Role of the Rag GTPases

in Amino Acid sensing

and mTORC1 signaling

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List of abbreviations

ALS	Amyotrophic lateral sclerosis
AMPK	Adenosine monophosphate- activated protein kinase
ARF1	ADP-ribosylation factor 1
ATF4	Activating transcription factor 4
ATP/ADP	Adenosine triphosphate/Adenosine diphosphate
CAD	Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase
CASTOR1	Cytosolic arginine sensor for mTORC1 subunit 1
CLEAR	Coordinated lysosomal expression and regulation
CoA	Coenzyme A
CRISPR-Cas	Clustered regularly interspaced short palindromic repeats-CRISPR associated protein
DEPTOR	Domain-containing mTOR-interacting protein
DNA	Deoxyribonucleic acid

DR	Dietary restriction
eIF	Eukaryotic initiation factors
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
FLCN	Folliculin
FNIP	Folliculin interacting proteins
GAP	Guanosine activating protein
GATOR	GTPase-activating protein
GCN	General control nonderepressible
GEF	Guanosine nucleotide exchange
GTP/GDP	Guanosine triphosphate/Guanosine diphosphate
GWAS	Genome-wide association studies
HEK	Human embryonic kidney
HIF	Hypoxia-inducible factor
IGF	Insulin-like growth factor
kDa	Kilodalton
LAMTOR	Lysosomal adaptor and MAPK and mTOR activator
LARS	Leucyl-tRNA synthetase
LSD	Lysosomal storage disease
MCOLN	Mucolipin-1
MiT	Microphthalmia
MPR	Mannose-6-phosphate receptor
MTHFD	Methylenetetrahydrofolate dehydrogenase
mTOR(C)	Mechanistic target of rapamycin (complex)
NADPH	Nicotinamide adenine dinucleotide phosphate

NAFLD	Non-alcoholic fatty liver disease
NPRL2	Nitrogen permease regulator 2-like protein
PDK	Phosphoinositide-dependent protein kinase
PI3K	Phosphoinositid-3-kinase
PRAS40	proline-rich akt substrate of 40 kDa
PTEN	Phosphatase and tensin homolog
QKO	Quadruple knock-out
Rag	Ras related GTPase
RAPTOR	Regulatory associated protein of mTOR
Rheb	Ras homolog enriched in brain
RICTOR	Rapamycin-insensitive companion of mTOR
SAM	S-adenosyl-methionine
SAR1B	Secretion associated Ras related GTPase 1B
SLC38A9	Solute carrier family 38 member 9
SREBP	Sterol regulatory element-binding protein
TARS	Threonyl-CoA synthetase
TCA	Tricarboxylic acid cycle
TF	Transcription factor
TOR	Target of rapamycin
TOS	TOR signaling
TRPML	Transient receptor potential cation channel, mucolipin subfamily
TSC	Tuberous sclerosis complex
ULK1	Unc-51 like autophagy activating kinase
uORF	Upstream open reading frame
V-ATPase	Vacuolar-type ATPase

Abstract

To sustain metabolism and homeostatic functions, cells need to acquire energy-rich nutrients from their environment. These are broken down in catabolic pathways to provide energy for ATP generation. Nutrients are also the building blocks of complex biomolecules and effectively define the composition of biomass. To balance energy metabolism and biomass production cells need to be able to switch from catabolic to anabolic metabolism. This switch is regulated by the kinase mTOR, which was identified as the target of the immunosuppressant Rapamycin. mTOR was shown to react cell-autonomously to amino acid availability by upregulating translation and downregulating autophagy. Beyond this, mTOR was demonstrated to upregulate various other anabolic pathways, with major implications for human disease and the ageing process. The proteins that facilitate mTOR activation in response to amino acids are the dimeric Rag GTPases, which are localized at the lysosomal surface. An active dimer is composed of a smaller and larger Rag monomer, however there are two paralog genes for the smaller (Rag A and RagB) and the larger (RagC and RagD) monomer. Although many regulators of the Rag GTPases have been identified, the role of paralog Rag GTPase genes has not been thoroughly investigated. Our hypothesis was that the paralog Rag GTPase proteins are non-redundant and facilitate different signaling events in the mTORC1 pathway. We tested this hypothesis by using gene editing tools to knock-out endogenous Rag GTPase genes, obtaining a quadruple knock-out cell line. We used this cell line for a reconstitution approach, in which we re-expressed all four possible Rag dimer combinations. We performed functional mTOR assays and were able to report novel, non-redundant functions of the paralogs. All dimer combinations rescued phosphorylation of the substrate S6K, which controls translation. However, only the Rag GTPase dimers containing the RagD paralog are able to

rescue the phosphorylation of lysosomal transcription factors TFEB and TFE3. We investigated TFE3-dependent transcription and were able to confirm a downregulation by RagD-, but not RagC-containing dimers. We studied the regulatory mechanism of substrate specificity and found stronger localization of RagD-containing dimers at the lysosomal surface. We identified the regions of the RagD protein that enable it to regulate the subset of lysosomal mTOR substrates. Moreover, we discovered that cancer-associated gain-of-function mutations enabled the paralog RagC protein to also facilitate lysosomal substrate phosphorylation. Finally, we demonstrated, that the RagB, but not the RagA paralog rendered mTOR activity resistant to amino acid starvation. We identified a novel mode of regulating mTOR substrate selectivity and amino acid response. Thus, we were able to uncover a whole new level of mTOR regulation by the paralogs of Rag GTPases with major implication for the pathways' function.

Zusammenfassung

Zellen sind auf die Aufnahme von energiereichen Nährstoffen aus der Umgebung angewiesen, um essentielle Stoffwechselfunktionen aufrechterhalten zu können. Die Nährstoffe werden in katabolen Stoffwechselwegen abgebaut um Energie in Form von ATP zu generieren. Gleichzeitig handelt sich bei den Nährstoffen um die Bausteine komplexer Biomoleküle, die für die Zusammensetzung sämtlicher Biomasse essentiell sind. Um Energiestoffwechsel und den Aufbau der Biomasse zu balancieren, müssen Zellen in der Lage sein bei ausreichendem Nährstoffangebot anabole Stoffwechselwege aktivieren zu können. Dieser Übergang wird durch die Proteinkinase mTOR kontrolliert, welche ursprünglich als pharmakologisches Ziel des Immunsuppressivums Rapamycin identifiziert wurde. mTOR wird auf zellautonome Weise durch Aminosäuren aktiviert, sodass die Translation durch mTOR hoch- und die Autophagie herunterreguliert werden kann. Darüber hinaus konnte eine Beteilung von mTOR an zahlreichen Stoffwechselwegen und eine zentrale Rolle im Alterungsprozess und bei der Entstehung von Krankreiten gezeigt werden. Die dimeren Rag GTPasen sind Proteine, die mTOR bei steigenden Leveln von Aminosäuren aktivieren und sich an der Oberfläche der Lysosomen befinden. Ein aktiver Rag GTPase Dimer besteht aus jeweils einem kleineren und einem größeren Rag GTPase Monomer. Das menschliche Genom enthält jedoch jeweils zwei paraloge Gene für das kleinere (RagA und RagB) und das größere (RagC und RagD) Rag GTPase Protein. Trotz ausgiebiger Forschung am mTOR Signalweg, ist die Rolle der paralogen Rag GTPasen niemals systematisch untersucht worden. Unsere Hypothese war, dass die paralogen Rag GTPase Proteine nicht beliebig austauschbar sind, sondern definierte regulatorische Rollen im mTOR Signalweg übernehmen. Wir haben diese Fragestellung in einer Zelllinie untersucht, deren endogene Rag GTPasen mittels CRISPR-Cas9 ausgeschaltet wurden. Die Zelllinie wurde mit den 4 möglichen dimeren Kombinationen der paralogen Rag

GTPasen rekonstituiert. Wir haben funktionale mTOR-Assays durchgeführt und dabei bisher unbekannte, spezifische Funktionen der paralogen Rag GTPasen gefunden. Alle vier Rag GTPase Dimere waren in der Lage die Phosphorylierung des mTOR Substrats S6K zu regulieren, welches die Translation kontrolliert. Jedoch waren nur die Dimere, welche das RagD Protein enthielten, in der Lage die Phosphorylierung der lysosomalen Transkriptionsfaktoren TFEB und TFE3 auszulösen. Eine Untersuchung der Transkription bestätigte, dass die Substrate des Transkriptionsfaktors TFE3 durch RagD negativ reguliert werden. Der regulatorische Mechanismus dieser Substratspezifität lag in einer verstärken Lokalisierung der RagD enthaltenden Dimere am Lysosom begründet. Wir konnten die Regionen des RagD Proteins identifizieren, die diese Art der Regulation ermöglichen. Außerdem konnten wir zeigen, dass in Krebszellen auftretende Mutationen im paralogen RagC Protein ebenfalls die negative Regulation der lysosomalen mTOR-Substrate ermöglichten. Weiterhin konnten wir zeigen, dass das RagB-, jedoch nicht das RagA-Paralog des kleineren Rag GTPase Monomers den mTOR Signalweg unempfindlich gegen Aminosäuremangel macht. Somit konnten wir die Existenz einer neuen Ebene der Regulation des mTOR-Signalweges nachweisen.

