Deciphering the molecular mechanisms of amino acid sensing by mTOR

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I. Abstract/Zusammenfassung

The mechanistic target of Rapamycin (mTOR) is a serine-threonine kinase and the master regulator of cell growth and metabolism. The mTOR complex 1 (mTORC1) integrates nutrient availability signals to promote anabolic and inhibit catabolic processes in the cell by phosphorylating multiple substrates. Dysregulation of the mTORC1 pathway is involved in ageing and disease. mTORC1 is recruited to the lysosomal surface by the Rag GTPases upon re-stimulation with amino acids after starvation, where it is activated by the small GTPase Rheb. Although the lysosome-centric model of mTORC1 activation is well-described, growing evidence suggests that amino acids do not activate mTORC1 only on the lysosomes. With this work, I sought to expand the model of mTORC1 activation, by showing that mTORC1 can be activated by amino acids towards specific substrates in a lysosome- and Rag-independent manner. Moreover, I sought to examine mTORC1 activity in unchallenged cells and explain its lysosomal localization when nutrients are abundant. The findings of this work shed light on a largely unexplored part of mTORC1 regulation and lead to a better understanding of the mechanism behind mTORC1 activation by amino acids.

Das Mechanistic Target of Rapamycin (mTOR) ist eine Serin-Threonin-Kinase und der Hauptregulator von Zellwachstum und -stoffwechsel. Der mTOR-Komplex 1 (mTORC1) integriert Signale der Nährstoffverfügbarkeit, um anabole und katabole Prozesse in der Zelle durch Phosphorylierung mehrerer Substrate jeweils zu fördern oder zu hemmen. Eine Dysregulation des mTORC1-Signalwegs ist an Alterung und Krankheit beteiligt. mTORC1 wird nach einer erneuten Stimulierung mit Aminosäuren nach einer Hungersnot durch die Rag-GTPasen an die lysosomale Oberfläche rekrutiert, wo es durch die small GTPase Rheb aktiviert wird. Obwohl das lysosomenzentrierte Modell der mTORC1-Aktivierung gut beschrieben ist, gibt es zunehmend Hinweise darauf, dass Aminosäuren mTORC1 nicht nur an den Lysosomen aktivieren. Mit dieser Arbeit habe ich versucht, das Modell der mTORC1-Aktivierung zu erweitern, indem ich gezeigt habe, dass mTORC1 durch Aminosäuren gegenüber spezifischen Substraten auf lysosomen- und Rag-unabhängige Weise aktiviert werden kann. Darüber hinaus habe ich versucht, die Aktivität von mTORC1 in unbelasteten Zellen zu untersuchen und seine lysosomale Lokalisierung zu erklären, wenn Nährstoffe im Überfluss vorhanden sind. Die Ergebnisse dieser Arbeit beleuchten einen weitgehend unerforschten Teil der mTORC1-Regulierung und führen zu einem besseren Verständnis des Mechanismus hinter der mTORC1-Aktivierung durch Aminosäuren.

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IV. Abbreviations

DEPTOR	DEP domain-containing mTOR-interacting protein
4E-BP1	eIF4E-Binding Protein 1
Arf1	ADP-ribosylation factor 1
ATG13	Autophagy-related 13
BafA1	Bafilomycin A1
BFA	Brefeldin A
bHLH	basic helix-loop-helix
CAD	Phosphatidic A+A1:B56cid+B8
CASTOR	Cytosolic Arginine Sensor for mTORC1
CHX	Cycloheximide
cKO	conditional Knock-Out
CLEAR	Coordinated Lysosomal Expression and Regulation (CLEAR)
eIF4E	eukaryotic translation initiation factor 4E
ER	Endoplasmic Reticulum
FIP200	focal adhesion kinase family-interacting protein of 200 kDa
FLCN	Folliculin
FNIP1 or FNIP2	Folliculin Interacting Protein 1 or 2
FRB domain	FKBP-rapamycin binding domain
GA	Golgicide A
GATOR	GTPase Activating Proteins Toward Rags
GEF	Guanine nucleotide Exchange Factor
GRASP55	Golgi Re-assembly and Stacking Rrotein 55
HEK	Human Embryonic Kidney cells
KD	Knock-down
KO	Knock-out
LC3	Light Chain 3
LRS	Leucyl-tRNA synthetase
LSD	Lysosomal Storage Disease
MEF	Mouse Embryonic Fibroblasts
MiT/TFE	Microphthalmia–Transcription Factor E
MITF	Melanocyte Inducing Transcription Factor
mLST8	mammalian Lethal with Sec13 protein 8
mSin1	mammalian SAPK Interacting protein 1

mTOR	mechanistic Target of Rapamycin
mTORC1	mechanistic Target of Rapamycin complex 1
mTORC2	mechanistic Target of Rapamycin complex 2
OPP	O-Propargyl-Puromycin
PA	Phosphatidic Acid
PLD	Phospholipase D
PRAS40	Proline-Rich Akt Substrate of 40 kDa
Protor-1/2	Protein observed with Rictor
Raptor	Regulatory associated protein of mTOR
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
S6K1	S6 Kinase 1
SAM	S-adenosylmethionine
siRNA	small interfering RNA
SLC38A9	member 9 of the solute carrier family 38 (SLC38A9)
SREBP	Sterol Regulatory Element Binding Protein
TFEB	Transcription Factor EB
TOP	Terminal OligoPyrimidine
TOS motif	TOR signaling motif
TSC	Tuberous Sclerosis Complex
ULK1	Unc-51 like autophagy activating kinase
UPS	Unconventional Protein Secretion
WB	Western Blot

1. Introduction

1.1 mTORC1 is a master regulator of cell growth and metabolism

The mechanistic Target of Rapamycin, or mTOR, was discovered during an effort to identify the target of the immunosuppressant and antifungal agent rapamycin, which was first purified in 1975 from soil bacteria found on Rapa Nui (Vézina et al., 1975). mTOR is a serine/threonine kinase and the catalytic subunit of two distinct complexes, with different localization patterns, different upstream regulators and different substrates and downstream targets. mTOR complex 1 (mTORC1) comprises of mTOR, the regulatory associated protein of mTOR (Raptor), the positive regulator mammalian lethal with Sec13 protein 8 (mLST8) and two inhibitory subunits, the proline-rich Akt substrate of 40 kDa (PRAS40) and the DEP domain-containing mTOR-interacting protein (DEPTOR) (Hara et al., 2002; Kim et al., 2003; Peterson et al., 2009; Sancak et al., 2007). mTOR complex 2 (mTORC2) comprises also of mTOR and mLST8, the rapamycin-insensitive companion of mTOR (Rictor), the mammalian SAPK interacting protein 1 (mSin1) and the protein observed with Rictor-1/2 (Protor-1/2). Rapamycin binds mTORC1 at the FKBP-rapamycin binding (FRB) domain (Fig. 1.1). mTORC2 is highly insensitive to rapamycin, due to the fact that Rictor partially covers the FRB domain of mTOR (Scaiola et al., 2020), but it can be inhibited by it after prolonged treatment in mammals (Sarbassov et al., 2006).

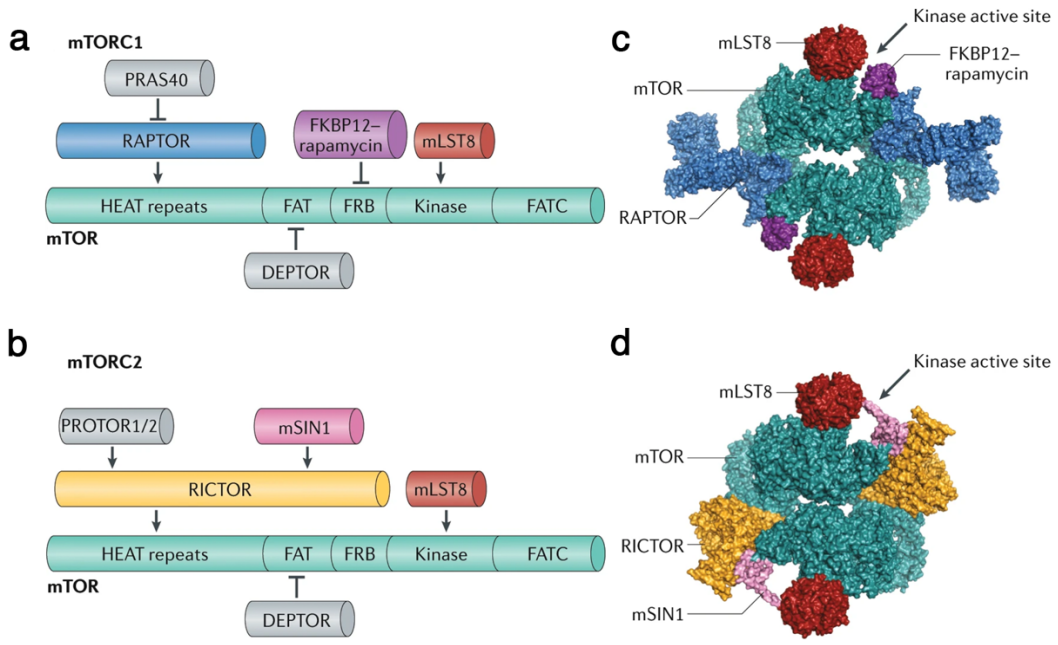

Figure 1.1 | mTORC1 and mTORC2 complex components

a., **b.** Schematic representation of the mTORC1 and mTORC2 complex components and topology of their binding on the mTOR protein **c.** A 5.9-Å reconstruction of mTORC1 (without PRAS40 and DEPTOR) in complex with FKBP12–rapamycin shown as a surface representation (Protein Data Base (PDB) ID: 5FLC) **d.** A 4.9-Å reconstruction of mTORC2 (without DEPTOR and PROTOR) is shown as a surface representation (PDB ID: 5ZCS) (modified from Liu and Sabatini, 2020).

Since its discovery it became clear that mTOR plays a central role in organismal metabolism and its dysregulation is the cause of several diseases. Moreover, mTOR has been closely connected to the progression of ageing and diseases related to ageing. mTORC1 integrates environmental stimuli to promote anabolic processes through the phosphorylation of a number of substrates (Fig. 1.2). mTORC1 responds to nutrient – amino acid, lipid, carbohydrate –growth factor and glucose availability. Amino acids, in particular, are the most robust stimulus for mTORC1 activation, as mTORC1 cannot be active when amino acids are scarce, even when growth factors are present. It is intuitive that mTORC1 can sense the nutrients available in the environment of the cell, to promote anabolic functions necessary for cell growth. In the context of my doctoral work, I focus on elucidating the mechanisms of mTORC1 regulation by amino acids.

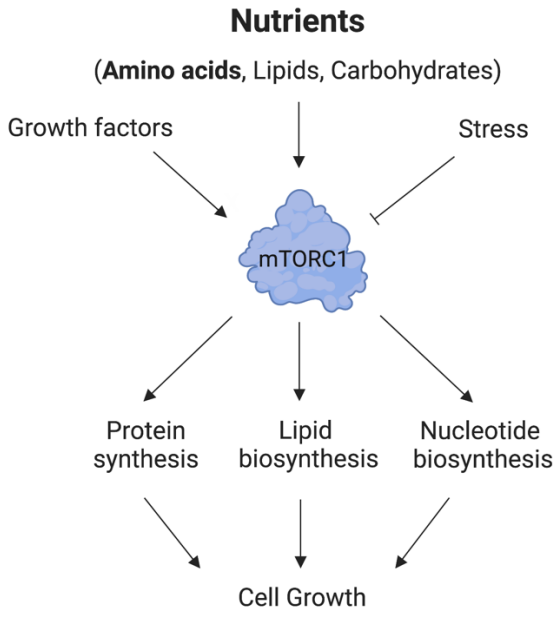

Figure 1.2 | mTORC1 integrates environmental stimuli to promote anabolic processes
Amino acids are the most robust stimulus for mTORC1 activation. mTORC1 promotes protein synthesis, lipid and nucleotide biosynthesis and glucose metabolism to regulate cell growth. (Created with BioRender.com)

The first proteins shown to be mTORC1 substrates and were mainly used over the years to assess mTORC1 activity are S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1) (Brunn et al., 1997; Burnett et al., 1998). S6K1 and 4E-BP1 contain a very characteristic and conserved TOR signalling (TOS) motif (Fig 1.3). This is found in the N-terminus of all known S6 kinases and in the C-terminus of the 4E-BPs (Schalm and Blenis, 2002) and it was shown to be necessary for the binding to Raptor and the subsequent phosphorylation by mTOR (Nojima et al., 2003; Schalm et al., 2003).

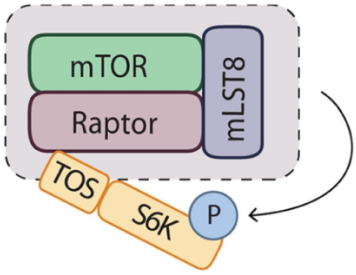

Figure 1.3 | TOS-mediated substrate recruitment mechanism

The recruitment of S6K1 (and 4E-BP1) by mTORC1 occurs via a TOS motif which is recognized by Raptor. (modified from Napolitano et al., 2022)

Although phosphorylation of T389 in S6K1 is activating and promotes the phosphorylation of its downstream targets, like S6 ribosomal protein, phosphorylation of 4E-BP1 is inhibitory and allows the formation of the translation initiation complex by releasing eIF4E (Ma and Blenis, 2009). The phosphorylation pattern of 4E-BP1 by mTORC1 is more complicated, as phosphorylation of Thr37/46 serves as a priming event, followed by phosphorylation on Thr70 and Ser65. It is important to mention that only Ser65 phosphorylation is sensitive to rapamycin treatment. Moreover, upon amino acid or growth factor starvation, S6K and 4E-BP1 are no longer phosphorylated by mTORC1.

It was shown already before the identification of S6K1 as an mTORC1 substrate, that rapamycin treatment inhibits its function to promote the translation of 5' Terminal OligoPyrimidine (TOP) motif-containing mRNAs (Jefferies et al., 1997). The TOP motif is very well conserved, the mRNAs containing it encode ribosomal proteins and transcription factors and their translation is regulated downstream of both S6K and 4E-BP1 to control cell growth (Iadevaia et al., 2008).

Apart from protein synthesis, mTORC1 regulates directly a series of other anabolic processes through its substrates. For instance, mTORC1 controls lipid biosynthesis by

promoting the function of the master transcriptional regulators of genes involved in *de novo* lipid and sterol biosynthesis, the members of the sterol regulatory element binding protein (SREBP) family, through the phosphorylation of S6K1 and Lipin1 (Düvel et al., 2010; Peterson et al., 2011; Porstmann et al., 2008). Moreover, there are reports that mTORC1 controls *de novo* pyrimidine biosynthesis via the S6K1-dependent phosphorylation of the carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase (CAD), the enzyme that catalyses the three first steps of pyrimidine biosynthesis.

1.2 mTORC1 regulation on the lysosomal surface

An important aspect of the regulation of mTORC1 activity is its subcellular localization. It was shown first that a group of small GTPases, called Rag GTPases, interact with Raptor and mediate amino acid signalling to mTORC1 (Kim et al., 2008; Sancak et al., 2008). There are four Rag proteins in mammals, which form obligate heterodimers. When amino acids are available, RagA/B GTP binds RagC/D GDP to form a heterodimer that activates mTORC1. The same studies that identified the importance of the Rag GTPases for the activation of mTORC1 showed that they colocalize with another small GTPase, the Ras homolog enriched in brain (Rheb), which was already demonstrated to be an activator of mTORC1 and important for the phosphorylation of its substrates S6K1 and 4EBP1 (Long et al., 2005).

Rheb localises in multiple membrane compartments in the cell and it was shown to activate mTORC1 on the lysosomal surface upon stimulation with amino acids. In fact, the Rag GTPases recruit mTORC1 to the lysosomal surface, where it is activated by Rheb (Sancak et al., 2010). The Rag GTPases were found to be tethered to the lysosomal surface by the LAMTOR (or Ragulator) complex (Sancak et al., 2010). The LAMTOR complex is a pentamer containing two heterodimers: Lamtor2/p14 forms a heterodimer with Lamtor3/MP1 and Lamtor4/p10 with Lamtor5/HBXIP, whereas Lamtor1/p18 wraps around the two heterodimers and anchors the complex to the lysosomal surface (Yonehara et al., 2017, Fig. 1.4). Upstream of Rheb is the inhibitory Tuberous Sclerosis Complex (TSC), the absence of which leads to mTORC1 hyperactivation towards those two mTORC1 substrates (Inoki et al., 2003; Tee et al., 2003). The TSC is the main upstream inhibitor of mTORC1 signalling and it is recruited to the lysosomal surface in response to nutrients through several different mechanisms, one of which also involves the Rag GTPases (Menon et al., 2014; Demetriades et al., 2014; Fitzian et al., 2021; Prentzell et al., 2021).

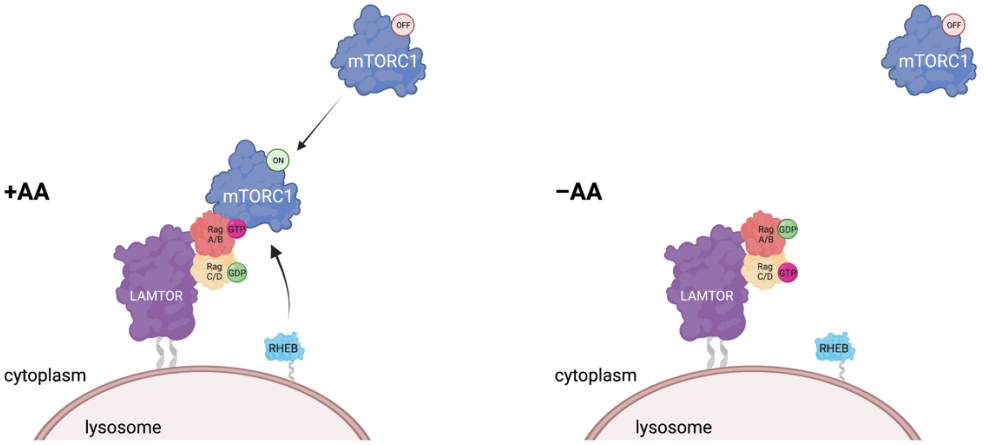

Figure 1.4 | Recruitment of mTORC1 to the lysosomal surface in response to amino acids
Upon stimulation with amino acids, mTORC1 is recruited to the lysosomal surface by active heterodimers of Rag GTPases (RagA/B^{GTP} with RagC/D^{GDP}), where it is activated by the small GTPase Rheb (left). Upon starvation of amino acids, the Rag GTPases take their inactive conformation (RagA/B^{GDP} with RagC/D^{GTP}), and mTORC1 de-localises to the cytoplasm where it remains inactive (Created with Biorender.com).

Loss of RagA in the early embryonic stages results to death on day E10.5 in mice (Efeyan et al., 2014). Moreover, it results to developmental malformations and reduced mTORC1 activity. However, in Mouse Embryonic Fibroblasts (MEF) derived from these mice, mTORC1 continues to phosphorylate S6K1 and 4EBP1 even upon amino acid and glucose starvation. RagB levels are increased upon RagA loss, but RagB are low in embryos and most adult tissues, so its loss does not result in a lethal phenotype. Another study shows that loss of both RagA and RagB in heart leads to cardiac hypertrophy in mice (Kim et al., 2014).

The initial discovery that mTORC1 is activated on the lysosomal surface was surprising, as for a long time after their discovery in 1950, lysosomes were considered to be simply the waste bags of the cell. However, nowadays we know that lysosomes are more complex organelles. The lysosomes are membrane-bound cell organelles, responsible for the degradation of macromolecules and other cellular components. They contain a large number of digestive enzymes in their lumen, such as glycosidases, proteases – for instance cathepsins – and sulfatases. The lysosomal enzymes are produced in the endoplasmic reticulum (ER) and are transported to the Golgi, where they get a manose-6-phosphate lysosomal tag. These enzymes are functional only in an acidic environment, like the lysosomal lumen. The maintenance of the lysosomal lumen pH is secured by the lysosomal v-ATPase, which is located on the lysosomal membrane and pumps H^+ into the lysosomal lumen (Forgac, 1998). An overview of the lysosomal properties is shown in Fig. 1.5.

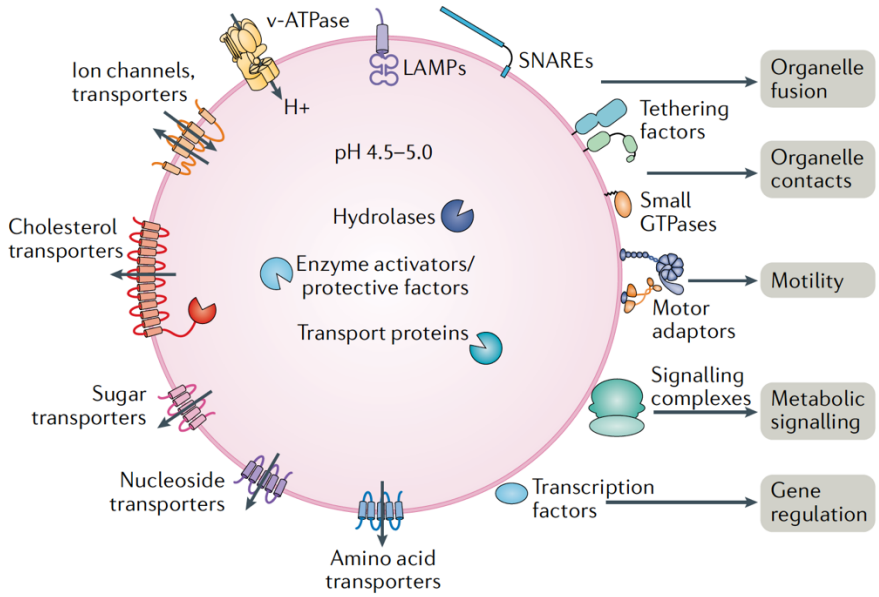

Figure 1.5 | Overview of the lysosomal properties

The lysosomal lumen contains mainly hydrolases, which degrade molecules like proteins and lipids to their building blocks. Enzyme activators and protective factors facilitate the function of these hydrolases. Transport proteins facilitate the transport of molecules like cholesterol outside of the lysosomal lumen. The acidic lysosomal lumen pH is secured by the v-ATPase, which is located on the lysosomal membrane. The lysosomal membrane contains also lysosome-associated membrane proteins (LAMPs), which among others protect the lysosomal membrane from degradation, SNAREs and tethering factors, which facilitate fusion with other organelles, motor adaptors, which facilitate motility through interaction with microtubule motors, signalling complexes like mTORC1, transcription factors, sugar, nucleoside and amino acid transporters, which release the degradation products, ion transporters which secure ion homeostasis in the lysosomes and cholesterol transporters (Ballabio and Bonifacino, 2020).

