly, NTD, SABA and DEP domains can be found in membrane-associated proteins. For example, a domain similar to NTD exists in the SNARE chaperone Sec18/NSF, the SABA domain—in Sec23 of COPII vesicles (again returning to the theme of coating complexes). The DEP domain, which has diverse functions in signal transduction, is involved in the interactions between the regulator of G protein signaling (RGS) proteins and their membrane-bound receptors, the GPCRs [44]. The DEP domain is also found in a DEPTOR subunit of mTORC1 [45].

NPRL2 and NPRL3 have a similar structure with N-terminal longin domains that heterodimerize (Figure 2A). C-terminal domains of NPRL2 and NPRL3 also form a large contact surface. The SABA domain in DEPDC5 interacts with the NPRL2 TINI domain (tiny intermediary of NPRL2 that interacts (with DEPDC5)). By the way, the domain nomenclature within the GATOR1 complex created a doubtful precedent, where protein domains are named after the first (SHEN) and the last (SABA-TINI) authors of the article that reported the structure [29].

#### 4.3. Posttranslational Modifications of SEA/GATOR

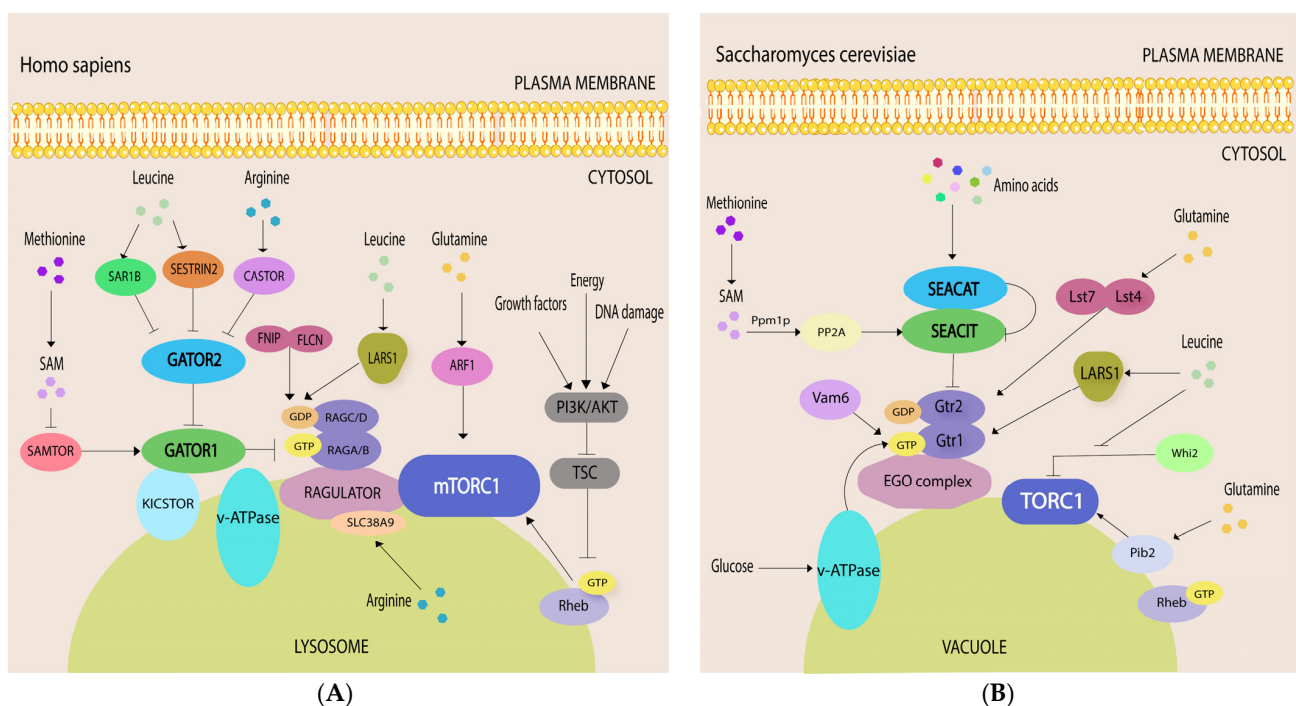
The majority of the information about post-translational modifications of the SEA/GATOR components came from whole proteome studies, essentially in yeast [46–51]. All the SEA and GATOR members are heavily phosphorylated and ubiquitinated (except of Sec13), with many modifications occurring at the disordered regions of proteins. However, there are still very few studies that explore the functional role of these modifications. Several papers, which describe the effect of ubiquitination, are mainly focused on the role of this modification on protein stability. Thus, Npr2 in yeast interacts with Grr1, the F-box component of the SCF<sup>Grr1</sup> E3 ubiquitin ligase [52]. Moderately unstable Npr2 is stabilized in *grr1Δ* mutants. In response to amino acids, CUL3-KLH22 E3 ubiquitin ligase induces K48 polyubiquitination on multiple DEPDC5 sites leading to its degradation [53]. Accordingly, DEPDC5 levels are increased during amino acid starvation. In the rich media, NPRL3 is more resistant to proteasome degradation than NPRL2 [54]. The data about the stability of SEA/GATOR proteins during amino acid starvation are contradictory and vary considerably in different species. For example, the level of practically all the SEA members in yeast decreases both during amino acid starvation and rapamycin treatment [13]. In *Drosophila* S2 cell lines, amino acid deprivation increases Nprl3 stability [55], although the reports in human cell lines indicate that the amount of NPRL2 and NPRL3 is not changed at least after 30 min of amino acid starvation [53]. It is reasonable to expect in the following years that we will gain more information about the role of posttranslational modifications not only on the stability of SEA/GATOR members, but also on their function.

## 5. Function of the SEA and GATOR in Nutrient Sensing and Responding

### 5.1. Overview of Amino Acid Axis of Signaling to mTORC1

One of the principal roles of SEA and GATOR as upstream regulators of mTORC1 is responding to amino acid availability [11,12] (Figure 3), although the role of both GATOR subcomplexes in glucose sensing has also been reported recently [56]. Effective functioning of the mTORC1 pathway with respect to cellular amino acid levels requires coordinated action of RAG guanosine triphosphatases (RAG-GTPases or RAGs) and their effectors, such as GTPase-activating proteins (GAPs), which stimulate GTP hydrolysis and guanine-nucleotide-exchange factors (GEFs). The major site of mTORC1 activation is the vacuole/lysosomal surface, where mTORC1 is recruited and induced in an RAG-GTPase dependent manner when amino acids are abundant [57,58]. There are the following four RAG GTPases: RAGA and functionally redundant RAGB; RAGC and functionally redundant RAGD (Figure 3A). They exist as obligate heterodimers, e.g., RAGA (or RAGB) with

RAGC (or RAGD). RAGs interact with a pentameric RAGULATOR complex, anchored to the lysosome [57–61]. RAGULATOR also interacts with v-ATPase, a protein pump at the lysosomal membrane. The guanine nucleotide loading is important for RAGs function. In the presence of amino acids, RAGs are active when RAGA/B is loaded with GTP, and RAGC/D is bound to GDP. Reversely, when amino acids are low, RAGs are inactive, and RAGA/B is loaded with GDP and RAGC/D is bound to GTP. Various GAPs and GEFs promote the conversion of RAGs from active to inactive form. This is where the SEACIT and GATOR1 complexes exert their major functions (see below). A RAG-independent induction of mTORC1 by amino acids both at the vacuole/lysosome and Golgi has also been described in yeast and humans [62–65], but will not be thoroughly discussed in this review since neither SEA nor GATOR seem to be involved in this mode of mTORC1 activation. Moreover, a recent study revealed that RAG-independent activation of mTORC1 by amino acids derived from protein degradation in lysosomes required HOPS complex and was negatively regulated by activation of the GATOR-RAGs pathway [37]. Thus, evolutionary related HOPS and GATOR2 [3] have similar but divergent roles in activating mTORC1 in response to different amino acid inputs.



**Figure 3.** Amino acid signaling. (A) mTORC1 signaling in *Homo sapiens*. (B) TORC1 signaling in *Saccharomyces cerevisiae*. Yeast and mammalian orthologues are designated with the same color. Arrows and bars represent activation and inhibition, respectively. See text for more details.

When amino acids are scarce, some amino acid sensors (see below) interact with and inhibit the GATOR2 complex, thus preventing inhibition of the GATOR1 by GATOR2 (Figure 3A). A mammalian-specific KICSTOR complex tethers GATOR1 to the lysosomal surface [66,67] where GATOR1 acts as a GAP for RAGA [12], thereby transforming RAGA to its inactive, GDP bound form, which further leads to mTORC1 suppression (Figure 3A).