1. Introduction

1.1. mTOR as a regulator of cell growth

1.1.1. The need for nutrient signaling

Nutrient availability is a central requirement for life and the functioning of virtually all biological processes. A hallmark of all living organisms, excluding only viruses, is metabolism which depends on the acquisition of environmental nutrients (Palm and Thompson, 2017). Unlike plants and some autotroph bacteria, the majority of organisms depend on the uptake of small, energy-rich, reduced compounds like amino acids, fatty acids and sugars. Their oxidation in **catabolic pathways** like glycolysis or the TCA cycle is needed to produce ATP to sustain metabolism and preserve cellular homeostasis. If cells do not suffer energy stress however, nutrients can be used to synthesize complex biomolecules like proteins, carbohydrates and lipids (González *et al.*, 2020). This switch to **anabolic pathways** requires nutrient sensing mechanisms that detect the levels of available nutrients. Nutrient signaling pathways then activate downstream biosynthetic pathways to promote **cell growth** (Liu and Sabatini, 2020). In unicellular organisms, which often experience large fluctuations in environmental nutrients, cell growth is mainly limited by nutrient availability. If enough nutrients are available, the cells can increase anabolism until they reach the size required for cell division, thereby also indirectly regulating cell proliferation (Palm and Thompson, 2017). The nutrients themselves are therefore the signal enabling cell growth. However, this cell-autonomous regulation of cell growth and proliferation is not suitable for body cells of multicellular organisms. Most mammalian cells remain quiescent, meaning their size and especially their number needs to be maintained rather than constantly expanded (Palm and Thompson, 2017). To maintain organismal homeostasis, higher organisms like mammals secrete hormones and growth factors, that license only special cell types to accumulate mass

and upregulate anabolism when nutrients are available (Liu and Sabatini, 2020). An example of this non-cell-autonomous regulation is the secretion of insulin by pancreatic beta cells allowing glucose uptake and glycogen synthesis in the muscle. Moreover, growth factors have crucial roles in activating cell proliferation, allowing cells to proceed in the cell cycle (Vasan and Cantley, 2022). Cell growth is tightly linked to cell proliferation, yet the processes are not identical. Importantly, cell growth is not merely a passive consequence of cell division that depends on nutrient availability. Rather it was shown that nutrients activate a separate pathway which actively regulates cell growth in unicellular and multicellular organisms, the **mTOR pathway** (Schmelzle and Hall, 2000). This realization was not always obvious and took decades of research on a variety of model organisms. The initial discovery of this pathway followed the study of a novel bacterial antifungal compound and immunosuppressant called Rapamycin.

1.1.2. The discovery of mTOR

Rapamycin is a macrolide isolated from a culture of *Streptomyces hygroscopicus* originally discovered in 1964 in a soil sample from the Easter Island Rapa Nui. It was named Rapamycin after the island and originally characterized as an antifungal compound that inhibited the growth of dermatophytes and opportunistic pathogens like *Candida albicans* (Vézina and Kudelski, 1975). Pharmaceutical interest in the compound rose when it was also shown to have immunosuppressant and anti-cancer properties, due to its inhibitory effect on cell proliferation. The investigation of its immunosuppressant function paved the way to understand its mechanism of action. Like previously known immunosuppressants FK506 and Cyclosporin A, Rapamycin binds to the small immunophilin FKBP12, thereby changing its binding properties. While the FK506-FKBP12 complex was known to inhibit the phosphatase calcineurin, Rapamycin induced FKBP12 binding to a different effector (Bierer *et al.*, 1990). The target of

rapamycin (**TOR**) was identified in a genetic screen for rapamycin resistant mutants (Heitman, Movva and Hall, 1991). The cloning of TOR1 and TOR2 genes revealed that these were high molecular weight lipid kinases of 281 and 283 kilodaltons respectively, both belonging of the PI3K family of kinases (Helliwell *et al.*, 1994). The mammalian 289kDa TOR homolog was cloned shortly after and named FRAP, RAFT or mTOR by its different co-discoverers, respectively (Sabatini *et al.*, 1994). The name mTOR (mammalian Target of Rapamycin) was eventually used canonically, although its full name was later changed to mechanistic target of rapamycin (Liu and Sabatini, 2020).

The identification of TOR1 and TOR2 as a kinases suggested their involvement in a previously uncharacterized signaling pathway (Helliwell *et al.*, 1994). This pathway was originally assumed to control cell division, since its inhibition caused a G1 phase arrest in the cell cycle, which would explain the antiproliferative effects of Rapamycin. Strikingly however, cell cycle arrest observed in Rapamycin-treated yeast was eventually shown to be a consequence of an inhibition of protein synthesis (Barbet *et al.*, 1996). Rapamycin treatment caused a response comparable to nutrient starvation, showing enlarged vacuoles and suggesting a role in nutrient signaling. Moreover, Rapamycin blocked protein synthesis by preventing TOR-dependent regulation of translation initiation. This regulation was suggested to be dependent on nutrient availability, presumably sensed upstream of TOR. Finally, mammalian mTOR was demonstrated to regulate translation initiation directly in response to **amino acid** availability in mammalian cells (Blommaart *et al.*, 1995; Hara *et al.*, 1998). In parallel to the effects of insulin addition or withdrawal, amino acid availability was shown to regulate translation through the mTOR pathway. The mTOR substrates facilitating this regulation were identified as translation regulators **S6K** and **4EBP1**. Phosphorylation of these substrates by mTOR was shown to react dynamically to amino acid availability (Hara *et al.*, 1998). Upon amino acid

withdrawal, S6K and 4EBP1 phosphorylation drops and cannot be restimulated by insulin. Conversely, amino acid re-addition leads to a rapid re-phosphorylation of 4EBP1 and S6K, which interestingly is strongest upon leucine and arginine addition.

1.1.3. mTOR as major regulator of anabolism and catabolism

As a central metabolic regulator, mTOR has attracted a lot of interest, considering the very fundamental need for cell growth. Importantly, for a cell to grow through promotion of anabolism, catabolic metabolism needs to be suppressed to prevent immediate degradation of the newly accumulated biomass. The vast majority of a cell's dry mass is built from proteins and the amino acids bound within them; thus, it is not surprising that mTOR was shown to inhibit protein degradation (Palm and Thompson, 2017). The first report again came from the yeast TOR, which negatively regulates **autophagy** upon nutrient stimulation (Noda and Ohsumi, 1998). Autophagy provides a means to degrade complex biomolecules to obtain nutrients for generating energy. Cellular components, including proteins, can be engulfed by double-membrane structures called autophagosomes (Lin and Hurley, 2016). These can be transported to degradative compartments, called **lysosomes**, where they are broken down by hydrolytic enzymes. Despite the presence of sufficient nutrients, autophagy could be pharmacologically induced by TOR inhibition by Rapamycin (Noda and Ohsumi, 1998). Thus, TOR was confirmed as the central regulator for autophagy initiation. The switch between anabolism and catabolism depends on the immediate response to nutrient availability by TOR, making it a **nutrient sensor** (González and Hall, 2017). The balancing of growth and autophagy and thus the fundamental homeostasis of cells maintaining their biomass depends on this pathway. Interestingly, the mTOR pathway not only regulates initiation, but also the more intermediate capacities for translation and autophagy. Unlike, 4EBP1, which directly binds to the translation initiation complex, the role of S6K is more indirect. The main S6K

substrate is the protein S6, which is assumed to increase ribosome function upon mTOR phosphorylation (Hannan *et al.*, 2003). Moreover, mTORC1 can phosphorylate LARP-1, thereby causing an upregulation of mRNAs containing the TOP motif (terminal oligopyrimidine tract) encoding ribosome components. Similarly, in the control of autophagy, mTORC1-dependent phosphorylation not only directly represses the kinase **ULK1**, but also controls the cellular capacities for autophagy by suppressing **lysosome biogenesis** (Lim and Zoncu, 2016)**.** The transcription factors of the **MiT-TFE** family (including **TFEB, TFE3**, TFEC and MITF) controlling lysosome biogenesis are direct mTORC1 substrates.