The lysosomes are involved in immune responses, membrane repair, cell adhesion and migration, regulation of transcription and translation and the regulation of cell metabolism by responding to signals coming from other cellular compartments. Due to their role in cellular homeostasis, dysregulation of lysosomes results to a number of lysosomal storage diseases (LSD), but also cancer and neurodegenerative disorders (Ballabio and Bonifacino, 2020). Despite extended studies, not all details about the lysosomal function, membrane composition and content are clarified. However, a wide signalling network around mTORC1 is described.

Since the identification of the lysosome-centric model of mTORC1 activation by amino acids and the Rag GTPases, a big part of the research around mTORC1 regulation focused exclusively on building the amino acid-sensing lysosomal machinery. The core amino acid-

sensing network that was described to control the activity of the Rag GTPases comprises the lysosomal v-ATPase (Zoncu et al., 2011), the GTPase activating proteins toward Rags 1 and 2 (GATOR1 and GATOR2) complexes (Bar-Peled et al., 2013), the folliculin/folliculin-interacting protein (FLCN/FNIP) complex (Petit et al., 2013; Tsun et al., 2013), the KICSTOR complex (Wolfson et al., 2017) as well as sensors for specific amino acids: SLC38A9 and cytosolic arginine sensor for mTORC1 subunits 1/2 (CASTOR1/2) for arginine (Chantranupong et al., 2016); Leucyl-tRNA synthetase (LRS) (Han et al., 2012), sestrins (Chantranupong et al., 2014; Wolfson et al., 2016) and SAR1B (Chen et al., 2021) for leucine and SAMTOR for methionine – sensed as S-adenosylmethionine (SAM) (Gu et al., 2017). More recently, the mitochondrial threonyl-t-RNA synthetase 2 (TARS2) was described as a threonine sensor for mTORC1 (Kim et al., 2021). Of those proteins, the GATOR1 complex is a GTPase activating protein (GAP), meaning it promotes hydrolysis of the GTP for RagA and RagB, whereas FLCN is a GAP for RagC and RagD. An overview of the lysosomal machinery is shown in Fig. 1.6.

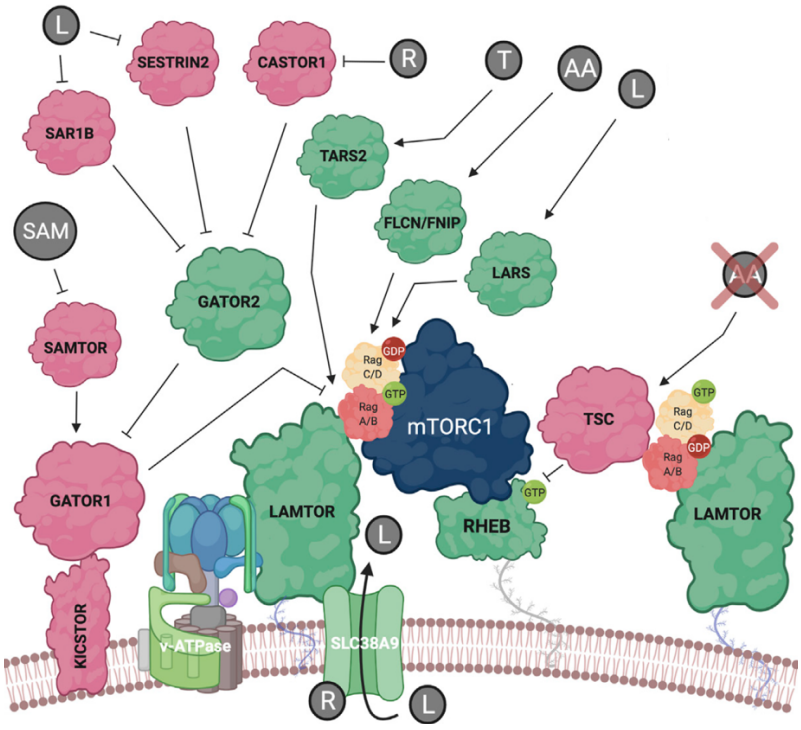

Figure 1.6 | Overview of the lysosomal machinery controlling mTORC1 activity

mTORC1 is recruited to the lysosomal surface by the Rag GTPases in response to amino acid stimuli, where it is activated by Rheb. The TSC complex upstream of mTORC1, which is also recruited to the lysosomes by the Rag GTPases, among others, inhibits Rheb in response to stress like amino acid starvation. The lysosomal amino acid sensing machinery comprises of a large number of proteins described as Rag regulators. These include GATOR1, which is a GAP for RagA/B and therefore has an

inhibitory function, and GATOR2, which inhibits GATOR1, KICSTOR, FLCN, which is a GAP for RagC/D, and sensors for specific amino acids: Sestrin2, SAR1B and LARS for leucine, Castor1 for arginine, TARS2 for threonine and SAMTOR for methionine, sensed as SAM (modified from Fernandes and Demetriades, 2021).

Most of the previous studies highlighting the importance of the Rag GTPases for mTORC1 activation focus on the acute re-stimulation of mTORC1 by amino acids, following amino acid starvation. However, work from our group and others showed that mTORC1 is still active in cells lacking the Rag GTPase in basal amino acid replete conditions (Demetriades et al., 2014; Jewell et al., 2015). Additionally, a more recent study showed that mTORC1 can be re-activated specifically by glutamine or asparagine independently of the Rag GTPases (Meng et al., 2020). Amino acid signalling in this case is mediated by a different small GTPase, the Golgi-located ADP-ribosylation factor 1 (Arf1), which regulates mTORC1 on the lysosomal surface. In line with this, glutamine has additionally been reported to signal to mTORC1 in a Rag-independent manner through Arf1 and Phospholipase D (PLD) (Bernfeld et al., 2018), regulating the levels of phosphatidic acid (PA), which facilitates the association between mTOR and Raptor (Xu et al., 2011). It is important to note that Arf1 and its respective GEFs plays an important role in many pathways inside the cell, including lipid droplet metabolism, clathrin-independent endocytosis, signalling at the plasma membrane, mitochondrial dynamics and transport along microtubules (Kaczmarek et al., 2017).

Interestingly the study introducing the lysosomal v-ATPase in the mTORC1 signalling pathway, describes additionally an inside-out mechanism for mTORC1 regulation by amino acids (Zoncu et al., 2011). In this model, amino acids need first to accumulate in the lysosomal lumen in order to initiate signalling to the Rag GTPases on the lysosomal surface to recruit mTORC1. Moreover, the member 9 of the solute carrier family 38 (SLC38A9) was later described as a component of the lysosomal amino acid sensing machinery signalling to mTORC1 (Rebsamen et al., 2015). SLC38A9 is a lysosomal amino acid transporter, the silencing of which suppresses arginine-induced mTORC1 re-activation. All these suggest that the lysosomal content is also an important factor, in addition to the environmental nutritional status, for mTORC1 activity.

1.3 Regulation of catabolic processes and the MiT-TFE family of transcription factors

As a master regulator of metabolism, mTORC1 does not control only anabolic processes in the cell. A big part of mTORC1 downstream functions comprises of the regulation of catabolic processes, namely autophagy, downstream of a different group of substrates.

Macroautophagy (herein referred to as autophagy) is a lysosome-dependent mechanism of cellular component degradation. These components can be single proteins or whole organelles. Although autophagy is triggered by nutrient withdrawal, housekeeping levels of autophagy are retained in cells growing in unchallenged conditions, supplemented with full medium and serum (Musiwaro et al., 2013). However, upon nutrient starvation the levels of autophagy increase and cellular components are engulfed by double-membraned vesicles called autophagosomes, the outer membrane of which finally fuses with the lysosomes to degrade their components. Macroautophagy is divided in five phases, which are initiation, membrane nucleation and pre-autophagosome or phagophore formation, elongation, autophagosome-lysosome fusion and cargo degradation (Fig 1.7) The autophagy-related (ATG) proteins are important players in all steps of macroautophagy (Deleyto-Seldas and Efeyan, 2021).

A widely used marker of autophagy is microtubule-associated protein 1 light chain 3 (LC3). There are three LC3 isoforms in humans which are modified during autophagy, LC3A, LC3B and LC3C. LC3 is cleaved right after its synthesis to form LC3-I. During autophagy, LC3-I is lipidated and converted to LC3-II, which associates with the autophagosome membrane (Fig 1.8). Therefore, the presence of LC3-II indicates autophagosome formation and is used as an indicator for autophagic activity (Kabeya et al., 2000; He et al., 2003; Kabeya et al., 2004).

mTORC1 phosphorylates directly Unc-51 like autophagy activating kinase (ULK1/2) and Autophagy-related 13 (ATG13), two proteins involved in the first steps of autophagy, in response to nutrient availability (Ganley et al., 2009). ULK1/2 and ATG13 form a complex with the focal adhesion kinase family-interacting protein of 200 kDa (FIP200) and ATG101, which stimulates phagophore nucleation. When amino acids are available, mTORC1 phosphorylates ULK1 and ATG13 to inhibit phagophore nucleation. Therefore, when active, mTORC1 inhibits catabolic processes in the cell. It is important to mention that mice expressing a constitutively active RagA mutant fail to survive postnatal day 1. This is because

they fail to induce autophagy upon amino acid depletion and use autophagy-derived amino acids for glucose production (Efeyan et al., 2013).

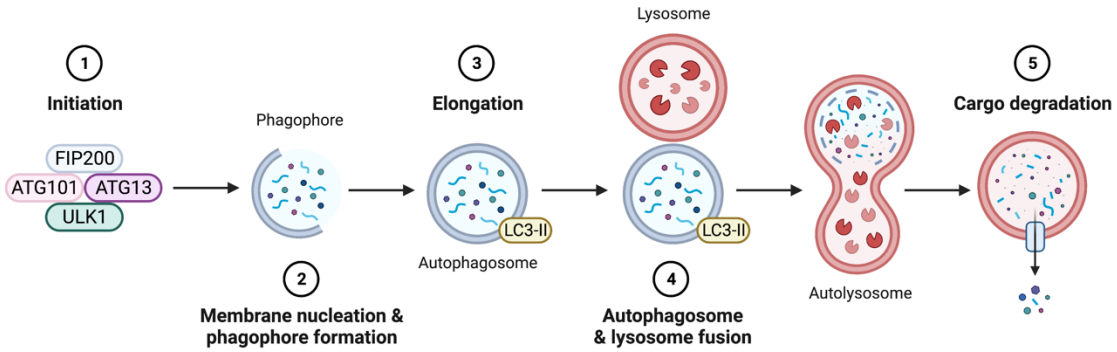

Figure 1.7 | The macroautophagy process

Macroautophagy is divided in five phases. ATG13 and ULK1, which are direct substrates of mTORC1, form a complex with FIP200 and ATG101 to stimulate membrane nucleation and phagophore formation. The phagophore is a double-membrane structure which engulfs cell components and divides to form double-membrane vesicles, the autophagosomes. The lipidated LC3-II is incorporated into the phagophore and autophagosome double membrane. In the next step, the autophagosomes fuse with acidic lysosomes, to form autolysosomes and degrade the autophagosome cargo and inner membrane. In the end, the lysosomes release the building blocks coming from cargo degradation. (Created with BioRender.com)

However, direct phosphorylation of proteins involved in autophagy is not the only way mTORC1 controls catabolic processes. The Transcription Factor EB (TFEB) is a member of the microphthalmia–transcription factor E (MiT/TFE) subfamily of basic helix-loop-helix (bHLH) transcription factors. This subfamily includes four members in humans: the melanocyte-inducing transcription factor (MITF), TFE3, TFEB, and TFEC. TFEB was first identified to regulate the transcription of a gene family containing the Coordinated Lysosomal Expression and Regulation (CLEAR) element, which encode lysosomal proteins. Under amino acid withdrawal or pharmacological mTORC1 inhibition, TFEB transitions from the cytoplasm to the nucleus, where it induces the transcription of the CLEAR genes (Palmieri et al., 2011; Sardiello et al., 2009). TFEB was later described as an autophagy and lysosomal biogenesis regulator in a study investigating the differences seen in gene expression upon starvation and mTORC1 inhibition. TFEB was first presented as part of an mTORC1-independent pathway regulating autophagy (Settembre et al., 2011), but a year later another study showed that TFEB is actually directly phosphorylated by mTORC1 on the lysosomal surface (Settembre et al., 2012). Interestingly, TFEB is recruited to the

lysosomes also by the Rag GTPases in response to amino acids (Martina and Puertollano, 2013).

The connection between TFEB and mTORC1 created new perspectives in the research of autophagy regulation downstream of mTORC1. mTORC1 controls the expression of genes involved in lysosomal biogenesis and autophagy from the surface of the main degradative organelle in the cell with the help of the same small GTPases that recruit mTORC1 to the lysosomes. When amino acids are available, mTORC1 phosphorylates TFEB on Ser211 at the lysosomal surface. This phosphorylation allows the binding of TFEB to the cytoplasmic 14-3-3 protein and its cytoplasmic retention (Martina et al., 2012; Roczniak-Ferguson et al., 2012). Upon amino acid starvation, TFEB is dephosphorylated by the phosphatase calcineurin, that responds to calcium signalling from the lysosomes and localises in the nucleus (Medina et al., 2015). However, upon re-supplementation with amino acids, TFEB leaves the nucleus and returns to the cytoplasm, where it binds the Rag GTPases and is again phosphorylated by mTORC1. It is important to note that TFEB in fact shuttles between the nucleus and the cytoplasm in normal fed conditions. The nuclear export signal (NES) in the N-terminus of TFEB, as well as the hierarchical phosphorylation of S142 and S138 in the proximity of it allow TFEB to leave the nucleus (Napolitano et al., 2018).

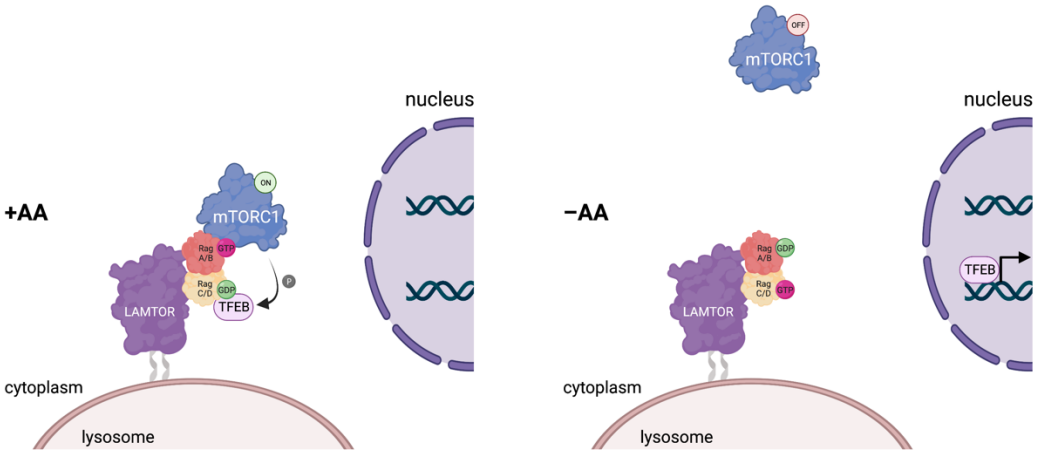

Figure 1.8 | Regulation of TFEB by mTORC1 on the lysosomal surface

In the presence of amino acids, mTORC1 phosphorylates the transcription factor TFEB, which is also recruited to the lysosomal surface by the Rag GTPases, to inactivate it and promote its cytoplasmic sequestration (left). However, upon removal of amino acids mTORC1 is inactive and TFEB localises in the nucleus, where it promotes the transcription of genes involved in lysosomal biogenesis and autophagy. (Created with BioRender.com)

TFEB belongs to the "non-canonical" substrates of mTORC1, as recruitment by the Rag GTPases and not a TOS motif is important for its phosphorylation by mTORC1. In fact, it was shown that the first 30 amino acids of TFEB are necessary for its binding to the Rag GTPases and its recruitment to the lysosomal surface (Martina and Puertollano, 2013). Moreover, recent studies prove that RagC and RagD specifically are responsible for the recruitment of TFEB to the lysosomal surface (Napolitano et al., 2020; Alesi et al., 2021; Gollwitzer et al., 2022; Cui et al., 2022). Finally, it was recently revealed that the Rag GTPases play an even more important role in the regulation of the TFE3 and MITF levels, as by recruiting them to the lysosome they mediate the ubiquitination and degradation of those transcription factors (Nardone et al., 2023).

RagC and RagD are the two Rag GTPases that are not only regulating mTORC1 and TFEB activation, but they are also regulated by them. RagC and RagD are important for the recruitment of TFEB to the lysosomal surface, but they are also transcriptionally regulated by TFEB (Di Malta et al., 2017). Moreover, mTORC1 promotes the full activation of RagC by amino acids and growth factors by directly phosphorylating it on S21 (Yang et al., 2019).

Although TFEB is the best-studied MiT-TFE transcription factor in the context of mTORC1 signalling, TFE3 was also shown to behave similarly upon amino acid starvation and re-supplementation and be phosphorylated directly by mTORC1 (Roczniak-Ferguson et al., 2012; Martina et al., 2014). TFE3 is also recruited by the Rag GTPases on the lysosomal surface and it promotes the transcription of genes containing the CLEAR element (Martina et al., 2014). Therefore, TFE3 is used as an alternative to TFEB, to assess mTORC1 activity towards its lysosomal substrates.

Moreover, the characterization of this pathway gave insights in the mechanisms behind lysosomal storage disorders (LSDs), which are caused by mutations on genes encoding for lysosomal proteins, mainly hydrolases (Ballabio and Gieselmann, 2009). The cell phenotype is characterised by enlarged lysosomes filled with undigested material and impaired autophagy flux. TFEB localization appears to be mainly nuclear in several LSDs (Sardiello et al., 2009). In other LSDs, like Pompe disease, TFEB overexpression leads to lysosomal clearance and rescue of the disease phenotype (Spampanato et al., 2013).

1.4 Unconventional regulation of mTORC1 substrates and mTORC1 localization

Since the identification of TFEB and TFE3 as mTORC1 substrates, there were hints that mTORC1 might not regulate all its substrates in a similar manner. A study from 2014 shows that in RagA/B KO MEFs, although S6K1 and 4EBP1 phosphorylation by mTORC1 was decreased, but no abolished, TFEB was always nuclear, regardless of the nutritional conditions (Kim et al., 2014). Loss of RagA/B led to impairment of autophagic flux and altered expression of lysosomal proteins. Moreover, RagA/B cKO hearts in this study presented abnormal accumulation of glycogen, phenocopying LSDs like Pompe.

As mentioned previously, FLCN promotes nucleotide hydrolysis of RagC or RagD, and forms a complex with FLCN-interacting protein 1 or 2 (FNIP1 or FNIP2) (Baba et al., 2006; Hasumi et al., 2008; Tsun et al., 2013). FLCN localises to lysosomes upon amino acid starvation and translocates to the cytoplasm upon amino acid re-supplementation. This depends on the nucleotide state of the Rag GTPases. However, upon FLCN loss in HEK293T cells, phosphorylation of S6K1 and 4EBP1 by mTORC1 is highly unaffected, but TFEB and TFE3 show enriched nuclear localization (Hong et al., 2010; Petit et al., 2013; Lawrence et al., 2019).

Vacuolar protein sorting 41 (VPS41) is part of the Homotypic fusion and Protein Sorting (HOPS) complex, which regulates fusion of autophagosomes and endosomes with lysosomes (van der Beek et al., 2019). When VPS41 is depleted, delivery of endocytic cargo and autophagic flux are impaired. Moreover, deletion of VPS41 results in inhibition of transport of lysosomal membrane proteins from the trans-Golgi network to the lysosomes (Swetha et al., 2011; Pols et al., 2013). Interestingly, a recent study showed that, upon VPS41 loss in patient fibroblasts or HeLa cells, mTOR is no longer lysosomal and TFE3 localises mainly in the nucleus, but S6K1 and 4EBP1 phosphorylation is not affected (van der Welle et al., 2021).

Finally, in TSC1/2 deficient human embryonic kidney T (HEK293T) or HeLa cells, mTORC1 is hyperactive towards S6K1 and 4EBP1, but TFEB is hypo-phosphorylated by mTORC1 and enriched in the nucleus (Alesi et al., 2021). However, a former study claimed that TFEB is not regulated downstream of the TSC-Rheb axis, as silencing of any of those genes did not have an effect on TFEB phosphorylation (Napolitano et al., 2020). In the same study it is shown that TFEB and S6K1 are differentially regulated by mTORC1 in response to different stimuli and depend on different upstream regulators. Rheb is necessary

for the phosphorylation of S6K1 and 4EBP1, but not for TFEB, and the Rag GTPases are necessary for TFEB phosphorylation, but not for the phosphorylation of the two cytoplasmic substrates.

