Nutritional and pharmacological modes of mTOR inhibition

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vorgelegt von
Filippo Artoni
aus Bologna, Italien

Berichterstatter: Dr. Constantinos Demetriades
(Gutachter) Prof. Dr. Marius Lemberg

Prüfungsvorsitzender: Prof. Dr. Mirka Uhlirova

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Abstract

mTOR signaling is a crucial pathway involved in numerous biological and disease processes. Hyperactive mTOR signaling has been linked to several diseases including cancer, diabetes, metabolic and neurological syndromes as well as the aging process. Inhibition of mTOR signaling is an important experimental and therapeutic tool to study mTOR biology and treat medical conditions involving hyperactive mTOR signaling. Several modes of mTOR inhibition exist including pharmacological inhibitors such as rapamycin and Torin1 as well as other interventions like amino acid (AA) starvation and genetic deletion of mTOR regulators. Despite the wide use of these interventions, a comprehensive understanding of the differential effects elicited by different modes of mTOR inhibition on cellular physiology is still lacking. Moreover, researchers outside the mTOR field tend to use these interventions interchangeably to probe a variety of biological questions, which can lead to incomplete or erroneous findings.

Here, to comprehensively study the differences between different modes of mTOR inhibition, we measured the effects that four interventions (rapamycin, Torin1, AA starvation and loss of RagA/B GTPases) elicited on the cellular proteome via a combination of proteomics and systems biology approaches. Our findings reveal that different modes of mTOR inhibition, while sharing a common set of protein targets, lead to unique proteomic signatures. Specifically, we find that Torin1 regulates mRNA splicing and lipogenesis-related proteins to a much greater extent than rapamycin. Additionally, we show how modeling AA starvation with Torin1 treatment may lead to inaccurate results, especially when studying AA-induced transcriptional responses. Moreover, we show that, while rapamycin, Torin1 and AA starvation are all associated with an increase in oxidative-related stress proteins, differential regulation of mitochondrial proteins suggests that different modes of mTOR inhibition induce ROS production via distinct mechanisms. Furthermore, we show how loss of RagA/B is characterized by a unique enrichment of lysosomal and cytoskeletal proteins, while also influencing a subset of the responses to AA starvation.

Finally, an expansion of this study shows that rapamycin's effects on both the cellular transcriptome and proteome are exclusively mediated via mTOR inhibition. Ultimately, these findings expand our knowledge of different modes of mTOR inhibition, while also providing ample material for the generation of novel hypotheses in the mTOR biology field. Ultimately, this study will also enable researchers interested in studying mTOR-related processes to select the most appropriate mode of mTOR inhibition depending on their specific biological question.

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I. Introduction

1.1| Mammalian Target of Rapamycin Complex 1 (mTORC1) and its functions

mTOR (Mechanistic Target of Rapamycin) is a highly-conserved, serine-threonine kinase that acts as a master regulator of cellular growth and metabolism[1-3]. In the presence of nutrients and growth factors, mTOR promotes cell growth by activating anabolic processes such as RNA transcription, translation and lipid synthesis[1-3]. Conversely, when cells are challenged by nutrient scarcity or other stressors, mTOR represses anabolism and favors catabolic processes such as autophagy[1-3].

1.1.1 mTORC1 molecular structure

At the molecular level, mTOR acts as the main component of two distinct complexes: mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). Each complex has evolved to sense different intra- and extra-cellular stimuli and control different downstream processes via phosphorylation of multiple substrates[4]. Each complex is comprised of different proteins that act as unique components and confer each complex their specific functions.

mTORC1 is constituted by mTOR and four additional subunits: regulatory-associated protein of mTOR (RAPTOR), mammalian lethal with SEC13 protein 8 (mLST8), proline-rich AKT substrate 40 kDa (PRAS40) and DEP-domain-containing mTOR-interacting protein (DEPTOR)[2,3] (Fig. 1.1).

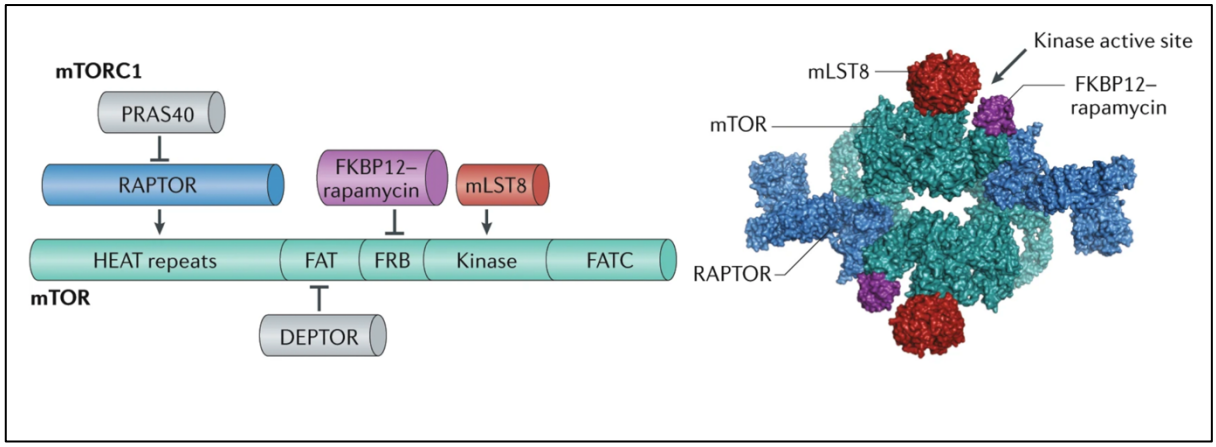


Figure 1.1 mTOR complex 1 (mTORC1) domains and structure.
Schematic representation of mTORC1 protein domains (left) and 3D structure as a dimer in its active form (right). Adapted from Liu & Sabatini, 2020.

substrate recognition via TOS (TOR signaling motifs) binding sequences, which are located on a subset of mTORC1 substrates[7]. RAPTOR knockdown via siRNA results into lowered phosphorylation levels of key mTORC1 substrate p70-S6 Kinase 1 (S6K) even when mTORC1 activity is stimulated by addition of leucine[5]. This suggests that RAPTOR plays an important role in modulating mTOR's catalytic activity in response to extracellular cues such as the presence of amino acids, which will be extensively discussed later.

RAPTOR-null cell lines and animals are not viable and therefore cannot be studied unless conditional or inducible knockouts are generated[8,9]. Tissue-specific RAPTOR knockout mice have been consistently shown to have developmental and/or metabolic abnormalities. For instance, loss of RAPTOR in preosteoblasts or skeletal muscle cells results in incomplete skeletogenesis or impaired neuromuscular junction (NMJ) stability, respectively[10,11]. Loss of RAPTOR in dontoblasts results into dentin malformation[12], while RAPTOR-deficient adipocytes show abnormal, enhanced mitochondrial respiration[13]. Taken together, the evidence indicates that RAPTOR is indispensable for proper mTORC1 functioning and plays a crucial role in mTORC1's ability to sense and respond to nutritional cues.

mLST8, another subunit of mTORC1, plays a more elusive role within mTORC1. It was initially reported to stabilize the interaction between mTOR and RAPTOR[14], though later studies showed that absence of mLST8 has no effects on mTORC1's activity[9]. Ultimately, further research will be required to establish the exact function of mLST8 within mTORC1. The other two subunits, PRAS40 and DEPTOR, have been identified as endogenous mTOR inhibitors[15,16]. PRAS40 has been shown to bind RAPTOR and inhibit S6K1 phosphorylation as well as Rheb-induced activation of mTORC1 signaling[16]. In the presence of insulin, AKT/PKB-mediated phosphorylation of PRAS40 interferes with PRAS40 binding to mTORC1 thus relieving repression of mTORC1 activity[16].

Consistently with its role of endogenous mTOR inhibitor, DEPTOR has been shown to be downregulated in most human cancers[15], which is thought to facilitate mTOR-driven cell growth and proliferation[17]. More recently, DEPTOR has been identified as a direct p53 target[18]. p53 directly binds and activates DEPTOR's promoter to repress cell

growth upon genotoxic stress. This is thought to facilitate cancer cell survival and chemoresistance. Paradoxically, high DEPTOR expression levels have been observed in multiple myeloma cells where they are thought to promote survival through continued activation of phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) signaling[15]. Taken together, these findings highlight how DEPTOR expression levels finely tune mTOR activity and affect cellular physiology in a highly context-dependent manner.

1.1.2 mTORC1's substrates

mTORC1's main substrates are the cytosolic p70-S6 kinase 1(S6K1)[19,20] and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)[21-23] as well as the lysosomal transcription factor EB (TFEB) and transcription factor 3 (TFE3)[24-26] (Fig. 1.2). Other important substrates include autophagy-related proteins 13 and 14 (ATG13/14)[27,28], unc-51 like autophagy activating kinase[27-29] (ULK1) and UV radiation resistance-associated protein (UVRAG)[30]. mTORC1 is also able to influence other processes such as lipid synthesis via Lipin1[31] and unconventional protein secretion via GRASP55[32].

S6K1 and 4E-BP1 are the main substrates through which mTORC1 controls protein synthesis[2]. mTORC1-dependent phosphorylation of S6K1 leads to activation of eukaryotic translation initiation factor 4B (eIF4B), which in turn promotes translation of mRNAs with complex 5′ UTRs via the 5′-cap-binding eukaryotic translation initiation factor 4F (eIF4F) complex[1]. Phosphorylated S6K1 (p-S6K1) also phosphorylates ribosomal protein S6 (S6), although the role of this protein in promoting translation remains more elusive. Other S6K1 targets include upstream binding factor (UBF) and transcription factor 1A (TIF-1A), both positive regulators of ribosomal RNA (rRNA) transcription[1], as well as programmed cell death protein 4 (PDCD4) whose role is

unclear, but has been observed in association with ribosomal proteins and is thought to be involved in apoptosis[33].