Since its discovery, the extent of metabolic pathways controlled by mTOR has extended far beyond protein synthesis and autophagy. There is a multitude of biosynthetic pathways that need to be upregulated in anabolic metabolism to make biomolecules needed for growth (Fernandes and Demetriades, 2021). None of these other regulatory mechanisms is as direct as the TOR regulation of protein synthesis, however. In order to increase their size and mass, growing cells need large amounts of lipids in order to expand the plasma and intracellular membranes. After amino acids, lipids make up the biggest part of cellular dry mass implicating a very likely role of lipogenesis regulation through mTOR (Palm and Thompson, 2017). A mechanism of transcriptional regulation of **lipid metabolism** could be identified with the discovery of Lipin1 phosphorylation (Peterson *et al.*, 2011). mTOR was demonstrated to phosphorylate Lipin1, thereby causing its cytoplasmic localization. The exclusion of Lipin1 from the nucleus leads to transcriptional remodeling promoting lipid and fatty acid biosynthesis pathways. Importantly, the transcriptional upregulation depends on another transcription SREBP, which is not a part of the core mTOR network. Although mTOR is generally accepted to be a positive regulator of lipid synthesis, the mechanism is much more indirect. Especially,

how the presence of fatty acid or their smaller building blocks Acetyl-CoA and Malonyl-CoA is signaled to mTOR remains unknown (Ben-Sahra and Manning, 2017a).

Like translation and most anabolic pathways, *de novo* lipogenesis is a very energy-demanding process. To make lipids, citrate is diverted from the mitochondrial TCA and shuttled to the cytosol, to synthesize Acetyl-CoA, which is then polymerized in a reaction requiring ATP and NADPH reducing equivalents (Röhrig and Schulze, 2016). To sustain anabolic metabolism, proliferating cells need a high flux through the glycolysis and TCA pathways. mTOR indirectly activates glycolysis through another transcription factor: HIF-1α (Düvel *et al.*, 2011). mTOR regulation of **mitochondrial biogenesis** on the other hand, seems to depend more on the translational upregulation downstream of 4EBP1 (Morita *et al.*, 2013). Finally, in rapidly proliferating cells, *de novo* synthesis of nucleotides is needed to provide building blocks for DNA replication. The synthesis of pyrimidine nucleotides was shown to be upregulated by direct mTOR phosphorylation of the biosynthetic enzyme complex CAD. (Robitaille *et al.*, 2013). **Purine metabolism** on the other hand, was shown to be upregulated transcriptionally by mTOR, mainly through MTHFD2 (Ben-Sahra *et al.*, 2016). Similar to the cases of SREBP and HIF1α, this transcriptional upregulation depends on another transcription factor, ATF4. The networks regulated by ATF4 and mTOR are even more overlapping, which might be due to an amino acid dependent activation mechanism (Ben-Sahra and Manning, 2017a). A chronic lack of amino acids causes the accumulation of uncharged tRNAs, which activate the kinase Gcn2. Like with mTOR inactivation, the consequence is a global inhibition of protein synthesis caused by phosphorylation of the translation initiation factor eIF2alpha (Harding *et al.*, 2003). Despite global translation inhibition, the translation of ATF4 can occur due to specific uORF (upstream open reading frames), which allows ATF4 to accumulate during prolonged amino

acid starvation. However, how certain targets are co-regulated by ATF4 and mTOR still awaits clarification.

1.1.4. mTOR in disease and ageing:

While most physiological processes are connected to nutrient signaling, the same is true for many pathologies. Some disorders like cancer and metabolic disease are tightly connected to mTOR pathway hyperactivity (Saxton and Sabatini, 2017). Inhibition of mTOR, especially with Rapamycin and its derivatives has thus been suggested as a treatment strategy. Since the mTOR kinase has been initially discovered as a drug target, there has been extensive work on medications and therapies targeting mTOR (Benjamin *et al.*, 2011). The role of mTOR in growth control immediately suggests a role in cancer, where uncontrolled cell proliferation is accompanied by cell growth. Similar to activated T-cells, cancer cells require a switch to anabolic metabolism. mTOR activation facilitates metabolic changes needed for cancer cell growth to provide lipids for cell membranes and nucleotides for DNA replication and transcription. Moreover, significant parts of the role of hyperactive mTOR depend on translation, especially downstream of 4EBP1 (Laplante and Sabatini, 2012). The branch of nutrient signaling which is mutated most often in cancer is growth factor signaling. Growth factor availability is signaled through the PI3K-Akt pathway, that also activates mTOR (see 2.1). Both, PI3K and mTOR inhibitors are approved cancer drugs (Vasan and Cantley, 2022). A cancer type that responds to mTOR inhibitors is metastatic **renal cell carcinoma (mRCC**). For other cancer types, mTOR inhibition has yielded rather disappointing results (see 2.1 and 2.4). The direct activation mechanism of mTOR by nutrients implicates the mTOR pathway in all kinds of disorders caused by nutrient overload. This is seen especially with increased food intake in the industrialized part of the world (Liu and Sabatini, 2020). Often summed up by the term metabolic disease, these range from obesity to diabetes, high blood pressure and

hypercholesterinemia. Consequences of metabolic disease include leading causes of death, like cardiovascular disease and organ failure. The hyperactivation of the mTOR pathway has been observed in the liver, where the upregulation of anabolic pathways, especially lipid biosynthesis, implicates mTOR in the pathology NAFLD (Gosis *et al.*, 2022). Finally, autophagy regulation facilitated by the mTOR pathway, is required for the clearance of toxic protein aggregates and defective organelles (Ballabio, 2016). A toxic accumulation of those products has been observed in many types of **neurogenerative disease** and is suspected to cause the death of nerve cells in diseases like Alzheimers's or Parkinson's disease (Klionsky *et al.*, 2021).

Besides its role in cancer and metabolic disease, there is a strong connection between the mTOR pathway and ageing. Organismal lifespan has been shown to be modulated by the mTOR pathway, which makes mTOR quite unique (Liu and Sabatini, 2020). Among the most complex biological processes, ageing is a number one risk factor for all the aforementioned pathologies (Harman, 1991). Dietary restriction (DR) has long been known to extend lifespan (McCay, Crowell and Maynard, 1989). The link between lifespan and reduced food intake has been studied genetically, especially with the characterization of long-lived mutants of *Caenorhabditis elegans* (Guarente and Kenyon, 2000). The genes regulating lifespan have been shown to be involved in nutrient signaling in the growth factor pathway. Also, mTOR inhibition by Rapamycin was shown to increase lifespan in various model organisms from flies to mice (López-Otín *et al.*, 2013). Understanding the regulation of mTOR and its substrates is key to developing new pharmacological strategies, beyond the use of Rapamycin. Especially in ageing and disease, the mTOR network can only be understood by investigation of its endogenous activation mechanisms and its interplay with the growth factor signaling branch.

1.2. The eukaryotic mTOR network:

1.2.1. Growth factor signaling and mTOR

A central reason, why Rapamycin has yielded disappointing results in cancer therapy was its selectivity for only one of two human mTOR complexes (Liu and Sabatini, 2020). Unlike in yeast, which expresses the different genes TOR1 and TOR2, the mammalian genome encodes for one mTOR protein, which is the active kinase of both complexes, mTORC1 and mTORC2. Both complexes differ in their composition of mTOR-associated/accessory proteins, of which two, **mLST8** and the inhibitory protein **Deptor** are present in both complexes (Liu and Sabatini, 2020). The core mTORC1 specific protein, **Raptor** (**r**egulatory **a**ssociated **p**rotein of m**TOR**) was discovered as an interactor of the mTOR kinase, required for its function in growth regulation and nutrient activation (Kim *et al.*, 2002). Later work showed that Raptor is of paramount importance for two key mTORC1 functions: activation and substrate specificity (see 2.2 and 2.3). Finally, mTORC1 contains the protein PRAS40, which is an inhibitor of its activity. Similar to DEPTOR, loss of PRAS40 causes hyperactivity of mTORC1 (Sancak *et al.*, 2007). Incidentally, the naming of the main **mTORC2** specific component reflects the insensitivity of the mTORC2 complex to rapamycin. **Rictor** (rapamycin-insensitive companion of mTOR) was identified as an mTOR interactor forming a different complex which neither binds to Rapamycin-FKBP12 nor phosphorylates S6K (Sarbassov *et al.*, 2005). Other components of mTORC2 complex include the proteins PROTOR1/2 and mSIN. Interestingly, the activation mechanism and substrate binding mode of mTORC2 have recently been shown to be facilitated by mSIN rather than Rictor (Liu *et al.*, 2015).