These findings add an additional layer of complexity to the regulation of cellular functions downstream of mTORC1. Furthermore, mTORC1 lysosomal localization was shown to be transient and lysosomal mTORC1 to be only a fraction of the total cellular pool (Lawrence et al., 2018).

It is known that mTORC1 has other functions on non-lysosomal locations. For instance, a recent study from our lab showed that mTORC1 phosphorylates the Golgi re-assembly and stacking protein 55 (GRASP55) on the Golgi to control unconventional protein secretion (UPS) in response to stress, including amino acid starvation (Nüchel et al., 2021). The phosphorylation pattern of GRASP55 closely follows the phosphorylation pattern of S6K1 and 4EBP1 in response to amino acids.

1.4 Aims of this study

To date, most studies highlighting the importance of the Rag GTPases and the lysosomal localization of mTORC1 for its regulation by amino acids have focused on the conditions of starvation and acute re-stimulation of mTORC1 by amino acids. Loss of the Rag GTPases under these conditions shows (i) partial insensitivity to starvation and (ii) significantly reduced re-activation of mTORC1 upon amino acid re-addition. However, how the Rag GTPases and the lysosomal localization of mTORC1 contribute to its activity in steady state conditions is not clear.

Starting from previous observations showing that mTORC1 may be active towards S6K1 and 4EBP1 in unchallenged cells in the absence of the Rag GTPases, I sought to confirm this phenotype in different cell types. Moreover, I examined in parallel the phosphorylation of TFEB and TFE3 by mTORC1, covering the whole spectrum of mTORC1 substrates, lysosomal or not lysosomal, which is a part ignored by many previous studies. Additionally, I investigated the effect of Rag KO on functions regulated downstream of those substrates.

Finally, this study reveals the reason why mTORC1 is located on the lysosomes of unchallenged cells in the first place and whether this localization is important for the regulation of all its downstream functions. Overall, this study sheds light on unexplored parts of mTORC1 regulation and presents a broader picture of the role of mTORC1 in the cell and not only as a part of the lysosomal machinery.

2. Results

2.1 mTORC1 is not lysosomal in Rag KO HEK293FT and MEF cells

To characterize mTORC1 localization in cells lacking functional Rag GTPase dimers, I used human embryonic kidney 293 FT (HEK293FT) RagA/B deficient cells generated in our lab. When RagA and RagB are depleted, RagC and RagD cannot form heterodimers and the proteins themselves are unstable. Immunofluorescence staining of mTOR in WT HEK293FT confirmed what is shown in previous studies, that mTOR co-localises with the lysosomal marker LAMP2 and forms lysosomal accumulations in basal, amino acid replete conditions (+AA), whereas it has a diffused cytoplasmic distribution upon amino acid starvation (-AA). Upon re-stimulation with amino acids, mTOR returns to the lysosomal surface (-/+AA). However, in RagA/B KO HEK293FT, mTOR is always cytoplasmic and does not co-localise with LAMP2 under any conditions (Fig. 2.1a, b).

This observation was also confirmed by modified lysosomal immunoprecipitation (Lyso-IP) experiments, based on the study by (Abu-Remaileh et al., 2017). WT or RagA/B KO cells expressing stably HA tagged TMEM192, a lysosomal membrane protein, were generated for this purpose. Lysosomes containing HA-TMEM192 were pulled down and the lysates were analysed with WB for the mTOR and Raptor components of the mTORC1 complex, as well as for lysosomal (Cathepsin D/CTSD, LAMP2) and cytoplasmic markers (GAPDH). Moreover, the non-lysosomal fraction was also collected and anaysed for the same proteins. As seen in Fig. 2.1c, mTOR and Raptor are found in the cytoplasmic fraction of both WT and RagA/B KO HEK293FT in amino acid replete conditions. In agreement with the immunofluorescence experiments, mTOR and Raptor are detected on the surface of intact lysosomes only from WT and not RagA/B KO HEK293FT cells. This means that mTORC1 does not localise on the lysosomes in cells deficient of the RagA/B, even when amino acids are available.

To answer the question whether the same phenotype is observed in the absence of RagC or RagD as well, immunofluorescence experiments were performed also on those cells in the before-mentioned nutritional conditions. Indeed, mTOR shows diffused cytoplasmic localization also in RagC/D KO HEK293FT cells even in amino acid replete conditions (Fig. 2.1d). This agrees with the literature and shows that mTORC1 is recruited on the lysosomal surface only in the presence of functional Rag dimers in human cells.

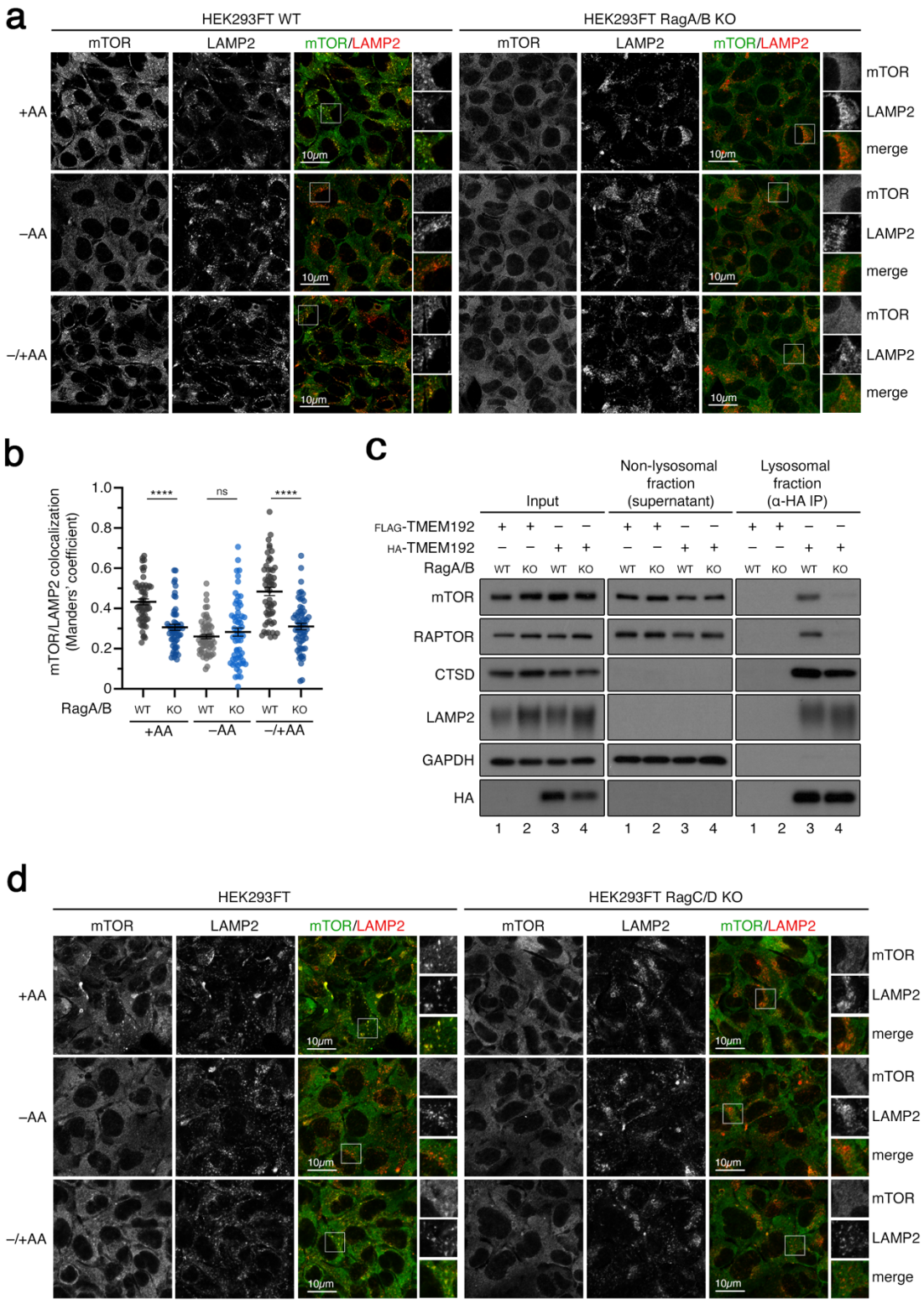

Figure 2.1 | mTORC1 is non-lysosomal in Rag KO HEK293FT cells

a. Colocalization analysis of mTOR with LAMP2 (lysosomal marker) in WT or RagA/B KO HEK293FT cells, treated as indicated in the figure, using confocal microscopy. Magnified insets shown to the right. Scale bars = 10 μm **b.** Quantification of colocalization (55-60 individual cells from 3-4 independent fields per condition). Data shown as mean ± SEM. **** $p<0.001$, ns: non-significant **c.** Lyso-IP experiments

in WT and RagA/B KO HEK293FT cells stably expressing HA-tagged TMEM192 (or FLAG-TMEM192 as negative control). Intact lysosomes were immunopurified by anti-HA IPs under native conditions, and the presence of LAMP2, cathepsin D (CTSD), mTOR and Raptor proteins in the lysosomal and non-lysosomal fractions, as well as in whole cell lysates, was analyzed by immunoblotting as indicated **d.** Colocalization analysis of mTOR with LAMP2 (lysosomal marker) in WT or RagC/D KO HEK293FT cells, treated as indicated in the figure, using confocal microscopy. Magnified insets shown to the right. Scale bars = 10 μm. Data shown are representative of 3 replicate experiments.

Previous studies used RagA/B KO MEFs to study the importance of the Rag GTPases. I used the same cells to explore mTOR localization in mouse cell lines. IF staining in MEFs confirmed that mTOR remains non-lysosomal under all nutritional conditions in RagA/B KO cells (Fig. 2.2a, b).

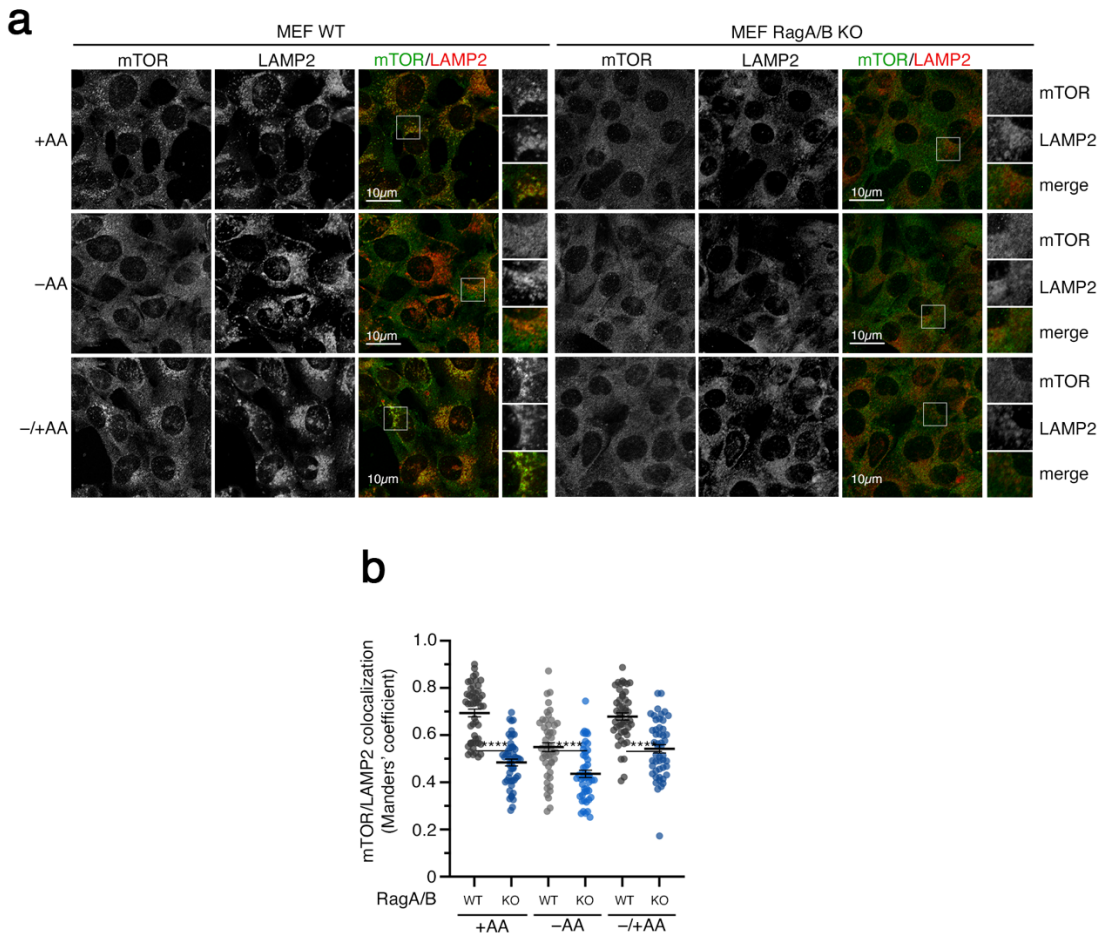

Figure 2.2 | mTORC1 is non-lysosomal in RagA/B KO MEFs

a. Colocalization analysis of mTOR with LAMP2 in WT or RagA/B KO MEF cells, treated as indicated in the figure, using confocal microscopy. Magnified insets shown to the right. Scale bars = 10 μm **b.** Quantification of colocalization (50 individual cells from 3 independent fields per condition). Data shown

as mean ± SEM. *** p<0.005, **** p<0.001, ns: non-significant. Data shown are representative of 3 replicate experiments.

2.2 mTORC1 phosphorylates S6K1 and 4EBP1 but not TFEB and TFE3 in RagA/B KO HEK293FT cells

The fact that mTORC1 is not lysosomal in the Rag KO cells is not idicative of its activity. To answer whether mTORC1 is active in cells lacking the Rag GTPases, I examined the phosphorylation of both its canonical and non-canonical substrates in all conditions mentioned above.

Surprisingly, western blot (WB) analysis in the HEK293FT showed that, in basal amino acid replete conditions, mTORC1 phosphorylates S6K1 and 4EBP1 in both WT and RagA/B KO cells, with phosphorylation of 4EBP1 in RagA/B KO cells being slightly reduced (Fig 2.3a). Upon starvation, the phosphorylation of those two substrates is abolished in WT cells, but there is still some leftover S6K1 phosphorylation in RagA/B KO cells. Upon re-stimulation, S6K1 and 4EBP1 phosphorylation levels are restored in WT but not in RagA/B KO cells. This means that although RagA/B seem to be dispensable for S6K1 and 4EBP1 phosphorylation by mTORC1 in unchallenged cells, they still play a role upon starvation and acute (30 min) restimulation with amino acids.

In contrast, TFEB and TFE3 phosphorylation by mTORC1 seems to be extremely affected by the absence of RagA/B, in line with the findings of previous studies. As shown in Fig. 2.3a, TFEB and TFE3 are not phosphorylated by mTORC1 under any conditions in the RagA/B KO cells, whereas the phosphorylation pattern follows that of S6K1 and 4EBP1 in WT cells in the different nutritional conditions.

To confirm that this phosphorylation is mTOR-dependent, we treated WT and RagA/B KO cells with Torin for 1h. As shown in Fig. 2.3b, Torin treatment abolished S6K1 phosphorylation in both cell lines, confirming that mTOR phosphorylates S6K1 also in the RagA/B KO cells.

To investigate whether mTORC2 activity is affected by the loss of the Rag GTPases, I examined the phosphorylation pattern of AKT, a well-characterized mTORC2 substrate, in basal, amino acid starvation and re-supplementation conditions. It is known already that mTORC2 does not respond to amino acid availability and my data confirm that

phosphorylation of AKT on S473 by mTORC2 is unaffected by the removal and re-addition of amino acids. Importantly, it is also unaffected by the loss of RagA/B (Fig. 2.3c).

As mentioned previously, the TSC complex, which also responds to amino acid availability, is an important upstream inhibitor of Rheb and consequently mTORC1. To investigate whether mTORC1 in the RagA/B KO cells is also regulated downstream of the TSC complex, I knocked down TSC2 and assessed S6K1 phosphorylation levels in WT and RagA/B KO cells. S6K1 phosphorylation was increased in both genotypes upon TSC2 knock down, which means that the TSC complex still regulates mTORC1 in the RagA/B KO cells (Fig. 2.3d). Moreover, TSC1 and TSC2 levels are similar between WT and RagA/B KO cells (Fig. 2.3e). This might explain the partial resistance to starvation in the Rag A/B KO cells, since the Rag GTPases are also responsible for the recruitment of TSC to the lysosomal surface.

Finally, overexpression of WT or active RagA (FLAG-RagA and FLAG-RagA Q66L respectively) induced TFEB phosphorylation by mTORC1 in the RagA/B KO HEK293FT (Fig. 2.3f). Overexpression of only RagA is enough to form dimers with RagC or RagD to recruit mTORC1 and TFEB on the lysosomal surface. This suggests that it is only the absence of the Rag GTPases and not a defect in mTOR function that abolishes TFEB and TFE3 phosphorylation in those cells.

In sum, these data show that in the absence of the Rag GTPases, mTORC1 is not lysosomal, but remains active towards its canonical substrates S6K1 and 4EBP1. Their phosphorylation is therefore largely Rag-independent. However, TFEB and TFE3 phosphorylation by mTORC1 is dependent on the presence of the Rag GTPases.

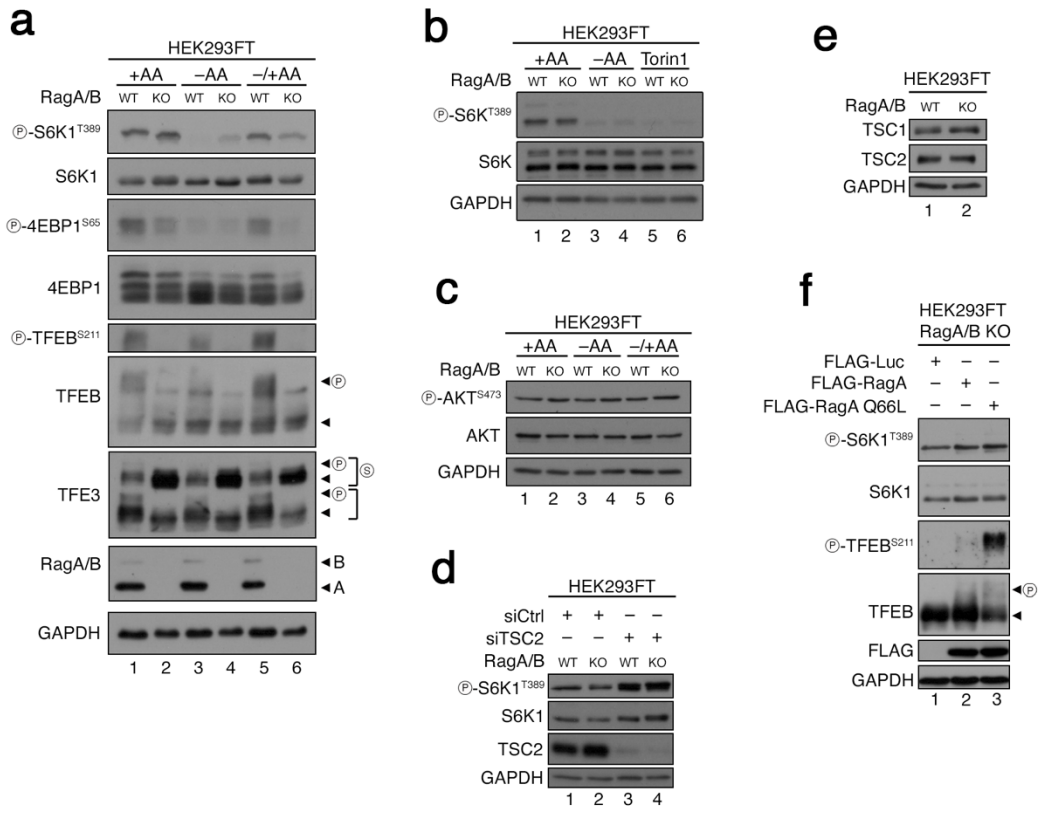

Figure 2.3 | mTORC1 phosphorylates S6K1 and 4EBP1 but not TFEB and TFE3 in RagA/B KO HEK293FT cells

a. Immunoblots with lysates from WT or RagA/B KO HEK293FT cells, treated with media containing or lacking AAs, in basal (+AA), starvation (–AA) or add-back (–/+AA) conditions, probed with the indicated antibodies **b.** Immunoblots with lysates from WT or RagA/B KO HEK293FT cells, treated with media containing (+AA) or lacking AA (–AA), or with Torin1, probed with the indicated antibodies **c.** Immunoblots with lysates from WT or RagA/B HEK293FT KO cells, treated with media containing or lacking AA, in basal (+AA), starvation (–AA) or add-back (–/+AA) conditions, probed with the indicated antibodies **d.** Immunoblots with lysates from WT or RagA/B KO HEK293FT cells, transiently transfected with siRNAs targeting TSC2 or a control RNAi duplex (siCtrl), probed with the indicated antibodies **e.** Immunoblots with lysates from WT or RagA/B KO HEK293FT cells probed with the indicated antibodies **f.** Immunoblots with lysates from RagA/B KO HEK293FT cells transfected with overexpression vectors containing either FLAG-Luc, or FLAG-RagA WT or the active RagA GTP form, FLAG-RagA Q66L, probed with the indicated antibodies. Arrowheads indicate bands corresponding to different protein forms, when multiple bands are present. P: phosphorylated form; S: SUMOylated form. Data shown are representative of 3 replicate experiments.