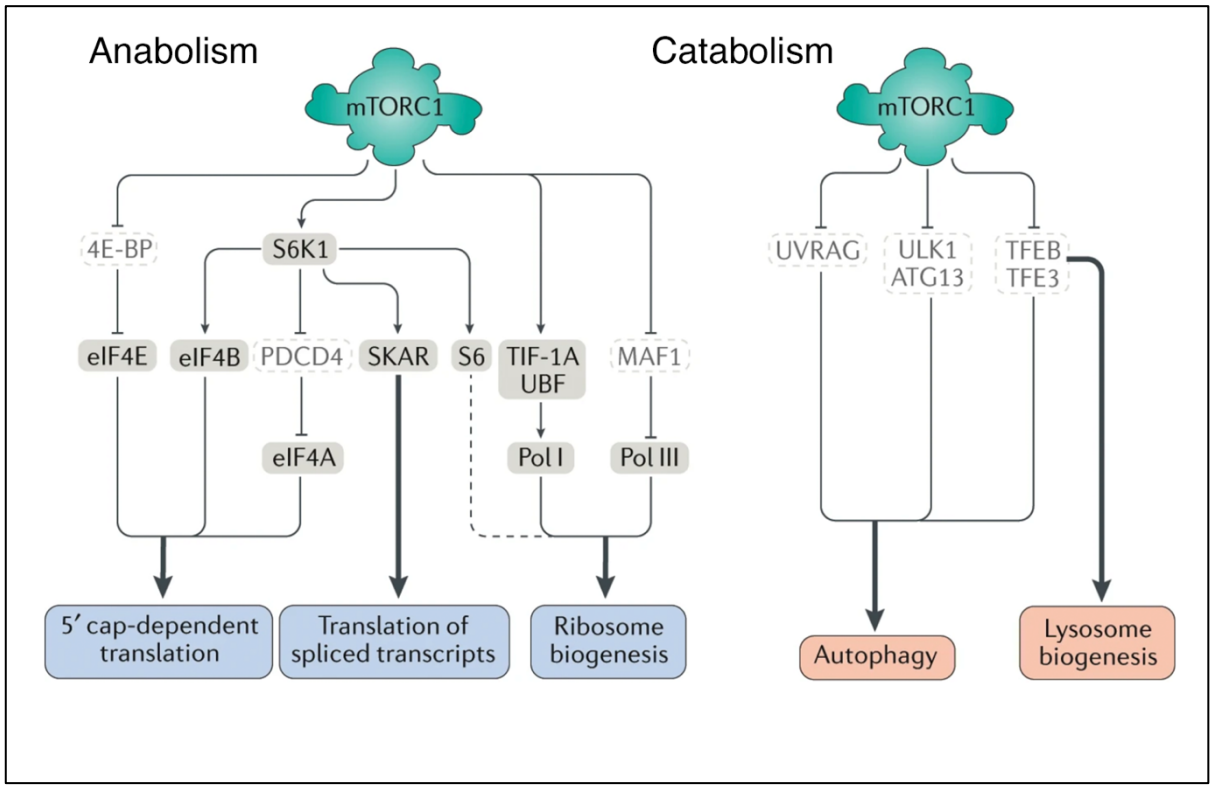


Figure 1.2 mTORC1 substrates.
Schematic representation of mTORC1 main substrates which are involved in regulating anabolic (left, blue) and catabolic (right, orange) processes. Substrates enclosed in dotted grey lines are inhibited by mTORC1 or by labeled downstream effectors. Substrates with solid grey background are activated by mTORC1 or other downstream effectors. Adapted from Liu & Sabatini, 2020.

Unlike S6K1's phosphorylation, mTORC1-dependent phosphorylation of 4E-BP1 is inhibitory[21]. When dephosphorylated, 4E-BP1 inhibits eukaryotic translation initiation factor 4E (eIF4E), which is required for 5′-cap-dependent translation[22,23]. Upon being phosphorylated, 4E-BP1 is no longer able to properly bind and inhibit eIF4E, which results into de-repression of 5′-cap-dependent translation. In simpler terms, 4E-BP1 phosphorylation promotes 5′-cap-dependent translation.

While the cytosolic substrates S6K and 4E-BP1 are primarily devoted to regulation of protein synthesis, TFEB and TFE3 control the expression of lysosomal genes[1,2]. TFEB shuttles between the lysosomal surface and the nucleus where it regulates the expression of lysosomal membrane proteins, hydrolases and autophagy-related genes. In nutrient-deprived conditions, calcineurin-dependent dephosphorylation of

TFEB leads to its nuclear import and promotion of lysosomal biogenesis-related genes. Conversely, abundance of nutrients promotes mTOR-driven, multi-site phosphorylation of TFEB at S142 and S138, which inhibits CRM1-dependent nuclear import of TFEB[34] thereby downregulating lysosomal biogenesis. This dynamic shuttling mechanism allows mTOR to upregulate catabolic processes such as autophagy to recycle amino acids and other essential molecules in nutrient-scarce conditions.

The closely-related TFE3 is less studied, but frequently, though not always, mirrors the phosphorylation status of TFEB and is also involved in regulating lysosomal biogenesis. More recently, TFE3 has also been reported to control glucose, lipid and mitochondrial metabolism[35]. Both TFEB and TFE3 have also been shown to be involved in the integrated stress response[36] and p53-dependent responses to DNA damage[37]. Other important transcription factors under mTORC1 control are activating transcription factor 4 (ATF4), sterol regulatory element binding protein (SREBP) and hypoxia inducible factor 1 subunit alpha (HIF1α), which are activated in response to low levels of nutrients, sterols and oxygen, respectively[2].

ATG13/14, ULK1 and UVRAG are autophagy genes which control various aspects of autophagy initiation and autophagosome formation. All three substrates function as positive regulators of autophagy and are repressed by active mTORC1 via phosphorylation. In the presence of nutrients, mTORC1 inhibits ULK1 and ATG13/14 via phosphorylation[27-29], thus preventing the cell from initiating autophagy. mTORC1 also phosphorylates UVRAG to negatively regulate autophagosome and endosome formation[30] in nutrient-abundant conditions. DAP1 (death associated protein 1) is yet another autophagy-related mTORC1 phosphorylation target, which instead acts as a repressor rather than an activator of autophagy when unphosphorylated by mTORC1[38]. The fact that, in the absence of nutrients, mTORC1's inactivity leads to activation of both positive and negative regulators of autophagy is consistent with a "gas and break" model of autophagy induction where overactivation of autophagy upon nutrient scarcity is prevented.

1.1.3 mTORC1 regulation

mTORC1's primary role is to integrate information about nutrient availability to coordinate cell growth. Evolution has developed elegant, multi-layer regulation mechanisms that enable mTORC1 to integrate multiple upstream inputs and fine tune the balance of anabolic and catabolic processes accordingly. In the presence of nutrients, mTORC1 is rapidly re-localized to the lysosomal surface where it docks to the LAMTOR-Rag complex in order to be activated by the small GTPase Ras homolog enriched in brain (RHEB)[39]. The Rag component of the LAMTOR-Rag complex is comprised of the two obligate heterodimers RagA/B and RagC/D. The GDP/GTP loading status of the Rags is crucial for mTORC1 activation and determines whether mTORC1 can be activated by Rheb[40]. Multiple upstream sensors converge onto the Rags and directly modify their GDP/GTP loading status depending on the availability of different nutrients, especially amino acids.

Different types of nutrients such as amino acids[40,41], insulin[42-45] and other growth factors[46,47] can be sensed by mTORC1 and lead to its activation via a complex cascade of upstream signals (Fig. 1.3). Many of these signals converge upon tuberous sclerosis complex (TSC), a major negative regulator of mTORC1 activity. TSC is a heterotrimeric complex composed by TSC1, TSC2 and TBC1D7 which inactivate Rheb via their GTPase-activating protein (GAP) activity. Together with AKT, TSC makes up the AKT/TSC signaling axis, which is able to sense the presence of insulin or IGF-1 (insulin-like growth factor 1) and activate mTORC1 accordingly. Specifically, upon sensing insulin, AKT phosphorylates TSC2 thereby releasing TSC from the lysosomal surface where its primary target Rheb is located[44,45]. This leads to Rheb de-repression and activation, which in turn allows mTORC1 activity to be stimulated.

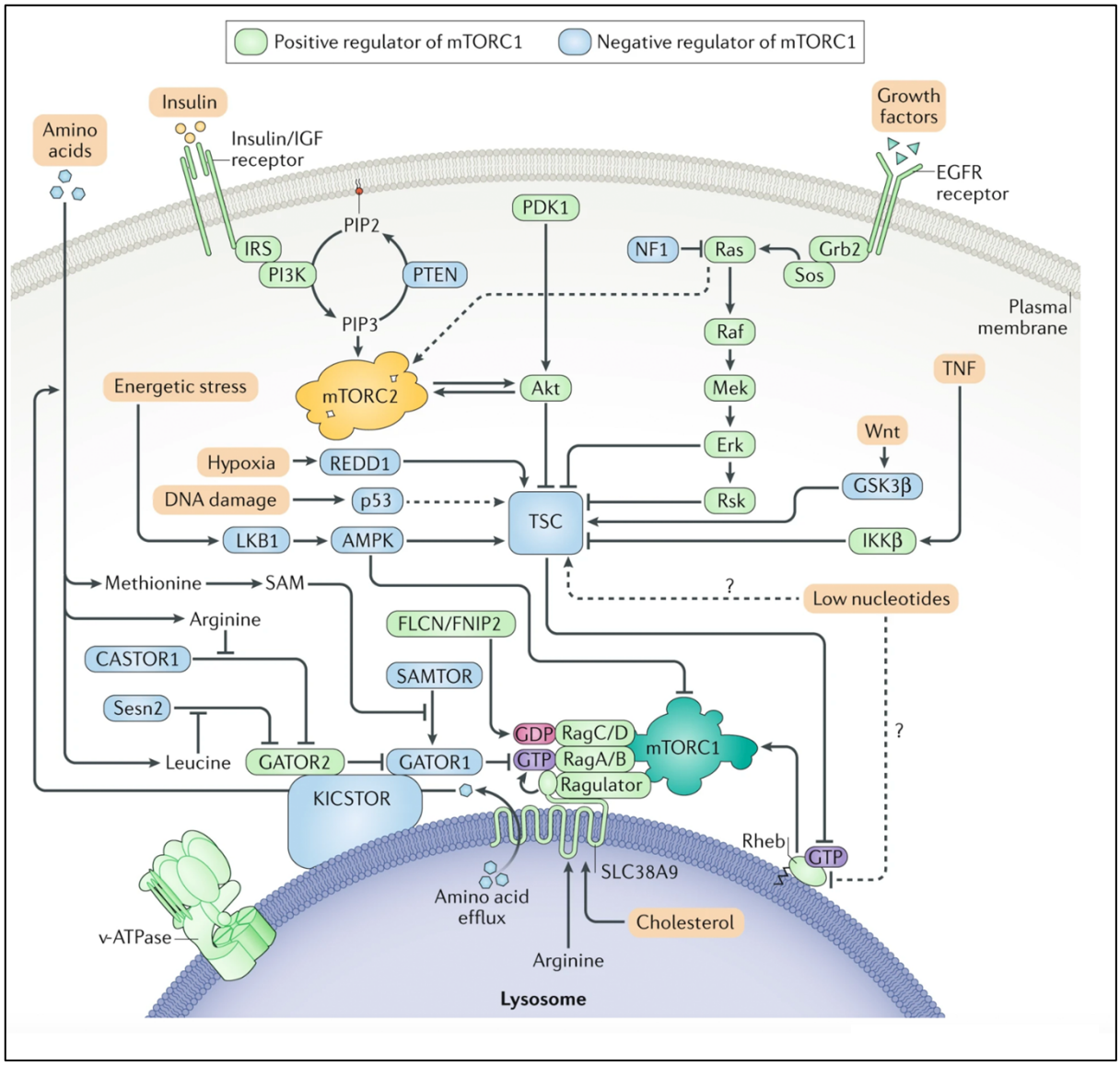


Figure 1.3 mTORC1 regulatory network.
Schematic representation of sensory and signaling regulatory network of mTORC1. Positive regulators of mTORC1 are in green while negative regulators are in blue. mTORC2 is included since, by signaling to mTORC1, it is an effective part of mTORC1's regulatory network. Obtained from Liu & Sabatini, 2020.