One key difference is that the mTORC1 complex responds to amino acids, while the mTORC2 complex does not and reacts only to growth factors (Liu and Sabatini, 2020). Growth factor signaling pathways are a typical eukaryotic feature, needed to license cells to accumulate mass

in response to nutrients. The amino acid stimulation of mTORC1 only reaches its full activity when growth factors are also present (Groenewoud and Zwartkruis, 2013). The main growth factor signaling pathway upstream of mTOR is the **PI3K-Akt pathway**. PI3K kinases are recruited to the plasma membrane upon binding of Insulin, IGFs or other growth factors to their respective receptors. At the plasma membrane, PI3Ks phosphorylate phosphatidylinositol lipids at the 3 positions, generating lipid species like phosphatidylinositol-3,4 biphosphate and phosphatidylinositol-3,4,5-triphosphate (Vasan and Cantley, 2022). These phosphoinositides recruit proteins containing the Pleckstrin homology domain, such as the serine/threonine kinase **Akt,** which facilitates downstream mTOR pathway activation. Interestingly, the main function of mTORC2 is the activation of Akt in growth factor signaling (Liu and Sabatini, 2020). For functional growth factor signaling, Akt is phosphorylated at Serine 308 by the phosphoinositide-dependent kinase PDK1. To acquire its fully active state however, Akt needs an additional phosphorylation at Serine 473, which strikingly was shown to be facilitated by mTORC2 (Sarbassov *et al.*, 2006). mTORC2 thus positively regulates Akt and like Akt itself, depends on upstream PI3Ks. The mSin subunit of mTORC2 was shown to also contain a Pleckstrin homology domain (Liu *et al.*, 2015). mTORC2 is thus mainly a member of the PI3K pathway of growth factor signaling, while nutrients are sensed by mTORC1. Activation of mTORC1 however also critically depends on the growth factor activation of PI3K-Akt (Liu and Sabatini, 2020). Upon dual S308 and S473 phosphorylation, Akt is able to leave its activation site at the plasma membrane and phosphorylate PRAS40 and **TSC2**, a central negative regulator of mTORC1.

The PI3K pathway is one of the most commonly mutated pathways in human cancers. Besides several PI3 kinases, the phosphoinositide phosphatase PTEN, a negative regulator of the PI3K-Akt pathway, is actually the second most mutated gene in cancers, after p53 (Vasan and

Cantley, 2022). Elevated growth factor levels come with an increased cancer risk and promote cancer cell proliferation. The PI3K-Akt also promotes expression of plasma membrane channels, that allows the import of glucose into the cell (Manning and Cantley, 2007). The PI3K pathway not only controls cell proliferation at the level of growth regulation and nutrient acquisition, but also controls cell division, cell survival, migration and many functions (Vasan and Cantley, 2022). The regulation of PI3K-Akt dependent processes independent of mTORC1 explains why cell proliferation in cancer cannot universally inhibited with Rapamycin (Benjamin *et al.*, 2011). Moreover, inhibition of mTORC1 causes feedback loops, that can further increase PI3K-Akt signaling (Hsu *et al.*, 2011). A disorder that is caused by mutations of an mTORC1 regulator downstream of PI3K is **Tuberous Sclerosis Complex (TSC).** This autosomal hereditary disease is characterized by tumor growth in many organs and severe cognitive disability (Henske *et al.*, 2016). mTORC1 is constantly hyperactivated in TSC, due to loss of function of the trimeric TSC1/TSC2/TBC1D7 complex (Liu and Sabatini, 2020). The regulatory target of the TSC complex is the small GTPase **Rheb**. Rheb is a small GTPse of the Ras-superfamily, which is widely distributed around cellular endomembranes, due to a membrane binding lipid anchor. TSC2 acts as GAP (GTPase activating protein) towards Rheb, inhibiting it by suppressing its active GTP bound state (Inoki *et al.*, 2003). Upon phosphorylation of TSC2 by Akt at Serine 939 and Threonine 1462, this inhibition gets released, which allows interaction of Rheb and the core mTORC1 complex. Although not a part of the mTORC1 complex itself, active GTP-bound Rheb is an essential accessory co-activator of mTORC1, required for its function. Rheb directly stimulates mTORC1 kinase activity by inducing a conformational change (Long *et al.*, 2005).

The TSC complex is major regulatory hub, which is able to sense multiple inputs affecting mTORC1 activity. The energy stress sensing kinase **AMPK (**Adenosine monophosphate-

activated protein kinase) phosphorylates TSC2 at residues Serine 1345 and Threonine 1227 to increase its activity and suppress mTORC1 during energy stress (González *et al.*, 2020). By promoting catabolic functions, AMPK is a major antagonist of mTORC1 and has been shown to additionally suppress mTORC1 through phosphorylation of conserved Serine residues 722 and 792 on Raptor (Gwinn *et al.*, 2008). Other conditions leading to mTORC1 inactivation through the TSC2 complex include hyperosmotic and pH stress (Plescher, Teleman and Demetriades, 2015; Demetriades, Plescher and Teleman, 2016). Many mTORC1 dependent downstream processes have been identified in TSC2 knock-out cells (Düvel *et al.*, 2011; Ben-Sahra and Manning, 2017b). However, amino acid sensing by mTORC1 is at least partially functional in TSC2 KOs and was thus assumed to depend on a PI3K-Akt-TSC2 independent pathway (Smith *et al.*, 2005). In the end, the mechanism that signals amino acid sufficiency to mTOR could only be resolved after the discovery of the **Rag GTPases**, a group of mTOR pathway regulators that play a major role in the nutrient activation axis (Sancak *et al.*, 2008).

1.2.2. The Rag GTPases are central regulators of mTORC1

The amino acid dependent activation was shown to operate by a unique mechanism, strikingly changing the subcellular localization of mTOR (Sancak *et al.*, 2008). The mTORC1 complex translocates from a still unknown compartment to the lysosomal surface upon amino acid stimulation (Sancak *et al.*, 2010). The withdrawal of amino acids on the other hand, not only causes rapid dephosphorylation of mTORC1 substrates, but also a loss of the lysosomal mTORC1 accumulations. This change of subcellular localization is dependent on the interaction of the mTORC1 component Raptor with a dimer of Rag GTPases, which are a subfamily of Ras-superfamily small GTPases with highly untypical features (Kim and Kim, 2016). They lack the characteristic lipid anchor and instead bind peripherally to the lysosomal surface, interacting with the lipid-anchored lysosomal **LAMTOR** complex. There are 4 human

genes encoding for Rag GTPases, RagA, RagB, RagC and RagD. They form obligate heterodimers of smaller Rag A or B monomers and larger Rag C or D monomers. Importantly, the GDP/GTP binding status of the Rag GTPases turned out to directly respond to amino acid availability (Sancak *et al.*, 2008). The active Rag dimer consists of a GTP-bound RagA/B and a GDP-bound RagC/D monomer. Upon amino acid withdrawal, the nucleotide binding status rapidly switches to the reverse configuration, with a GDP-bound RagA/B and a GTP-bound RagC/D monomer, which causes loss of Raptor binding and lysosomal mTOR (Tsun *et al.*, 2013). This fast, dynamic and cell-autonomous mechanism facilitates direct nutrient activation of mTOR by amino acids. The Rag GTPases do not alter mTOR kinase activity, but recruit it to the lysosomal surface to interact with membrane-bound Rheb. If mTORC1 is artificially tethered to the lysosomal surface, mTORC1 kinase activity will become insensitive to withdrawal of amino acids, but not growth factors (Sancak *et al.*, 2010). With Rheb additionally responding to the PI3K-TSC axis, mTORC1 regulation is thus regulated by two separate axes, both required for an active lysosomal mTORC1 complex. This model of a 'coincidence detector' explained the different inputs of growth factors and amino acids (Groenewoud and Zwartkruis, 2013). More recent studies provide evidence for significant crosstalk between the two branches however, including a role of TSC2 in nutrient signaling as well as mTORC1 dependent RagC phosphorylation upon growth factor stimulation (Demetriades, Doumpas and Teleman, 2014; Yang *et al.*, 2018).