2.3 mTORC1 phosphorylates S6K1 and 4EBP1 but not TFEB and TFE3 in RagC/D KO HEK293FT and RagA/B KO MEF cells

As seen in Fig. 2.1 and 2.2, mTOR is not lysosomal in the RagC/D KO HEK293FT or the RagA/B KO MEFs. To examine whether the signalling patterns observed in the RagA/B KO HEK293FT are a general effect of the loss of the Rag GTPase, western blot analysis was also performed in the additional two cell lines. As expected by the importance of RagC and RagD for the recruitment of TFEB to the lysosomal surface and its phosphorylation by mTORC1, loss of RagC/D resulted in complete abolishment of TFEB phosphorylation under all nutritional conditions (Fig. 2.4a). On the contrary, S6K1 and 4EBP1 phosphorylation by mTORC1 is mildly affected by the loss of RagC/D in basal, amino acid replete conditions. However, similarly with what is observed in the RagA/B KO HEK293FT, the phosphorylation levels of S6K1 and 4EBP1 do not reach the basal levels upon re-stimulation with amino acids in the RagC/D KO cells.

RagA/B KO MEFs show a similar phenotype, with S6K1 phosphorylation upon starvation being even more resistant (Fig. 2.4b). TFEB and TFE3 are also not phosphorylated in those cells under any conditions. Unfortunately, the antibody that recognizes phosphorylated S211 on TFEB is human-specific, but conclusions about the phosphorylation pattern of TFEB can be also drawn by the migration pattern of the total protein (Fig. 2.4b).

These data show that the effect of Rag GTPase loss in TFEB and TFE3 regulation is not limited only in one model of Rag GTPase loss and non-lysosomal mTORC1 appears to be active towards S6K1 and 4EBP1 in different Rag loss-of-function human and mouse cell lines.

2.4 The regulation of mTORC1 substrates in RagA/B KO cells is connected to their localization

As mentioned previously, the recruitment of TFEB and TFE3 to the lysosomal surface is necessary for their phosphorylation by mTORC1, whereas this is not the case for the regulation of the canonical substrates S6K1 and 4EBP1. Indeed, lyso-IP experiments show that no S6K1 and 4EBP1 are detected on the lysosomal fraction of WT or RagA/B KO HEK293FT, whereas they are both present in the non-lysosomal fraction (Fig. 2.5a). TFEB is detected in the lysosomal fraction of the WT cells, as expected, but it is not present in the lysosomal fraction of the RagA/B KO HEK293FT. This goes hand in hand with the observation that TFEB is not phosphorylated in the RagA/B KO cells.

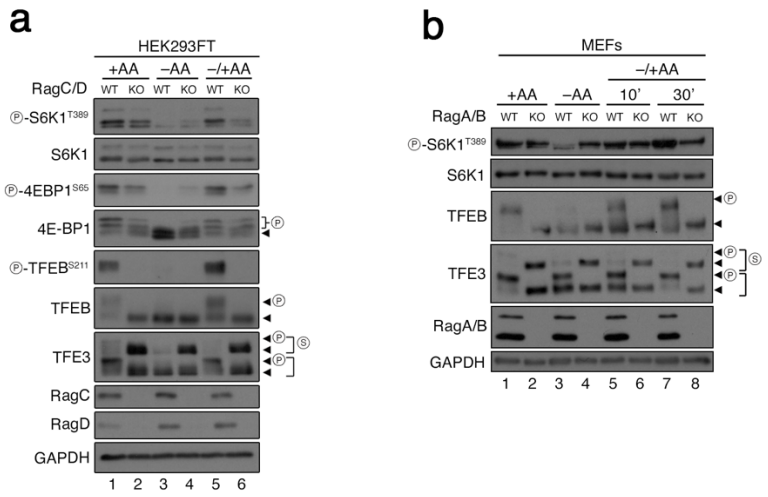

Figure 2.4 | mTORC1 phosphorylates S6K1 and 4EBP1 but not TFEB and TFE3 in RagC/D KO HEK293FT and RagA/B KO MEF cells

a. Immunoblots with lysates from WT or RagC/D KO HEK293FT cells, treated with media containing or lacking AA, in basal (+AA), starvation (–AA) or add-back (–/+AA) conditions, probed with the indicated antibodies **b.** As in **a.**, but with WT or RagA/B KO MEFs. Arrowheads indicate bands corresponding to different protein forms, when multiple bands are present. P: phosphorylated form; S: SUMOylated form. Data shown are representative of 3 replicate experiments.

So far, the details about the subcellular distribution of S6K1 remain unknown. There are two S6K1 isoforms, p70 S6K1 and p85 S6K1. It was previously reported that p70 S6K1 is mainly cytoplasmic, whereas p85 S6K1 contains a nuclear localization signal (Reinhard et al., 1992). Indeed, IF experiments with staining of endogenous S6K1 in HEK293FT cells showed diffused localization which covers the cytoplasm and the nucleus. S6K1 does not appear to accumulate on lysosomes or co-localise with the lysosomal marker LAMP2 and the signal distribution is similar between WT and RagA/B KO HEK293FT cells (Fig. 2.5b). The specificity of the S6K signal in IFs was confirmed by transient knockdown with small interfering RNA (siRNA) for S6K1 (Fig. 2.5c).

In sum, S6K1 and 4EBP1 regulation by mTORC1 is independent from its lysosomal localization or the Rag GTPases and their subcellular distribution is connected to this regulation.

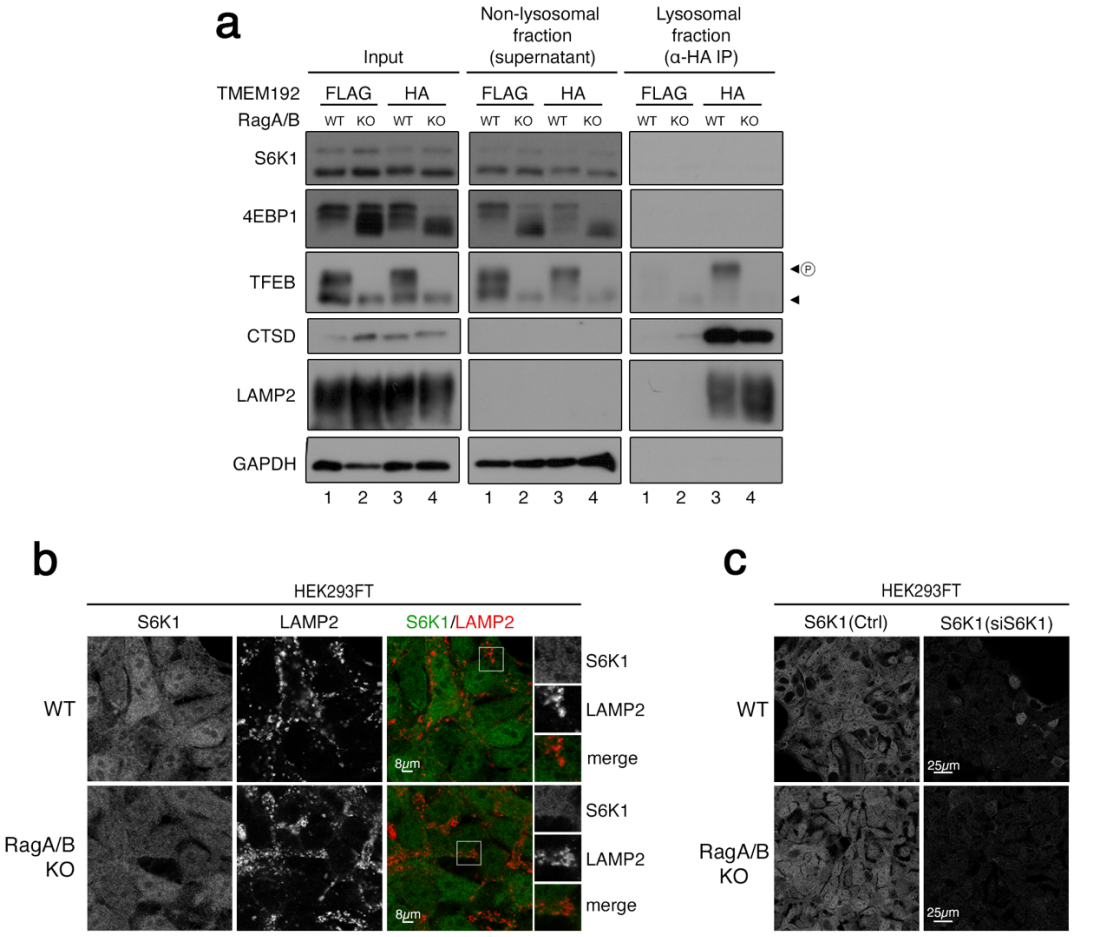

Figure 2.5 | The regulation of mTORC1 substrates in RagA/B KO cells is connected to their localization

a. Lyso-IP experiments in WT and RagA/B KO HEK293FT cells stably expressing HA-tagged TMEM192 (or FLAG-TMEM192 as negative control). Intact lysosomes were immunopurified by anti-HA IPs under native conditions, and the presence of LAMP2, cathepsin D (CTSD), S6K1, 4EBP1 and TFEB proteins in the lysosomal and non-lysosomal fractions, as well as in whole cell lysates, was analyzed by immunoblotting as indicated **b.** Colocalization analysis of S6K1 with LAMP2 in HEK293FT WT cells, using confocal microscopy. Magnified insets shown to the right. Scale bars = 8 μm **c.** S6K1 specificity analysis, using confocal microscopy. Cells were transiently transfected with siRNAs targeting S6K1 or a control RNAi duplex (siCtrl). Scale bars = 25 μm

2.5 TFEB and TFE3 are constitutively nuclear in the RagA/B KO cells

Regarding TFEB and TFE3 regulation, their phosphorylation by mTORC1 determines their subcellular localization. Therefore, I investigated their subcellular localization in RagA/B KO cells, where TFEB and TFE3 are never phosphorylated by mTORC1.

Even when mTORC1 is lysosomal and active, TFEB and TFE3 still shuttle between the lysosomal surface, the cytoplasm and the nucleus. As expected, TFEB and TFE3 show a diffused cellular localization in WT HEK293FT, covering part of the cytoplasm and the nucleus (Fig. 2.6a, c). However, in RagA/B KO HEK293FT, TFEB and TFE3 appear mainly nuclear (quantified in Fig. 2.6b, d). This agrees with the observation that mTORC1 does not phosphorylate TFEB and TFE3 in the absence of the Rag GTPases.

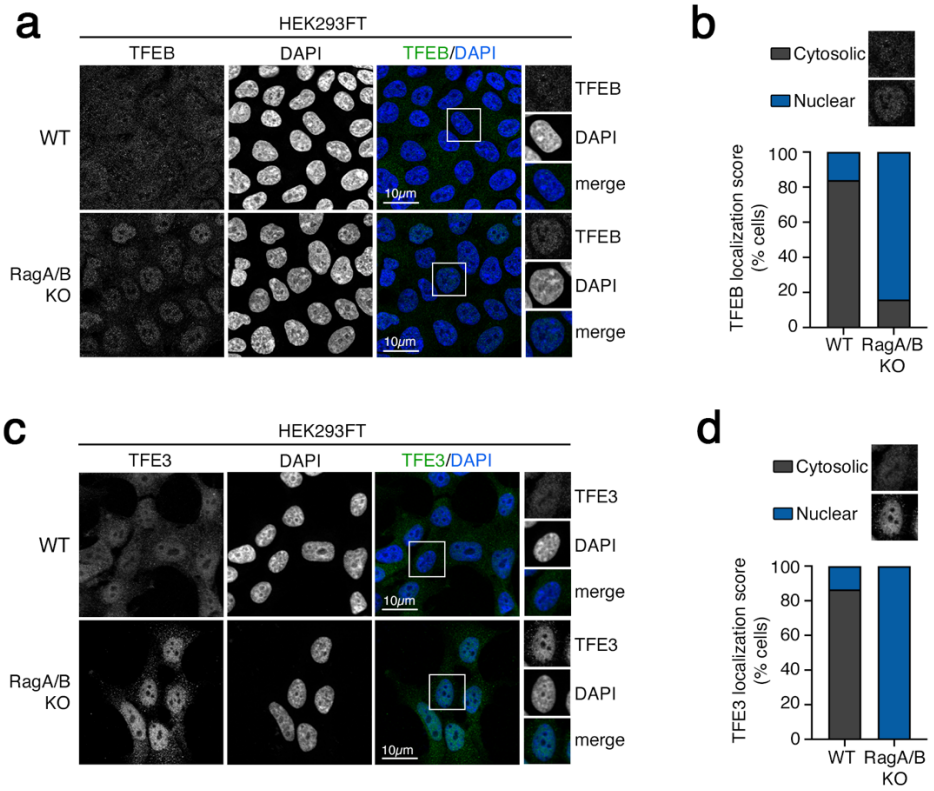

Figure 2.6 | TFEB and TFE3 are constitutively nuclear in the RagA/B KO cells

a. TFEB localization analysis in WT or RagA/B KO HEK293FT cells, using confocal microscopy. Nuclei stained with DAPI. Magnified insets shown to the right. Scale bars = 10 μm **b.** Scoring of TFEB localization. Individual cells were scored for nuclear or cytoplasmic TFEB localization as indicated in the example images (nWT = 65 cells, nABKO = 102 cells) **c.** As in **a.** but for TFE3 localization **d**. Scoring of TFE3 localization (nWT = 52 cells, nABKO = 52 cells). Data shown are representative of 3 replicate experiments.

2.6 LAMTOR1 KD has the same effect with Rag loss on mTOR localization and signalling

As mentioned in 1.2, the Rag GTPases are tethered to the lysosomal surface by the LAMTOR complex. The LAMTOR complex consists of 5 proteins, LAMTOR1, 2, 3, 4 and

5, with LAMTOR1 surrounding the other LAMTOR monomers and anchoring the complex to the lysosomal surface (Su et al., 2017).

In an effort to recapitulate the phenotype I observed in the loss of the Rag GTPases with other methods, I knocked-down LAMTOR1 in HEK293FT cells (Fig. 2.7a). Silencing of LAMTOR1 had the same effect with the loss of the Rag GTPases in those cells, with mTOR presenting a diffuse non-lysosomal localization in basal, amino acid replete conditions (Fig. 2.7b, c). Moreover, upon LAMTOR1 KD, S6K1 and 4EBP1 phosphorylation by mTORC1 is not affected, whereas TFEB is not phosphorylated by mTORC1 even when amino acids are present (Fig. 2.7d).

This confirms the hypothesis that mTORC1 can be active away from lysosomes in conditions where amino acids are available. Furthermore, it is again indicated that mTORC1 lysosomal localization is absolutely necessary only for the regulation of its lysosomal substrates, like TFEB.

2.7 Cells have basal lysosomal degradation even when amino acids are sufficient

The importance of mTORC1 localization to the lysosomal surface is connected to the regulation of catabolic processes when amino acids are scarce. However, all previous experiments presented here show that mTORC1 localises on the lysosomal surface in steady-state conditions where amino acids are available. Furthermore, here I demonstrate that in those conditions, mTORC1 can be active towards its key substrates for protein synthesis away from the lysosomal surface. Therefore, a question that rises is why mTORC1 localizes to the lysosomal surface in basal conditions in the first place.

As seen in Fig. 2.8a and reflected on the quantification in Fig. 2.8b, LC3B puncta appear after of BafA1 treatment in WT HEK293FT in basal, amino acid replete conditions. This suggests that autophagosome formation and trafficking takes place even in unchallenged cells, meaning that lysosomes continue their degrative activity even when amino acids are sufficient. Moreover, WB analysis shows that an LC3B-II band appears upon Baf1A treatment in those cells, confirming the IF data (Fig 2.8c). Finally, the same phenotype is observed also on WT MEFs upon BafA1 treatment, which means that this is a general and conserved phenomenon (Fig 2.8d, e).

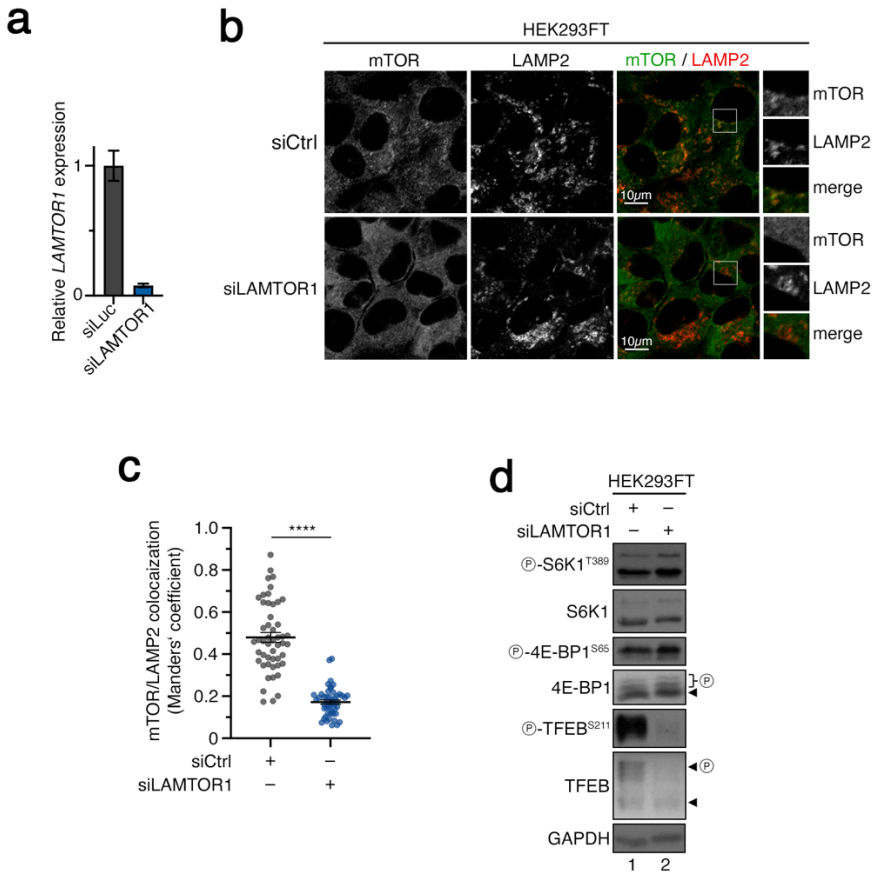

Figure 2.7 | LAMTOR1 KD has the same effect with Rag loss on mTOR localization and signalling

a. Expression analysis of LAMTOR1 confirms successful knockdown in HEK293FT cells. Data shown as mean ± SD **b.** Colocalization analysis of mTOR with LAMP2 in HEK293FT WT cells, using confocal microscopy. Cells were transiently transfected with siRNAs targeting LAMTOR1 or a control RNAi duplex (siCtrl). Magnified insets shown to the right. Scale bars = 10 μm **c.** Quantification of colocalization (50 individual cells from 3 independent fields per condition). Data shown as mean ± SEM. **** $p<0.001$ **d.** Immunoblots with lysates from HEK293FT WT cells, transiently transfected with siRNAs targeting LAMTOR1 or a control RNAi duplex (siCtrl), cultured under basal conditions, and probed with the indicated antibodies. Arrowheads indicate bands corresponding to different protein forms, when multiple bands are present. P: phosphorylated form. Data shown are representative of 3 replicate experiments.

These data suggest that mTORC1 potentially responds to amino acids released from the lysosomes even in unchallenged cells. This might explain why mTORC1 localises on the lysosomal surface to inhibit TFEB and TFE3.

2.8 Inhibition of the lysosomal v-ATPase has the same effect with the loss of the Rag GTPases

To confirm our hypothesis that amino acid release from the lysosomes signal to mTORC1 even in unchallenged cells, Baf1A treatment was used in IF experiments to assess mTOR localization. Inhibition of the lysosomal v-ATPase results in the alkalinization of the lysosomal lumen and therefore the inactivation of lysosomal enzymes that can function only in acidic environment (Yoshimori et al., 1991). Moreover, it inhibits release of amino acids from the lysosomal lumen.

Although in basal, amino acid replete conditions (+AA), mTOR co-localises with the lysosomal marker LAMP2 on the lysosomal surface, 6h Baf1A treatment in WT HEK293FT cells resulted to the de-localization of mTOR from the lysosomal surface (Fig. 2.9a, b). Moreover, mTOR does not localise on the lysosomes under any nutritional condition in cells treated BafA1, resembling the same phenotype that loss of the Rag GTPases has.