Growth factors such as epidermal growth factor (EGF) are sensed via the epidermal growth factor receptor (EGFR), which inhibits TSC via mitogen-activated protein kinase (ERK/MAPK)[46] and p90-ribosomal S6 kinase (RSK)[47]-mediated phosphorylation. Fibroblast growth factor 21 (FGF21) and, more recently, fibroblast growth factor 1 (FGF1) have also been reported to activate mTORC1 via MAPK signaling[48,49]. Wnt and tumor necrosis factor (TNF) are other signaling effectors that influence mTORC1 activity via TSC repression[50,51] though the exact mechanisms have yet to be uncovered.

To avoid repetition, the central role of amino acids in the regulation of mTORC1 activity will be introduced later in the context of amino acid starvation (AA starvation) (**section 1.4.4**). The role of Rag GTPases in amino acid sensing and mTORC1 activation will also be extensively introduced at a later point in the context of RagA/B DKO cells (**section 1.4.5**). The last mTORC1 regulator discussed here is 5' adenosine monophosphate-activated protein kinase (AMPK). As a major regulator of cell growth and metabolism, mTORC1 has evolved to sense the cell's energy status by directly interacting with AMPK, a master kinase complex that is highly sensitive to levels of adenosine monophosphate (AMP) and is activated when energy stores are low. AMPK has a dual mode of action of mTORC1 inhibition in that it can directly phosphorylate RAPTOR, while also activating TSC2[52,53]. This enables robust inhibition of mTORC1 activity when the cell is under energetic stress.

In summary, mTORC1, a master regulator of cellular growth and metabolism, is comprised of five subunits: mTOR kinase, RAPTOR, mLST8, PRAS40 and DEPTOR. Each subunit has different structural and regulatory functions and can influence mTOR's kinase activity towards its downstream targets. Some of these key substrates are the cytosolic S6K and 4E-BP1 as well as the lysosomal TFEB. Phosphorylation of these and more substrates transduces signals to further downstream effectors, which in turn induce adaptive changes in different cellular functions such as protein synthesis and autophagy. mTORC1 activity towards each of its substrates is tightly regulated by a large upstream, sensing apparatus which modulates the level of mTOR activity according to the availability of nutrients and growth factors.

1.2| Mammalian Target of Rapamycin Complex 2 (mTORC2) and its functions

1.2.1 mTORC2 molecular structure

Like mTORC1, mTORC2 is nucleated by mTOR and mLST8, core components of both complexes. In mTORC2, RAPTOR is replaced by rapamycin-insensitive companion of mTOR (RICTOR), a different scaffolding protein which gives mTORC2 its identity. MAPK-interacting protein 1 (mSIN1), DEPTOR and protein associated with RICTOR 1 or 2 (PROTOR1/2) all interact with RICTOR and form the rest of the complex[2] (Fig. 1.4).

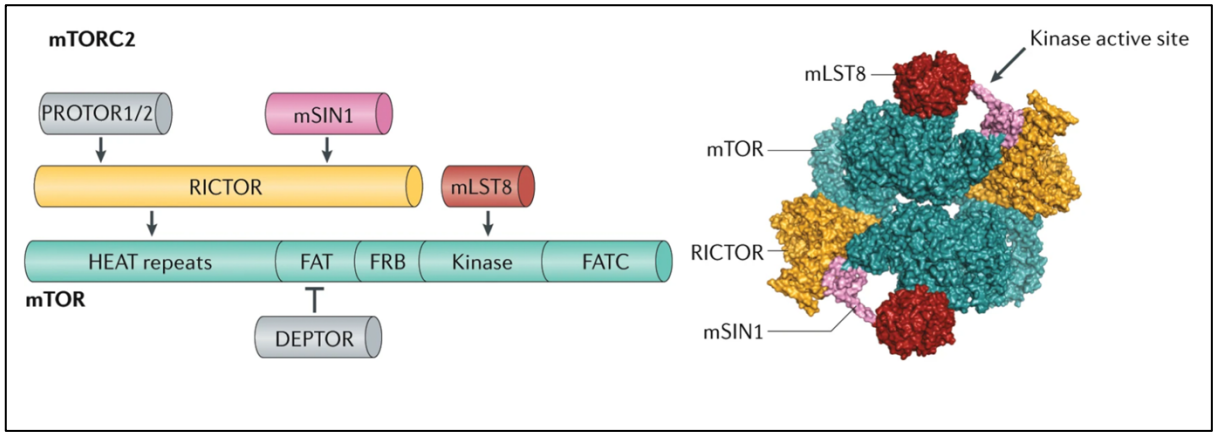


Figure 1.4 mTORC2 domains and structure.
Schematic representation of mTORC2 protein domains (left) and 3D structure as a dimer in its active form (right). Adapted from Liu & Sabatini, 2020.

As the name implies, RICTOR is rapamycin insensitive, or better, the binding of RICTOR to mTOR makes mTORC2 rapamycin insensitive[54]. mTORC2 is known to homodimerize into a lozenge-like shape in its active form. Steric hindrance caused by this supramolecular shape is thought to prevent Rapamycin from binding mTOR's FRB domain, thus rendering mTORC2 insensitive to acute Rapamycin treatment[54]. A more thorough explanation of Rapamycin's influence on mTORC2 is available in **section 1.4.1**. RICTOR is essential for mTORC2 function as RICTOR knockdowns lead to dramatic reductions in AKT activation[55], one of the major substrates and readouts of mTORC2 activity. In vivo ablation of RICTOR results into impaired AKT-FOXO and PKCα signaling[9] as well as reduced lifespan of male mice[56], underscoring RICTOR's importance for proper mTORC2 functioning.

PROTOR1/2, though not essential for mTORC2 assembly and activity towards AKT and PKCα[57], is essential for proper serum- and glucocorticoid-induced protein kinase 1 (SGK1) and N-myc downregulated 1 (NDRG1) phosphorylation, at least in mouse kidneys[57]. PROTOR1/2 directly binds RICTOR[58] and its expression was recently shown to be upregulated by long non coding RNA LINC01133 in triple-negative breast cancer where it acts as a PI3K-AKT signaling shunt[59].

1.2.2 mTORC2's substrates

As mTORC1, mTORC2 exerts its functions via phosphorylation of multiple, downstream substrates including AKT[55], SGK[60] and PKCα (protein kinase C alpha)[54] (Fig. 1.5). AKT is a central component of the PI3K pathway and mediates the cellular response to insulin by inhibiting forkhead box O 1/3a (FOXO1/3a) transcription factors (TFs)[61], thus promoting cell survival and proliferation. FOXO1/3a TFs are pro-apoptotic[62,63] and induce cell cycle arrest[63-65], an undesirable outcome in the presence of insulin, which signals the presence of nutrients that can be used for cell growth. By inhibiting FOXO1/3a TFs, AKT ensures that anabolic processes are promoted in response to insulin thus stimulating cell growth and proliferation. This is also achieved by activation of NADK (NAD kinase)[66], which powers many anabolic processes including fatty acid and nucleotide biosynthesis by converting NAD+ into NADP+.

AKT's functions are not limited to inhibition of FOXO TFs and activation of NADK. To promote glucose homeostasis, AKT inhibits glycogen synthase kinase 3b (GSK3b)[67], which ultimately results into increased glycogen synthesis. Finally, AKT enables signaling between mTORC2 and mTORC1 allowing integration and coordination of the two complexes. This is accomplished via AKT-driven phosphorylation of TSC2[68] which leads to mTORC1 de-repression and activation. Interestingly, S6K1, a direct substrate of mTORC1, has been reported to affect mTORC2 activity via phosphorylation of RICTOR[69]. Additionally, more evidence has shown that mTORC2 can phosphorylate AKT[70] possibly affecting its ability to phosphorylate TSC2 and therefore regulate mTORC1 activity. Lastly, AKT might directly affect mTORC2 activity via mSIN1 phosphorylation[71]. Taken together, these findings suggest the presence of one or more signaling feedback loop(s) between mTORC1 and mTORC2. Further

implications of a potential mTORC1/mTORC2 feedback loop will be discussed in the context of rapamycin-mediated inhibition of mTORC1 (**section 1.4.1**).