The mechanism that leads to the rapid change of the Rag GTPases' nucleotide states was shown to depend on guanosine activating proteins (GAPs). The GAP that regulates the nucleotide binding state of the small RagA/B monomer was shown to be a complex of three cytosolic proteins Nprl2, Nprl3 and Depdc5, which was named the **GATOR1** complex (Bar-Peled *et al.*, 2013). By activating the GTP hydrolysis of the smaller RagA/B monomer, the GATOR

complex is able to suppress mTORC1 activity. A second, pentameric complex was identified as a negative regulator of GATOR1 and called **GATOR2**. GATOR2 binds to GATOR1 upon amino acid stimulation, thereby releasing the inhibition of the Rag GTPases (Bar-Peled *et al.*, 2013). Due to the unusual dimeric form, a second GAP was discovered to activate on the Rag dimer in parallel to GATOR1. **Folliculin-FNIP2 (FLCN-FNIP)** was identified as the GAP for the larger RagC/D monomer, which is needed for its activation, considering that the active form contains the GDP nucleotide (Tsun *et al.*, 2013). The presence of two major complexes has raised major interest in the regulation of the Rag GTPase nucleotide cycling between the different states. The GATOR1-GATOR2-axis seems to be dominant over the FLCN-FNIP2 axis and GATOR1 seems to be required for recruitment of FLCN-FNIP to the lysosomal Rag dimer (Meng and Ferguson, 2018a). Both, the structures of the Rag-binding GATOR1 complex and the FLCN-FNIP2 complex have been resolved (Shen *et al.*, 2018; Lawrence *et al.*, 2019). Upstream regulators and interactors of the GATOR1 and Folliculin-FNIP axes in turn, have uncovered a huge upstream network of cytosolic and lysosomal regulators unveiling a Rag GTPase-centric signaling network (Fernandes and Demetriades, 2021). The sensor function of mTORC1 has been linked to the Rag GTPases almost exclusively.

Similar to loss of function mutations in the growth factor signaling branch, mutations in the Rag GTPase dependent nutrient signaling branch have been associated with mTOR-dependent disease phenotypes. GATOR1 inactivating mutations have been found in human cancer cell lines, which showed high mTORC1 activity, uncoupled from amino acid sensing activity (Bar-Peled *et al.*, 2013). Interestingly, mutations in GATOR1 have been linked to focal epilepsy, suggesting a role of mTOR regulation in neurons (Peng, Yin and Li, 2017). Folliculin had been identified as a locus for the Birt-Hogg Dubé syndrome years before it had been connected to mTOR (Dal Sasso *et al.*, 2015). Another link between cancer and the regulatory components

of the amino acid sensing branch was discovered in Follicular Lymphoma cancer cells (Okosun *et al.*, 2016). Genome-wide association studies (GWAS) have uncovered recurrent mutations in the GTP/GDP binding domain of RagC, which were shown to lock RagC in the active state. Follicular Lymphoma mutants were shown to activate mTORC1 kinase activity. Interestingly, similar GTPase activating mutations have been found in RagD and linked to a novel type of hereditary disease, affecting cardiomyocytes and kidney tubules (Schlingmann *et al.*, 2021). Unlike the widespread mutations in the PI3K pathway that cancer cells acquire to be able to proliferate, mutations of the Rag signaling network seem to be more cell type specific.

1.2.3. The substrates of mTORC1

The central role of the lysosomal Rag GTPases was discovered a decade after confirmation of the amino acid dependent regulation of 4EBP1 and S6K dependent translation (Hara *et al.*, 1998). Both, 4EBP1 and S6K are not lysosomal but cytosolic proteins, however (Liu and Sabatini, 2020). The ribosomes are the site of translation, thus mTORC1 should be expected to phosphorylate its substrates in their proximity, which would mean cytosolic or ER localization. This discrepancy is remarkable, since it is the Raptor component involved in Rag GTPase binding and lysosomal translocation that also enables mTORC1 substrate specificity (Liu and Sabatini, 2020). Raptor facilitates a protein-protein interaction that is vital for the selection of mTORC1 substrates. S6K, 4EBP1 and a variety of other mTORC1 substrates contain the **TOS**-motif (**TO**R **S**ignaling), which allows them to bind to the Raptor-containing mTORC1 complex to be phosphorylated (Schalm *et al.*, 2003). In 4EBP1, phosphorylation causes a dissociation of the small inhibitory protein from the eukaryotic translation initiation factor eIF4E. Interestingly mTOR causes several sequential phosphorylation events, first on Threonine 37 and Threonine 46, then on Serine 65 (Böhm *et al.*, 2021). The TOS-motif containing S6K was shown to be a cytoplasmic protein phosphorylating a ribosomal substrate S6 (Blommaart *et al.*,

1995). The role of phosphorylated S6 protein at the ribosome has remained mysterious however (Liu and Sabatini, 2020). The autophagy initiating kinase ULK1 on the other hand does not contain a TOS-motif (Dunlop *et al.*, 2011). ULK1 phosphorylates various substrates to initiate the early stages of autophagy. mTOR phosphorylation of ULK1 negatively regulates autophagy, preventing the formation of the Beclin1-Vps34-complex needed for downstream phosphoinositide phosphorylation required for autophagy (Park *et al.*, 2016). ULK1 localizes to punctae, thought to be early autophagosome formation sites upon amino acid starvation. The exact site of its rather diffuse localization in nutrient sufficiency and thus mTORC1 phosphorylation remains unknown however (Dite *et al.*, 2017). The MiT-TFE transcription factors **TFEB and TFE3** on the other hand, show a dynamic shuttling between nucleus and cytosol, which is directly controlled by mTORC1 (Settembre *et al.*, 2012). Unlike all other mTORC1 substrates, TFEB and TFE3 bind directly to the Rag GTPases, which allows their phosphorylation at the lysosome. TFEB is phosphorylated by mTORC1 at Serine residues Serine 142 and Serine 211. Phosphorylated TFEB binds to 14-3-3 proteins, which retain it in the cytosol, suppressing its activity. TFEB and TFE3 have been shown to control lysosome biogenesis, with a high degree of redundancy (Puertollano *et al.*, 2018). Concerning lysosomal mTORC1 substrates, TFEB will be considered as an example for MiT-TFE transcription factors (see 3.1). TFEB and TFE3 have been recently implicated in **substrate specific mTORC1 mechanisms**, involving differential regulation of cytosolic and lysosomal substrates. Their phosphorylation by mTORC1 depends exclusively on the Rag GTPases and FLCN-FNIP and not on growth factors and Rheb, like it is the case for cytosolic substrates (Napolitano *et al.*, 2020).

1.2.4. Inhibitors of mTOR

Rapamycin and the first generation of Rapamycin-derived drugs, the **Rapalogs** have received admission for clinical use. Although effective in the treatment of metastatic renal cell cancer, their overall impact in cancer therapy has been modest (Laplante and Sabatini, 2012). Besides the selectivity of Rapamycin for mTORC1, a major limitation for the antiproliferative effects of Rapamycin is the Rapamycin resistance of the 4EBP1 substrate. Although 4EBP1 phosphorylation at Serine 65 is sensitive to Rapamycin, this is not the case for Threonine 37 and Threonine 46 (Böhm *et al.*, 2021). The limitations of Rapamycin led to the development of second generation mTORC1 inhibitors, which are ATP-competitive inhibitors of the mTOR kinase activity (Thoreen *et al.*, 2009). Another approach was the development of dual-inhibitors for PI3K and mTOR (Benjamin *et al.*, 2011). The competitive inhibitor **Torin** is widely used in research and of huge value for investigating the effects of complete mTOR inhibition (Thoreen *et al.*, 2009). Despite their ability to inhibit both, mTORC2 and 4EBP1, the use of second-generation inhibitors in the clinic is likewise limited. Unlike Rapamycin, they tend to inhibit other kinases of the PI3K family, which, in combination with their higher effective dose, causes toxic side-effects and decreases their therapeutic index (Thoreen, 2021) Although there are ongoing clinical studies with ATP-competitive inhibitors, the Rapalogs remain the most widely used class of mTOR inhibitors in the clinic (Rodrik-Outmezguine *et al.*, 2016).

Another use of Rapamycin and Rapalogs would be as a treatment for metabolic disease or even as anti-aging drugs. However, several potential side-effects have to be taken into consideration. For once, a limitation for the treatment of age-related disease is immunosuppression. Besides, chronic rapamycin treatment has been shown to cause other side effects, including glucose intolerance, insulin resistance and a deregulated lipid metabolism (Kennedy and Lamming,

2016). Interestingly these adverse metabolic effects have been shown to be mTORC2 dependent (Lamming *et al.*, 2012). Although Rapamycin insensitive, the mTORC2 complex tends to be destabilized during chronic Rapamycin treatment (Sarbassov *et al.*, 2006). The compromised Akt activation impairs the cells' ability to import glucose. A novel approach that could circumvent some of these limitations led to the third generation of mTORC1 inhibitors or **Rapalinks** (Rodrik-Outmezguine *et al.*, 2016). These novel inhibitors connect rapamycin to an ATP-competitive mTOR inhibitor with a linker. The bivalent molecule retains the specify for mTORC1, yet combined with full inactivation of the kinase activity by the ATP-competitive inhibitor. Newly developed Rapalinks indeed show very high specificity for mTORC1 at concentrations that do not destabilize mTORC2, while being able to fully suppress 4EBP1 phosphorylation (Lee *et al.*, 2021).