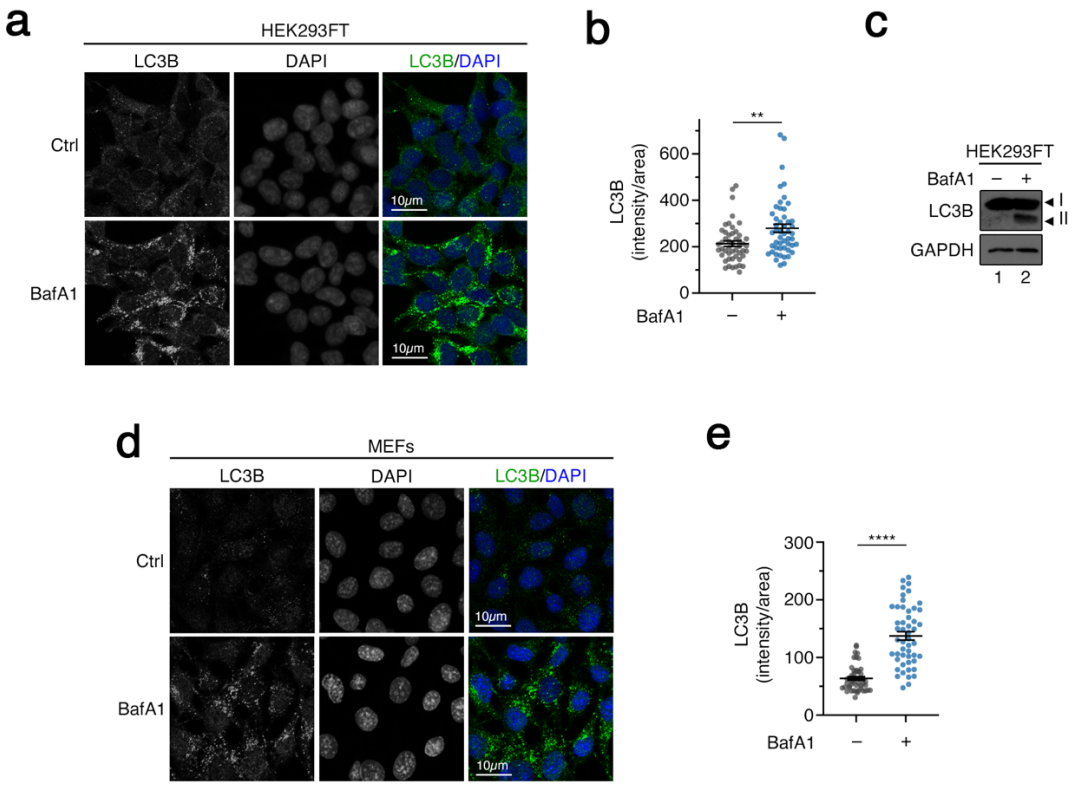

Figure 2.8 | Cells have basal lysosomal degradation even when amino acids are sufficient

a. Basal lysosomal degradation in HEK293FT cells indicated by accumulation of LC3B upon 6h BafA1 treatment **b.** Quantification of LC3B signal (50 individual cells from 5 independent fields per condition)

c. WB analysis of lysates from control or BafA1-treated HEK293FT WT cells, probed with antibody for LC3B. LC3B-II migrates faster than the non-lipidated form **d.** Basal autophagy in MEF cells indicated by accumulation of LC3B upon 6h BafA1 treatment **e.** Quantification of LC3B signal in the MEFs (48-50 individual cells from 5 independent fields per condition) Data shown in **b**, **e** as mean ± SEM. ** $p<0.01$, **** $p<0.001$, ns: non-significant. Data shown are representative of 3 replicate experiments.

In addition, WB analysis revealed that S6K1 and 4EBP1 are phosphorylated by mTORC1 in basal conditions in cells treated with BafA1 and this phosphorylation responds to the removal and re-addition of amino acids (Fig. 2.9c). However, in agreement with all previous observations, TFEB is not phosphorylated by mTORC1 under any nutritional condition.

BafA1 treatment dissociates the lysosomal from the non-lysosomal pool of mTORC1, without affecting directly the presence of the Rag GTPases. It is clear from these data that lysosomal mTORC1 is dependent on lysosomal function to regulate TFEB and TFE3, which are master regulators of lysosomal biogenesis.

2.9 Lysosomal protease inhibition has the same effect with Rag loss, without affecting RagA/B localization

BafA1 does inhibit the function of the enzymes inside the lysosomal lumen and the release of amino acids from the lysosomal lumen, but is not specific for protease inhibition. In order to specify whether the phenotype we observe is due to the inhibition of protein degradation, I used a combination of drugs which inhibit specifically the lysosomal proteases. These are Pepstatin A (PepA), an inhibitor of acid proteases (aspartyl peptidases) like Cathepsin D, and E64, a cysteine protease inhibitor. The combination of those two drugs inhibits sufficiently lysosomal protein degradation.

After 16h of treatment with the two inhibitors, mTOR does not localise on the lysosomal surface in HEK293FT cells, resembling the effect of BafA1 (Fig. 2.10a, b). Moreover, non-lysosomal mTORC1 can phosphorylate S6K1 and 4EBP1 in response to amino acids, but does not phosphorylate TFEB in any nutritional condition in HEK293FT cells treated with the inhibitors (Fig. 2.10c). This suggests that indeed, amino acid release from the lysosomes is necessary for mTORC1 to be recruited to the lysosomal surface and phosphorylate TFEB.

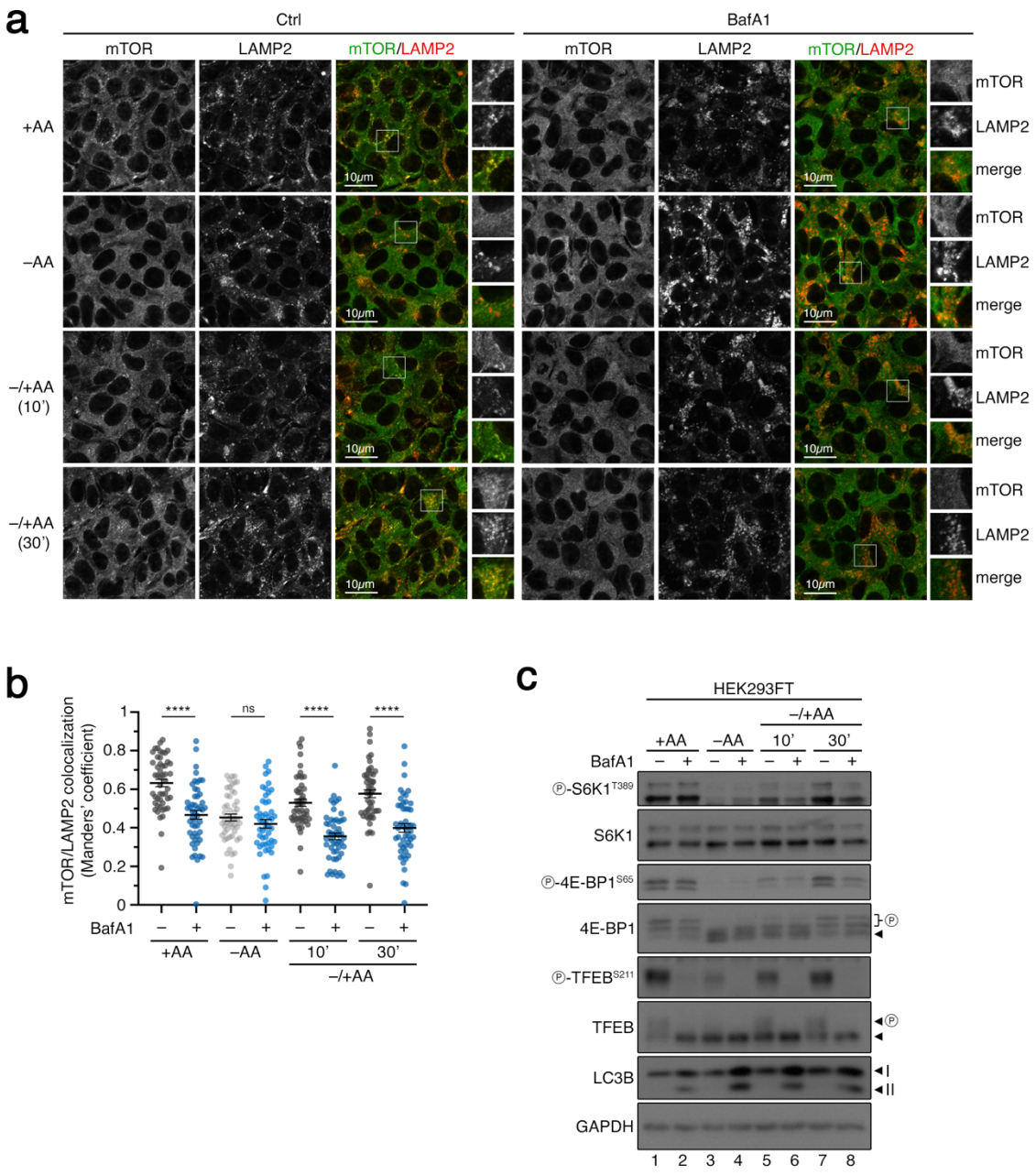

Figure 2.9 | Inhibition of the lysosomal v-ATPase has the same effect with the loss of the Rag GTPases

a. Colocalization analysis of mTOR with LAMP2 in HEK293FT WT treated as indicated in the figure, using confocal microscopy. Magnified insets shown to the right. Scale bars = 10 μm **b.** Quantification of colocalization (50 individual cells from 5 independent fields per condition). Data shown as mean ± SEM. ** p<0.01, **** p<0.001, ns: non-significant **c.** Immunoblots with lysates from HEK293FT WT cells, treated with media containing or lacking AAs, in basal (+AA), starvation (–AA) or add-back (–/+AA) conditions, and BafA1 as shown, probed with the indicated antibodies. Arrowheads indicate bands corresponding to different protein forms, when multiple bands are present. P: phosphorylated form. Data shown are representative of 3 replicate experiments.

Both in the case of Rag KO and LAMTOR KD, the Rag GTPases are not expected to localise on the lysosomal surface, which affects, apart from mTORC1 localization, the localization of TFEB and TFE3 as well. To examine whether the Rag GTPases remain lysosomal upon treatment with PepA + E64, I performed an IF experiment, staining with an antibody that recognizes both RagA and RagB. RagA and RagB appear to be lysosomal in both control and treated cells, as they co-localise strongly with LAMP2 (Fig. 2.10d). This suggests that the treatment affects the degradative capacity of the lysosomes without directly affecting the localization of the Rag GTPases. Therefore, inhibiting the lysosomal function only towards protein degradation can de-localise mTOR and activate TFEB, without affecting the activity of the non-lysosomal pool of mTORC1.

2.10 Non-lysosomal mTORC1 is regulated by specific amino acids in the RagA/B KO HEK293FT cells

As mentioned in 1.2, previous studies have shown that mTORC1 is regulated on the lysosomal surface by specific amino acids. So far, the presence of arginine (R), leucine (L) and methionine (M) was shown to be important for mTORC1 activation on the lysosomal surface. To examine the effect that starvation of specific amino acids has on non-lysosomal mTORC1, I prepared cell culture media based on DMEM (see Methods) lacking specific groups of amino acids. The amino acid groups, based on their biochemical properties, were: HRK (histidine, arginine, lysine – positively charged amino acids), MLIGV (methionine, leucine, isoleucine, glycine, valine – non polar, aliphatic amino acids), STC (serine, threonine, cysteine – polar, uncharged amino acids) and WFY (tryptophan, phenylalanine, tyrosine – aromatic amino acids).

First, my control experiment in WT HEK293FT cells shows that, upon treatment with media lacking the groups of arginine, methionine and leucine (-HRK, -MLIGV), S6K1, 4EBP1 and TFEB phosphorylation is decreased (Fig. 2.11a, b). However, the starvation of those amino acids does not have the same effect on the RagA/B KO cells (Fig. 2.11c, d). In fact, starvation of the STC group has the strongest effect on mTORC1 activity towards S6K1 in the RagA/B KO cells, suggesting that one or more of these three amino acids are important for the regulation of non-lysosomal mTORC1.

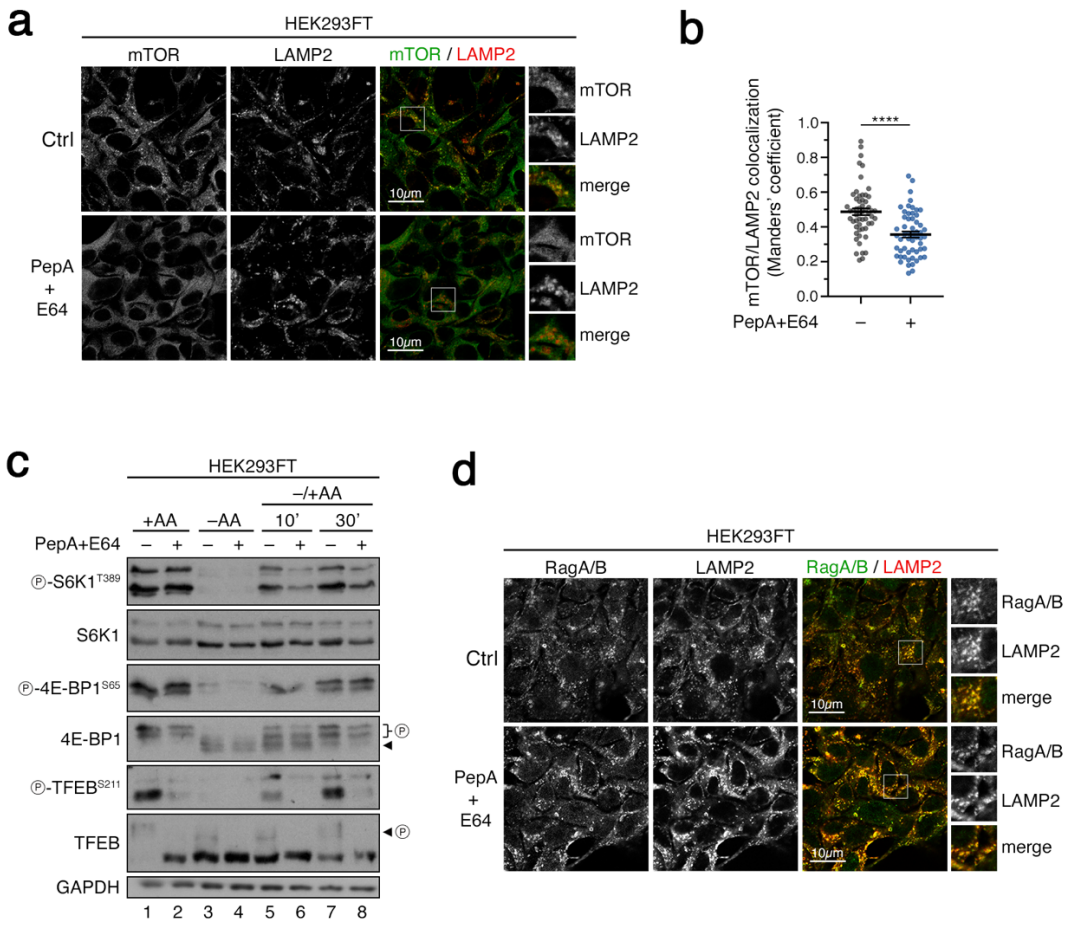

Figure 2.10 | Lysosomal protease inhibition has the same affect with Rag loss, without affecting RagA/B localization

a. Colocalization analysis of mTOR with LAMP2 in HEK293FT WT cells, treated as indicated in the figure, using confocal microscopy. Magnified insets shown to the right. Scale bars = 10 μm **b.** Quantification of colocalization (56 individual cells from 3 independent fields per condition). Data in shown as mean ± SEM. **** $p<0.001$. **c.** Immunoblots with lysates from HEK293FT WT cells, treated with media containing or lacking AA, in basal (+AA), starvation (–AA) or add-back (–/+AA) conditions, and protease inhibitors (PepA+E64) as shown, probed with the indicated antibodies. Arrowheads indicate bands corresponding to different protein forms, when multiple bands are present. P: phosphorylated form. **d.** Colocalization analysis of RagA/B with LAMP2 in HEK293FT WT cells, treated as indicated in the figure, using confocal microscopy. Magnified insets shown to the right. Scale bars = 10 μm. Data shown are representative of 3 independent experiments.

I next sought to identify which individual amino acids signal to non-lysosomal mTORC1 in the RagA/B KO cells. For this purpose, I prepared media lacking only single amino acids. Starvation of serine and threonine has the strongest impact on mTORC1 activity towards S6K1 (Fig. 2.11e, f). Cysteine appears also to be important for non-lysosomal mTORC1, but to a lesser extent.

These data shed light on the regulation of non-lysosomal mTORC1 by amino acids and open new directions regarding the identification of possible new sensors for the pathway.

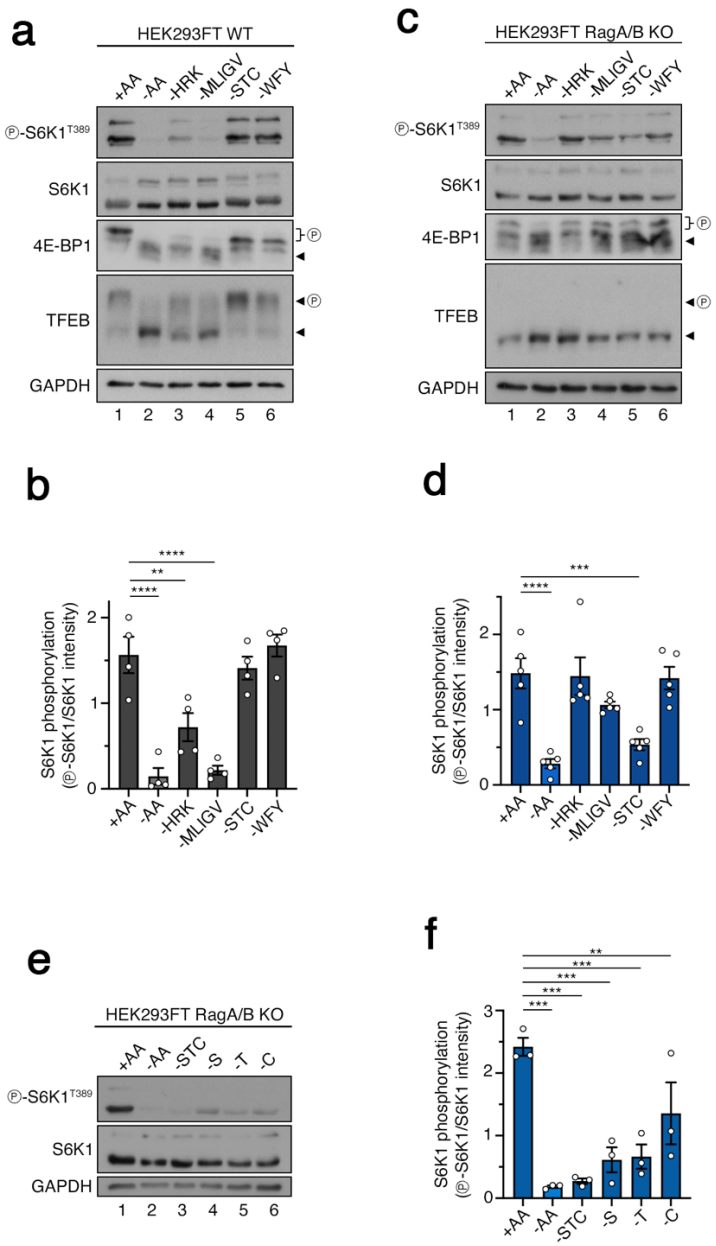

Figure 2.11 | Non-lysosomal mTORC1 is regulated by specific amino acids in the RagA/B KO HEK293FT cells

a. Immunoblots with lysates from WT HEK293FT cells, treated with media containing or lacking the AA groups shown in the figure, probed with the indicated antibodies **b.** Quantification of mTORC1 activity (p-S6K1^{T389}/S6K1). n = 4 **c.** As in **a.**, but for RagA/B KO HEK293FT cells **d.** Quantification of mTORC1 activity (p-S6K1^{T389}/S6K1). n = 5 **e.** Immunoblots with lysates from RagA/B KO HEK293FT cells, treated with media containing or lacking the AAs shown in the figure, probed with the indicated antibodies **f.** Quantification of mTORC1 activity (p-S6K1^{T389}/S6K1). n = 3. Arrowheads indicate bands corresponding to different protein forms, when multiple bands are present. P:

phosphorylated form. Data shown are representative of at least 3 replicate experiments. Data in **b.**, **d.**, **f.** shown as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.005, **** p<0.001, ns: non-significant.

2.11 Arf1 is not necessary for mTORC1 regulation in RagA/B KO cells

The fact that mTORC1 is not lysosomal, but remains active towards a group of substrates in the RagA/B KO cells suggests that other Rag-independent mechanisms of mTORC1 regulation exist. To investigate whether our phenotype is mediated by the small GTPase Arf1, which localises on the Golgi (see also 1.2), I knocked-down Arf1 in the RagA/B KO HEK293FT (Fig. 2.12a). Knockdown of Arf1 does not have an effect on S6K1 or 4EBP1 phosphorylation in the RagA/B KO cells (Fig. 2.12b). In fact, mTORC1 remains active towards these two substrates and their phosphorylation responds to amino acid starvation and re-supplementation, meaning that the underlying mechanism of mTORC1 regulation in in unchallenged cells is different. However, the response of S6K1 phosphorylation in the starvation and re-supplementation conditions is blunted upon Arf1 KD.

Moreover, treatment with Golgicide A (GA) and Brefeldin A (BFA), two inhibitors for Arf1 which cause Golgi fragmentation, had no effect on S6K1 phosphorylation by mTORC1 in WT or RagA/B KO HEK293FT (Fig. 2.12c – the effect of the two drugs is shown in Fig. 2.12d, where the Golgi appears fragmented in both WT and RagA/B KO cells).

2.12 *De novo* protein synthesis levels are not affected by the loss of the Rag GTPases

Since S6K1 and 4EBP1 phosphorylation is not affected in amino acid replete conditions in the Rag KO cells, I wondered whether this is reflected on their downstream functions. Therefore, I compared *de novo* protein synthesis levels in WT and RagA/B KO HEK293FT cells by performing an OPP assay and assessing the protein synthesis levels with IF imaging. This assay uses a puromycin analog, o-propargyl-puromycin (OPP), which is integrated at the newly synthesised peptide chains and causes premature chain termination during translation. Addition of a picolyl azide fluorophore leads to production of fluorescence signal (here green). It is important to notice that the subcellular localization of the puromycylated peptides does not reflect the location of their active translation. Ribosomal proteins, for instance, often contain a nuclear transport signal that sends even nascent peptide chains to the nucleus (Enam et al., 2020).