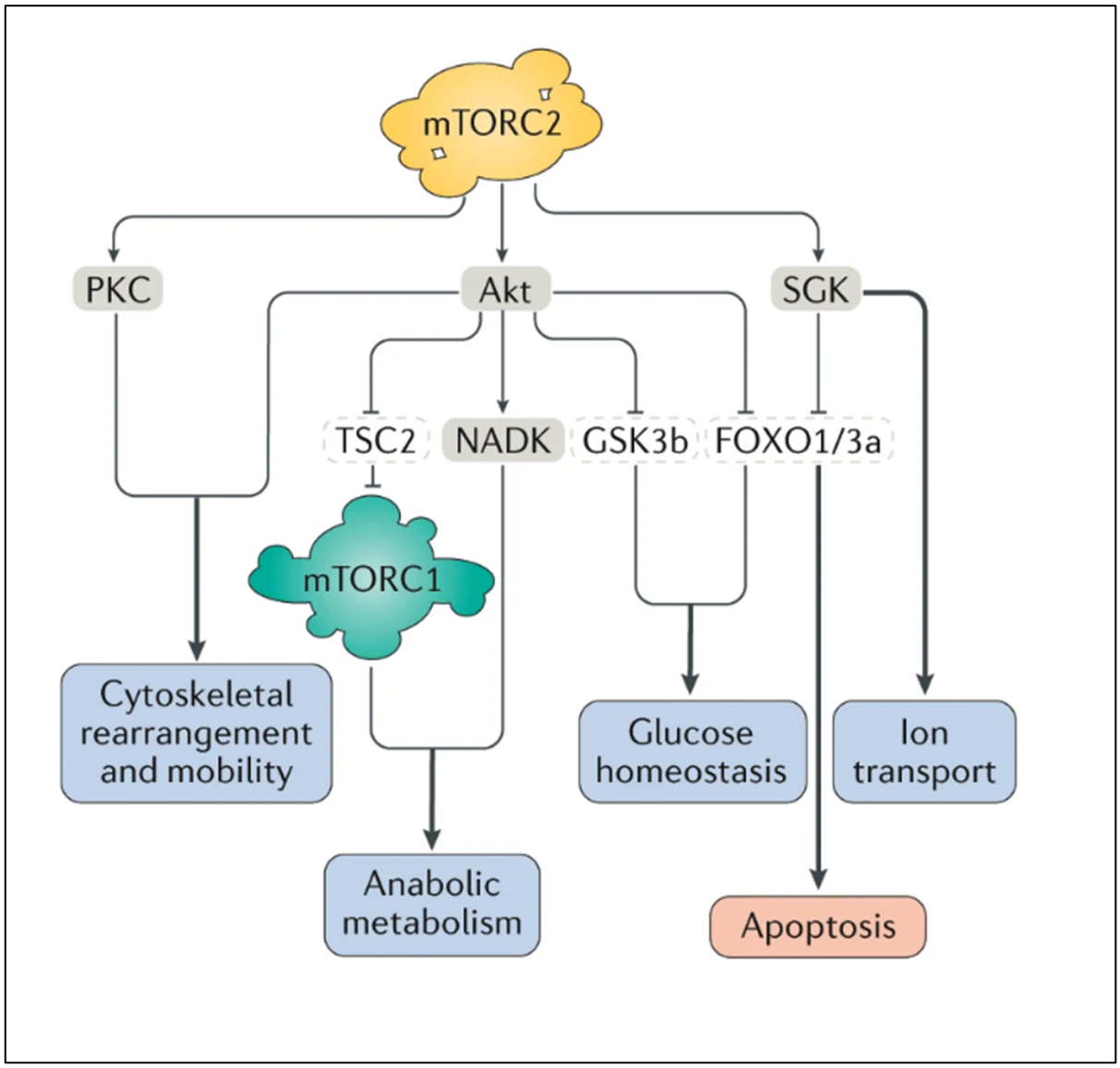


Figure 1.5 mTORC2 substrates.
Schematic representation of mTORC2 main substrates which are involved in regulating anabolic (blue) and catabolic (orange) processes. Substrates enclosed in dotted grey line are inhibited by mTORC1 or by labeled downstream effectors. Substrates with solid grey background are activated by mTORC1 or other downstream effectors. Adapted from Liu & Sabatini, 2020.

Like AKT, SGK negatively regulates the activity of FOXO1/3a TFs[72] thus promoting anabolism. Additionally, SGK inhibits NDRG1, a tumor suppressor gene with many functions, via phosphorylation at multiple sites[73]. NDRG1 is involved in a variety of biological processes including apoptosis, inflammation, angiogenesis, cytoskeleton remodeling as well as cell proliferation, differentiation and adhesion[74]. NDRG1's

versatile nature implies that mTORC2 has wide reach in regulating a multitude of processes, many of which are precluded to mTORC1.

Last, but not least among mTORC2 substrates, PKCα plays a unique role among all other mTOR downstream effectors since, rather than regulating metabolism, it controls the actin cytoskeleton[75], thus influencing processes like cell migration. This has significant clinical relevance since chemotaxis and migration are key processes involved in cancer progression and metastasis making mTORC2 a possible therapeutic target. Together with NDRG1 and other PKCs, PKCα enables mTORC2 to appropriately remodel the cytoskeleton and direct cell motility in response to specific growth factors and other signaling molecules.

1.2.3 mTORC2 regulation

mTORC2's regulatory network (Fig. 1.6) is far less understood than mTORC1's, however it is clear that the PI3K pathway plays a major role in transducing signals, such as the presence of growth factors like IGF1, to mTORC2. Competing models of mTORC2's regulation have been proposed (Fig. 1.6)[76]. "Model 1" of mTORC2 activation is localization-dependent postulating that mTORC2 is actively recruited to the plasma membrane by PI3K-produced PI(3,4,5)P_3. The model further proposes that mTORC2 undergoes an activating conformational change upon PI(3,4,5)P3 binding to mSIN1. In parallel, PI(3,4,5)P3 also recruits AKT to the plasma membrane where mTORC2 is then able to phosphorylate it[76].

"Model 2" envisions an actively-constituted mTORC2 that is statically localized to the plasma membrane. In this model, PI(3,4,5)P3 facilitates AKT recruitment to the plasma membrane where AKT phosphorylates mSIN1 to further increase mTORC2 activity, which in turn leads to mTORC2-driven phosphorylation of AKT. This forms a feedback loop through which AKT promotes its own activation via mTORC2 upon growth factor stimulation[76].

Besides the plasma membrane, mTORC2 has been observed in association with mitochondria, ER, MAM (mitochondria-associated ER membranes) and lysosomal membranes suggesting the existence of separate mTORC2 pools that are differentially

regulated. For instance, mTORC2 has been observed activating AKT at the lysosomal membrane to antagonize PH Domain And Leucine Rich Repeat Protein Phosphatase 1 (PHLPP1) and inhibit chaperone-mediated autophagy[77]. Another study has shown that mTORC2 affects MAM-associated proteins 1,4,5-trisphosphate receptor (IP3R) and hexokinase 2 (HK2) via AKT to regulate mitochondrial function[78]. Ultimately, much more research is needed to fully dissect mTORC2's functions and regulation in each subcellular compartment/organelle.

Another layer of mTORC2 regulation is mediated by the action of small GTPases such as rac family small GTPase 1 (RAC1) and Ras. RAC1 has been shown to activate mTORC2 in response to growth factors[79] together with RAB35, member RAS oncogene family (RAB35)[80]. Ras-GTP activates mTORC2 at the plasma membrane via direct binding to mSIN1 and mTOR[81]. Mutations leading to constitutively-active Ras are oncogenic further suggesting a possible role of hyperactive mTORC2 in tumorigenesis and metastasis. Other small GTPases activate mTORC2 in response to different stimuli which are outside the scope of this thesis and thus will not be extensively introduced.

Finally, mTORC2 signaling is integrated with other large signaling branches such as YES1-associated transcriptional regulator (YAP), phosphatase and tensin homolog (PTEN), Wnt, transforming growth factor beta (TGF-β) and AMPK signaling which allow it to dynamically respond to extra- and intracellular stimuli[76]. AMPK positively regulates mTORC2 activity during times of energetic stress via phosphorylation of mTOR and RICTOR to promote cell survival[82]. AMPK may also indirectly activate mTORC2 via the mTORC1/2 feedback loop upon inactivating mTORC1. Other aforementioned signaling pathways, though not less important, will not be extensively introduced given that they were not affected by any of the different modes of mTOR inhibition that were part of this study.

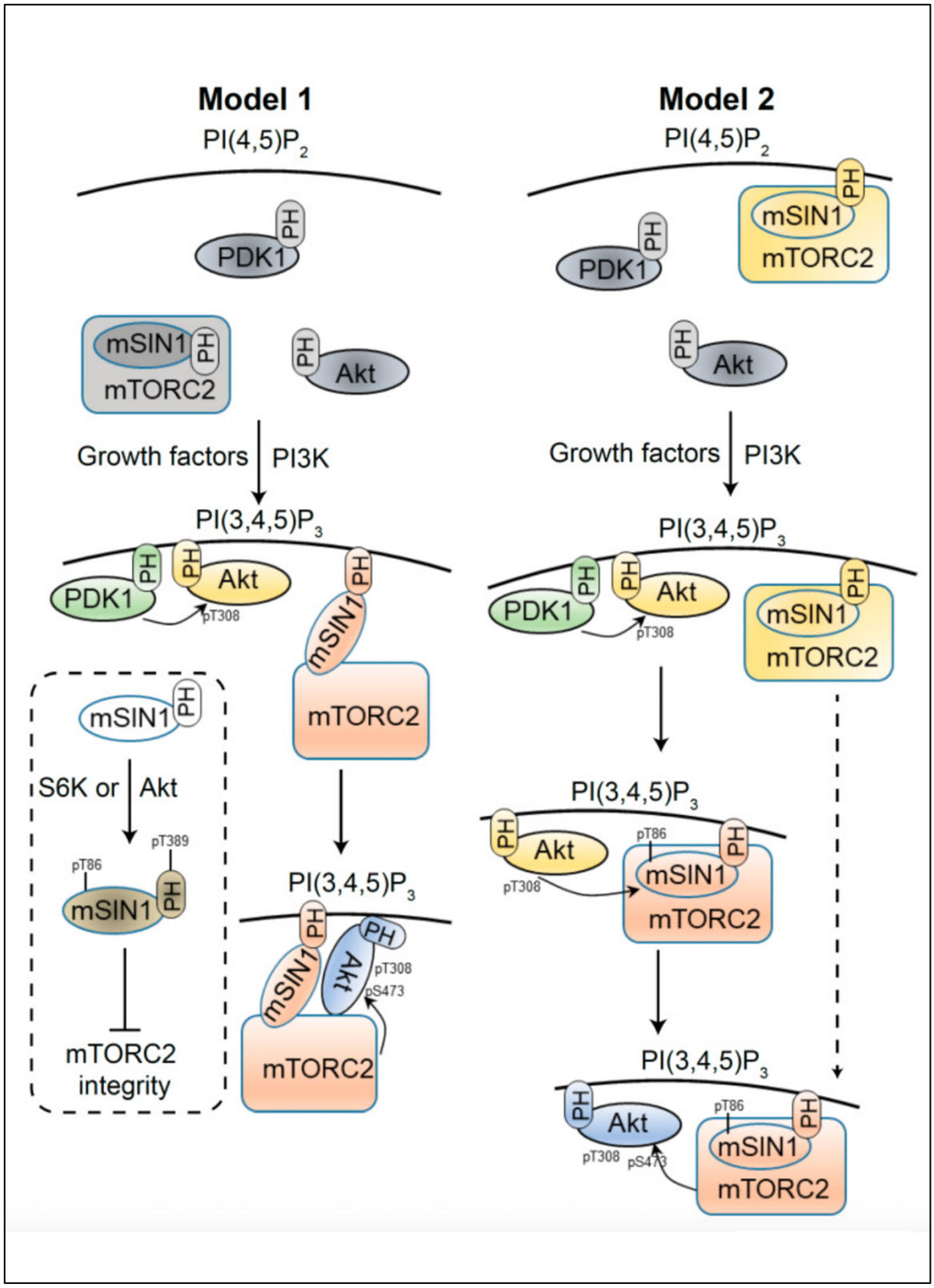


Figure 1.6 Putative models of mTORC2 regulation.
Schematic representation of potential mechanisms of mTORC2 activation. Model 1 (left) emphasizes the central role of $PI(3,4,5)P_3$ in activating mTORC2 via mSIN1 inhibition and recruiting both mTORC2 and AKT to the plasma membrane. Model 2 (right) proposes a static model of mTORC2 localization at the plasma membrane and emphasizes AKT's role in activating mTORC2 upon growth factor stimulation. Adapted from Fu & Hall, 2020.