The development of third generation mTOR inhibitors was partly guided by the rationale to increase their effectiveness to derepress 4EBP1, thereby increasing specificity for one downstream pathway of mTORC1. This approach might yield even more specific inhibitors in the future. Another pathway downstream of mTORC1 is the autophagic-lysosomal pathway. A component that derepresses autophagy might show potential in treating neurodegeneration (Ballabio, 2016). Interestingly, some novel approaches aim at specifically targeting lysosomal mTORC1 substrates. Overall, the appreciation of the central role of mTORC1 at lysosomes was the key to understanding the nutrient-dependent mTOR network. Thus, the central role of lysosomes in the cell biology of mTORC1 will be discussed.

1.3. Cell biology of mTORC1

1.3.1. Lysosomes

Lysosomes are single membrane organelles with a low luminal pH that derive from the secretory pathway. Their main function is degradation of complex biomolecules, including proteins, glycoproteins, glycans but also entire organelles or intracellular parasites. Lysosomes mostly receive their cargo from endocytic vesicles and most importantly, from autophagosomes. In order to degrade a multitude of different biomolecules, lysosomes contain a large variety of **acidic hydrolases** such as proteases, glycosidases, lipases and nucleases, which catalyze hydrolytic reactions in the acidic lysosomal lumen (Lim and Zoncu, 2016). The targeting of proteins to the lysosomal lumen depends on the **Mannose-6-phosphate** pathway. Proteins are post-translationally modified by addition of Mannose-6-phosphate in the Golgi apparatus, allowing Mannose-6-phosphate receptor (MPR)-facilitated vesicle transport to the lysosomal lumen (Lim and Zoncu, 2016). The low luminal pH of 4,5 - 5,5 is maintained by ATP-dependent import of protons through **V-ATPase,** an integral protein complex of the lysosomal membrane. The pH-gradient over the lysosomal membrane is also required for some of the lysosomal membrane transporters, allowing exchange of ions and small organic molecules in and out of lysosomes. Lysosomes have been thoroughly studied, especially in the context of **lysosomal storage disease (LSD**) (Huizing and Gahl, 2020). Most LSD originate from mutations in genes encoding lysosomal hydrolases. Loss of lysosomal enzyme function can cause lysosomal accumulation of unprocessed metabolites (Ballabio, 2016). These toxic accumulations can be treated by enzyme replacement therapy (ERT), where recombinant lysosomal enzymes are given to patients. Among other symptoms, patients with LSDs often show neurological deficiencies (Ballabio, 2016). The role of lysosomal function in neurons is especially relevant beyond LSD. The clearance of toxic protein aggregates like Huntingtin, Tau

or alpha synuclein through autophagy has been suggested as a strategy to alleviate neurogenerative disease (Klionsky *et al.*, 2021).

A potential way to increase lysosome function could be to target the transcription factor **TFEB** (Sardiello *et al.*, 2009). TFEB has been identified as a master regulator of lysosomal biogenesis and function. Many genes encoding lysosomal components and enzymes are regulated by the CLEAR motif palindromic 10–base pair (bp) GTCACGTGAC motif which TFEB and the closely related transcription factor **TFE3** bind to. TFEB activation was observed in Lysosomal Storage Disease and suggested to be a regulatory mechanism of an **autophagic-lysosomal pathway** in response to lysosomal challenges. Strikingly, TFEB overexpression has been shown to alleviate the amount of accumulated substrate in disease models of neurodegenerative disease, where it induces clearance of protein aggregates (Decressac *et al.*, 2013; Lim and Zoncu, 2016).

The view of lysosomes has recently changed from organelles involved only in degradation, to what has been called a 'signaling platform' (Lim and Zoncu, 2016). The presence of the LAMTOR-Rag GTPase machinery at the lysosomal surface puts mTORC1 at the center of signaling at the lysosomal surface. The Rag GTPase dependent regulation of TFEB was a breakthrough finding, especially considering that unlike S6K or 4EBP1, TFEB is recruited to the lysosomal surface to be phosphorylated by mTORC1 (Settembre *et al.*, 2012). Interestingly, an additional mechanism that causes TFEB activation by dephosphorylation has been shown to depend on lysosomal calcium release through the protein mucolipin-1 (MCOLN1/TRPML1). Calcium release activates the phosphatase calcineurin, which dephosphorylates TFEB at Serine 142 and 211. This mechanism is assumed to be activated during starvation conditions and interestingly, upon physical exercise (Medina *et al.*, 2015).

Furthermore, this activation is independent of mTORC1 phosphorylation. Finally, there have been reports showing that the major mTORC1 antagonist AMPK is also activated at the lysosomal surface in a LAMTOR-dependent mechanism, in response to glucose starvation and metformin (Ma *et al.*, 2022).

1.3.2. The mTORC1 nutrient sensors:

The localization of mTORC1 at lysosomes has been assumed to be connected to the autophagic-lysosomal pathway (Sancak *et al.*, 2010). Autophagy leads to the production of free amino acids, as a result of lysosomal protein degradation. Lysosomal amino acids are released to the cytosol for protein synthesis and mTORC1 reactivation. mTORC1 activity and autophagy have indeed been shown to oscillate during phases of longer starvation (Yu *et al.*, 2010). Reactivation of mTORC1 was first assumed to work through an **inside-out** mechanism. Amino acids accumulate within the lysosome, where their presence is sensed and causes the Rag GTPases on the lysosomal surface to switch to the active state. Amino acid activation was reported to be dependent on V-ATPase and a later report implicated LAMTOR as a guanosine nucleotide exchange (GEF) and activator factor of RagA/B (Zoncu *et al.*, 2011; Bar-Peled *et al.*, 2012). Although these components were later confirmed to be necessary for Rag GTPase activation, they are not sufficient for inside-out sensing, since none of them bind directly to amino acids. Another lysosomal transmembrane protein, the solute like carrier protein **SLC38A9** was eventually demonstrated to contain a binding site for the amino acid **arginine** (Rebsamen *et al.*, 2015). SLC38A9 is an amino acid transporter, which not only transports arginine, but other essential amino acids derived from lysosomal autophagy (Wyant *et al.*, 2017). The mechanism of SLC38A9 was demonstrated to be direct regulation of the Rag GTPase nucleotide state (Shen and Sabatini, 2018). This confirmed SLC38A9 and the Rag GTPases as essential components needed for the local reactivation of mTORC1 pathway.

Strikingly, SLC38A9 together with NPC1 also senses cholesterol by direct protein-metabolite interaction, thereby extending the Rag GTPase nutrient sensing to lipids (Castellano *et al.*, 2017)

In addition to the SLC38A9, cytosolic amino acid sensors connect amino acid levels and the Rag GTPase nucleotide state through the GATOR1-GATOR2 axis. The inhibitory GATOR1 complex can be recruited to the lysosomal surface by another complex named KICSTOR (Wolfson *et al.*, 2017). During nutrient sufficiency however, GATOR2 binds to GATOR1 to suppress it and prevent its inhibitory action GAP activity towards the Rag GTPases. The interactome of the cytosolic GATOR2 protein has uncovered a cytosolic amino acid sensing network. CASTOR1 is an inhibitor of GATOR2, which like SLC38A9 directly binds to arginine (Chantranupong *et al.*, 2016). Upon arginine binding, CASTOR1 dissociates from GATOR2 enabling it to activate mTORC1 by GATOR1 suppression. A similar mode of action was described for **Sestrin2**, which was characterized as a GATOR2 dependent **leucine** sensor (Wolfson *et al.*, 2016). Incidentally, leucine and arginine had been reported to be strongest activators of mTORC1 activity among all amino acids almost two decades earlier. Very recently, another leucine sensor (**SAR1B**) has been shown to similarly inhibit GATOR2 (Chen *et al.*, 2021).