In the presence of amino acids, RAGULATOR and v-ATPase undergo a conformational change that results in RAGULATOR exerting GEF activity towards RAGA or RAGB [60]. RAGULATOR can also trigger GTP release from RAGC [68]. In parallel, upon arginine binding arginine sensor SLC38A9, which resides at the lysosome, stimulates GDP release from RAGA [68]. A complex between folliculin (FLCN) and folliculin-interacting protein (FNIP) 1 and/or 2 is a GAP for RAGC/D [69]. In addition, leucyl-tRNA synthetase (LeuRS or LARS1 or LRS) also has GAP activity towards RAGD [70]. Active RAGULATOR-RAG

stimulates recruitment of mTORC1 to the lysosomal membrane where it is fully activated by small GTPase, RHEB, loaded with GTP [71]. RHEB is under the control of another signaling node—the TSC complex, composed of TSC1, TSC2 and TBC1D7, where TSC2 acts as a GAP to inhibit RHEB. TSC is a nexus of multiple physiological stimuli (e.g., energy status, growth factors, DNA damage) that signal to mTORC1 through PI3K-AKT network [72]. RAG GTPases regulate the recruitment of TSC to the lysosome and its ability to interact with and inhibit RHEB in response to amino acid starvation, growth factors removal and to other stresses that inhibit mTORC1 [73–75]. Both RAGs and RHEB are necessary for mTORC1 activation at the lysosome, as the lone presence of either one is not sufficient. Accordingly, only when both the RAG GTPases and RHEB are inactive mTORC1 fully released from the lysosome [73].

The RAGs and RAGULATOR are conserved both in fission and in budding yeast (Figure 3B) [76,77]. Thus, the orthologue of RAGA/B, a protein called Gtr1 in yeast, forms a heterodimer with Gtr2, which is an orthologue of RAGC/D. Similar to mammals, in order to activate TORC1, GTP-bound Gtr1 and GDP-loaded Gtr2 interact with trimeric *S. cerevisiae* Ego1-Ego3 complex (Lam1-Lam4 in fission yeast) analog of RAGULATOR. Iml1/Sea1 from the SEACIT serves as a GAP for Gtr1 in the absence of amino acids [11]. Interestingly, LARS1 in yeast is the GEF for Gtr1 [78], while in mammalian cells LARS1 was shown to be a GAP for RAGD [70], although a GAP activity was not confirmed in a later study from different laboratory [69]. Lst4-Lst7 complex, an orthologue of mammalian FLCN/FNIP, is GAP for Gtr2 [79]. The GEF for Gtr2 in yeast and for RAGC/D in mammals is still not known.

There are some notable differences between yeast and humans during amino acid signaling to mTORC1 (Figure 3). First, many amino acid sensors (e.g., SAMTOR, SESTRINs) are absent in yeast (see below) [1]. Second, v-ATPase in yeast, which interacts with Gtr1, seems to activate TORC1 in response to glucose [80]. Third, RHEB orthologue in yeast *S. cerevisiae* seems not to be involved in TORC1 signaling, although it is required for arginine and lysine uptake [81]. Fourth, *S. cerevisiae* does not have TSC homologues, thus the entire branch of TSC/RHEB signaling is not conserved in this particular yeast. In contrast, *S. pombe* has both RHEB and TSC, which are involved in mTORC1 activation. How *S. cerevisiae* achieves full TORC1 activation at the vacuole without TSC/RHEB branch is currently not well understood.

## 5.2. GATOR2 Interactions with Leucine Sensors SESTRINs and SAR1B and Arginine Sensor CASTOR1

Cytosolic leucine can be sensed by the proteins from the SESTRIN family (SESTRINs 1–3) [82–84], by small GTPase SAR1B [85] and by leucyl-tRNA synthetase [70,86,87]. Arginine is sensed by CASTOR1 protein homodimer in the cytoplasm [88,89] and by SLC38A9 together with TM4S5F protein at the lysosomal membrane [90–92].

GATORs can interact directly with several amino acid sensors (Figure 3A). During leucine or arginine starvation, SESTRIN2 [82], SAR1B [85] or CASTOR1, respectively [88,89] interact with and inhibit the GATOR2 complex. WDR24 and SEH1L are essential for interaction with SESTRIN2, but it is not known which component of GATOR2 interacts with SESTRIN2 directly [93,94]. SAR1B directly binds MIOS, but not other GATOR2 subunits [85]. WDR24, SEH1L and MIOS were sufficient for interaction with CASTOR1 [89]; the CASTOR1 N-terminal domain is involved into direct interaction with MIOS [95]. Binding sites for SESTRIN2 and CASTOR1 are located at different parts of GATOR2 [89]. These interactions prevent inhibition of the GATOR1 by GATOR2 [96] and as a consequence, lead to mTORC1 inhibition. Neither SESTRIN2 nor CASTOR1 interact with GATOR1 [89,93,97].

In the presence of amino acids, interaction of leucine to the defined binding pocket in monomeric SESTRIN2 [83] or arginine with its binding pocket at the homodimeric CASTOR1 [88,95,98] results in dissociations of these sensors from GATOR2 and relieves mTORC1 inhibition. It is important to note, however, that SESTRIN2-GATOR2 interactions were initially observed in the cell-lines cultured in leucine-rich conditions [93,97], even if amino acid starvation enhanced this interaction. In vitro addition of leucine reduces the

SESTRIN1-GATOR2 or SESTRIN2-GATOR2 interactions, but it does not affect SESTRIN3-GATOR2 interaction [82,84]. Interestingly, SESTRIN2 and SAR1B detect different parts of leucine; SAR1B recognizes the amino group and side chain of leucine [85], while SESTRIN2 interacts with leucine's amino and carboxyl groups [83].

Interactions of SESTRINs to GATOR2 depends on a cell type and physiological conditions. Thus, in the skeletal muscle of rats, SESTRIN1 is the most abundant isoform, and SESTRIN2 expression is much lower relative to either SESTRIN1 or SESTRIN3. Accordingly, oral administration of leucine to fasted rats induced SESTRIN1-GATOR2 disassembly, but did not affect the interaction of other SESTRIN isoforms with GATOR2 [84]. This suggests that in the rat skeletal muscle, it is probably SESTRIN1 that has a primary role as a leucine sensor and leucine-induced activation of mTORC1 in skeletal muscle happens via SESTRIN1 release from GATOR2. SESTRINs-GATOR2 interactions can also be age dependent. Thus, in the skeletal muscle of young pigs, SESTRIN2 is more abundant than SESTRIN1 but the GATOR2 amounts are the same. Accordingly, during amino acid starvation the abundance of the SESTRIN2-GATOR2 complex reduced more in younger pigs [99].

Recently, GATOR2 was reported to be required for SESTRIN2-induced AKT activation and AKT translocation to plasma membrane [94]. In addition, GATOR2 physically bridges SESTRIN2 with mTORC2 where WDR59's interaction with mTORC2's component RICTOR is essential for the communication between GATOR2 and mTORC2, and WDR24 is crucial for GATOR2-SESTRIN2 interaction. In HeLa cells, GATOR2 promotes AKT activation and facilitates AKT-dependent inhibitory phosphorylation of TSC2 [75]. Thus, although an exact molecular function of GATOR2 has not yet been defined, it is clear that GATOR2 might have a large repertoire of various activities. Solving the structure of GATOR2 alone and in complex with its interactors will provide essential information about how these multiple functions can be exerted.

### 5.3. GATOR1 Interaction with SAM Sensor, SAMTOR

The SAM sensor, SAMTOR, binds to GATOR1 during SAM or methionine deprivation, and negatively regulates mTORC1 activity [100]. The component of GATOR1 that interacts with SAMTOR is currently unknown. In the presence of SAM, this metabolite occupies its binding pocket in SAMTOR, which disrupts the interaction of an amino acid sensor with GATOR1, promoting mTORC1 activity. SAMTOR and GATOR1 interactions are dependent on KICSTOR (see below). When SAMTOR is bound to SAM, it dissociates from GATOR1-KICSTOR, thus inhibiting GATOR1 and promoting mTORC1 activation [101]. On the other hand, methionine starvation promotes interaction between SAMTOR and the GATOR1-KICKSTOR complex, but weakened the interaction between GATOR1 and GATOR2, thus leading to mTORC1 suppression [100]. SAM levels can be affected by the availability of vitamin B12. Mice NPRL2 KO embryos have significantly reduced methionine levels and demonstrate phenotypes reminiscent of B12 deficiency [20]. It is currently unknown whether methionine can be sensed directly. Interestingly, leucine can also signal to mTORC1 through its metabolite, acetyl-coenzyme A, but in a RAG-independent and cell-specific manner [102].

In a recent study, Jewell laboratory investigated the potency of each amino acid to stimulate mTORC1 in MEF or HEK293 cells [65]. Ten amino acids were able to re-stimulate mTORC1 and promote its lysosomal localization. Glutamine and asparagine signal to mTORC1 through a RAG-independent mechanism via ADP-ribosylation factor ARF1. Eight amino acids (alanine, arginine, histidine, leucine, methionine, serine, threonine and valine) filter through RAGs. While three cytoplasmic sensors for leucine, arginine and methionine (SAM) have been identified, it is not known whether the other five amino acids also have their specific sensors and whether they will interact with GATORs.