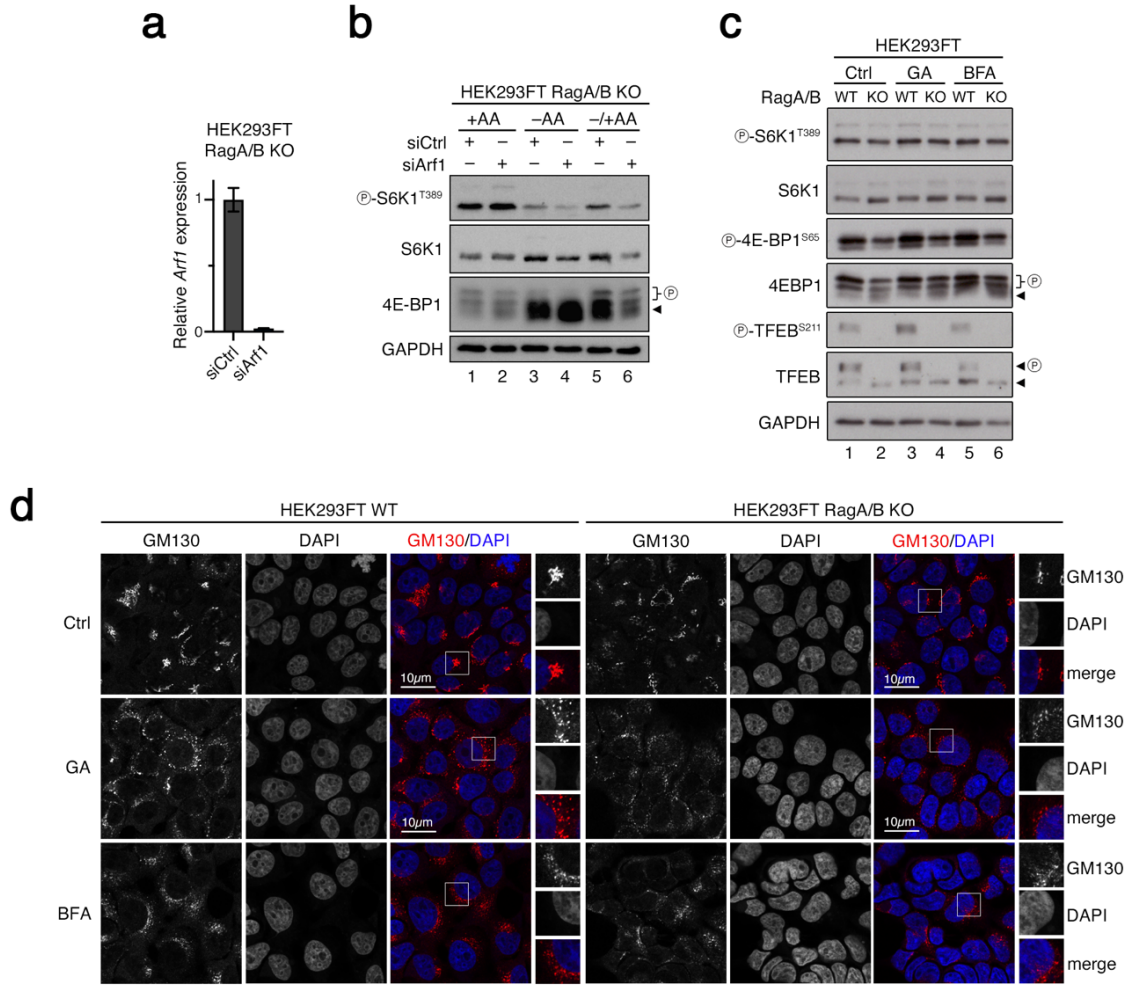

Figure 2.12 | Arf1 is not necessary for mTORC1 regulation in RagA/B KO cells

a. Expression analysis of Arf1 confirms successful knockdown in RagA/B KO HEK293FT cells. Data shown as mean ± SD **b**. Immunoblots with lysates from RagA/B KO HEK293FT cells, transiently transfected with siRNAs targeting Arf1 or a control RNAi duplex (siCtrl), and treated with media containing or lacking AA, in basal (+AA), starvation (–AA) or add-back (–/+AA) conditions, probed with the indicated antibodies **c**. Immunoblots with lysates from WT or RagA/B KO HEK293FT cells, treated with GA or BFA as shown in the figure, probed with the indicated antibodies. Arrowheads indicate bands corresponding to different protein forms, when multiple bands are present. P: phosphorylated form. **d**. Golgi morphology using GM130 (Golgi marker) in HEK293FT WT and RagA/B KO cells, using confocal microscopy. Cells treated with Golgicide A (GA) or Brefeldin A (BFA) as indicated. Magnified insets shown to the right. Scale bars = 10 μm. Data shown are representative of 3 replicate experiments.

As shown in Fig. 2.13a, the OPP signal in RagA/B KO cells is comparable to the that in WT cells. Treatment with Cycloheximide (CHX), an inhibitor of translational elongation, abolishes completely protein synthesis in both cell lines, proving the specificity of the OPP signal. This is reflected also on the quantification of multiple fields, as the differences between the two cell lines are not significantly different (Fig. 2.13b).

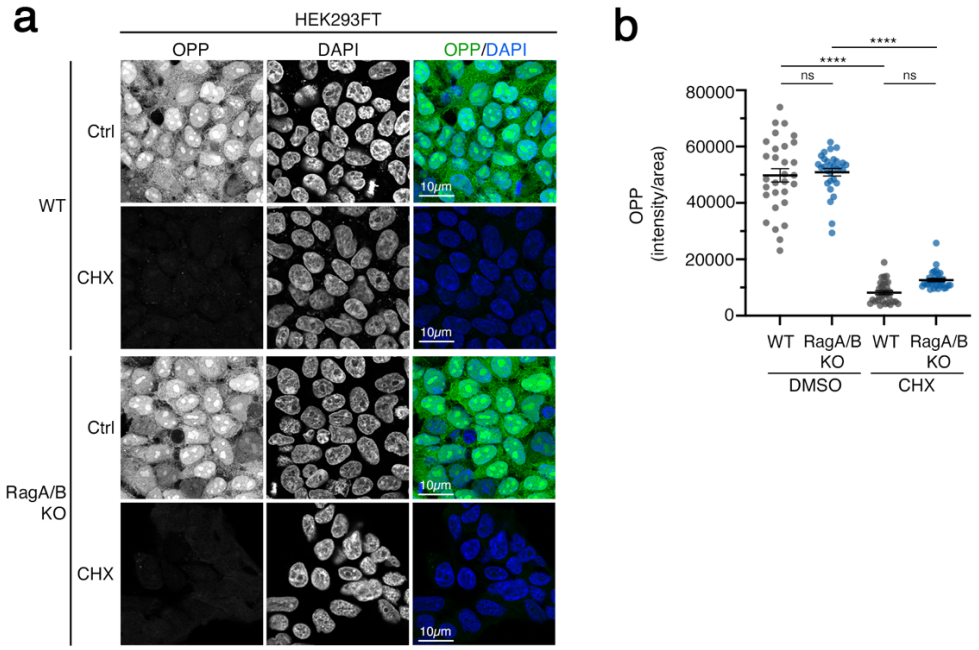

Figure 2.13 | *De novo* protein synthesis levels are not affected by the loss of the Rag GTPases
a. *De novo* protein synthesis (OPP incorporation) assay with WT or RagA/B KO HEK293FT cells. Nuclei stained with DAPI. Cycloheximide (CHX) inhibitor used as negative control. Scale bars = 10 μm **b.** Quantification of OPP signal (field intensity of 30 independent fields). Data shown are representative of 3 replicate experiments. Data shown as mean ± SEM. **** $p<0.001$, ns: non-significant.

2.13 Functions downstream of TFEB and TFE3 are regulated differentially between WT and RagA/B KO HEK293FT cells

Since TFEB and TFE3 are regulated differentially between the WT and RagA/B KO cells, I next looked into the effect of Rag loss on their downstream functions. As mentioned, TFEB and TFE3 are transcription factors. Therefore, I analysed the expression of genes, the transcription of which is regulated by the MiT-TFE transcription factors. As expected from the fact that TFEB and TFE3 are constituitively nuclear in the RagA/B KO cells, the transcriptional levels of all genes examined were significantly increased in the RagA/B KO HEK293FT compared to WT cells (Fig2.14a).

As mentioned in 1.3, the target genes of the MiT-TFE transcription factors code for proteins involved in lysosomal biogenesis or for lysosomal components. In this way, TFEB and TFE3 control degradation processes in the cell. To investigate whether the increased transcriptional levels of genes involved in lysosomal biogenesis result in higher number of lysosomes in the RagA/B KO cells, lysotracker staining was performed. Indeed, RagA/B KO HEK293FT cells have significantly increased number of lysosomes compared to the WT cells (Fig. 2.14b, c). Furthermore, LC3B staining revealed that autophagosome number is also increased in RagA/B KO cells, as more LC3B puncta are observed (Fig 2.14d, e).

These data suggest that differential mTORC1 localization is connected, though different groups of substrates, to differential regulation of its downstream functions. In unchallenged cells growing in amino acid replete conditions, mTORC1 phosphorylates S6K1 and 4EBP1 independently of the Rag GTPases and lysosomes to promote protein synthesis, whereas it phosphorylates TFEB and TFE3 in a Rag and lysosome-dependent manner to limit lysosomal biogenesis and catabolic processes in cells.

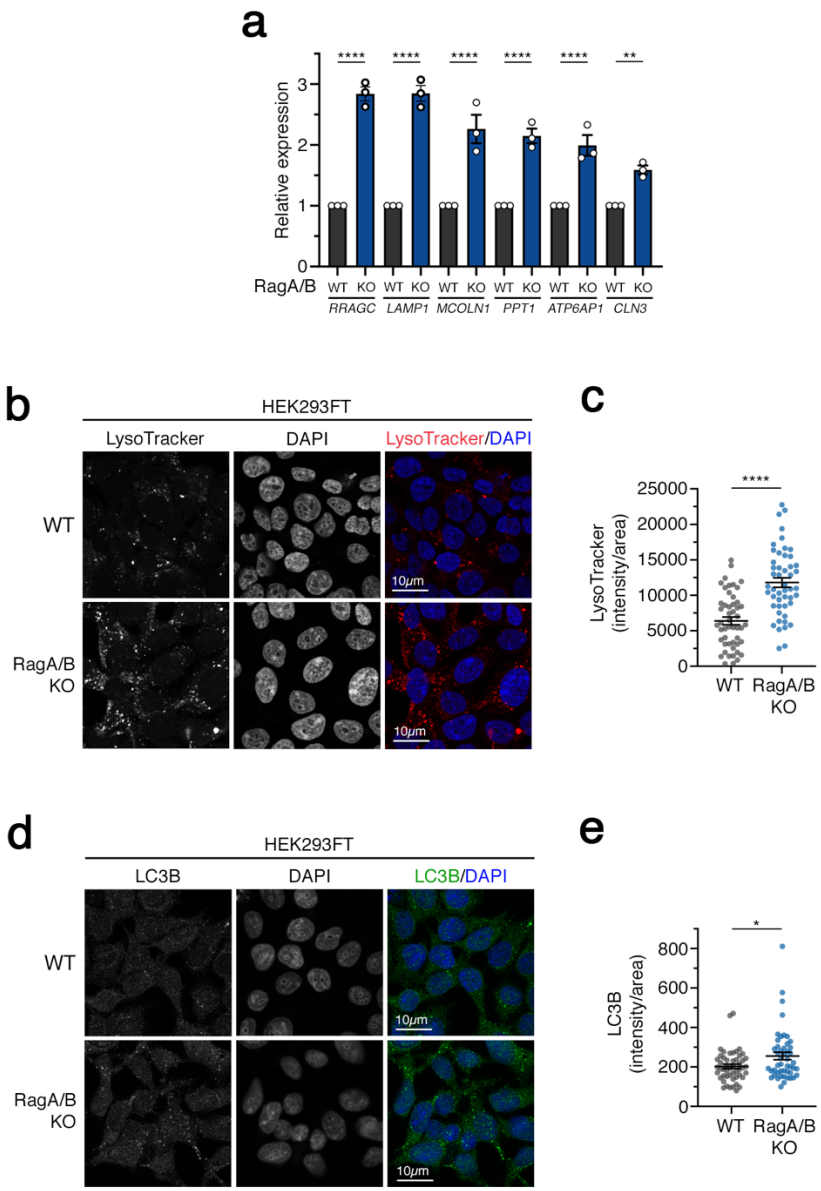

Figure 2.14 | Functions downstream of TFEB and TFE3 are regulated differentially between WT and RagA/B KO HEK293FT cells

a. Expression analysis of TFEB/TFE3 target genes in HEK293FT WT or RagA/B KO cells **b.** LysoTracker staining in HEK293FT WT or RagA/B KO cells. Nuclei stained with DAPI. Scale bars = 10 μm **c.** Quantification of LysoTracker signal (50 individual cells from 5 independent fields per condition) **d.** LC3B staining in HEK293FT WT or RagA/B KO cells. Nuclei stained with DAPI. Scale bars = 10 μm **e.** Quantification of LC3B signal (50 individual cells from 5 independent fields per condition). Data in **a.**, **c.**, **e.** shown as mean ± SEM. * $p<0.05$, ** $p<0.01$, **** $p<0.001$, ns: non-significant. Data shown are representative of 3 replicate experiments.

3. Discussion

3.1 Key findings of this study

Active mTORC1 promotes anabolic and inhibits catabolic processes in the cell through the phosphorylation of numerous substrates, to control the cellular metabolism in response to nutrients. Amino acids are the most robust signal for mTORC1 activation. In this work I focus on an unexplored pathway of mTORC1 regulation by amino acids, going beyond the well-described Rag GTPase- and lysosome-centric model for mTORC1 activation.

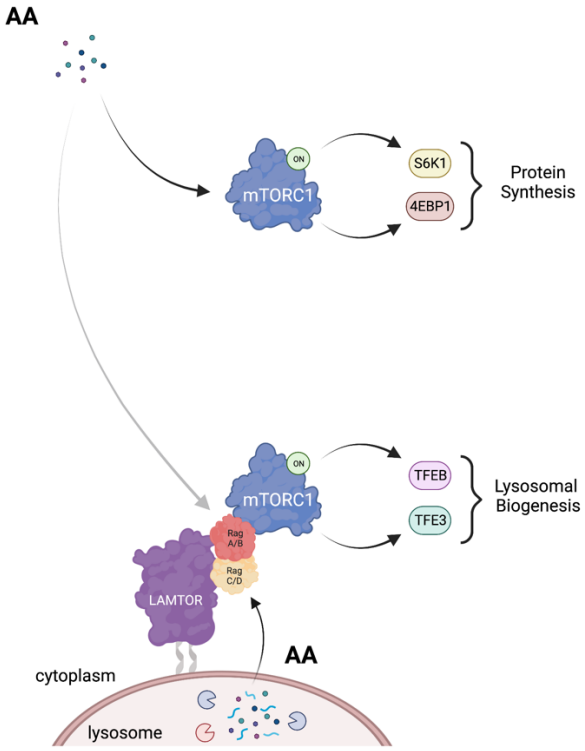

Figure 3.1 | Working model

mTORC1 is recruited to the lysosomal surface by the RagGTPases to control TFEB and TFE3 phosphorylation and regulate lysosomal biogenesis. Lysosomal mTORC1 activity depends mostly on amino acids coming from protein degradation in the lysosomal lumen. Extracellular amino acids can affect mTORC1 activity on lysosomes, but mTORC1 de-localises from the lysosomal surface when the lysosomal pool of amino acids is depleted, even when extracellular amino acids are abundant. mTORC1 phosphorylates S6K1 and 4EBP1 in response to extracellular amino acids in a Rag GTPase- and lysosome-independent manner to promote protein synthesis. Lysosomal amino acid depletion does not affect non-lysosomal mTORC1 activity. (Created with BioRender.com)

By using different biochemical and microscopy methods, Rag KO cell lines and specific drug treatments, I show that mTORC1 recruitment to the lysosomal surface is not necessary for its activation by amino acids and the phosphorylation for its best-characterized substrates

S6K1 and 4EBP1, which are responsible for anabolic processes, namely protein synthesis. However, de-localization of mTORC1 from the lysosomal surface prevents TFEB and TFE3 phosphorylation, leading to the activation of catabolic processes, namely lysosomal biogenesis, even when amino acids are available. This indicates that mTORC1 can be active towards specific substrates and inactive towards others simultaneously and demonstrates that mTORC1 regulation is more complicated than previously thought.

In addition, this study answers a pivotal question in the mTOR field: why mTORC1 is lysosomal in basal, amino acid replete conditions. Although lysosomal localization of mTORC1 has been discussed in the context of amino acid starvation and re-supplementation, to connect mTORC1 to its downstream catabolic functions through its localization on the catabolic organelle in the cell, it was still unclear why mTORC1 needs to be there even when exogenous amino acids are sufficient. With this study it became clear that lysosomes perform basal degradation even in nutrient rich conditions and the amino acids which are constantly released from the lysosomes signal to mTORC1.

3.2 Importance of the Rag GTPases

As mentioned previously, since their discovery, the Rag GTPases were considered to be crucial for mTORC1 regulation by amino acids. Indeed, we know that upon re-supplementation with amino acids, the Rag KO cells show a blunted response when it comes to S6K1 and 4EBP1 phosphorylation. Therefore, in the studies where mTORC1 activation was assessed in starvation and acute amino acid addback conditions (10-30 min), mTORC1 activity appears highly compromised in the absence of the Rag GTPases. Moreover, Rag KO mice die around embryonic day E10.5 (Efeyan et al., 2014). However, this is still a later time point compared to mTOR and RAPTOR KO, which die on E3.5 and E6.5 respectively (Gangloff et al., 2004; Murakami et al., 2004; Guertin et al., 2006). This suggests that although the Rags are indeed necessary for proper embryonic development, their loss does not equal complete loss of mTORC1 activity. Now we know that loss of the Rag GTPases could probably lead to (i) failure to inactivate mTORC1 via TSC, which leads to partial resistance to starvation when it comes to p-S6K1 and p-4EBP1 and (ii) constitutive activation and nuclear localization of TFEB and TFE3. The latter is connected to lysosomal storage diseases, meaning that their constant activation has severe effects on organismal homeostasis (Parenti et al., 2021).

Moreover, MEF cells extracted from RagA KO mice show persistent mTORC1 activity towards S6K1 and 4EBP1 and MEF cells extracted from double RagA/B KO mice have

only slightly reduced mTORC1 activity towards those substrates (Efeyan et al., 2014; Kim et al., 2014).

3.3 Previous work on the Rag-independent regulation of mTORC1 by AAs

The fact that mTORC1 is still active towards its canonical substrates in Rag KO cells suggests that additional, Rag-independent mechanisms for mTORC1 activation should exist in these cells. A series of studies over the past years sought to identify those mechanisms.

As mentioned previously, Arf1 has been reported to mediate mTORC1 activation by amino acids in RagA/B KO MEF and HEK293A cells (Jewell et al., 2015). In this study, stimulation of RagA/B KO cells by prolonged glutamine re-addition following amino acid starvation leads to re-activation of mTORC1 and phosphorylation of S6K1 and 4EBP1 after amino acid starvation. This does not happen with other amino acids, like leucine. Furthermore, this activation is described to be mediated through the Arf1 GTPase, since upon KD of Arf1, glutamine fails to induce activation of mTORC1 in the RagA/B KO cells. However, Arf1 is known to promote COPI-mediated vesicle budding, to regulate the formation of clathrin-coated vesicles at the trans-Golgi network and to stimulate the assembly of spectrin and the actin cytoskeleton on Golgi membranes (D'Souza-Schorey and Chavrier, 2006). These functions are going beyond mTORC1 signalling and silencing of Arf1 can affect cell physiology on multiple levels. Therefore, whether the role of Arf1 in glutamine sensing towards mTORC1 re-activation is direct is not clear, and it cannot be excluded that Arf1 affects mTORC1 also via other cellular functions that are unrelated to glutamine sensing.

Furthermore, a follow-up study from the same group suggested that asparagine can also activate mTORC1 in a Rag-independent manner via Arf1 (Meng et al., 2020). However, asparagine is not contained in the normal DMEM, which contains specifically the following 15 amino acids: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, tyrosine, cysteine, arginine, glutamine, glycine, and serine. In order to study asparagine effect on mTORC1 activity, the authors maintained HEK293A cells for several weeks in DMEM supplemented with asparagine, before performing amino acid withdrawal and re-supplementation experiments. Moreover, they starve the RagA/B KO cells of amino acids for 2-3h and then they stimulate them with individual amino acids in a 5x concentration compared to what is found it the normal DMEM recipe.

In the current study I starved both WT and RagA/B KO HEK293FT cells of specific groups of amino acids for 1h only, to assess their importance for mTORC1 activation. It is

important to mention that starvation of these amino acids had an effect on mTORC1 activity even though glutamine was still present in the medium. This suggests that additional amino acids besides glutamine are important for mTORC1 activation towards S6K1 and 4EBP1 in RagA/B KO cells.

Finally, knocking down Arf1 in our RagA/B KO model does not have a strong effect in mTORC1 activity as assessed by the canonical substrates. Furthermore, treatment with the Arf inhibitor BFA does not have an effect on S6K1 phosphorylation in basal conditions either, neither in the WT or in the RagA/B KO HEK293FT. This means that mTORC1 activity in basal, amino acid replete conditions is not mediated by Arf1 in the RagA/B KO HEK293FT cells. The partially blunted response to starvation and re-supplementation upon Arf1 KD in the RagA/B KO cells could be due to the other functions Arf1 mediates in the cells.