Other amino acid sensors have been described, most notably SAMTOR, which instead of directly binding to methionine, signals the levels of its metabolite S-adenosyl-methionine (SAM) to GATOR1 (Gu *et al.*, 2017). Also, there have been reports of amino acid sensors that bind to the Rag GTPases but not GATOR1 (Han *et al.*, 2012; Kim *et al.*, 2021). Finally, there have been various reports of **Rag GTPase-independent mechanisms**, of amino acid sensing. Glutamine, for example has been shown to facilitate lysosomal mTORC1 recruitment via the

small GTPase Arf1, independently of LAMTOR (Jewell *et al.*, 2015). Overall, the appreciation of the central role of lysosomes, was the key to understanding the nutrient-dependent mTOR network. Strikingly however, the most central components of this network, the Rag GTPases themselves have still not been sufficiently investigated.

1.3.3. Rag paralogs

There have been breakthrough discoveries concerning amino acid sensing in the network upstream of the Rag GTPases. Yet, a central feature of the Rag GTPase pathway has not been thoroughly investigated. In yeast and even *Drosophila melanogaster*, there is only one small Rag GTPase and one large Rag GTPase monomer each (Kim and Kim, 2016). Unlike invertebrates however, the mammalian genome encodes for 4 Rag GTPase genes. The mammalian Rag GTPases are the result of two gene duplications in mammalian evolution. Gene duplication events often allow one paralog to mutate freely and acquire specific functions. In the case of the Rag GTPases, the paralogs still have a very high sequence homology (Kim and Kim, 2016). The amino acid sequences of the two paralogs of the smaller RagGTPase monomers, RagA and RagB are 97,1 % identical. The Rag paralogs RagC and RagD share a sequence identity of 81,1%. The paralogs are traditionally considered redundant, yet there are a number of reports that suggest otherwise. One key phenomenon has been described in several independent reports and yet never been systematically investigated: mTORC1 regulators are identified as Rag GTPase interacting proteins, but show preference for one Rag GTPase paralog over the other. For instance, RagA, but not RagB was shown to bind to TSC2, which would explain its role in amino acid sensing (Demetriades, Doumpas and Teleman, 2014). Concerning the GATOR1 complex, the component NPRL2 has been shown to preferentially bind to RagD, which could have implications for the Rag GTPase nucleotide binding state (Kwak *et al.*, 2016). Moreover RagD, but not RagC was reported to interact with

Leucyl-tRNA synthetase (LARS) which has been reported to be a Folliculin-independent GAP for RagD in leucine sensing (Han *et al.*, 2012). Interestingly, amino acid binding by an aminoacyl-tRNA synthetase would provide a direct mode for the metabolite-protein interaction. Incidentally, it was also reported that Threonine is sensed by Threonyl-CoA synthetase (TARS), through a binding mechanism involving preferably RagC (Kim *et al.*, 2021). These paralog-specific interactions might define novel branches of nutrient signaling. They also could be very relevant for different cell-type specific differences in amino acid sensing, since Rag paralog expression is cell type-specific (Kim and Kim, 2016). Depending on Rag paralog expression, the composition of active Rag GTPase dimers, localized at the lysosome might determine the cell type-specific response to nutrients.

1.4. Aims of this thesis

In the context of my PhD thesis, I decided to investigate the question of redundancy between the Rag paralogs experimentally. Instead of following up on previously reported Rag GTPase interactors, a more systematic approach was pursued, using an established model system. The cell line HEK293FT, had previously been used for investigating cell-autonomous mTORC1 recruitment to the lysosome in response to amino acid stimulation. I used CRISPR-Cas9 to generate a Rag GTPase quadruple knock-out (**QKO**) cell line. This loss of function model is used for reconstitution studies. Cell lines rescued with the four possible dimer combinations of Rag GTPase paralogs are tested in functional mTORC1 assays like mTOR localization and mTORC1 substrate phosphorylation. It is thus investigated, whether amino acid signaling through mTORC1 is regulated at the level of Rag GTPase dimer composition. To understand the mechanism of mTORC1 regulation by Rag paralogs, interactions with key mTORC1 components are analyzed. To understand the functional consequences, cellular downstream

processes like lysosome biogenesis are investigated. Finally, cancer-associated mutants are tested to investigate their mode of action.

1.5. Statement of contribution

The format of my PhD thesis is a monography including the publication: 'A Rag GTPase Dimer Code Defines the Regulation of mTORC1 by Amino Acids.' by authors: Peter Gollwitzer, Nina Grützmacher (equal contribution), Sabine Wilhelm, Daniel Kümmel, and Constantinos Demetriades. The publication was featured in the scientific journal *Nature Cell Biology*, Volume 24 Issue 9, September 2022. The publication is included in full length with all extended data.

The publication is the immediate outcome of my main PhD project with the working title: 'Non-redundant functions of the Rag GTPase dimers in Amino Acid sensing'. I have been working on the question, whether the Rag GTPase are functionally redundant or not since the beginning of my PhD in June 2017. I presented and discussed the main hypothesis and experimental strategy in my early project proposal, my TAC meetings and reports and on the occasion of lab meetings and retreats. In exchange with my supervisor, Dr. Constantinos Demetriades and my TAC, I made the decision to investigate this question by generating stable, genetically modified cell lines. I believed that major technical limitations, that previously prevented me from testing the main hypothesis, could be overcome by the use of monoclonal cell lines. Using this approach, I generated the data that supported the non-equivalence of Rag GTPase dimers and drew the main conclusions that are included in the publication.

I worked in close co-operation with my colleague Nina Grützmacher to generate the data for the publication. My contribution includes a majority of the genetics and cell culture work in the publication. I generated knock-out cell lines and designed CRISPR-Cas9 vectors and sequencing primers for validating those knock-outs. Moreover, I generated stable, monoclonal

cell lines and tested if they express near-equal levels of the HA-tagged Rag GTPases. Aided by my colleague Sabine Wilhelm, I spent considerable amounts of time expanding and freezing several clones of every reconstituted cell line in the publication. I also performed the cloning of the majority of expression vectors and plasmids used in the publication, including the cloning of vectors for reconstitution with HA-tagged Rag GTPases by puromycin selection. Moreover, I cloned vectors for expressing chimeric Rag GTPases and Rag GTPases containing cancer-associated mutants.

I developed several tools for this project, mainly by modifying established protocols. I had the idea for the 'LysoRag IP' experiment in Figure 3c/d, which I performed together with Nina Grützmacher based on a modified LysoIP protocol. I established Co-Immunoprecipitation of FLAG-tagged p18 and HA-tagged Rag GTPases as an assay for the Rag-LAMTOR interaction. Together with Nina Grützmacher, I generated the IP data in Figures 3e and 4f and the Supplementary Figures. I selected the TFE3 targets UAP1L1 and GPNMB from unpublished RNA sequencing data from our group. I generated the confocal imaging data in the publication and drew regions of interest (ROIs) for Co-Localization analysis. Of the Western blot data, I generated the data for the Figures: 1b, 3c, 3e, as well as ED1d and ED3b. Finally, I gave my thoughts and comments to the text parts of the manuscript, written by Dr. Constantinos Demetriades.

2. Results

The results of this PhD thesis are described in the following publication: Gollwitzer P, Grützmacher N, Wilhelm S, Kümmel D, Demetriades C.

A Rag GTPase dimer code defines the regulation of mTORC1 by amino acids.

Nat Cell Biol. 2022 Sep;24(9) :1394-1406.

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PMCID: PMC9481461

3. Discussion

3.1. The key results concerning Rag paralogs were confirmed independently

We report here that contrary to the previous belief, the Rag GTPases are not redundant, but have non-equivalent functions. Strikingly, it is two different functions of the canonical Rag GTPases, that depend on the presence of different Rag GTPase paralogs in a functional Rag GTPase dimer. While the difference between RagC and RagD affects substrate specificity, the difference between RagA and RagB affects the response to amino acid starvation. Importantly, in parallel to our study, another report by Figlia et *al*. independently confirmed our main results (Figlia *et al.*, 2022). Their study contained reconstitution experiments of RagGTPase double knockout (**DKO**) HEK293T cells, instead of our quadruple knock-out **QKO.** Due to the obligate heterodimerization of small and large Rag GTPases, a double knockout of either category is sufficient however, to fully abrogate Rag GTPase function. Strikingly, in RagC/D double knockouts, reconstitution with RagD leads to a stronger rescue of TFEB phosphorylation compared to RagC. This result strengthens our confidence in the finding that the main function of RagD is facilitating the mTORC1-dependent phosphorylation of TFEB and TFE3 substrates. Interestingly, RagD has been identified as a transcriptional target of TFEB, a few years ago (Di Malta *et al.*, 2017). An upregulation of RagD levels by TFEB would provide an autoregulatory feedback loop, limiting TFEB activity in the nucleus. The authors of this study reported that transcriptional upregulation of RagD increased general mTORC1, using S6K as a substrate. Indeed the TFEB-RagD feedback loop has been observed by others, yet under the assumption of a general upregulation of mTORC1 activity (Li *et al.*, 2019). Neither we, nor Figlia and colleagues observed increased S6K phosphorylation upon reconstitution with RagD when compared to RagC. Thus, the new findings suggest, that this feedback loop

has either a dual role in different cell types or is more likely part of the autophagic-lysosomal pathway controlling lysosome biogenesis.