### 5.4. SEACIT and Amino Acid Sensing in Yeast

Amino acid sensing in yeast differs significantly from the mammalian system (Figure 3B). SESTRINs, CASTOR1 and SAMTOR are not conserved in *S. cerevisiae* and

*S. pombe*, which presumes that the interaction of these amino acid sensors with GATOR complexes arose later in the evolution. Nevertheless, Npr2 does participate in methionine sensing in *S. cerevisiae*, but in a very different way than in mammals. Under normal growth conditions, Ppm1p methyltransferase methylates two subunits of yeast protein phosphatase 2A (PP2A), which promotes Npr2 dephosphorylation, TORC1 activation and suppression of autophagy [103]. Low methionine level leads to a decreased SAM, which blocks PP2A methylation and its phosphatase activity. As a result, Npr2 accumulates in phosphorylated form, which most probably changes the integrity of the SEACIT complex due to increased interaction between phosphorylated Npr2 and Iml1/Sea1 [9]. Therefore, SEACIT is no longer able to repress TORC1 effectively, resulting in autophagy activation. Interestingly, Npr2-deficient yeast grown in a minimal medium, containing ammonium as a sole nitrogen source and lactate as a nonfermentable carbon source, metabolize glutamine into nitrogen-containing metabolites and maintain high SAM concentrations [104].

As in mammals, yeast also have amino acid sensing pathways parallel to SEA-GTR signaling [105]. For example, Pib2, which resides at the vacuole membrane, interacts with TORC1 complex in a glutamine-sensitive manner, suggesting that Pib2 acts as a part of a putative glutamine sensor [64]. Although both Pib2 and EGO are required for TORC1 tethering to the vacuolar membrane and its activation, they form different complexes with TORC1, ruling out a possibility that the SEA complex can participate in Pib2-dependent amino acid sensing. Even if Pib2 does not have apparent ortholog in mammals, PLEKHF1 protein shares high sequence similarity with Pib2 domains, important for TORC1 activation. However, PLEKHF1 is not involved in the glutamine-dependent regulation of mTORC1 [65]. In addition, Whi2, localized at the cell periphery, specifically senses low amino acid levels in general and leucine levels in particular, and suppresses TORC1 activity independently of the SEA complex [106,107]. The Whi2 homologue in mammals, KCTD11, acts as a negative regulator of mTORC1 during amino acid deprivation [106].

All these recent findings demonstrate that amino acid sensing mechanisms are way more diverse, because not only amino acids themselves, but also their metabolites can be sensed in a RAG-dependent, RAG-independent and cell-specific manner.

Many questions about amino acid sensing ultimately related to SEA and GATOR functions remain unanswered. Does every amino acid have its own sensor? Will all the sensors that work through RAGs interact with GATORs? What are the determinants of the interaction of amino acid sensors with one or another GATOR complex? In other words, why do SESTRIN2 and CASTOR1 interact with GATOR2, and SAMTOR with GATOR1? What are the factors that determine sensing of the same amino acid by different sensors? For example, why does leucine need three sensors (SESTRIN2, SAR1B and LARS1) that function in the same cell types, in the same subcellular location (cytosol), through the same pathway (RAG-dependent)? Leucine can also signal through its catabolite acetyl-CoA and activate mTORC1 via EP-300-mediated acetylation of RAPTOR [102]. Can other amino acids signal both themselves and their metabolites through different sensors? For example, the methionine metabolite SAM is sensed by SAMTOR, does a methionine sensor exist? Amino acid sensing also happens at Golgi, where GATORs, SESTRINS, CASTOR1 and SAMTOR have not been found thus far. How is amino acid sensing is achieved at Golgi? What is the repertoire of cell-type specific sensors? The primary role of aminoacyl tRNA synthetases is binding to cognate amino acids and their attachment onto appropriate tRNAs. Some of them, such as cytosolic LARS1 [70,78] and mitochondrial TARS2 (but not cytosolic TARS) [108], are also implicated in the upstream regulation of mTORC1 pathway. Are other aminoacyl tRNA synthetases also involved in mTORC1 regulation? What are the details of a crosstalk between general amino acid sensing through GCN2 and sensing through the mTORC1 pathway? Finally, what are the main determinants of amino acid sensing in yeast given that many mammalian amino acid sensors discovered thus far do not have yeast homologous, yet the GATOR-RAG-RAGULATOR (SEA-GTR-EGO) system is conserved?

### 5.5. SEACIT/GATOR1 as GAP for EGO/RAG

Two papers published simultaneously in 2013 reported the results that have dramatically increased the significance of the SEA/GATOR complex in the regulation of mTORC1 pathway. The laboratory of Claudio de Virgilio found that in *S. cerevisiae*, the SEA subcomplex, which was subsequently named SEACIT (SEAC subcomplex inhibiting TORC1 signaling) [14], acts as a GAP for Gtr1 and, thus, inhibits TORC1 [11]. In a parallel study, David Sabatini's laboratory characterized for the first time the human homologue of the SEA complex, and also found the GAP activity of the SEACIT analogue, which received the GATOR1 name (GTPase activating protein activity towards RAGA) [12]. In both yeast and humans, SEACAT/GATOR2 acts upstream of SEACIT/GATOR1, suppressing its GAP activity, thus being "an inhibitor of an inhibitor", although how exactly this suppression is achieved is completely unknown.

A molecular mechanism of how SEACIT/GATOR1 acts as a GAP has been addressed in several functional and structural studies, but a complete consensus of how exactly the GAP function is exerted has not yet been achieved. Indeed, in an initial study by the de Virgilio group, it was demonstrated that in *S. cerevisiae*, Iml1/Sea1 can co-precipitate with Gtr1 in the presence but substantially less in the absence of other SEACIT subunits. Yet, in the in vitro binding and GAP essays, Iml1/Sea1 could directly bind to Gtr1 and promote GTP hydrolysis in the absence of Npr2 and Npr3. GAPs often supply a catalytic amino acid residue (Arg, Asp or Gln) in their active sites, thus forming an "arginine finger" or "Gln/Asn thumb" that can be inserted into nucleotide-binding pocket of a GTPase [109]. In the highly conserved Iml1/Sea1 domain, essential for its GAP activity (aa 929-952), a conserved Arg<sup>943</sup> was critical for GAP activity both in vitro and in yeast cells. Human DEPDC5 could partially complement TORC1 inhibition defect in *iml1Δ* cells, suggesting a conserved role of Iml1/Sea1 and DEPDC5 across the species. Therefore, when the cryo-EM structure of GATOR1 (Figure 2A) and GATOR1 in the complex with RAG GTPases was solved, it came as a surprise because it revealed a very unexpected mode of interaction between GTPases and GAPs [29].

For the structural studies, GATOR1 was copurified with RAG GTPase heterodimer, containing wild type RAGA and mutant RAGC, which can bind GTP, but not GDP. In addition, this heterodimer was loaded with GDP and non-hydrolysable GTP analogue (GppNHp) to create the most favorable nucleotide-binding configuration for interaction with GATOR1. The structure demonstrated that the overall conformation of the GATOR1 in a complex with RAG GTPases is similar to a free GATOR1 (see above). The SHEN domain of DEPDC5 can contact directly with a site proximal to nucleotide binding pocket of GTP analogue-bound RAGA. However, quite surprisingly, this interaction did not appear to be responsible for the stimulation of GTP hydrolysis. The kinetic analysis of GTP hydrolysis of DEPDC5 alone with RAGA or NPRL2/NPRL3 dimer with RAGA revealed that it is rather NPRL2/NPRL3, which has GAP activity. Moreover, a conserved Arg<sup>78</sup> localized on the loop of NPRL2 longin domain is the "arginine finger", responsible for GAP activity [110]. However, this Arg<sup>78</sup> is located far away and is opposite to the RAGs binding interface of DEPDC5. Moreover, an earlier study from Wang laboratory showed that amino acid stimulation enhances the interaction of RAGA with both endogenous DEPDC5 and NPRL3 [111]. To explain these rather contradictory observations, a two-state model of GATOR1 interaction with RAG GTPases was proposed: in the inhibitory mode, DEPDC5 SHEN domain interacts strongly with RAGs and GAP activity of GATOR1 is weak; alternatively, a low affinity interaction dependent on NPRL2/NPRL3 stimulates GAP activity. Such bi-modal activity has not been previously observed between a GAP and a GTPase. Moreover, before this study, longin domains were found to be highly represented in many GEFs, where they would serve as adaptable platforms for GTPases [42]. In addition, in a structure of *Chaetomium thermophilum* Mon1-Ccz1-Ypt7 complex, Mon1-Ccz1 GEF contacts its cognate GTPase Ypt7 through a face of a conserved longin domain heterodimer [112]. NPRL2 and NPRL3 also form a heterodimer using their longin interaction domains; therefore, it is quite intriguing why in case of Mon1-Ccz1 longin heterodimer supports a GEF



activity, while NPRL2/NPRL3 longin domains assist to GAP function. One of the plausible explanations might involve a possibility that NPRL2–NPRL3 interaction with RAGs can be sterically compromised by GATOR2, because it is NPRL3, which is necessary and sufficient for interaction with GATOR2. Finally, to add even more complexity, one (and the only) study reported that NPRL2 interacts with RAGD in amino acid scarcity, and with Raptor during amino acid sufficiency to activate mTORC1 [113]. Although the authors explain this behavior by suggesting that NPRL2 may not solely exist as a part of GATOR1, these findings require more clarifications.

It is evident that more structural studies will be necessary to explain this peculiar mode of interaction between GATOR1 with RAG GTPases. For example, a structure of RAGs–NPRL2–NPRL3 would allow to observe the conformation of the active GAP, a task that will not be very easy, given a weak association of NPRL2/NPRL3 heterodimer with RAGs in the absence of DEPDC5. In addition, solving a structure of yeast SEA complex, where the association between SEACAT (GATOR2) and SEACIT (GATOR1) is much stronger and where GAP activity seems to be performed by Iml1/Sea1 (DEPDC5), rather than by other components of the complex, would be absolutely central for the elucidating how SEACIT/GATOR1 exert its GAP function.