A different study suggested that PLD-derived PA is required for glutamine to activate mTORC1 in the absence of RagA/B (Bernfeld et al., 2018). This regulation takes place downstream of Arf1. Supplementation with PA can induce S6K phosphorylation even upon 24h of glutamine starvation in both WT and RagA/B KO HEK293A, suggesting a Rag-independent underlying mechanism. Moreover, a follow-up study from the same lab supports that exogenously supplied PA vesicles can drive lysosomal localization of mTORC1 even in the absence of amino acids, the Rag GTPases, growth factors and Rheb (Frias et al., 2020). However, phosphatidic acid is necessary for the complex assembly of both mTORC1 and mTORC2 (Toschi et al., 2009). This means that any intervention on PA production can have severe effects on mTORC1 signalling, which are not related to its other regulators but to the general function of the complex.

3.5 Regulation of non-lysosomal mTORC1 by specific amino acids

The current work supports the existence of a non-lysosomal pool of mTORC1 which is active in basal conditions inside the cell and responds to amino acid starvation and re-supplementation. According to my data, mTORC1 is never lysosomal on the RagA/B KO cells. This means that external amino acids signal and regulate specifically the non-lysosomal pool of mTORC1 in the RagA/B KO cells.

A number of studies focused on the regulation of mTORC1 by glutamine in the RagA/B KO cells. However, glutamine appears to be important due to its general role in cellular metabolism. Although a non-essential amino acid, glutamine is considered to be

conditionally essential, as it is an important source of carbon and/or nitrogen for protein, lipid, and nucleotide biosynthesis. This applies at a higher extend to cancer cells. Therefore, the fact the glutamine starvation affects mTORC1 activity towards S6K1 in the RagA/B KO cells is not necessarily because of direct signalling of glutamine to mTORC1. Moreover, in the current study I show that starvation of other individual amino acids for 1h has a strong effect on non-lysosomal mTORC1 activity, even when glutamine is included in the cell culture medium.

One of the amino acids having the most robust effect on non-lysosomal mTORC1 activity according to my data is threonine. The mitochondrial threonyl-tRNA synthetase (TARS2) was described to activate mTORC1 in response to increased threonine levels (Kim et al., 2021). TARS2 was found to interact with the LAMTOR complex and the Rag GTPases in HEK293T cells. Moreover, mTORC1 activity, as assessed by the canonical substrates, was reduced upon threonine starvation and further reduced when knocking down TARS2. Furthermore, mTORC1 recruitment to the lysosomal surface was abolished upon TARS2 silencing, which led the authors to the conclusion that TARS2 is necessary for the threonine-mediated activation and lysosomal recruitment of mTORC1. Finally, they claim that TARS2 can sense the cytoplasmic free threonine, but not threonine coming from protein degradation in lysosomes. Although this mechanism appears to be Rag-dependent, the threonine starvation in the study by Kim et al. has an effect on mTORC1 activity after 6-12 hours, whereas my starvation experiments take place in 1h. This means that cytoplasmic mTORC1 in the RagA/B KO cells responds much faster to threonine starvation, which suggests the existence of an additional sensor for threonine.

So far, no serine sensor for the mTORC1 pathway is identified. This could be due to the fact that most researchers focus on Rag-dependent mechanisms to build the regulatory network around mTORC1. The possibility that a specific sensor does exist, but functions independently of the Rag GTPases and the lysosomal machinery is further strengthened by my experiments, where serine starvation has such a strong effect on mTORC1 activity in the RagA/B KO cells.

3.4 Dissociation of lysosomal and non-lysosomal substrate regulation

In the current study I show that mTORC1 regulates differentially its substrates depending on their location and the source of amino acids. mTORC1 regulates S6K1 and 4EBP1 in response to mainly extracellular amino acids in a lysosome-independent manner, whereas it regulates TFEB and TFE3 on the lysosomal surface and mainly in response to amino acids

coming from the lysosomal lumen. A series of previous studies implied that there is differential regulation of the lysosomal and non-lysosomal substrates of mTORC1, but they did not investigate where this regulation takes place.

A recent study dissociated the two groups of mTORC1 substrates by showing that S6K1 and 4EBP1, but not TFEB phosphorylation is sensitive to Rheb activity induced by growth factor signalling (Napolitano et al., 2020). TFEB phosphorylation requires instead the activity of FLCN-RagC/D, in line with a previous study (Lawrence et al., 2019). Moreover, it is shown that S6K1 and 4EBP1 phosphorylation is independent from the Rag GTPases, but the authors suggest that mTOR regulates the phosphorylation of these substrates from the lysosomal surface. Although part of their results is confirmed by my work, my data make clear that mTORC1 localization to the lysosomal surface is not necessary for its activity towards specific substrates.

A different study showed that, when expressing variants of *VPS41* or deleting subunits of the HOPS complex (see also 1.4), mTORC1 is not lysosomal, TFEB and TFE3 phosphorylation is abolished, autophagy is constantly active, but phosphorylation of S6K1, 4EBP1 and ULK1 by mTORC1 remains unaffected (van der Welle et al., 2021). The authors mention that "it is tempting to speculate that impaired endocytic cargo delivery deprives lysosomes of nutrients, thereby causing a state of starvation". The authors could not explain why S6K1 and 4EBP1 are still phosphorylated. However, this observation is similar with what I observe in this work. Indeed, inhibition of amino acid release from the lysosomes does not affect S6K1 and 4EBP1, although it affects lysosomal mTORC1 in a way similar to external amino acid starvation.

Finally, in $TSC^{-/-}$ cells it is shown that, although mTORC1 is hyperactive towards S6K1 and 4EBP1 as expected, it does not phosphorylate TFEB, which localises always in the nucleous and induces lysosomal biogenesis (Alesi et al., 2021). This is particularly relevant for the Tuberous Sclerosis Complex disease, as the dissociation of the substrate regulation can lead to the development of tailored therapeutic treatments.

3.5 Activation of mTORC1 at the lysosome vs. non-lysosomal sites

Although there is extensive knowledge about the activation of mTORC1 by amino acids to the lysosomal surface, little is known about mTORC1 in other subcellular locations. Although mTORC1 lysosomal localization has been connected to its activation by the lysosome-localised Rheb, Rheb localization expands to other intracellular membranes as

well. Interestingly, a large fraction of Rheb appears to localise on the Golgi and is able to activate mTORC1 on the Golgi-lysosome contact sites in MEF cells (Hao et al., 2018). Therefore, the possibility that mTORC1 is activated on the Golgi surface by Rheb cannot be excluded. Moreover, a different study argued that Rheb localization on the lysosomal surface is not always necessary for mTORC1 activation, Rheb can localise on the Golgi or the ER and even weak membrane interactions can lead to mTORC1 activation by Rheb in HeLa cells (Angarola and Ferguson, 2019). This is one more piece of evidence that mTORC1 can be activated in multiple different cellular locations apart from lysosomes.

Two recent studies showed that mTORC1 phosphorylates substrates on the Golgi in normal growth conditions. mTORC1 was shown to directly phosphorylate GRASP55, a Golgi-localised protein, to regulate its localization and downstream function in UPS (Nüchel et al., 2021). Cellular stress or pharmacological inhibition of mTORC1 in WI-26 cells causes GRASP55 translocation from the Golgi to autophagosomes and multivesicular bodies, to initiate UPS. Phosphorylation of GRASP55 by mTORC1 responds to protein starvation, like the phosphorylation of S6K1 and 4EBP1. Moreover, mTOR is shown to localize with GRASP55 on the Golgi, confirming that mTORC1 is possibly active not just on the cytoplasm, but on the surface of other cellular organelles too. Furthermore, a second study revealed a different mTORC1 substrate on the Golgi surface. The N-terminal kinase-like protein SCYL1 was found to be directly phosphorylated by mTORC1 on the Golgi, to control organelle distribution and extracellular vesicle secretion in MCF-7 breast cancer cells (Kaeser-Pebernard et al., 2022). SCYL1 was also shown to be rapidly dephosphorylated upon amino acid starvation, confirming the fact that mTORC1 can respond to amino acids in association with the Golgi.

Finally, a recent study provided evidence that mTORC1 localises at membrane ruffles together with Rheb, where it responds to amino acid starvation and re-supplementation (Makhoul et al., 2023). The regulation of mTORC1 at the membrane ruffles is mediated by Arf5, a small GTPase localised on the Golgi, as well as the membrane ruffles. This protein appears in general to be important for the rapid response of mTORC1 to exogenous amino acids.

To sum up, several studies over the past few years separate mTORC1 activation by amino acids from its lysosomal localization. Moreover, it becomes clear that the lysosomal localization and the Rag GTPases are responsible only for a small fragment of the broad

spectrum of functions downstream of mTORC1. This change of the consensus will shed light to ignored parts of the mTORC1 pathway and reveal unknown players.

3.6 Physiological relevance

Although it is intuitive for mTORC1 to control TFEB and TFE3 on the lysosomal surface, since TFEB and TFE3 themselves control lysosomal biogenesis, no clear connection was made between protein degradation in lysosomes and mTORC1 activity. With this work I show that even in basal, amino acid replete conditions, inhibition of lysosomal degradation causes the de-localization of mTORC1 from the lysosome and the abolishment of TFEB and TFE3 phosphorylation. Since mTORC1 dysregulation is connected to the development of cancer, neurodegenerative and cardiovascular disease, as well as to ageing, this knowledge could give a better insight of the molecular mechanisms behind disease and lead to the development of a better tailored therapeutic treatment.

mTORC1 is hyperactive in up to 80% of human cancers (Menon and Manning, 2008) and amino acid signalling to mTORC1 is connected to cell growth in several cancer types. Therefore, it is important to decipher the molecular mechanisms behind individual amino acid sensing by mTORC1. Several studies show that individual amino acids can influence the progression of specific cancer types. For instance, leucine deprivation appears to cause caspase-dependent apoptotic death of melanoma cells (Sheen et al., 2011). Furthermore, acute lymphoblastic leukemia cells are not able to synthesize asparagine, therefore treatment with L-asparaginase to lower the levels of circulating asparagine is used as a therapeutic strategy (Avramis, 2012). The knowledge that the pool of mTORC1 responsible for protein synthesis responds potentially to additional groups of amino acids creates new perspectives for this research.

TFEB/TFE3 localization appears to be important in the context of several diseases. The Birt–Hogg–Dubé syndrome is characterised by mutations on the FLCN gene and TFEB nuclear localization. Deletion of TFEB in FLCN KO background mice rescued the kidney tumor phenotype observed in the disease (Napolitano et al., 2020). Moreover, TFEB appears to be "the key driver of renal tumorigenesis" in the Tuberous Sclerosis Complex disease (Alesi et al., 2021).

Interestingly, a recent study suggested that treatment of human lung carcinoma A549 and cervical cancer HeLa cells with drugs like BafA1 could mimic lysosomal storage disorders (Fedele and Proud, 2020). TFEB and TFE3 nuclear localization is a phenotype observed in

some LSDs (Sardiello et al., 2009). On the other hand, overexpression and constant nuclear localization of TFEB in Pompe disease models, where glycogen accumulates to the lysosomes due to a deficiency of glycogen-degrading lysosomal enzyme, leads to lysosome clearance and rescue of the pathological phenotype (Spampanato et al., 2013). To date, the therapeutic strategy for this disease is treatment with the missing enzyme, but its delivery to the lysosomes is not always effective.

Finally, this work could potentially lead to better understanding of the mechanisms behind ageing. It is already well-described how branched chained amino acids (BCCA), leucine, isoleucine and valine, which signal to mTORC1 in a Rag-dependent manner, contribute to the ageing phenotype. However, two of the hallmarks of ageing, which currently get more attention in the field, are loss of proteostasis and disabled macroautophagy (López-Otín et al., 2023). TFEB plays a critical role in both functions, as a master regulator of lysosomal biogenesis. Impairment of TFEB regulation in all levels can lead to cell senescence. For instance, it was shown that in older naïve $CD4^+$ T cells, failure of re-activation of FOXO1, which induces TFEB transcription, causes impaired lysosomal proteolytic activity (Jin et al., 2020).

Furthermore, TFEB appears to play an important role in age-induced neurological disorders. TFEB expression declines in aged brains and its overexpression leads to alleviation of senescence markers and memory loss in mice (Wang et al., 2021). In addition, in regard to Alzheimer's disease, which is linked to the ageing brain, TFEB overexpression in primary astrocytes improves tau uptake and lysosomal activity *in vitro* (Martini-Stoica et al., 2018). Moreover, it reduces pathology in the hippocampus of tauopathy model mice and decreases tau spreading in the hippocampus of a tau spreading model mouse. Taken together, these data highlight the importance of TFEB in age-related diseases and make crucial the delineation of the molecular mechanisms behind TFEB regulation.

3.6 Open questions

Over the years, extended research around the regulation of the Rag GTPases lead to the discovery of the proteins that built the complex lysosomal machinery network I described in 1.2. Although it is possible that more proteins regulating lysosomal mTORC1 will be discovered in the future, the first aim should be the identification of amino acid sensors that signal to non-lysosomal mTORC1. Even though it is clear that mTORC1 localises at a wide range of membranes in the cell, the previous focus on the lysosome-centered mTORC1 regulation did not allow extended research for amino acid sensors in other locations. Small

steps are made towards this direction, but a whole new world is yet to be discovered. As mentioned, Arf5 seems to be important for mTORC1 regulation on the membrane raffles, but it remains unclear how it senses amino acid availability (Makhoul et al., 2023). Moreover, in this work I show that non-lysosomal mTORC1 is regulated by specific amino acids, namely serine and threonine, but no sensors for those are described. Moving the focus to non-lysosomal mTORC1 will reveal a new machinery for mTORC1 regulation, which might prove relevant for disease too.

Despite the fact that TFEB and TFE3 were characterized early as mTORC1 substrates, most studies assess mTORC1 activity by investigating only S6K1 and 4EBP1 phosphorylation. However, mTORC1 phosphorylates a large number of additional proteins to regulate different functions (Battaglioni et al., 2022). Therefore, it is important to highlight towards which substrates mTORC1 is active in different contexts. Moreover, and taking into consideration the recent advances in the field, it is certain that more substrates in different subcellular locations will be described in the future.

Amino acids enter the cells and exit the lysosomes through transporters on the plasma membrane and the lysosomal surface respectively. Although previous studies connected specific groups of amino acids to specific transporters in both locations, no lysosomal transporters are identified for instance for amino acids such as serine and threonine (Rudnik and Damme, 2021). Moreover, even though many transporters are described over the years, the way they regulate mTORC1 signalling on the lysosome or in other locations is still unclear.

To summarise, every new discovery in the mTOR field opens the door to more questions, as it enhances the complexity of the mTOR signalling network. Although the current work answers why mTORC1 localises on the lysosomes in basal conditions and how the different subcellular pools of mTORC1 regulate differentially lysosomal and non-lysosomal substrates, the identification of additional amino acid sensors, the characterization of the regulatory machinery and the downstream substrates of mTORC1 in non-lysosomal locations and the connection of mTORC1 regulation to the function of specific amino acid transporters are questions which will occupy scientists in the future.

4. Materials & Methods

4.1 Cell culture

All cell lines were grown at 37 °C, 5% CO_2. Human female embryonic kidney HEK293FT cells (#R70007, Invitrogen; RRID: CVCL_6911) and immortalized mouse embryonic fibroblasts (MEFs) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (#41965-039, Gibco), supplemented with 10% fetal bovine serum (FBS) (#F7524, Sigma; #S1810, Biowest). All media were supplemented with 1x Penicillin-Streptomycin (#15140-122, Gibco).

HEK293FT cells were purchased from Invitrogen. Wild-type control and RagA/B KO immortalized MEFs were a kind gift of Kun-Liang Guan (described in (Jewell et al., 2015). The identity of the HEK293FT cells was validated by the Multiplex human Cell Line Authentication test (Multiplexion GmbH), which uses a single nucleotide polymorphism (SNP) typing approach, and was performed as described at www.multiplexion.de. All cell lines were regularly tested for Mycoplasma contamination, using a PCR-based approach and were confirmed to be Mycoplasma-free.

4.2 Cell culture treatments

Amino acid (AA) starvation experiments were performed as described previously (Demetriades et al., 2014, 2016). In brief, custom-made starvation media were formulated according to the Gibco recipe for high-glucose DMEM (Table 4.1), specifically omitting either all amino acids or specific amino acid groups, as indicated in the figures. For vitamin supplementation, MEM vitamin solution in 1x concentration was added (#11120-037, Thermo Fisher Scientific). Other components of the custom media are listed on Tables 4.2 and 4.3. The media were filtered through a 0.22-μm filter device and tested for proper pH and osmolality before use. For the respective AA-replete (+AA) treatment media, commercially available high-glucose DMEM was used (#41965039, Thermo Fisher Scientific). All treatment media were supplemented with 10% dialyzed FBS (dFBS) and 1x Penicillin-Streptomycin (#15140-122, Gibco). For this purpose, FBS was dialyzed against 1x PBS through 3,500 MWCO dialysis tubing. For basal (+AA) conditions, the culture media were replaced with +AA treatment media 60-90 min before lysis or fixation. For amino-acid

starvation (-AA), culture media were replaced with starvation media for 1 hour. For AA add-back experiments, cells were first starved as described above and then starvation media were replaced with +AA treatment media for 30 min, unless otherwise indicated in the figures.

Components	Molecular Weight	Concentration (mg/L)	mM
Amino Acids			
Glycine	75.0	30.0	0.4
L-Arginine hydrochloride	211.0	84.0	0.39810428
L-Cystine 2HCl	313.0	63.0	0.20127796
L-Glutamine	146.0	584.0	4.0
L-Histidine hydrochloride-H_2O	210.0	42.0	0.2
L-Isoleucine	131.0	105.0	0.8015267
L-Leucine	131.0	105.0	0.8015267
L-Lysine hydrochloride	183.0	146.0	0.7978142
L-Methionine	149.0	30.0	0.20134228
L-Phenylalanine	165.0	66.0	0.4
L-Serine	105.0	42.0	0.4
L-Threonine	119.0	95.0	0.79831934
L-Tryptophan	204.0	16.0	0.078431375
L-Tyrosine disodium salt dihydrate	261.0	104.0	0.39846742
L-Valine	117.0	94.0	0.8034188
Vitamins			
Choline chloride	140.0	4.0	0.028571429
D-Calcium pantothenate	477.0	4.0	0.008385744
Folic Acid	441.0	4.0	0.009070295
Niacinamide	122.0	4.0	0.032786883
Pyridoxine hydrochloride	206.0	4.0	0.019417476
Riboflavin	376.0	0.4	0.0010638298
Thiamine hydrochloride	337.0	4.0	0.011869436
i-Inositol	180.0	7.2	0.04
Inorganic Salts			
Calcium Chloride ($CaCl_2$) (anhyd.)	111.0	200.0	18.018.018
Ferric Nitrate ($Fe(NO_3)_3$*9H2O)	404.0	0.1	2,48E+03
Magnesium Sulfate ($MgSO_4$) (anhyd.)	120.0	97.67	0.8139166
Potassium Chloride (KCl)	75.0	400.0	5.3333335
Sodium Bicarbonate ($NaHCO_3$)	84.0	3700.0	4.404.762
Sodium Chloride (NaCl)	58.0	6400.0	110.344.826
Sodium Phosphate monobasic ($NaH2PO_4$-H2O)	138.0	125.0	0.9057971
Other Components			
D-Glucose (Dextrose)	180.0	4500.0	25.0

Table 4.1 | The Gibco recipe for high-glucose DMEM

Supplier	Name	Catalog number
Applichem	CaCl2-2H20	A1873,1000
Sigma	Iron(III) nitrate nonahydrate	F8508-100G
Sigma	Magnesium sulfate heptahydrate	13142-1KG
Roth	Potassium Chloride	6781.1
Sigma	Sodium bicarbonate	S5761-1KG
Sigma	Sodium chloride	31434-5KG-R
Roth	Sodium dihydrogen phosphate monohydrate	K300.2
Applichem	D-Glucose	A1422,1000

Table 4.2 | List of inorganic components used for the custom media

Supplier	Amino acid	Catalog number
Sigma	L-Arginine	A8094-25G
Sigma	L-Cystin	30200-25G
Sigma	L-Glutamine	49419-25G
Sigma	L-Histidine	H8000-25G
Sigma	L-Isoleucine	I2752-25G
Sigma	L-Leucine	L8912-25G
Sigma	L-Lysine Hydrochloride	L5626-100G
Sigma	L-Methionine	M9625-25G
Sigma	L-Phenylalanine	P5482-25G
Sigma	L-Proline	P0380-100G
Alfa Aesar	L-Serine	J62187
Sigma	L-Threonine	T8625-10G
Sigma	L-Tryptophan	T0254-25G
Applichem	L-Tyrosine	A1677.1000
Sigma	L-Valine	V0500-25G

Table 4.3 | List of amino acids used for the custom media

For Bafilomycin A1 (#BML-CM110-0100, Enzo) treatments, the drug was added to a final concentration of 100 nM in the media for 6 hours before lysis or fixation. Treatment with E64 (#2935.1, Roth) and Pepstatin A (#P5318, Sigma) to block lysosomal protease activity was performed by adding a combination of E64 (25 μM) and Pepstatin A (50 μM) in the media for 16 hours before lysis or fixation, and in the last 90 min before lysis the AA add-back protocol was conducted. For experiments including treatments with +AA and –AA media, Bafilomycin or E64+PepA were also kept in the treatment media. To inhibit mTOR kinase activity, Torin1 (#14379, Cell Signaling Technology) was added in the culture media

(final concentration 250 nM) for 1 hour. Akt inhibition was achieved by addition of the Akt inhibitor VIII (#ENZ-CHM125, Enzo) in the culture media for 30 min (final concentration of 10 μM). Golgicide A (#345862, Sigma) and Brefeldin A (#BUF075, Biorad) were added in the culture media at final concentrations of 10 μM and 10 μg/ml, respectively, for 1 hour.