Other than that, Figlia et al. worked almost exclusively on the discrepancy between RagA and RagB in the mTORC1 dependent response to amino acid starvation. Again, independently confirming our results, they identify the 33 amino acid N-terminal tail as the determinant of amino acid starvation resistance. Moreover, they suggest a mechanism that could answer some of the questions remaining open in our work. They demonstrate that RagB has reduced GATOR1 binding, mostly to the GATOR component DEPDC5. They hypothesize based on structural models that the N-terminus might interfere with formation of the lysosomal GATOR1-Rag GTPase complex and prevent GATOR1 from acting as a GAP. The differences in DEPDC5 binding are moderate however and Figlia et *al*. suggest that *in vivo* an additional long splice variant of RagB compromised in GTP binding additionally inhibits the GATOR1 complex. Figlia et *al*. used p-S6K as a readout which, in our RagB/D reconstituted cells still reacted to some degree to amino acid starvation, other than TFEB and TFE3. This discrepancy might be due our exclusive reconstitution with a dimer of RagB/RagD, while in their system RagB is able to dimerize with both RagC and RagD. This interpretation would be in line with our conclusions about the Rag paralogs. While the RagA vs. RagB difference affects kinetics, the RagC vs. RagD difference affects substrate specificity. While our reconstitution renders TFEB and TFE3 insensitive to amino acid starvation, Figlia et al. achieve the same for S6K. Finally, based on the high level of RagB and its longer splice variant in neurons, Figlia et al. suspect RagB to mainly play a role in the central nervous system. The inability to completely inactivate mTORC1 in response to amino acids might be of physiological relevance. A baseline mTORC1 activity in the absence of amino acids might be required to sustain essential mTORC1 functions, most likely translation or inhibition of autophagy. Interestingly,

autophagy induction has previously been shown to be suppressed in the brain, potentially in in order to preferentially supply neurons with nutrients from other tissues (Helfand *et al.*, 2003). Suppression of autophagy in neurons might be caveat for therapies aimed at inducing the autophagic-lysosomal pathway to combat neurodegeneration. A baseline level of translation on the other hand might be vital to preserve cell survival in the brain.

3.2. The mechanism of substrate selectivity depends on LAMTOR binding

The mechanism of substrate specify depends on the lysosomal localization of the Rag dimer to the lysosome. The stronger LAMTOR-binding of RagD compared to RagC is causing the increased lysosomal localization of the entire mTORC1. The shuttling of the Rag GTPase dimer on and off the LAMTOR complex has been reported before, yet has never been linked to substrate specificity (Bar-Peled *et al.*, 2012). Instead, weakening of the LAMTOR interaction has been shown to occur upon amino acid stimulation. Interestingly, the cancer mutants in the RagC GTP-binding domain have been shown to be unable to detach from the LAMTOR complex (Lawrence *et al.*, 2018). Considering that the cancer-associated mutants were originally shown to stimulate S6K phosphorylation, some of these reports seem contradictory (Okosun *et al.*, 2016). The data obtained from the reconstituted QKO cells, suggest that the cancer mutants do not affect p-S6K but phenocopy the RagD-specific effects towards TFE3 and TFEB. The looser association of RagC with lysosomes might reflect the shuttling aspect, while RagD and the cancer mutants seem to be locked at the lysosome. This suggests a mechanism involving lysosomal substrates TFEB and TFE3 in the development of Follicular Lymphoma. The effects of the point mutations might be highly specific for B-cells however and so far, there seems to be no indication for a causal role of TFEB or TFE3 phosphorylation in Follicular Lymphoma (Ortega-Molina *et al.*, 2019).

If the cancer-mutants are locked in the active, GDP-bound state, does that mean that RagD is also constantly GDP-locked? The data from the Co-IP experiments of FLAG-tagged Rag GTPases clearly suggests otherwise. RagC and RagD have similar affinities for mTOR and Raptor, while the cancer mutants were shown to have strongly increased Raptor binding (Okosun *et al.*, 2016). Also, the RagC/D GAP Folliculin binds with similar affinities, arguing against a constitutive activation of RagD. The only exception seems to be LAMTOR, which implies that RagD simply changes localization, but not its nucleotide state. Interestingly, a recent report might still suggest some secondary effects on RagD nucleotide state regulation. LAMTOR was originally described as a GEF, that activates the smalls Rag GTPases RagA and RagB (Bar-Peled *et al.*, 2012). However, a mostly overlooked publication from the same group clarified that is incorrect and was caused by a misinterpretation of their data (Shen and Sabatini, 2018). Instead, the LAMTOR complex acts as a so-called 'non-canonical GEF', which destabilizes the GTP-bound inactive form of RagC/D, thus acting by a completely different mechanism. Indeed, the authors claim that LAMTOR has a similar activating effect on RagC/D as the GAP Folliculin. GDP-loading of RagC/D has been shown to be the key activator of TFEB phosphorylation, which might be achieved for RagD through LAMTOR binding (Li *et al.*, 2022).

3.3. Lysosomal and non-lysosomal mTOR are regulated differently

Finally, the RagA/C reconstituted cells suggest that an mTORC1 complex with decreased lysosomal localization is still equally active when it comes to S6K-phosphorylation. This is in clear contradiction to the model of amino acid dependent mTORC1 activation on the lysosomal surface. Nevertheless, recent concepts of substrate-specific mTORC1 pathways might help to understand of this observation. While the active RagC/D nucleotide state is strongly inhibitory towards TFEB activity, its effect on S6K phosphorylation is minimal, as can be seen with the

cancer mutants. The only aspect, besides p-TFEB, that it equally boosts is mTOR localization. Several recent publications have demonstrated that the opposite effect can be achieved by knock-down of the RagC/D GAP Folliculin, which leads to TFEB-dephosphorylation without affecting S6K (Wada *et al.*, 2016; Napolitano *et al.*, 2020). The loss of function of folliculin causes Birt-Hogg-Dubbé syndrome, and constantly nuclear TFEB has been shown to cause major phenotypes of this disease. It is more and more accepted that the Rag GTPases are part of an alternative mTORC1 pathway downstream of Folliculin.

This Folliculin-Rag-TFEB pathway apparently needs a tight balance. While its inactivation causes Birt-Hogg-Dubbé syndrome, its hyperactivation is associated with Follicular Lymphoma. Despite many unknowns, there are attempts to pharmacologically target it. Nuclear translocation of TFEB could alleviate neurodegenerative disease by boosting autophagic-lysosomal pathway. One suggestion would be to develop a drug that destabilizes the active folliculin complex (**AFC**), thus preventing Folliculin to act on RagC/D and promoting nuclear TFEB translation (Hurley, 2022). Interestingly, novel pharmacological agents have been shown to impair Folliculin GAP activity by sequestering Folliculin to membrane sites containing the ATG8 protein GABARAP (Goodwin *et al.*, 2021). This mechanism seems to be independent of mTOR and amino acids, but appears be the very first example of an alternative endogenous mechanism upstream of Folliculin-Rag-TFEB. Critically, GABARAP recruits Folliculin to lysosomes, to form the inhibitory lysosomal folliculin complex (**LFC**), due to a newly discovered LC3-interaction (LIR)-motif. The LFC also forms during amino acid starvation, yet there have been no reports of an amino acid sensing mechanism affecting Folliculin without previously affecting RagA/B nucleotide loading through GATOR1-GATOR2 (Meng and Ferguson, 2018b).

The reduced lysosomal localization of mTORC1 in RagA/C reconstituted cell seems to diminish its response to amino acid starvation and re-addition. Importantly however, the response is only diminished, but not lost even in the complete absence of the Rag GTPases. Hence, there are not only amino acid independent mechanisms of the Rags, like GABARAP recruiting Folliculin, but also Rag-independent mechanism of amino acid sensing. Glutamine is able to activate mTORC1 through the small GTPase Arf1, however this mechanism still seems to involve lysosomes (Jewell *et al.*, 2015). The mechanisms that regulate non-lysosomal amino acid sensing by mTORC1 are currently under investigation in our group. Nevertheless, this work has shown despite almost 15 years of research, the Rag GTPases are still worth investigating.

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