#### 5.6. SEA/GATOR Recruitment to the Vacuolar/Lysosomal Membrane

In yeast, both TORC1 and SEA complex localize at the vacuole membrane regardless of the presence or absence of amino acids [3,77,114,115]. Iml1/Sea1 did not require other SEA components to localize to the vacuole membrane in both budding and fission yeast [11,28]. In contrast, Npr2 and Npr3 mutually depend on each other and on Iml1/Sea1 for vacuolar localization [11,28]. Importantly, the deletion of any of the SEACIT components during nitrogen starvation caused the re-localization of Tor1 to the cytoplasm [13].

In mammalian cells, mTORC1 is recruited to the lysosome in the presence of the amino acids, where it is fully activated by RHEB [116]. In addition, the activation of mTORC1 by RHEB can happen at the surface of other organelles, because both RHEB and mTORC1 have been detected at the Golgi apparatus, the peroxisome, the plasma membrane and ER [62,117,118]. Stably expressed GFP-tagged components of GATOR1 (NPRL2 and DEPDC5) and GATOR2 (MIOS and WDR24) localize to the lysosome regardless of the amino acid levels [12,67], although a recent study revealed that during amino acid starvation, WDR24, MIOS and mTOR can be found at a rough ER membrane [119]. Similarly, *Drosophila* GATOR2 components Mio and Seh1 localize to lysosomes in both fed and starved flies. Mammals, however, developed additional mechanisms to maintain GATORs at the lysosomal membrane, which include an interaction with the protein complex KICSTOR, that is not present in non-vertebrates and the regulation of GATOR1–RAGA interaction via ubiquitination.

The mammalian-specific KICSTOR complex identified in 2017 plays a key role in the localization of GATOR1 to its GTPase substrates [66,67]. KICSTOR consist of four proteins, KPTN, ITFG2, C12orf66 and STZ2, whose initial letters gave the complex its name. *C. elegans* only encode a homologue of SZT2, while yeasts and *Drosophila* lack entire KICKSTOR [15,67]. Both GATOR1 and GATOR2 associate with KICKSTOR in an amino-acid insensitive manner. STZ2 is responsible for the interaction of KICKSTOR with GATOR1, since STZ2 knockouts impaired the localization of GATOR1 to the lysosomes, but not GATOR2 or RAG GTPases. SZT2 is also necessary for the coordinated GATOR1 and GATOR2 binding and for GATOR1-dependent inactivation of mTORC1 at the lysosome. SZT2 contains several regions that allow interaction with GATOR1 and GATOR2 [66]. SZT2–DEPDC5 interactions can occur in the absence of other GATOR components [29]. SZT2 does not bind GATOR2 in the absence of NPRL3, once again underlining a crucial role of this protein in GATOR1–GATOR2 interactions. In addition, lysosomal localization of WDR59 is abolished in the absence of SZT2. Thus, KICKSTOR, and especially its largest component 380 kDa SZT2, may facilitate interaction between GATOR1 and GATOR2 and maintain both subcomplexes together. In contrast, in *S. cerevisiae*, both SEA subcomplexes can form a stable complex without other mediating proteins. It is intriguing why, during

evolution, mammals acquired a large protein complex to maintain interactions between GATOR1 and GATOR2, which otherwise are quite stable in lower eukaryotes.

GATOR1 is also implicated to the recruitment to the lysosomal surface of another GAP—FLCN/FNIP. GATOR1-dependent control of the RAGA nucleotide state drives FLCN recruitment to lysosomes when amino acids are scarce [120]. Indeed, when amino acids are low, the GAP activity of GATOR1 promotes the GDP-RAGA/B conformation and FLCN/FNIP is recruited to the lysosome to act as a GAP towards RAGC/D. In this study, only knockout of *NPRL3* in HeLa cells were verified, and it is not known whether knockout of other GATOR1 components would have the same effect. Nevertheless, these findings help to resolve the apparent contradiction reported earlier, that FLCN-FNIP heterodimer binds to RAGA/B, but acts as a GAP for RAGC/D [69,121]. Cryo-EM structures of the human FLCN-FNIP-RAG-RAGULATOR complex containing an inactive form of the RAG heterodimer confirmed that the FLCN-FNIP2 heterodimer binds to the GTPase domains of both RAGA and RAGC [122,123].

GATOR1-RAGA interactions are controlled by several kinases and E3 ubiquitin ligases, which are not present in lower eukaryotes. For example, an oncogenic non-receptor tyrosine kinase, SRC, disrupts GATOR1-Rags interactions, promoting mTORC1 recruitment and activation at the lysosomal surface [124]. Currently, it is not known what the mechanisms that activate SRC in response to amino acids are and whether GATOR1 subunits or RAGs can be phosphorylated by SRC. On the other hand, DEPDC5 can be phosphorylated by Pim1 kinase at S1002 and S1530, and by AKT also at S1530 [125]. This phosphorylation seems not to affect the ability of DEPDC5 to interact with neither NPRL2 nor SZT2, but elevated Pim1 expression during amino acid starvation overcame mTORC1 suppression.

Two lysosome localized E3 ligases, RNF152 and SKP2, mediate K63-linked polyubiquitination of RAGA at different sites, which promote GATOR1 recruitment to RAGA and the consequent inactivation of mTORC1 [111,126]. Remarkably, SKP2 ubiquitinates RAGA at K15 during prolonged amino acid stimulation [126], while, quite opposite, RNF152 ubiquitinates RAGA at a different set of lysines (K142, 220, 230, 244) during amino acid starvation [111]. SKP2 provides a negative feedback loop, where RAGA ubiquitination and GATOR1 recruitment restrict mTORC1 activation upon sustained amino acid stimulation. Inversely, during amino acid starvation, it is RNF152-dependent RAGA ubiquitination, which enhances GATOR1–RAGA interaction. Interestingly, RNF152 can also ubiquitinate RHEB, sequestering RHEB in its inactive RHEB-GDP form and promoting its interaction with TSC2, which leads to mTORC1 inactivation [127]. Thus, RNF152 acts a negative mTORC1 regulator in both amino acid and growth factor branches of mTORC1 signaling.

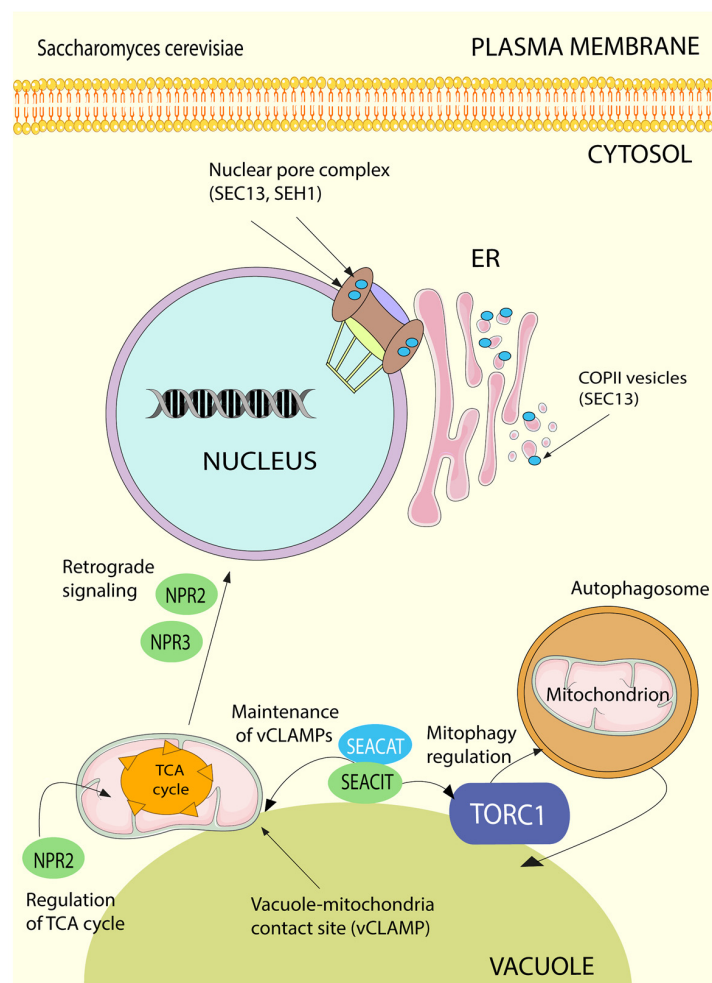
### 5.7. SEA/GATOR in Autophagy

One of the major functions of mTORC1 is in the regulation of autophagy, which is induced when mTORC1 is inhibited. Thus, it is not surprising that deletions of SEACIT/GATOR1 components suppress autophagy in yeast [3,9,13,103,104,128,129], *Drosophila* [130], *C. elegans* [131] and mammals [129,132]. Just as the opposite, mutations in GATOR2 may promote autophagy, which can happen even in the absence of nutrient starvation, as it is a case of *wdr24* mutants in *Drosophila* [133]. In contrast, deletions of SEACAT members in yeast seem not to have a drastic effect on autophagy initiation and flux [3]. Interestingly, the nitrogen starvation deletion of *SEA1* or double deletion of *NPR2* and *NPR3* resulted in the inhibition of vacuolar fusion [13]. As the inactivation of TORC1 during nitrogen deprivation promotes vacuole coalescence [134], deletions of any of the SEACIT members increase TORC1 activity during starvation, and, therefore, induce vacuolar fragmentation and defects in autophagy.