In summary, mTORC2 is a master regulator of cellular physiology which, in addition to regulating metabolic functions such as glucose homeostasis, is in charge of rearranging the cytoskeleton and promoting cell survival in response to external and internal stimuli. It is composed of six subunits: mTOR kinase, mLST8, RICTOR, mSIN1, DEPTOR and PROTOR1/2. These subunits confer mTORC2 specificity towards its main substrates AKT, SGK and PKC as well as help regulating mTOR's kinase activity in response to different stimuli. Some of the major regulators of mTORC2 are the PI3K/insulin pathway, AKT and small GTPases. Through different signaling feedback loops, mTORC2 communicates with mTORC1 to coordinate the regulation of cell metabolism. In this way, the two complexes can be seen as close and complementary partners that allow cells to sense and react to the full spectrum of stimuli that require adaptive changes in cellular physiology.

1.3| mTOR signaling in disease and aging

mTOR signaling has revealed itself to be a major player in a variety of diseases ranging from cancer, diabetes, metabolic syndrome, neurological disorders and aging[1-3,83]. In particular, hyperactive mTOR signaling has been associated with most of these conditions, thus therapeutic interventions tend to focus on approaches to downregulate mTOR signaling, with the possible exception of age-induced muscle wasting (cachexia), which is likely to benefit from increased mTOR activity[84].

Mutations in mTOR signaling components, especially negative regulators such as DEPTOR, GATOR1, FLCN and TSC have been linked to multiple cancer types[15,85-87]. Mutations leading to constitutively active Rag GTPases have also been identified in lymphomas[88]. Misregulation of upstream signaling pathways such as the PI3K and MAPK pathways can also lead to mTOR hyperactivation and is observed in many human cancers[2]. Rapamycin and analogous compounds (rapalogs), such as Everolimus and Temsirolimus, are frequently used to manage the progression of certain cancer types such as renal cell carcinoma and some forms of lymphoma[89,90]. However, rapalogs have had only limited success as chemotherapeutic agents since they tend to inhibit tumor growth rather than leading to full remission.

One application for which Rapalogs have proven to be particularly useful is the treatment of tuberous sclerosis complex (TSC) syndrome, a rare congenital disease caused by lesions in TSC1 and TSC2 genes[87]. TSC patients have hyperactive mTOR signaling and display a variety of symptoms including the development of benign tumors in the kidneys and other vital organs like the heart, lungs and brain[87]. Other symptoms are neurological abnormalities which can result in seizure disorders and intellectual disability[87]. Skin abnormalities and lung diseases such as lymphangioleiomyomatosis (LAM) are other common symptoms observed in TSC patients[87]. The varied clinical presentations of TSC syndrome underscore the multi-faceted role of mTOR in the regulation of many processes across the body. In fact, TSC patients seem to have symptoms related to almost all diseases associated with hyperactive mTOR signaling with the possible exception of diabetes and accelerated aging.

Diabetes and metabolic syndrome are also affected by misregulation of mTOR signaling. mTORC2 is responsible for regulating glucose homeostasis throughout the body via AKT signaling[91]. Moreover, GSK3 has been shown to regulate glucose transporter GLUT1 expression levels via TSC2 and mTOR[92]. Given mTORC1's role in controlling de novo lipid synthesis via the SREBP pathway[31], excessive mTORC1 activity is also detrimental for conditions like obesity. Paradoxically, chronic rapamycin treatment, which theoretically should ameliorate the effects of hyperactive mTOR signaling, can sometimes lead to the development of a diabetes-like metabolic syndrome[93]. This is thought to happen via a mTORC1/2 feedback loop, mediated by S6K and AKT, through which mTORC2 becomes able to stimulate mTORC1 activity with the help of AKT[94]. This is one of Rapamycin's most troubling side effects, which limits its use as an anti-aging drug. Luckily, intermittent rapamycin treatments have been shown to be effective for lifespan extension without causing diabetes-like symptoms[93]. Interestingly, recent binary pharmacology approaches have been able to target rapamycin delivery exclusively to the brain[95], which could represent a future treatment strategy to suppress hyperactive mTOR-driven seizures without the risk of developing metabolic side effects in other body tissues.

The last two settings in which hyperactive mTOR has been shown to play a significant role are neurological/developmental disorders and aging. Congenital hyperactive mTOR signaling leads to neuronal mis-wiring[96], which manifests itself after birth primarily as epilepsy and other seizure disorders. Mutations in upstream, negative mTOR regulators like GATOR1 subcomplex subunit DEPDC5 have also been shown to cause familial focal epilepsy[97]. mTOR signaling is involved in a variety of neuronal processes including firing-dependent synaptic translation and pruning as well as remodeling of dendritic spines[2]. These processes are essential for long-term potentiation, learning and memory[2], which partially explains the high rates of intellectual disability observed in TSC syndrome and related conditions. Interestingly, acute treatment with rapalogs can suppress seizures in TSC patients, indicating that reducing mTOR hyperactivity after birth is a viable therapeutic strategy despite the presence of prenatal neuronal miswiring[87]. However, rapamycin treatment has been shown to inhibit remodeling of dendritic spines[98], which might be an acceptable side effect for patients with poorly controlled seizures, but might prevent wide adoption of rapamycin treatment for anti-aging purposes.

Downregulation of mTOR signaling via dietary/caloric restriction and/or treatment with mTOR inhibitors is now widely accepted to slow down aging in a variety of model organisms[1,99,100], although aggressive calorie restriction is challenging to achieve in humans[101] and might not represent a realistic strategy. Most studies aimed at extending animal health- and lifespan have used rapamycin or rapalogs to achieve mTOR inhibition. To avoid repetition, these will be introduced in **section 1.4.1**. Given the potential that different nutritional and pharmacological modes of mTOR inhibition have for improving human health, it is imperative to gain a thorough understanding of their effects on cellular physiology. One of the main aims of this thesis was to expand our knowledge of such nutritional and pharmacological interventions by studying the effects of starvation regimens (AA starvation), mTOR inhibitors (rapamycin and Torin1) and genetic deletion of mTOR regulators (knockout of Rag GTPases) *in vitro*. These findings will allow us to better understand the impact of these different interventions at a cellular level, while providing a guide map for further investigation of specific biochemical pathways affected by different modes of mTOR inhibition. Future research focusing on mTOR biology might one day enable us to develop better therapeutic interventions to treat diseases like cancer, diabetes and aging described in this introduction.

1.4| Modes of mTOR inhibition

As already mentioned, mTOR inhibition represents a potential therapeutic strategy for the treatment of several diseases. For this study, we decided to focus on four modes of mTOR inhibition (rapamycin, Torin1, AA starvation and RagA/B double knockout), which represent a wide range of treatment modalities and affect multiple mTOR substrates (Fig. 1.7). **Sections 1.4.1-5** introduce each mode of mTOR inhibition in more detail.

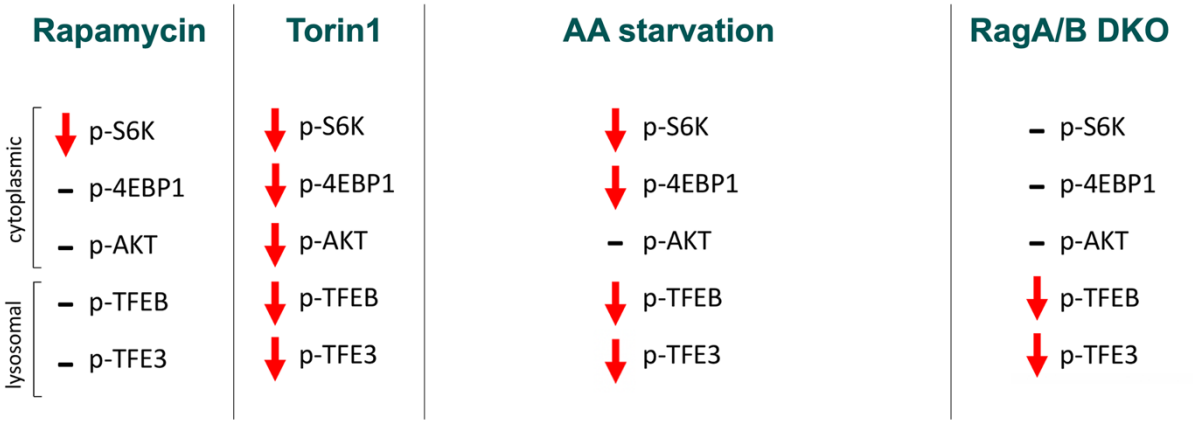


Figure 1.7 Modes of mTOR inhibition reduce phosphorylation levels of different mTOR substrates.
Schematic representation of the effects of different modes of mTOR inhibition (rapamycin, Torin1, AA starvation and RagA/B DKO) on the phosphorylation levels of mTOR substrates (S6K, 4EBP1, AKT, TFEB, TFE3). Down-pointing red arrows represent reductions in phosphorylation levels, while hyphens represent no change in phosphorylation levels.

1.4.1 Rapamycin: an allosteric inhibitor of mTORC1 activity

Rapamycin (Sirolimus) is a naturally-derived macrolide that exhibits anti-fungal, immunosuppressive, anti-cancer and anti-aging effects[1,102]. Rapamycin is a first-generation, allosteric mTOR kinase inhibitor. Its mechanism of action is unique in that it forms a drug-protein complex with cytosolic immunophilin FK506-binding protein 12 (FKBP12) in order to inhibit its target, mTOR. After docking to mTOR's FRB domain, the Rapa-FKBP12 complex hinders access to mTOR's catalytic site thus preventing the phosphorylation of the larger mTORC1 substrate S6K[103]. 4E-BP1's phosphorylation is only mildly affected by Rapamycin treatment[104], while other mTORC1 substrates such as TFEB are completely insensitive to Rapamycin[1]. Unlike ATP-competitive inhibitors, Rapamycin primarily inhibits mTORC1 activity without affecting mTORC2 unless administered chronically and even then only in certain cell

types[105]. mTORC2's varying levels of rapamycin sensitivity across different cell lines are not fully understood, but the expression levels of FKBP proteins, in particular FKBP12 and FKBP51, seem to determine which cell lines have rapamycin-sensitive mTORC2 complexes. High levels of FKBP12 sensitize mTORC2 to rapamycin while high levels of FKBP51 desensitize it[105]. Direct manipulation of FKBPs' levels has also been shown to either de- or sensitize mTORC2 to rapamycin suggesting a direct causal link between FKBPs' levels and mTORC2's level of rapamycin sensitivity[105].

Additional explanations have been offered to explain mTORC2's varying responses to rapamycin. For instance, it has been hypothesized that rapamycin may "lock" a large percentage of mTOR molecules into mTORC1 configurations, thus preventing the formation of new mTORC2 complexes[106]. Ultimately, mTORC2's context-dependent sensitivity to rapamycin is still a matter of active investigation and warrants further research, especially in light of the mTORC2-dependent metabolic side effects[2] observed in animals chronically treated with the drug[2].