4.3 Antibodies

A list of all antibodies used in this study is found in Table 4.4. The H4B4 and ABL-93 antibodies against LAMP2, were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology. H4B4 was deposited to the DSHB by August, J.T. / Hildreth, J.E.K. (DSHB Hybridoma Product H4B4) (Mane et al., 1989). ABL-93 was deposited to the DSHB by August, J.T. (DSHB Hybridoma Product ABL-93) (Chen et al., 1985).

Antibody	Supplier	#
Rabbit monoclonal anti-phospho-p70 S6 Kinase (Thr389) (D5U1O)	Cell Signaling Technology	97596
Rabbit polyclonal anti-S6 Kinase	Cell Signaling Technology	9202
Rabbit monoclonal anti-phospho-TFEB (Ser211) (E9S8N)	Cell Signaling Technology	37681
Rabbit polyclonal anti-TFEB (for human TFEB)	Cell Signaling Technology	4240
Rabbit polyclonal anti-TFEB (for mouse TFEB)	Bethyl Laboratories	A303-673A
Rabbit polyclonal anti-TFE3	Cell Signaling Technology	14779
Rabbit polyclonal anti-phospho-Akt (Ser473)	Cell Signaling Technology	9271
Rabbit polyclonal anti-Akt	Cell Signaling Technology	9272
Rabbit monoclonal anti-phospho-4E-BP1 (Ser65) (D9G1Q)	Cell Signaling Technology	13443
Rabbit polyclonal anti-phospho-4E-BP1 (Thr37/46)	Cell Signaling Technology	9459
Rabbit polyclonal anti-4E-BP1	Cell Signaling Technology	9452
Rabbit monoclonal anti-mTOR (7C10)	Cell Signaling Technology	2983
Rabbit monoclonal anti-LC3B (D11) XP	Cell Signaling Technology	3868
Rabbit polyclonal anti-SQSTM1/p62	Cell Signaling Technology	5114
Rabbit polyclonal anti-TSC2	Cell Signaling Technology	4308
Rabbit polyclonal anti-phospho-Tuberin/TSC2 (Thr1462)	Cell Signaling Technology	3611
Rabbit monoclonal anti-RAPTOR (24C12)	Cell Signaling Technology	2280
Rabbit monoclonal anti-RagA (D8B5)	Cell Signaling Technology	4357
Rabbit monoclonal anti-RagC (D8H5)	Cell Signaling Technology	9480
Rabbit polyclonal anti-RagD	Cell Signaling Technology	4470
Rabbit polyclonal anti-CathepsinD	Cell Signaling Technology	2284
Rabbit polyclonal FLAG DYKDDDDK Tag Antibody	Cell Signaling Technology	2368
Rat monoclonal anti-HA (3F10)	Roche	11867423001
Rabbit monoclonal anti-GAPDH (14C10)	Cell Signaling Technology	2118
Mouse monoclonal anti-α-Tubulin	Sigma-Aldrich	T9026

Rat monoclonal anti-LAMP2 (ABL-93)	Developmental Studies Hybridoma Bank	ABL-93
Mouse monoclonal anti-LAMP2 (H4B4)	Developmental Studies Hybridoma Bank	H4B4

Table 4.4 | List of antibodies

4.5 Plasmids and molecular cloning

The pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid was a gift from Feng Zhang (Addgene plasmid #62988) and described in (Ran et al., 2013). The pLJC6-3xHA-TMEM192 and pLJC6-2xFLAG-TMEM192 plasmids (Wyant et al., 2017) were obtained from Addgene (plasmids #104434 and #104435; deposited by the Sabatini lab). All restriction enzymes were purchased from Fermentas/Thermo Scientific. The integrity of all constructs was verified by sequencing.

4.6 mRNA isolation, cDNA synthesis and quantitative real-time PCR

Total mRNA was isolated from cells using a standard TRIzol/chloroform-based method (#15596018, Thermo Fisher Scientific), according to manufacturer's instructions. For cDNA synthesis, mRNA was transcribed to cDNA using the RevertAid H Minus Reverse Transcriptase kit (#EP0451, Thermo Fisher Scientific) according to manufacturer's instructions. The cDNAs were diluted 1:10 in nuclease-free water and 4 µl of diluted cDNA were used per reaction, together with 5 µl 2x Maxima SYBR Green/ROX qPCR master mix (#K0223, Thermo Fisher Scientific) and 1 µl primer mix (2.5 µM of forward and reverse primers). Reactions were set in technical triplicates in a StepOnePlus Real-Time PCR system (Applied Biosystems). Relative gene expression was calculated with the 2-ΔΔCt method, with RPL13a as an internal control, and normalized to the expression of the gene in the respective siCtrl or WT sample. All qPCR primers used in this study are listed in Table 4.5.

4.7 Plasmid DNA transfections

Plasmid DNA transfections in HEK293FT cells were performed using Effectene transfection reagent (#301425, QIAGEN), according to the manufacturer's instructions.

4.8 Generation of stable cell lines

For the generation of stable cell lines expressing HA-tagged TMEM192 (lyso-IP lines) or FLAG-tagged TMEM192 (negative control lines for HA lyso-IPs), WT HEK293FT cells were transfected using the respective expression vectors. Forty-eight hours post-transfection, cells were selected with 3 µg/mL puromycin (#A11138-03, Thermo Fisher

Scientific). Single-cell clones that express similar TMEM192 levels were used in lyso-IP experiments.

Name	Sequence
RRAGC_for	5' ACTGCCGACCTTGGAAAACC 3'
RRAGC_rev	5' GGGAACTGTCTGTTGCAATGT 3'
LAMP1_for	5' TGGGCGTCTCTAATGTCTGC 3'
LAMP1_rev	5' CAGGATCACCCCGAATGTCA 3'
MCOLN1_for	5' CTATCATGTGAAGTTCCGCTC 3'
MCOLN1_rev	5' GTCACAAACATGTCGTCCC 3'
PPT1_for	5' CTCTCAGTACGTTGCCCTCTG 3'
PPT1_rev	5' ACTGTAGGCCAGTGGGATTTG 3'
ATP6AP1_for	5' TTCTAACCTAGAGAATGCCCTG 3'
ATP6AP1_rev	5' AGAGTGCTGACTGCATACC 3'
CLN3_for	5' TGGACAGTGTTCAAGGGTC 3'
CLN3_rev	5' GTCCCTGGTTAATGAAATACTCG 3'
LAMTOR1_for	5' CAAAGCTCTCAATGGAGCC 3'
LAMTOR1_rev	5' AATGATGTTGCTGGCTGTC 3'
ARF1_for	5' GCCAGTGCCTTCCACCTGTC 3'
ARF1_rev	5' GCCTCGTTCACACGCTCTCTG 3'
RPL13a_for	5' CCGCCCTACGACAAGAAA 3'
RPL13a_rev	5' AGGCGCCCCAGATAGG 3'

Table 4.5 | List of qPCR primers

4.9 Generation of knockout cell lines

The HEK293FT RagA/B KO, RagC/D KO, HEK293FT HA-TMEM192 RagA/B KO and FLAG-TMEM192 RagA/B KO cell lines were generated using the pX459-based CRISPR/Cas9 method, as described elsewhere (Ran et al., 2013). The sgRNA expression vectors were generated by cloning appropriate DNA oligonucleotides (Table 4.6) into the BbsI restriction sites of the pX459 vector (#62988, Addgene). An empty pX459 vector was used to generate matching control cell lines. In brief, transfected cells were selected with 3 μg/ml puromycin (#A11138-03, Thermo Fisher Scientific) 48 hours post-transfection. Single-cell clones were generated by single cell dilution and knockout clones were validated by immunoblotting.

Name	Sequence
RagA-gRNA-5UTR-s	5' caccgATTACATTGCTCGCGACACC 3'
RagA-gRNA-5UTR-as	5' aaacGGTGTCGCGAGCAATGTAATc 3'
RagB-gRNA-5UTR-s	5' caccgCTGCCTATTCTCATCGCCTA 3'
RagB-gRNA-5UTR-as	5' aaacTAGGCGATGAGAATAGGCAGc 3'
RagB-gRNA-3CDS-s	5' caccgTACATCCAACACTTATGTGA 3'
RagB-gRNA-3CDS-as	5' aaacTCACATAAGTGTTGGATGTAc 3'
RagC-gRNA-5CDS-s	5' caccgATCGGCCGCGCCGTAACTGC 3'
RagC-gRNA-5CDS-as	5' aaacGCAGTTACGGCGCGGCCGATc 3'
RagC-gRNA-3UTR-s	5' caccgTAGTCTGAATCCCAGCGTCG 3'
RagC-gRNA-3UTR-as	5' aaacCGACGCTGGGATTCAGACTAc 3'
RagD-gRNA-5UTR-s	5' caccgTGACTCCTCCGCCGGCGGGC 3'
RagD-gRNA-5UTR-as	5' aaacGCCCGCCGGCGGAGGAGTCAc 3'
RagD-gRNA-3UTR-s	5' caccgAGATTGGAGCTACAAGCTCC 3'
RagD-gRNA-3UTR-as	5' aaacGGAGCTTGTAGCTCCAATCTc 3'

Table 4.6 | Oligos for sgRNA expression from the pX459 vector for CRISPR/Cas9 KO cell line generation

4.10 Gene silencing experiments

Transient knockdown of GNPTAB, LAMTOR1, TSC2, MIOS and ARF1, were performed using pools of 4 siGENOME gene-specific siRNAs (Horizon Discoveries). An siRNA duplex targeting the R. reniformis luciferase gene (RLuc) (#P-002070-01-50, Horizon Discoveries) was used as control. Transfections were performed using 20 nM siRNA and the Lipofectamine RNAiMAX transfection reagent (#13778075, Thermo Fisher Scientific), according to the manufacturer's instructions. Cells were harvested or fixed 72 hours post-transfection and knockdown efficiency was verified by immunoblotting or quantitative real-time PCR.

4.11 Cell lysis and immunoblotting

For standard SDS-PAGE and immunoblotting experiments, cells from a well of a 12-well plate were treated as indicated in the figures, washed once with serum-free DMEM, and lysed in 250 µl of ice-cold Triton lysis buffer (50 mM Tris pH 7.5, 1% Triton X-100, 150 mM NaCl, 50 mM NaF, 2 mM Na-vanadate, 0.011 gr/ml beta-glycerophosphate), supplemented with 1x PhosSTOP phosphatase inhibitors ((#04906837001, Roche) and 1x

cOmplete protease inhibitors (#11836153001, Roche), for 10 minutes on ice. Samples were clarified by centrifugation (14000 rpm, 15 min, 4 °C) and supernatants transferred to a new tube. Protein concentration was determined using a Protein Assay Dye Reagent (#5000006, Bio-Rad). Normalized samples were boiled in 1x SDS sample buffer for 5 min, 95 °C (6x SDS sample buffer: 350 mM Tris-HCl pH 6.8, 30% glycerol, 600 mM DTT, 12.8% SDS, 0.12% bromophenol blue).

Protein samples were subjected to electrophoretic separation on SDS-PAGE and analysed by standard Western blotting techniques. In brief, proteins were transferred to nitrocellulose membranes (#10600002 or #10600001, Amersham), stained with 0.2% Ponceau solution (#33427-01, Serva) to confirm equal loading. Membranes were blocked with 5% skim milk powder (#42590, Serva) in PBS-T [1x PBS, 0.1% Tween-20 (#A1389, AppliChem)] for 1 hour at room temperature, washed three times for 10 min with PBS-T and incubated with primary antibodies (1:1000 in PBS-T, 5% bovine serum albumin [BSA; #10735086001, Roche] rotating overnight at 4 °C. The next day, membranes were washed three times for 10 min with PBS-T and incubated with appropriate HRP-conjugated secondary antibodies (1:10000 in PBS-T, 5% milk) for 1 hour at room temperature. Signals were detected by enhanced chemiluminescence (ECL), using the ECL Western Blotting Substrate (#W1015, Promega); or SuperSignal West Pico PLUS (#34577, Thermo Scientific) and SuperSignal West Femto Substrate (#34095, Thermo Scientific) for weaker signals. Immunoblot images were captured on films (#28906835, GE Healthcare; #4741019289, Fujifilm).

4.12 Lysosomal purification (Lyso-IP) assays

To biochemically isolate intact lysosomes and associated proteins, we developed a modified lyso-IP method, based on the protocol previously described by the Sabatini group (Abu-Remaileh et al., 2017), which allows us to also assess the non-lysosomal fractions. In brief, cells were seeded on a 15 cm dish until they reached 80-90% confluency, washed 2x with ice-cold PBS and scraped in 1 mL ice-cold PBS, containing 1x PhosSTOP phosphatase inhibitors (#04906837001, Roche) and 1x cOmplete protease inhibitors (#11697498001, Roche). Cells were then pelleted by centrifugation (1000 x g, 2 min, 4°C) and resuspended in 1 mL ice-cold PBS containing phosphatase and protease inhibitors. For input samples, 25 µl of the cell suspension were transferred in a new tube and lysed by the addition of 125 µl of Triton lysis buffer (50 mM Tris pH 7.5, 1% Triton X-100, 150 mM NaCl, 50 mM NaF, 2mM Na-vanadate, 0.011 gr/ml beta-glycerophosphate), supplemented with 1x PhosSTOP phosphatase inhibitors (#04906837001, Roche) and 1x cOmplete protease inhibitors

(#11836153001, Roche) on ice for 10 min. Lysed input samples were then cleared by centrifugation (14000 x g, 15 min, 4°C) and the supernatant was transferred to new tubes containing 37.5 µl of 6x SDS sample buffer and boiled for 5 min at 95°C. For the lysosomal and non-lysosomal fractions, the remaining cell suspension was homogenized with 20 strokes in pre-chilled 2 mL hand dounce homogenizers kept on ice. The homogenate was cleared by centrifugation (1000 x g, 2 min, 4°C) and incubated with 100 µl pre-washed Pierce anti-HA magnetic beads (#88837, Thermo Fisher Scientific) on a nutating mixer for 3 min at room temperature. After incubation with the beads, the supernatant was transferred to a new tube and centrifuged at high speed (20000 x g, 10 min, 4°C) to remove membranes and other organelles and retrieve the non-lysosomal fraction. Twenty-five microliters of the cleared supernatant were transferred in a new tube, mixed with 125 µl Triton lysis buffer, and incubated for 10 min on ice. Next, 37.5 µl 6x SDS sample buffer was added and samples were boiled. For the lysosomal fraction, beads were washed three times with ice-cold PBS containing phosphatase and protease inhibitors using a DynaMag spin magnet (#12320D, Invitrogen). After the last wash, lysosomes were eluted from the beads by addition of 50 µl Triton lysis buffer and incubation for 10 min of ice. Isolated lysosomes were then transferred to a new tube, 12.5 µl 6x SDS sample buffer was added and samples were boiled.

4.13 Immunofluorescence and confocal microscopy

Immunofluorescence/confocal microscopy experiments were performed as described previously (Demetriades et al., 2014; Fitzian et al., 2021). In brief, cells were seeded on glass coverslips (coated with fibronectin), treated as described in the figure legends, and fixed with 4% paraformaldehyde (PFA) in 1x PBS (10 min, RT), followed by two permeabilization/washing steps with PBT (1x PBS, 0.1 % Tween-20). Cells were blocked in BBT (1x PBS, 0.1% Tween-20, 1% BSA) for 45 minutes. Staining with anti-mTOR (#2983, Cell Signaling Technology) and anti-LAMP2 (#H4B4, Developmental Studies Hybridoma Bank) primary antibodies diluted 1:200 in BBT solution was performed for 2h at room temperature. Staining with anti-TFEB (#4240, Cell Signaling Technology) or anti-TFE3 (#14779, Cell Signaling Technology) antibodies (1:200 in BBT) was performed by incubation for 16 h at 4°C. After staining with primary antibodies, cells were washed three times with PBT. Next, cells were stained with highly cross-adsorbed fluorescent secondary antibodies (Donkey anti-rabbit Alexa Fluor 488, Donkey anti-mouse TRITC; both from Jackson ImmunoResearch) diluted 1:200 in BBT for 1 hour. Nuclei were stained with DAPI (#A1001, VWR) (1:2000 in PBT) for 5 min and coverslips were washed three times with

PBT solution before mounting on glass slides with Fluoromount-G (#00-4958-02, Invitrogen).

For LC3B staining, cells were fixed with 100% methanol for 15 minutes at -20°C, permeabilized with 0,1% Triton™-X100 (#A4975, AppliChem) for 5 minutes and blocked for 1 hour in LC3B blocking solution (1x PBS, 5% FBS, 0,3% Triton X-100). Coverslips were incubated overnight at 4 °C with anti-LC3B antibody (#3868, Cell Signaling Technology) in LC3B staining solution (1x PBS, 1% BSA, 0,3% Triton X-100). Slides were washed three times in 1x PBS, incubated with Donkey anti-rabbit Alexa Fluor 488 (Jackson ImmunoResearch) (1:500, in 1x PBS, 1% BSA, 0,3% Triton X-100) for 1 hour at room temperature. Coverslips were then washed twice with 1x PBS, stained with DAPI (1:2000 in 1x PBS) and mounted on glass slides with Fluoromount-G (#00-4958-02, Invitrogen).

All images were captured on an SP8 Leica confocal microscope (TCS SP8 X or TCS SP8 DLS, Leica Microsystems) using a 40x oil objective lens. Image acquisition was performed using the LAS X software (Leica Microsystems). Images from single channels are shown in grayscale, whereas in merged images, Alexa Fluor 488 is shown in green, TRITC in red and DAPI in blue.

4.14 LysoTracker staining

For LysoTracker staining experiments, cells were seeded in fibronectin-coated coverslips and grown until they reached 80-90% confluency. Lysosomes were stained by the addition of 100 nM LysoTracker Red DND-99 (#L7528, Invitrogen) in complete media for 1 hour in standard culturing conditions. Cells were then fixed with 4% PFA in PBS for 10 min at room temperature, washed and permeabilized with PBT solution (1x PBS, 0.1% Tween-20), and nuclei stained with DAPI (1:2000 in PBT) for 10 min. Coverslips were mounted on slides using Fluoromount-G (#00-4958-02, Invitrogen). All images were captured on an SP8 Leica confocal microscope (TCS SP8 X or TCS SP8 DLS, Leica Microsystems) using a 40x oil objective lens. Image acquisition was performed using the LAS X software (Leica Microsystems).

4.15 Quantification of colocalization

Colocalization analysis in confocal microscopy experiments was performed as in (Demetriades et al., 2016; Fitzian et al., 2021) using the Coloc2 plugin of the Fiji software (Schindelin et al., 2012). An average of 50 cells from 3-5 independent representative different images captured from each experiment was used and Manders' colocalization coefficient

(MCC) with automatic Costes thresholding (Manders et al., 1993; Costes et al., 2004; Dunn et al., 2011) was calculated in individual cells. The area corresponding to the cell nucleus was excluded from the cell region of interest (ROI) to prevent false-positive colocalization due to automatic signal adjustments. MCC is defined as a part of the signal of interest (mTOR), which overlaps with a second signal (LAMP2).

4.16 Quantification of LC3B and LysoTracker intensities

Staining intensity was calculated using the Fiji software. ROIs were determined for approximately 50 cells per condition over 5 independent representative images and integrated density was calculated, representing the sum of the values of all pixels in the given ROI. Exact numbers of individual cells analysed per experiment are indicated in the figure legends.

4.15 Scoring of TFEB/TFE3 localization

Subcellular localization of TFEB and TFE3 was performed by scoring the distribution of signal in the cytoplasm and the nucleus. Five independent fields were analysed per experiment. Exact numbers of individual cells analysed per experiment are indicated in the figure legends.

4.16 OPP assay

To test de novo protein synthesis, OPP (O-propargyl-puromycin) incorporation assays were performed using the Click-iT Plus OPP Protein Synthesis Assay kit (#C10456, Thermo Fisher Scientific), according to the manufacturer's instructions. In brief, cells seeded in fibronectin-coated coverslips until they reached 80-90% confluence. Control samples were treated with 100 μM cycloheximide (#239765, Sigma) for 4 hours before fixation to block translation. Click-iT OPP component A (20 μM) was added to the culture media for 30 minutes, cells were fixed for 10 min at room temperature with 4% PFA, and washed twice with PBT. Next, cells were incubated with Click-iT Plus OPP reaction cocktail for 30 minutes at room temperature protected from light, followed by one wash with Click-iT Reaction Rinse Buffer and further DAPI staining as described for immunofluorescence. All samples were imaged on an SP8 Leica confocal microscope (TCS SP8 X or TCS SP8 DLS, Leica Microsystems) using a 40x oil objective lens. Image acquisition was performed using the LAS X software (Leica Microsystems).

4.17 Statistical analysis

Statistical analysis and presentation of quantification data was performed using GraphPad Prism (version 9.1.0). Data in graphs shown as mean ± SEM. Significance was calculated using Student's t-test (for pairwise comparisons) or one-way ANOVA with post hoc Holm-Sidak test (pairwise comparisons to controls). Sample sizes (n) and significance values are indicated in figure legends (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, ns: non-significant).

V. Contributions

Figure	Data generated by
2.1	DDA, SAF
2.2	DDA
2.3	DDA
2.4	DDA, SAF
2.5	DDA, SAF
2.6	DDA, SAF
2.7	DDA
2.8	SAF
2.9	SAF
2.10	DDA
2.11	DDA
2.12	DDA, SAF
2.13	DDA
2.14	DDA, SAF

DDA	Danai Dimitra Angelidaki
SAF	Stephanie A. Fernandes

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