Recently a bi-directional feedback loop, which regulates autophagy and involves SEACAT, has been described [50]. In order to control autophagy, TORC1 phosphorylates and inhibits the Atg1 kinase essential for autophagy initiation, but Atg1, in turn, can phosphorylate SEACAT components. Although it is currently not known whether that phosphorylation acts positively or negatively on TORC1 activity, this finding uncovers the

important node of convergence between TORC1 and Atg1, with the SEACAT being both the regulator and effector of autophagy.

The SEA complex is also important for specific types of autophagy. Thus, yeast with deletions of SEACIT complex members failed to activate selective degradation of mitochondria via mitophagy (Figure 4) [135,136]. Given the conservation of the SEA/GATOR function, it is reasonable to assume a similar role of GATOR in mammals, although the involvement of GATOR in specific types of autophagy in mammals has not yet been described.



**Figure 4.** Functions of the *S. cerevisiae* SEA complex and its components beyond nutrient response. Indicated are Seh1 and Sec13 as components of the nuclear pore complex and Sec13 as part of COPII vesicles. Npr2 and Npr3 regulate retrograde signaling. Npr2 is also involved in the regulation of TCA cycle. Finally, SEA complex is involved in the maintenance of the vacuole-mitochondria contact sites (vCLAMPs) and is important for mitophagy.

## 6. SEA and GATOR Functions beyond Nutrient Responding

### 6.1. SEA/GATOR Evolution Origin

SEA/GATOR has always been “living double lives” with a number of its components having diverse “moonlighting” functions beyond their role in the regulation of nutrient sensing and responding (Figures 4 and 5). Although the majority of these functions seem to be related to the SEA/GATOR role in the regulation of mTORC1, others are clearly associated with totally different pathways. Accordingly, despite the fact that the main localization site of SEA/GATOR is a vacuole/lysosomal membrane, some of its components can be found in the nucleus, ER, mitochondria, plasma membrane, etc., depending on the functions that they fulfil in different cell types, stages of cell cycle progression and physiological conditions [24,54,94,135,137–140]. The most outstanding examples are Seh1

and Sec13, which together are the members of the Nup84 subcomplex in the nuclear pore complex, with Sec13 also being a component of COPII coated vesicles [3]. This “double life” of Seh1 and “triple life” of Sec13 witnesses the evolution of the endomembrane system. Indeed, the progression from prokaryotic to eukaryotic cells was accompanied by the acquisition of membranous structures, eventually transformed into organelles, which often adopted preexisting molecules and adjusted them for new needs via duplication and neofunctionalization [33]. During this transformation, a central role was played by ancient protocoatomers, which facilitated membrane bending. Not only Seh1 and Sec13, but the entire SEACAT/GATOR2 complex belongs to the large family of protocoatomer-derived complexes that form transport vesicles (COPI, COPII, clathrin), membrane-associated coats (nuclear pore complexes), tethering complexes (HOPS/CORVET) and other membrane associated structures, such as SEACAT/GATOR itself [3,31,34]. These various assemblies have a number of structural similarities, including a hallmark feature—a presence of N-terminal  $\beta$ -propeller, formed by WD40 repeats, and C-terminal  $\alpha$ -solenoid composed of  $\alpha$ -helices (HEAT repeats). In that view, Sea4/MIOS is the most well preserved protocoatomer descendant, while Sea2/WDR59 and Sea3/WDR24 diverged more profoundly, losing many  $\alpha$ -helices, but still preserving N-terminal  $\beta$ -propellers.

GTPases, with their corresponding GEFs and GAPs, are other important elements of membrane-associated assemblies. SEACIT/GATOR1 carries this functional feature of endomembrane system, being a GAP for RAGA GTPase. In addition, longin domains present in two components of the SEACIT/GATOR1 can also be found in small GTPases and many other proteins involved in assembly, fusion and tethering of membranes [141]. Here, again, paralogs Npr2/NPRL2 and Npr3/NPRL3 evidence that evolution progressed through duplication and divergence, because both proteins seem to have additional functions, apart from mTORC1 regulation.

Remarkably, the entire vacuole/lysosome-associated mTORC1 pathway machinery contains multiple structural elements typical for classical endomembrane systems [30]. For example, the mTORC1 complex has a  $\beta$ -propeller subunit mLST8, structurally very close to Seh1 and Sec13. Similar to other coatomers, another mTORC1 subunit, Kog1/RAPTOR, contains HEAT repeats and  $\beta$ -propeller, but in a “Lego game of evolution” these structural elements switch places with HEAT repeats situated at the N-terminus and  $\beta$ -propeller at the C-terminus. By the way, in the mTORC1 complex, RAPTOR interacts with the HEAT domain of mTOR. Finally, the abundance of small GTPases, GAPs and GEFs that control mTORC1 witness the common evolution origin of the core endomembrane system and its regulators.

## 6.2. Regulation of Mitochondrial Biogenesis and Quality Control

The mTORC1 pathway plays an essential role in mitochondrial biogenesis, mitochondrial genome repair, the phosphorylation of mitochondrial proteins and the regulation of mitophagy, the selective degradation of mitochondria by autophagy. As a central controller of the mTORC1 pathway, SEA/GATOR is also involved in the regulation of mitochondria function and quality control (Figure 4, Figure 5). The analysis of synthetic genetic interactions in *S. cerevisiae* revealed already in 2011 that SEA genes interact with many mitochondrial genes, with Npr2 located close to the mitochondrial gene cluster [3,142,143]. About 20% of proteins that co-precipitate with SEA components are mitochondrial proteins [13,135] and, inversely, enriched mitochondrial fractions contain SEA proteins [137]. Both C-terminal GFP tagged Iml1/Sea1 and Sea4 can be localized to the mitochondria [135]. Moreover, treatment with rapamycin significantly increases the amount of cells with cytoplasmic and mitochondrial localizations of Iml1/Sea1, although a fraction of Iml1/Sea1 can still be observed at the vacuole [138]. Similarly, in HEK 293T cells NPRL2 can be localized to the mitochondria and many mitochondrial proteins can be found in the proteome of NPRL2 and NPRL3 [54]. Recently, SESTRIN2, which interacts with GATOR2 during leucine starvation (see above), was also found to be localized to mitochondria and silencing of GATOR2 genes considerably reduced the mitochondrial pool of SESTRIN2 [144]. Finally, Sec13 was shown to be interacting with mitochondrial antiviral signal protein (MAVS, also

known as VISA) [145,146]. MAVS is localized on the outer membrane of mitochondria, with a small proportion present at mitochondria-associated membranes (MAMs). Sec13 overexpression increases MAVS aggregation and facilitates interferon  $\beta$  production, while low levels of Sec13 result in a weaker host antiviral immune response. Currently, it is not clear whether other proteins from nuclear pore complex or COPII or GATOR2 are also involved in these interactions.

					
Amino acid signaling to mTORC1 (SEA/GATOR)	✓	✓	✓	✓	✓
General autophagy (SEACIT/GATOR1)	✓	✓	✓	?	✓
Mitophagy (SEACIT/GATOR1)	✓	?	?	?	?
Retrograde signaling (NPR2, NPR3)	✓	?	?	?	?
Vacuole-mitochondria contact sites (SEA)	✓	?	?	?	?
Nuclear pore complex components (SEC13, SEH1)	✓	✓	✓	✓	✓
COPII vesicles (SEC13)	✓	✓	✓	✓	✓
DNA damage response (NPR2/NPRL2)	?	?	✓	?	✓
Cell division and cell cycle regulation (SEA/GATOR)	?	?	✓	?	✓

**Figure 5.** Multiple functions of the SEA/GATOR complex.

The deletion of SEA/GATOR components affects mitochondria functions. The total abundance of SEA proteins is increased during respiratory growth and decreased upon nitrogen starvation, *sea2* deletion impairs respiration capacity in *S. cerevisiae* [147]. *npr2* $\Delta$  cells have defective mitochondrial-housed metabolic pathways, such as synthesis of amino acids, and an impaired tricarboxylic acid (TCA) cycle activity. *npr2*-deficient cells showed decreased pools of nitrogen-containing intermediates of the TCA cycle and nucleotides. Yet, *npr2* $\Delta$  yeast use TCA cycle intermediates for replenishment of biosynthetic pathways to sustain the hypermetabolic state due to mTORC1 constant activation, suggesting a role of SEACIT in the regulation of cataplerotic reactions of the TCA cycle depending on the amino

acid and nitrogen status of the cell [148]. This was later supported by another study that demonstrated that skeletal-muscle-specific NPRL2 loss in mice promoted aerobic glycolysis by altering the tuning between the amino acid sensing pathway and TCA cycle function. NPRL2-mKO mice also had less oxidative muscle fibers and more glycolytic muscle fibers, a hallmark of aerobic glycolysis, which highlights the functional role of NPRL2 in vivo in the regulation of glucose entry into the TCA cycle [149].