Third-generation mTOR inhibitors such as Rapalink-1 combine rapamycin's specificity for mTORC1 with the potency of catalytic inhibitors to achieve total inhibition of mTORC1 activity and reduction in phosphorylation levels of rapamycin-resistant substrates such as 4E-BP1[107,108]. Though Rapalink-1 is advertised as being highly specific towards mTORC1 and ineffective against mTORC2, caution should be used when experimenting with this inhibitor as the dose required to achieve mTORC1 inhibition without affecting mTORC2 varies greatly between cell lines[108]. Given Rapalink-1's unique mode of action, we considered including it among the modes of mTOR inhibition investigated in this study, however, after performing dose curves in multiple cell lines, we were unable to find a concentration that induced strong inhibition of all mTORC1 substrates without affecting mTORC2 activity (data not shown).

Rapamycin's effects are thought to be largely due to mTOR inhibition. At higher doses, Rapamycin is used as an immunosuppressive agent due to its ability to misregulate interleukin-2 (IL-2) signaling, T-cell differentiation and proliferation[109]. Rapamycin does this by impairing lymphocytes' ability to properly sense and integrate antigenic as well as cytokine signals[109,110]. Normally, antigenic stimulation leads to mTOR activation and differentiation of CD4+ T cells into specific T helper cell subtypes. In

parallel, mTOR inhibits the induction of regulatory T cells and promotes an effector fate over a memory one for CD8+ cells[109,110]. Rapamycin-induced mTOR inhibition is thought to disrupt these delicate regulatory mechanisms leading to immune dysfunction.

For these reasons, historically, rapamycin was mainly used for prevention of graft-vs-host disease (GVHD) following kidney transplantation[102,111]. However, the last two decades have brought rapamycin back into the spotlight after a series of studies demonstrated its powerful anti-aging effects across multiple species including worms[112], flies[113-115] and mice[116-118]. A robust body of evidence has shown that low-dose Rapamycin treatment extends both health- and lifespan across different treatment regimens. Indeed, late-life, temporary and intermittent rapamycin treatment have all been shown to extend lifespan in different model organisms with varying degrees of success[116,118,119].

One of the most novel and promising applications of Rapamycin is in treating aging and age-related diseases in humans. Pre-treatment with Rapamycin was shown to enhance flu vaccine efficacy in the elderly[120] suggesting a possible rejuvenation of the immune system. Currently, the randomized, placebo-controlled Participatory Evaluation of Aging with Rapamycin for Longevity Study (PEARL) is testing Rapamycin in humans to assess its impacts on human aging and longevity (ClinicalTrials.gov ID: NCT04488601). This is an important step for the field since PEARL is the first study explicitly designed to test whether Rapamycin has anti-aging effects in humans. Previous studies have only focused on treating specific age-related diseases such as dermal atrophy[121] and advanced macular degeneration[122], rather than focusing on rapamycin's more general potential for comprehensive prevention of age-related diseases. Another notable study currently underway is the Dog Aging Project[123], which aims to monitor the effects of rapamycin treatment in aging companion dogs. Preliminary evidence suggests that dogs treated with rapamycin for 10 weeks have improved cardiovascular function[124]. This study will provide new insights in the biology of mammalian aging and, perhaps even more importantly, will help increase the general public's growing acceptance of anti-aging medicine.

1.4.2 Expansion: Rapamycin's specificity as an mTOR inhibitor

For reasons discussed in the following section, we decided to further expand our study of one mode of mTOR inhibition, namely Rapamycin. The following section introduces the rationale underlying our decision to further study Rapamycin, specifically its specificity for mTOR and its effects on cellular physiology. The reader may want to skip to **section 1.4.3** to resume reading the main's project introduction.

Rapamycin is one of the most promising candidates for healthspan extension in humans, thus it is critical to fully understand its mechanism of action and level of specificity to mTOR. Most kinase inhibitors are plagued by low target selectivity[125-127]. This limits their usefulness and often leads to undesired side effects. Rapamycin is one of the most well-studied mTOR inhibitor, but its specificity towards mTOR has never been thoroughly tested in an unbiased manner. Rapamycin has been reported to activate TRPML1 (TRP cation channel 1), also known as MCOLN1 (mucolipin 1), independently of mTOR[128]. This was observed only at high doses (μM range) and *in vitro*, casting doubt on whether TRPML1 activation would happen *in vivo* where these concentrations are not achieved[89,90,129,130]. Nevertheless, this finding suggests that Rapamycin may bind unspecifically to substrates other than mTOR. Additionally, another study conducted in mice suggested that rapamycin may block exercise-induced ribosomal RNA (rRNA) accumulation in skeletal muscle cells independently of mTOR[131], further suggesting that rapamycin might have mTOR-independent effects.

Finally, Rapamycin's mechanism of action is unique in that it has to form a drug-protein complex with FK506-binding proteins (FKBPs), most commonly with FKBP12, to inhibit mTORC1 activity[103]. FKBPs are chaperone proteins that exhibit proline isomerase activity and facilitate the proper folding of a variety of "client" proteins. FKBP12 has been shown to interact and influence the activity of multiple targets including ryanodine receptors (RyRs), inositol 1,4,5-trisphosphate receptors (IP3Rs) and TGFβ family type I receptors[132-135]. FKBP12.6, FKBP51 and FKBP52 have their own natural targets and modulate the pharmacology of rapamycin in a cell type-specific manner[136]. Thus, it is conceivable that rapamycin might exert mTOR-independent effects by interfering with the interaction with FKBPs and their natural

targets. Given the evidence presented in this section, we decided to ask the following biological question:

Q. Does rapamycin's mechanism of action rely solely on mTOR inhibition?

To address this, we set forth the following research aims:
AIM I: discover whether rapamycin has mTOR-independent effects.
AIM II: deepen our understanding of rapamycin's mechanism of action.

To conclusively establish whether Rapamycin had any mTOR-independent effects, we generated a rapamycin-resistant (RR) cell line by mutating MTOR at its endogenous locus and assessed rapamycin's effects on the cellular transcriptome and proteome via an unbiased, multi-omics approach. The results of this investigation are collected in **section 2.8** and are part of a published paper available at: **https://doi.org/10.1111/acel.13888**

1.4.3 Torin1: an ATP-competitive inhibitor of mTORC1/2 activity

Torin1 is an ATP-competitive, catalytic inhibitor of mTOR, which is able to inhibit both mTORC1 and mTORC2 activity. Torin1, originally quinoline 1, was discovered in 2010 as a highly potent, selective mTOR inhibitor[137]. It quickly gained popularity among researchers for its ability to inhibit phosphorylation of both mTORC1 and mTORC2 substrates, thus overcoming many of rapamycin's limitations. One of the first discoveries made with Torin1 was to reveal how phosphorylation levels of mTORC1 target 4E-BP1 were partially resistant to rapamycin since they could be further reduced by Torin1[104]. In addition to 4E-BP1, Torin1 was shown to strongly inhibit phosphorylation of virtually all mTORC1/2 targets including S6K, TFEB, TFE3 and AKT. Since then, Torin1 has been implemented in numerous studies probing different biological questions and has now become a staple in the mTOR biology field[2,107,111]. Despite numerous efforts to enhance Torin1's clinical utility, such as the discovery of Torin2[138], a Torin1 analog optimized to have better bioavailability, ATP-competitive inhibitors have struggled in clinical trials as chemotherapeutic agents [2,83]. For now, they remain powerful tools with which to study mTOR biology and related processes in the lab.

1.4.4 Amino acid starvation: a nutritional mode of mTOR inhibition

Amino acid (AA) starvation is one of the most well-studied and physiologically-relevant methods to achieve mTOR inhibition. Different types of AA or nutrient starvation have been studied including AA starvation augmented by removal of insulin and/or growth factors. For the purposes of this thesis, amino acid starvation is defined by removal of all amino acids in the presence of 10% dialysed FBS, which ensures full amino acid removal while preserving growth factors.

Amino acid-mediated mTORC1 activation requires a cascade of complex signaling processes. First, amino acids have to be taken up from the extracellular space into the cell via specialized amino acid transporters. These transmembrane proteins are part of the solute carrier family and utilize both facilitated diffusion and active transport, including Na+-dependent transport, to shuttle amino acids across cell membranes. Amino-acid transporters are sensitive to AA size and charge and thus are specific to classes of amino acids rather than to individual ones. Importantly, AA transporters can be symporters, antiporters and uniporters. Some examples include PROT (SLC6A7), a cation symporter responsible for proline's transport; LAT1 (SLC7A5/SLC3A2), a large neutral AA antiporter that preferentially exchanges neutral-branch and aromatic AAs; SLC7A11, an antiporter that exchanges cysteine for glutamate in a chloride-dependent fashion and CAT1 (SLC7A1), a cationic AA uniporter[139]. It is noteworthy that AA transporters' expression levels are tightly regulated according to the demands of each cell type and their misregulation has been observed in various cancers[140].

After amino acids have been imported into the cell, an array of sophisticated AA sensors detects their presence and transduces information about their abundance to downstream effectors. Some of the most prominent AA sensors include the leucine-sensing SESTRIN2 and SAR1B, which play a crucial role in activating mTORC1 via GATOR2 de-repression, the arginine-sensing CASTOR1/2 and the lysosomal arginine-sensing and leucine transporter SLC38A9[2]. Other amino acids including leucine and threonine can even be sensed by their respective tRNA synthetases: leucyl-tRNA Synthetase 1 (LARS1)[141] and mitochondrial threonyl-tRNA synthetase 2 (TARS)[142]. Ultimately, most AA sensors converge on the obligate heterodimers RagA/B and RagC/D, essential components of the LAMTOR-Rag complex, whose

GDP/GTP loading status controls mTORC1 activation at the lysosomal surface[2]. Additional AA sensors such as the methionine-sensing S-adenosylmethionine sensor upstream of mTORC1 (SAMTOR) detect the presence of their target AAs indirectly by sensing related metabolites such as the methionine-derived S-adenosylmethionine (SAM)[143].

Another key signaling axis that is activated in response to AA starvation is the mTORC1-ATF4 signaling axis. ATF4 is a transcription factor and key effector of the integrated stress response, which controls the expression of AA transporters and components of the translation machinery[144]. Upon stressors such as AA starvation, mTORC1 de-stabilizes ATF4 mRNA and inhibits its translation via 4E-BPs[144]. Recently, RNA-Seq data analysis revealed that the expression of multiple AA-related genes including many AA transporters (SLC7A11, SLC3A2, SLC7A5, SLC7A1) is under mTOR and ATF4's control[144]. Overall, the response to AA starvation is mediated by a combination of specialized AA sensors, which converge on Rag GTPases, and ATF4 transcriptional activity.

1.4.5 RagA/B double knockout (DKO): genetic deletion of AA-sensing machinery

Animal and *in vitro* Rag knockout models have been invaluable tools for expanding our knowledge of several mTOR-regulated biological processes including embryonic development[145], autophagy[146] and nutrient-sensing mechanisms[147-150]. RagA/B double knockout (DKO) cells, lacking both Rag A and Rag B, cannot form any functional Rag dimer and thus cannot regulate mTORC1 activity at the lysosomal surface. This results in cells which incompletely inactivate mTOR upon AA starvation and respond to AA re-addition in a delayed fashion[45]. Phosphorylation levels of cytosolic substrates S6K and 4E-BP1, which do not require Rag-facilitated transport to the lysosomal surface for activation, are not strongly affected under steady-state conditions in RagA/B DKO cells[151]. However, phosphorylation levels of lysosomal substrates TFEB and TFE3 are markedly reduced in RagA/B DKO cells, which highlights the importance of Rag GTPases in regulating the activity of lysosomal/autophagy-related proteins. More recently, RagA/B, as opposed to RagC/D were shown to be the primary effectors of the AA starvation response with RagA/B

expressing cells maintaining mTORC1 and lysosomal activity even upon AA starvation[150]. In summary, RagA/B DKO represents a unique mode of mTOR inhibition which primarily affects lysosomal substrates (TFEB, TFE3) and function rather than cytosolic substrates (S6K, 4E-BP1) and processes such as protein synthesis. This enables us to have a unique tool with which to investigate branches of mTOR signaling that most other interventions don't target in isolation.

1.5| Biological questions and research aims

Inhibition of mTOR signaling is a crucial experimental and therapeutic tool, which has allowed scientists in the past two decades to greatly expand our knowledge of mTOR biology and effectively treat conditions such as graft-vs-host disease and TSC syndrome. Researchers outside the mTOR field are often unaware of the subtle differences between mTOR inhibitors and other interventions aimed at reducing mTOR signaling. For instance, the pivotal study describing mTORC1 regulation of what we now call "ATF4-mediated starvation response" utilized Torin1 as opposed to AA starvation for its original discovery[144]. Many studies use rapamycin and Torin1 interchangeably to validate phenotypes, while others still use Torin1 to simulate AA starvation under the assumption that these lead to a similar response. More importantly, we still don't have a comprehensive understanding of the ways in which mTOR inhibitors and other modes of mTOR inhibition differentially affect cellular physiology. To address this gap in knowledge, we sought to answer one main biological question:

Q. Are all modes of mTOR inhibition equivalent to one another?

To answer this, we formulated the following research aims:
AIM I: discover whether different modes of mTOR inhibition lead to similar proteomic profiles.
AIM II: identify key proteomic signatures for each mode of mTOR inhibition.

To achieve these aims, we decided to use a combination of proteomics and systems biology approaches to characterize the impact that four important modes of mTOR

inhibition (rapamycin, Torin1, AA starvation, RagA/B DKO) had on the cellular proteome.

II. Results

2.1| Different modes of mTOR inhibition share a set of common yet differentially-regulated targets

To investigate how different modes of mTOR inhibition diverge in their effects on cellular physiology, we treated HEK293FT cells with either rapamycin, Torin1 or amino acid-free media for 24 hours and performed whole-cell proteomics. In parallel, we also performed whole-cell proteomics on previously-generated RagA/B DKO cells. Rapamycin and Torin1 24-hour treatments were normalized to their respective time-matched DMSO controls, amino acid starved cells were normalized to cells left in basal media and RagA/B DKO were treated with DMSO only (Fig. 2.1).

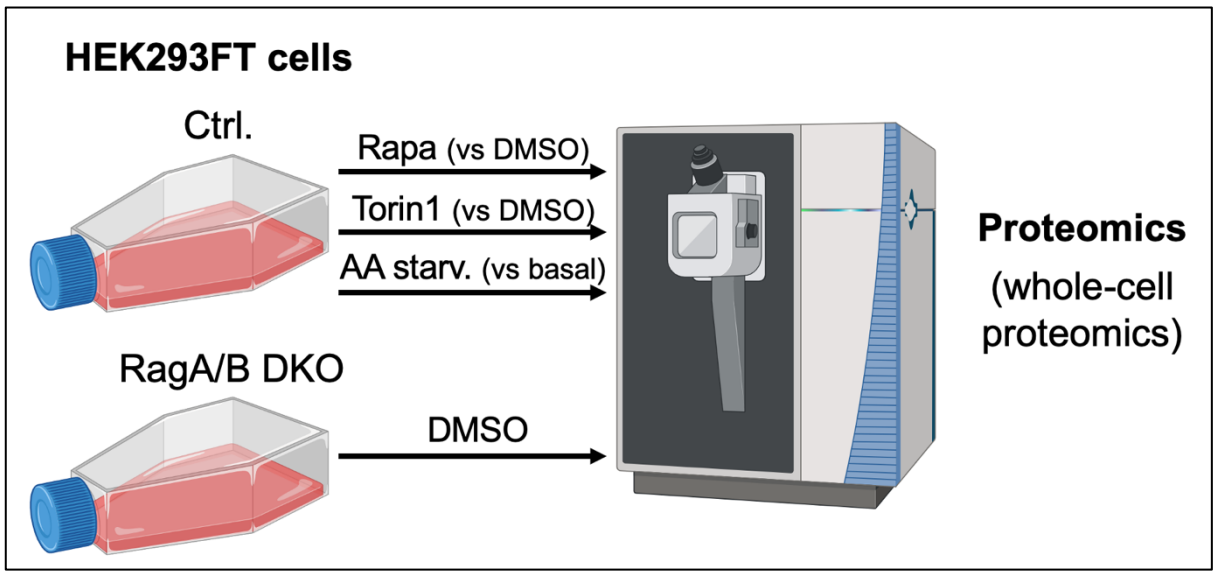


Figure 2.1 Experimental setup for proteomics of different modes of mTOR inhibition.
Schematic representation of drug treatments (24 hrs) representing different modes of mTOR inhibition (rapamycin: 20 nM; Torin1: 250nM) performed on control (ctrl.) HEK293FT cells. RagA/B DKO cells were treated with DMSO (24 hrs). Whole-cell total proteomics was performed for each sample.

The treatments led to expected changes in the phosphorylation levels of several direct mTOR targets (S6K, 4E-BP1, TFEB, AKT) as evidenced by western blot analysis (Fig. 2.2A). Rapamycin reduced phosphorylation levels of S6K, while Torin1 completely abrogated phosphorylation levels of S6K, 4E-BP1 and AKT, while also reducing TFEB phosphorylation levels (Fig. 2.2A). AA starvation reduced TFEB, S6K and 4E-BP1 phosphorylation, while RagA/B DKO primarily reduced TFEB phosphorylation levels, while also having a mild effect on p-4E-BP1 (Fig. 2.2A). Our proteomics experiments detected more than 7600 proteins ($p<0.05$) whose total levels changed in response to

the four modes of mTOR inhibition under investigation (Fig. 2.2B). Scatter-plot representations of proteomics data revealed that each mode of mTOR inhibition reshaped the cellular proteome in a unique way (Fig. 2.2B). Moreover, annotation of strongly up- and downregulated proteins revealed the presence of both unique and shared protein targets across the different modes.

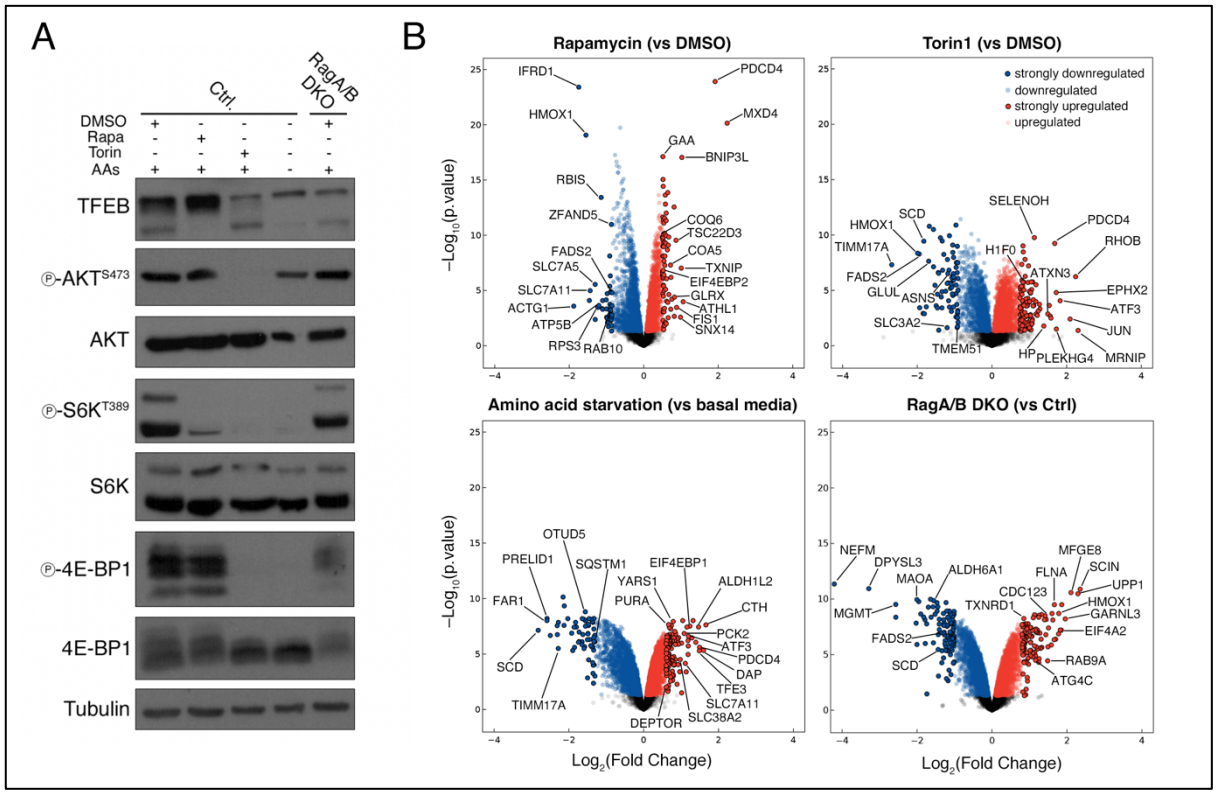


Figure 2.2 Different modes of mTOR inhibition affect specific mTOR targets and reshape the cellular proteome in unique ways.
A) Western blot analysis of phosphorylation levels of mTORC1 (S6K, 4E-BP1, TFEB) and mTORC2 (AKT) substrates showing unique patterns of inhibition for each mode of mTOR inhibition. B) Scatter plots representing whole-cell proteomics data of control HEK293FT cells treated with Rapamycin (20 nM), Torin1 (250 nM) and AA-depleted media for 24 hours. RagA/B DKO cells were treated with DMSO for 24 hours. Up- and downregulated proteins are colored in red and blue respectively. Strongly-regulated proteins are marked with black edges. Top proteins of interest are labeled with their respective names.