The function of GATOR1 proteins in mitochondrial health seems not to be limited to NPRL2. A heterozygous mutation in the CTD domain of DEPDC5 gene found in an autistic child was correlated with a significant decrease in mitochondrial complex IV activity and decrease in the overall oxygen consumption rate in peripheral blood mononuclear cells. Therefore, this variant of DEPDC5 can be directly related to an altered mitochondrial function in autistic disease [150]. Mice with skeletal-muscle specific deletion of DEPDC5 showed increased mitochondrial respiratory capacity and TCA cycle activity [151].

SEACIT is also involved in the communication of the mitochondria with other organelles. The mitochondria-to-nucleus communication pathway, known as the retrograde signaling, is triggered by mitochondrial dysfunctions in order to alter the expression of nucleus-encoded mitochondrial genes to effect metabolic reprogramming and to restore cellular fitness [152,153]. *npr2Δ* and *npr3Δ* yeast strains failed to activate the retrograde signaling pathway when grown in media containing ammonia as nitrogen source [10,148]. In order to recruit the substrates for biochemical reactions and export resulting products mitochondria rely on direct transport with organelles through contact sites [154]. The vacuole and mitochondria contact sites, vCLAMPs, are important for lipid exchange [155] and may also serve for the sensing of the integrity and functionality of mitochondria (Figure 4) [135]. Importantly, SEACIT is required for the maintenance of vCLAMPs and the deletion of any SEACIT members drastically reduces the amount of vCLAMPs in yeast cells [135]. Whether GATOR1 has the same functions in mammalian cells remains to be discovered.

### 6.3. GATOR1 and DNA Damage Response

The notion that Npr2/NPRL2 might have a role in DNA damage response appeared when it was found that mutations in this protein, both in yeast and human, confer resistance to the anticancer drugs cisplatin and doxorubicin (Figures 4 and 5) (see below) [156,157]. These compounds induce high levels of DNA damage, which eventually lead to cell cycle arrest and apoptosis [158,159]. Study of the role of NPRL2 in DNA damage response in non-small-cell-lung cancer cells treated with cisplatin [160] demonstrated that the ectopic expression of NPRL2 activates the DNA damage checkpoint pathway in cisplatin-resistant and NPRL2-negative cells, leading to cell cycle arrest in the G2/M phase and induction of apoptosis. Upon ectopic expression, NPRL2 promotes ROS production via NADPH oxidase (NOX) 2 activation [54]. Overexpressed NPRL2 accumulates in the nucleus, where it interacts with the apoptosis initiation factor, AIF. In addition, NPRL2 expression provokes the phosphorylation of tumor suppressor p53, which, in turn, activates a DNA-damage checkpoint pathway via p21 and CDC2. An excessive amount of NPRL2 results in cell cycle arrest in G1 phase in cells with constitutively p53 and to CHK2-dependent S or G2/M in p53-negative cancer cell lines [54,161]. Currently, it is not known whether these functions are performed by NPRL2 as a part of GATOR1 complex, or separately. *Drosophila* GATOR is also critical to the response to meiotic double strand DNA breaks (DSB) during oogenesis, since depletion of each GATOR1 component fails to repair DSB with *nprl3* mutants showing increased sensitivity to genotoxic stress both in germline and somatic cells [162].

### 6.4. GATOR in Cell Division and Cell Cycle Regulation

GATOR2 is important for both mitotic and meiotic division (Figure 5). Depletion of MIOS in HeLa cells resulted in mitotic defects, such as spindle assembly defects and delay or failure in cytokinesis [163]. MIOS regulates mitotic events through Aurora A kinase and Polo-like kinase 1 (Plk1), which control the localization and function of mitotic spindle. MIOS is important for spindle formation, subsequent chromosome segregation and proper

concentration of active Plk1 and Aurora A at centrosomes and spindle poles. SEH1, which forms a complex with MIOS (see above), targets GATOR2 to mitotic chromosomes, required for the localization of chromosomal passenger complex and functions in chromosome alignment and segregation by regulating the centromeric localization of Aurora B [164]. This function of GATOR2 nevertheless seems to be related to its role in mTORC1 activation, because depletion of MIOS causes reduced mTORC1 activity at centromeres in mitotic cells [163].

In *Drosophila*, Mio localizes to oocyte nucleus at the onset of prophase and meiosis I, and is required for the maintenance of the meiotic cycle during oocyte maturation [24]. *Drosophila* Seh1 is also involved in the maintenance of meiotic cycle and regulation of microtubule dynamics in ovarian cysts [36]. Depletion of *iml1* in the female germ line delays mitotic/meiotic transition and ovarian cysts undergo an extra mitotic division [18]. Thus, GATOR1 downregulates TORC1 activity to promote the mitotic/meiotic transition in ovarian cysts, while inhibition of GATOR1 by GATOR2 prevents the constitutive downregulation of TORC1 at the later stages of oogenesis.

#### 6.5. The Role of GATOR in Development

Animal development and growth is closely related to the ability to respond to different nutrient cues. Therefore, it is not surprising that GATOR components are important at different stages of embryonic and somatic development. Various studies in *Drosophila* by Lilly's group demonstrated that mutations of *mio*, resulting in the production of truncated protein, suppresses oocyte growth and differentiation [24]. Seh1 in *Drosophila* is also required in oogenesis, but is dispensable for somatic development [36]. Both Mio and Seh1 promote TORC1 activation in female fly's germ lines, but play a relatively minor role in the activation of TORC1 in many somatic types [18]. Wdr24, which is also required for ovary growth and female fertility, promotes TORC1-dependent cell growth not only in germ line, but also in somatic tissues of *Drosophila* [133]. *nprl2* mutations in *Drosophila* decrease the lifespan in flies, which have an accelerated gastrointestinal tract aging process [165].

In *C. elegans*, NPRL2 and NPRL3 are required for postembryonic development, which is supported by the availability of a specific sphingolipid. When *C. elegans* larvae are placed in the environment lacking this lipid, they suspend growth and cell division, which can be overcome by resupplying the lipid. When this lipid is absent, postembryonic growth and development can be re-initiated by activating TORC1 or inhibiting NPRL2/3 [17]. NPRL3 represses intestinal TORC1 activity at least in part by regulating apical membrane polarity, which is probably the main reason of larval development defects in worms that are not supplied with a sphingolipid [166]. In addition, *nprl3*-deficient worms grow slowly due to the lack of the ability to sense vitamin B2 deficiency in their food [131]. NPRL3 deficiency in worms' intestines triggers a gut protease activity, which derives in abnormal behavior and growth impairing [131].

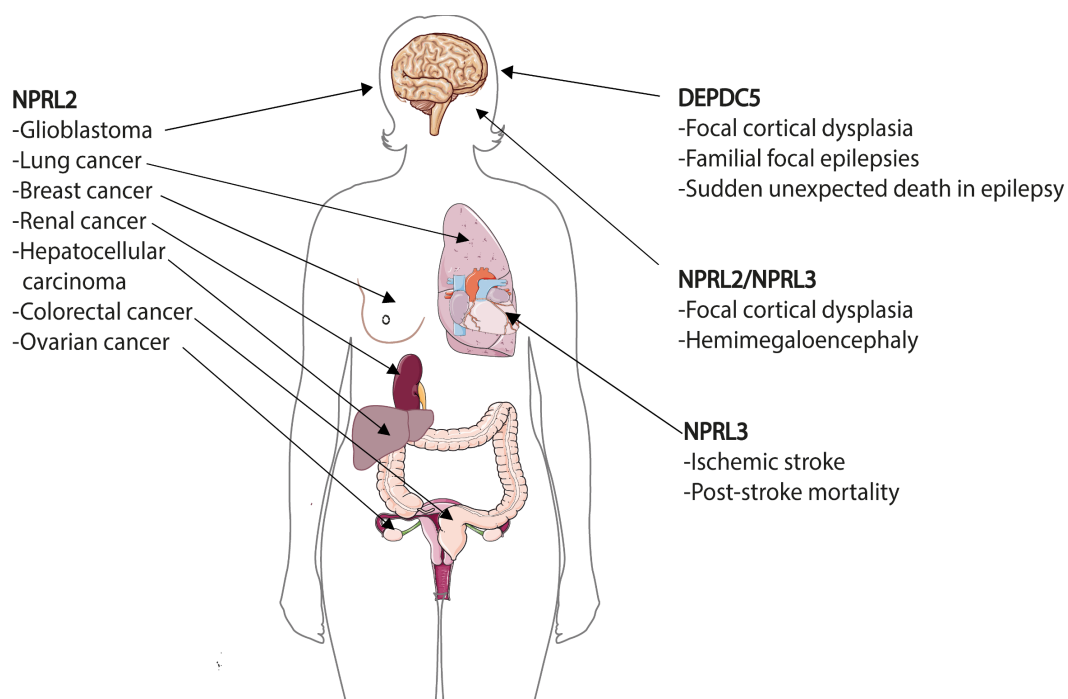
### 7. Deletion Phenotypes of the SEA/GATOR Components across Different Species

In unicellular yeast *S. cerevisiae*, SEA genes (apart from Sec13) are non-essential [3] and in rich media, SEA deletion mutants grow practically with the same rate as wild type yeast [3]. In fission yeast *S. pombe*, deletion of any GATOR1 as well as GATOR2 component Sea3 results in a severe growth defect [22,28]. Homozygous deletions of *nprl2* and *nprl3* in *Drosophila* are semi-lethal and deletions of *iml* are lethal, with GATOR1 activity required for animals to transit the last stage of pupal development [130]. In addition, *nprl2* null flies have a significantly reduced lifespan [165]. Similarly, *depdc5* knockout in zebrafish resulted in premature death at 2–3 weeks post-fertilization [167]. In mice homozygous knockouts of Seh1 [168], Wdr59 and Wdr24 are embryonically lethal [169]. Constitutive knockout homozygous and heterozygous GATOR1 rodent models differs significantly. Thus, GATOR1 homozygous animals *Nprl2*<sup>-/-</sup> mice [20], *Nprl3*<sup>-/-</sup> mice [41], *Depdc5*<sup>-/-</sup> rats [21] and *Depdc5*<sup>-/-</sup> mice [170] are embryonically lethal. Mice embryos deficient for NPRL2 expression show a compromised liver hematopoiesis, which has a

negative impact on embryonic viability [20]. Although mutations in GATOR1 genes are associated with epileptic disorders and brain malformations, heterozygous *Depdc5*<sup>+/-</sup> rats and mice did not present spontaneous epileptic seizures, but *Depdc5*<sup>+/-</sup> rats have subtle cortical malformations [21,170]. Several tissue specific knockouts have also been investigated. Neuron-specific conditional homozygous *Depdc5* knockout mice lived till adulthood, but had larger brains and exhibited a decreased survival [171]. The hepatic deletion of *Depdc5* in mice resulted in mild liver inflammation and decreased fat level [172]. Skeletal muscle-specific *Depdc5* depletion in mice resulted in muscle hypertrophy, but neither the physical nor contractile muscle function of these mice improved [151]. Similarly, mice with *Nprl2* deletion in skeletal muscles had larger muscle fibers and exhibited altered running behavior [149]. In conclusion, deletions of SEA/GATOR components in every organism studied thus far provoked severe defects on growth and viability.

### 8. GATOR in Human Diseases

During the last decade it became increasingly evident that alternations in the expression of GATOR genes can cause various human diseases (Figure 6). Mutations of GATOR2 components can be found in various cancers according to The Cancer Genome Atlas (TCGA) and Cancer Cell Line Encyclopedia (CCLE), COSMIC and cBioPortal databases, although their recurrent mutation frequency is very low [173]. None of the GATOR2 mutations in these cancers were studied on the molecular level and currently there are no data about the involvement of GATOR2 components in other human pathologies [174]. One of the reasons of the low pathogenicity of GATOR2 mutations could be that they would cause an increased, but most probably not complete, suppression of the mTORC1 pathway, which can rather be associated with healthier conditions.



**Figure 6.** Deregulation of GATOR1 components in different human diseases. Expression of GATOR1 components is downregulated in many cancers and GATOR1-related neurological disorders.

In striking contrast to GATOR2, many pathological mutations in GATOR1 genes have been reported. These mutations are mainly related with two main types of human diseases—cancer and epilepsy. Although the alternations in sequence and gene expression associated with these pathologies have been reported for all three GATOR1 genes, there are striking differences that mark some kind of “preferences” of a gene for a pathology.



Thus, DEPDC5 mutations are more frequent in epilepsies in comparison with mutations in other GATOR1 members. NPRL2 mutations can be found more often in different types of cancers and are associated with resistance to anticancer drugs cisplatin and doxorubicin. Even though NPRL3 is a paralogue of NPRL2, its alternations in cancer are less recurrent. Instead NPRL3 appeared to be required for the normal development of the cardiovascular system. Below we will describe alternations of GATOR1 expression in different diseases.

### 8.1. Epilepsies and Brain Malformations—DEPDC5 and Others

In 2013, DEPDC5 was reported as the first gene implicated in familial focal epilepsies by Baulac and Scheffer groups [175,176]. In the following years, it became clear that mutations in DEPDC5 are also related with brain malformations, notably with focal cortical dysplasia (FCD), which is a major cause of drug-resistant epilepsy [177] and can be associated with sudden unexpected death in epilepsy (SUDEP) [178]. In 2016, mutations related with focal epilepsies, familial cortical dysplasia and SUDEP were also reported for Nprl2 and Nprl3 [174,179,180]. Since then, more than 140 variants of GATOR1 genes have been found in up to 37% of patients with familial focal and in other forms of epilepsies [181]. These variants include loss-of-function mutations (67%), missense mutations (27%), splice site changes (4%), frameshifts and copy number variants (~1%). Interestingly, the distribution of mutations in an epilepsy cohort differs drastically from the overall distribution of GATOR1 mutations listed in the gnomAD database, where loss-of-function represents only 4% of variants, with the majority (88%) being missense mutations. Importantly, histopathological analysis of brain tissues resected from individuals with GATOR1 gene mutations demonstrate the hyperactivation of mTORC1 pathway, suggesting that mTORC1 signaling plays an important role in brain development [174,179,180].

Nearly 85% of GATOR1 mutations in epilepsies account for changes in DEPDC5 with both somatic and germline mutations detected all through the gene without clustering. Initially, it was not clear how germline *Depdc5* mutations can cause FCD, especially taking into account that these mutations are often dominantly inherited from an asymptomatic carrier parent [181] and that in rodent models *Depdc5*<sup>+/-</sup> constitutive heterozygous mutations do not exhibit an epileptic phenotype [21,170]. The discovery of second hit somatic mutations in trans, which led to a biallelic inactivation in a subset of brain cells, explained this phenomenon [182,183]. Nprl2 and Nprl3 mutation are less frequent (6% and 9%, respectively), which might be partially related with the fact that their involvement in epilepsies and brain malformations has been tested in a low number of people [26,181]. Cases with simultaneous mutations in different GATOR1 genes have not been described thus far. Several *Nprl2* or *Nprl3* variants found in individuals with FCD or hemimegalencephaly (HME) have been reported recently [184,185]. Interestingly, *NPRL3* single nucleotide polymorphism has been associated with ischemic stroke susceptibility and post-stroke mortality [186], which can be related with increased mTOR activity, that is known to accelerate brain recovery after stroke. The role of NPRL3 in this disease is most probably related with its function in focal epilepsies that might occur in ischemic cerebrovascular disorders [187]. Finally, genetic alternations of KICSTOR complex, required for GATOR1-mediated repression of mTORC1 signaling (see above), have also been linked to epilepsies and brain malformations [188–190].

Thus, it is evident that GATOR1 plays an essential role in cortical formation and development. Mutations of GATOR1 components became important features of “mTORopathies”—a set of pathological conditions characterized by brain malformations, neurological disorders and mTORC1 hyperactivity due to either gain-of-function mutations in a pathway activators (e.g., *AKT*, *RHEB*, *MTOR* itself) or loss-of-function mutations of inhibitors (e.g., *TSC1*, *TSC2*) [191,192]. However, mutations of GATOR1 genes seem to result in a broader spectrum of neurological disorders than other “mTORopathic” genes. Not only are these mutations highly related with medically intractable epilepsies and, especially SUDEP, but they are also observed in autism spectrum disorders [150] and could be implicated in

Parkinson's disease [193]. Therefore, it was recently proposed to name GATOR1-related neurological disorders as GATORopathies [194].

### 8.2. Cancer and Anticancer Drug Resistance—NPRL2 and Others

Among GATOR1 components, NPRL2 was the first that was suggested to be a tumor suppressor [195] almost a decade before the GATOR1 complex was described for the first time. NPRL2 has the higher cancer-associated recurrent mutational frequency out of all the GATOR1 genes [173]. For example, missense mutations in metastatic breast cancers are twice more frequent in Nprl2 (1.55%), than in Nprl3 or Depdc5 (0.78%) [196]. Low levels of NPRL2 expression have mostly been detected in solid tumors (Figure 6), including hepatocellular carcinoma [197], glioblastoma [12], as well as in renal [198,199], ovarian [12,199], colorectal [199–202], breast [199,203] and lung cancers [157,160,199,204,205]. Paradoxically, NPRL2 might also have functions as an oncogene. Recent studies in castration resistant prostate cancer (CRPC) revealed that poor prognosis is associated with high expression of NPRL2 [206].

Alternations of NPRL2 expression is also related to the resistance to a number of anticancer drugs. The most recurrent cases are associated with the resistance to cisplatin and doxorubicin, which has been initially observed in Npr2 deletion mutants in yeast [156] and further confirmed in human lung cancer cell lines [157,160]. The reason of this resistance is still not clear, but it could be related with a role of NPRL2 in DNA damage response (see above) [54,160]. Overexpression of NPRL2 in colon cancer cells increases the sensitivity to a topoisomerase I inhibitor irinotecan (CPT-11) by activation of the DNA damage checkpoints [207]. Genomic alternations of all three GATOR1 components have recently been associated with the resistance to PI3K $\alpha$  inhibitors in primary and metastatic breast cancer [208]. This resistance is explained by the sustained activation of the mTORC1 pathway due to the loss of function mutations of GATOR1 components. In this case, it is reasonable to expect that concomitant mTOR blockage by rapalogs or mTOR pan-inhibitors might overcome resistance. Inversely, CRPC cells, where NPRL2 expression is elevated, are resistant to everolimus [209].

Surprisingly, during the last decade, not a single article reported a study about the involvement of NPRL3 in cancer and drug resistance, even if in the COSMIC database there are almost three times more somatic cancer mutations listed for NPRL3 than for its paralogue NPRL2.

A low frequency DEPDC5 inactivation mutation has been observed in glioblastoma and ovarian cancer, but was not further investigated [12]. DEPDC5 downregulation was also observed in tumors of breast cancer patients [53], where it is strongly correlated with the upregulation of KLHL22 E3-ubiquitin ligase, responsible for DEPDC5 polyubiquitylation and degradation (see above). Recently, DEPDC5 inactivation was discovered in gastrointestinal stromal tumors (GIST), one of the most common human sarcomas. Chromosome 22q deletions are observed in ~50% of GIST and recurrent genomic inactivation of DEPDC5 (>16%) makes it the bona-fide tumor suppressor contributing to GIST progression via increased mTORC1 pathway signaling [210]. This is in striking contrast with >250 non-GIST sarcomas where DEPDC5 aberrations are infrequent (~1%). Interestingly, cancer occurrence in epilepsy probands with germline GATOR1 variants is very low and at present it is considered that there is no link between epileptic germline GATOR1 variants and cancer [181].

Currently, >2000 somatic mutations in different tumors are listed for GATOR1 genes in the COSMIC database, none of them have been studied in detail. It is reasonable to expect that in the following years we should gain more information about the molecular mechanisms associated with the tumorigenesis provoked by these mutations.

### 8.3. Cardiovascular Diseases—NPRL3

In striking contrast to other GATOR1 components, and especially to its paralogue NPRL2, NPRL3 seems to be less important for epilepsy and cancer. Rather it appears as a

crucial gene, necessary for the normal development of the cardiovascular system [41]. Mice with the deletion of NPRL3 promoter often have severe embryonal cardiac defects and die in late gestations. A single nucleotide polymorphism of NPRL3 was reported in sickle cell anemia [211], a disease characterized by various hemoglobin abnormalities. These defects are explained by the fact that the introns of NPRL3 contain super-enhancers required for high level expression of the genes encoding the  $\alpha$ -globin subunits of hemoglobin in humans and mice [212,213]. These regulation elements appeared to be deeply preserved during evolution. A recent genomic study revealed that the NPRL3 gene carrying a strong regulatory element became linked to at least two different globin genes in ancestral vertebrate, just before the divergence between jawless and jawed vertebrates [214]. Each of these ancestral globin genes evolved in the modern hemoglobin genes, but kept their enhancers in NPRL3, which provide an explanation to a long-standing enigma of how globin genes linked to the same adjacent gene undergo convergent evolution in different species.

Therefore, the pathologies associated with NPRL3 mutations are related with the disturbances of the transcriptional elements in the *Nprl3* gene rather than with the function of the protein product in the mTORC1 pathway. Similarly, the higher recurrence of NPRL2 mutations in cancers and DEPDC5 mutations in epilepsies could be related with the specific moonlighting functions of these GATOR1 members beyond the regulation of the mTORC1 pathway.

## 9. Conclusions

Since its discovery ten years ago, the SEA/GATOR complex has been recognized as an important regulator of the mTORC1 pathway that deals with the cell's response to amino acid and glucose availability, DNA damage, mitochondria impairment, etc. Many studies have also revealed the role of the SEA/GATOR complex in human diseases, especially in cancer and epilepsies. Despite the growing number of discoveries involving the SEA/GATOR complex in many organisms, a lot of questions concerning its function and the mechanisms leading to pathologies are still left unanswered. For example, the role of the GATOR complex in amino acid sensing and response has been already clarified in great detail in several studies; however, it is still unknown whether the SEA complex in yeast can perform sensing functions, given that many amino acid sensors interacting with GATOR are not conserved in yeast. The functions of the SEA complex in autophagy and in the formation of organelle contact sites have been extensively studied in yeast. Whether the GATOR complex has these functions in higher eukaryotes is currently unknown (Figure 5). Finally, the most intriguing problem at the moment concerns the molecular function of the SEACAT/GATOR2 complex, an enigma that has remained unresolved despite these 10 years of research and discoveries. Without any doubt, having a high-resolution structure of this subcomplex with or without its partners (SESTRINs, CASTOR2 and others) will be crucial for understanding its function. It will be also important to figure out the principles of interaction between the two SEA/GATOR subcomplexes in different organisms, which can shed light on how evolution shaped this assembly to adapt for the particular needs of various species. SEA members appeared earlier than GATOR members, similar to crocodiles, which are slightly older than alligators [215]. In the same way with crocodiles and alligators, SEA and GATOR are similar to each other in terms of size, structure (appearance) and function (behavior). On the other hand, both SEA and GATOR have a number of subtle yet significant differences that might be able to explain how they each adjusted to operate optimally in different organisms and environments. For example, as with crocodiles, which are bigger than alligators, SEA components are also bigger than their human homologues. Therefore, it will not be surprising if the structural studies reveal that the shape of the SEA complex will slightly differ from that of the GATOR complex, as the V-shape crocodiles' snout differs from larger U-shape snout of alligators. Despite the slight difference in shape, both reptiles use their snouts to effectively catch and hold the food. Similarly, SEA and GATOR complexes, despite several structural differences, can still respond to the presence of nutrients during regulation of the mTORC1 pathway.

We are, therefore, confident that the next decade of SEA/GATOR research will lead to new exciting discoveries of the structure and function of this complex, that can better characterize its implication in health and diseases.

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**Titre :** *Le rôle du complexe GATOR1, régulateur en amont de la voie mTORC1, dans la fonction mitochondriale*

**Mots clés :** mTORC1 – Mitochondrie – SEA/GATOR

**Résumé :** Le mammalian Target of Rapamycin (mTOR) est une serine-thréonine kinase, sous-unité principale des deux complexes, mTOR complex 1 (mTORC1) et mTORC2. La voie de mTORC1 est une des plateformes centrales de la signalisation cellulaire. Ce complexe répond à une variété de signaux, tels que les niveaux d'énergie, d'oxygène et de nutriments, ainsi que des facteurs de croissance et de dommage de l'ADN. La voie de mTORC1 a un rôle clé dans la régulation de la fonction mitochondriale, dans le contrôle de la biogénèse et la dynamique mitochondriale et la dégradation sélective des mitochondries endommagées ou non-fonctionnelles. La majorité des détails concernant ces mécanismes de régulation, est encore inconnue. Un des principaux régulateurs en amont de la voie mTORC1 en réponse aux acides aminés est le complexe GAP-activity towards RAGs (GATOR), composé du DEPDC5, NPRL2 et NPRL3. Ces 3 composants sont notamment classés comme des suppresseurs de tumeurs. Une interaction entre la mitochondrie et le complexe SEACIT (homologue de GATOR1), a été trouvée chez la levure dont les souches avec des délétions pour les protéines de ce complexe,

ont montré une déficience du métabolisme oxydatif et des mécanismes de la dégradation mitochondriale. Cependant, savoir si ces fonctions du SEACIT sont conservées chez les mammifères est une question ouverte. Dans ce travail de thèse, nous montrons que les protéines du complexe GATOR1 peuvent être localisées dans la mitochondrie. Dans les cellules HEK 293, le knockdown des protéines GATOR1 déclenche des altérations significatives de la morphologie mitochondriale, et aussi sur des mécanismes de fission et fusion mitochondriale, change le taux de consommation d'oxygène et impact la polarisation de la membrane mitochondrial. En conclusion, nos résultats montrent que, dans un modèle de cellules humaines, la voie mTORC1 peut en partie moduler la fonction mitochondriale à travers le complexe GATOR1.

**Title :** The role of GATOR1 complex, upstream regulator of mTORC1 pathway, in mitochondrial function

**Keywords :** mTORC1 – Mitochondria – SEA/GATOR

**Abstract :** The mammalian Target of Rapamycin (mTOR) is a serine-threonine kinase and the core subunit of two complexes, mTOR complex 1 (mTORC1) and mTORC2. mTORC1 pathway is one of the central hubs of cellular signaling. It responds to a variety of signals, such as energy, oxygen and nutrient levels, growth factors, DNA damage, etc. mTORC1 pathway has a key role in regulation of mitochondrial function and controls mitochondrial biogenesis, dynamics, and selective degradation of damaged or non-functional mitochondria. Many details of this regulation remain unclear. One of the main upstream regulators of mTORC1 in response to amino acid availability is the GAP-activity towards Rags (GATOR1) complex composed of DEPDC5, NPRL2 and NPRL3. All three components of GATOR1 are tumor suppressors. An interaction between mito-

chondria and GATOR1 homologue (SEACIT complex) was demonstrated in yeast, which have impaired oxidative metabolism and mitochondria degradation upon deletion of SEACIT proteins. Whether these functions are conserved in mammals is unknown. Here, we demonstrate that mammalian GATOR1 components are localized at the mitochondria. Knockdown of GATOR1 proteins in HEK 293 cells trigger a significant alteration of mitochondrial morphology, and mitochondrial fusion and fission events, impacts oxidative metabolism, and triggers dysfunctions in mitochondrial membrane polarization. Taken together, our results show that in human cells mTORC1 pathway modulates mitochondrial function in part via the GATOR1 complex.