To determine which proteins were commonly regulated across interventions, we generated Venn diagrams depicting all proteins that changed in a statistically-significant manner ($p<0.05$). We identified 833 core proteins that were commonly targeted by all four conditions (Fig. 2.3A). AA starvation and RagA/B DKO shared the greatest number (2501) of commonly affected proteins (Fig 2.3B), while Rapamycin and Torin1 shared the fewest (1690) (Fig. 2.3C). However, only 155 and 136 proteins, respectively, were regulated in the same direction by all four treatments (Fig. 2.3D,E).

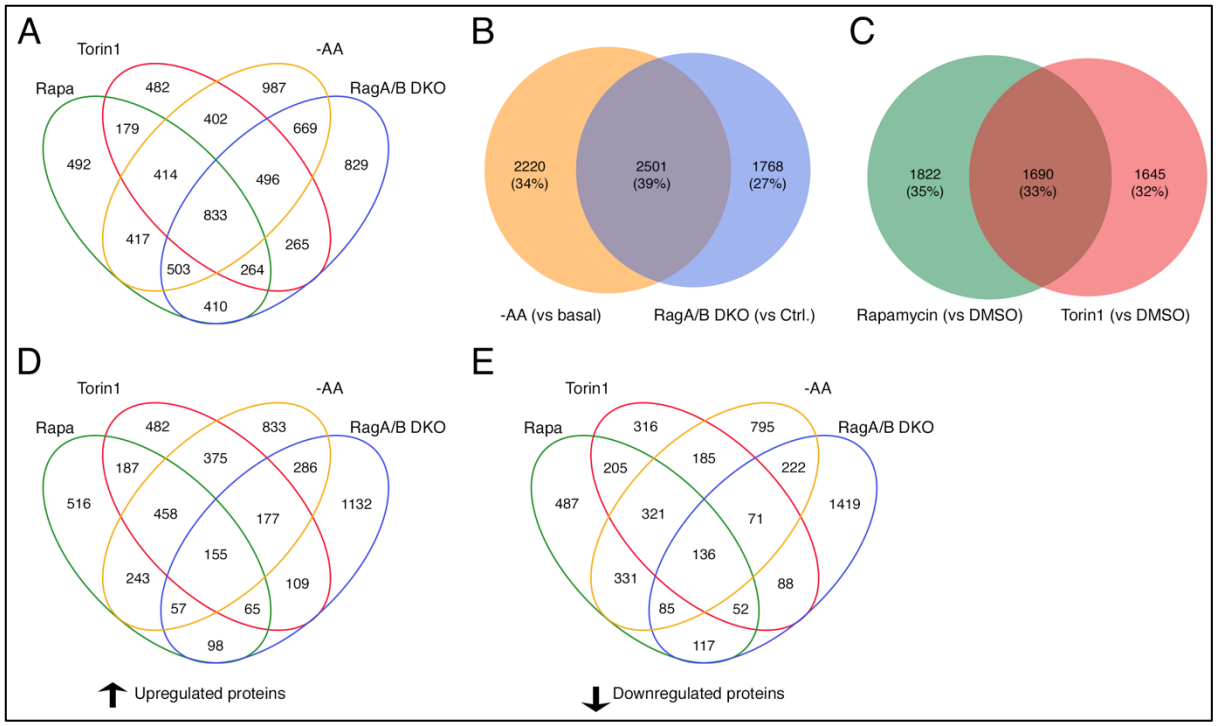


Figure 2.3 Venn diagram comparisons of proteins targeted by different modes of mTOR inhibition.
A) Venn diagram including all statistically-significant proteins ($p<0.05$) detected across different modes of mTOR inhibition. B) Venn diagram of all proteins whose levels changed in a statistically-significant way ($p<0.05$) detected in AA starvation and RagA/B DKO cells. C) Venn diagram of all statistically-significant ($p<0.05$) proteins detected in rapamycin and Torin1. D) Venn diagram of all statistically-significant ($p<0.05$) proteins upregulated in the same direction ($log_2FC>0$) by all interventions. E) Venn diagram of all statistically-significant ($p<0.05$) proteins downregulated in the same direction ($log_2FC<0$) by all interventions.

This highlighted the need for a deeper analysis of the data in order to make accurate comparisons of the different methods of mTOR inhibition.

We thus decided to generate scatter plots (Fig. 2.4) comparing two conditions at a time. To do this, we assigned each condition to either the x or y-axis and plotted the respective log_2 fold change (log_2FC) values for all proteins affected by two particular interventions. This enabled us to intuitively visualize whether protein levels changed in the same or the opposite direction across two conditions by simply assessing the distribution of data points across the four quadrants of the Cartesian plane. Data points located in either the I or III quadrants represented proteins whose abundance changed in the same direction, while data points located in the II or IV quadrants represented proteins whose abundance changed in opposite directions. Conditions that shared a large number of affected proteins, and that therefore appeared very similar at first glance, turned out to be very different when analyzed with this method.

For instance, AA starvation and RagA/B DKO, which shared the greatest number of affected proteins, did not regulate their shared targets in the same direction. This was clearly shown by the scatter plot comparing the two conditions (Fig. 2.4), which exhibited a line of best fit with a negative slope (-0.02) and an associated R^2 value of 0.0005, which suggested no correlation between the two conditions. Rapamycin and Torin1, with a positive slope of +0.76 and a R^2 value of 0.31, were the two interventions with the strongest positive correlation (Fig. 2.4). Protein level changes induced by AA starvation were also positively correlated with the ones observed in Rapamycin (slope: +0.79, R^2: 0.27) and Torin1 (slope: +0.48, R^2: 0.28), while RagA/B DKO-induced changes were not correlated with the ones from any other treatment (Fig. 2.4). Overall, “acute” treatments like Rapamycin, Torin1 and AA starvation exhibited the greatest level of correlation despite their different mechanisms of action and their unique effects on the phosphorylation levels of mTOR substrates.

In order to visualize total protein level changes of some of the most common mTOR-related proteins, we generated special scatter plots referred from here on as “dot plots” (Fig. 2.5). Interestingly, some of mTOR’s direct phosphorylation targets exhibited large changes in total protein levels in addition to the expected changes in their phosphorylation status. For instance, the cytosolic PDCD4 was strongly upregulated by all treatments except RagA/B DKO (Fig. 2.5). Other direct, cytosolic targets such as DAP and EIF4E-BP1 (4E-BP1) were highly upregulated exclusively in AA starvation (Fig. 2.5). Interestingly, perhaps one of the most canonical, direct mTOR target RPS6KA1 (S6K) was only mildly upregulated, but was affected in all four conditions (Fig. 2.5).

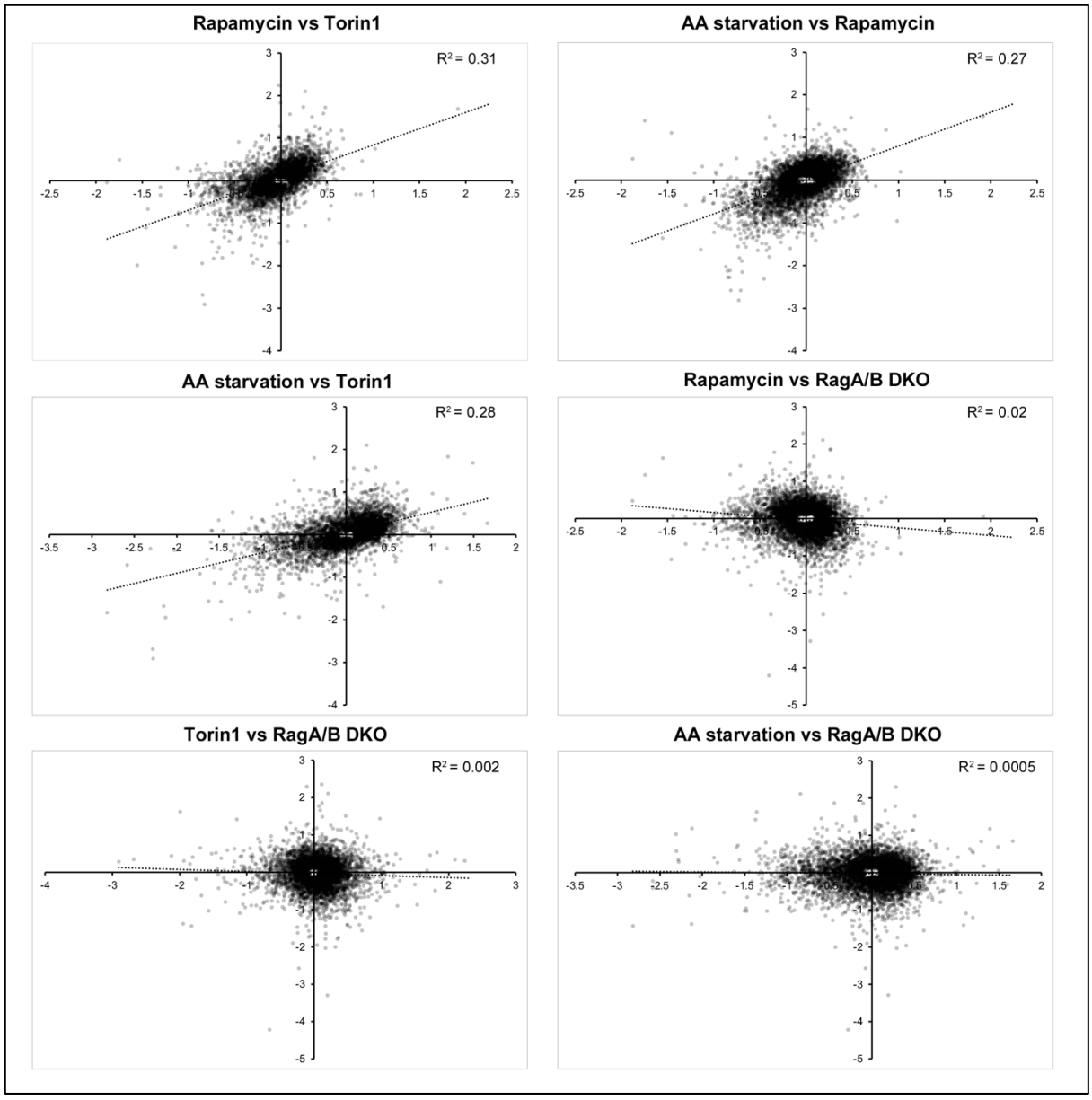


Figure 2.4 Two-way comparisons of modes of mTOR inhibition.
Scatter plots of protein level changes ($p<0.05$ in at least one of the two compared interventions) shared by labeled modes of mTOR inhibition. Each scatter plot compares two interventions at a time. Each data point's x and y coordinates correspond to log_2FC values from the compared interventions. R^2 values are displayed at the top right corner of each graph.

Lysosomal mTOR targets such as TFE3 were upregulated only in AA starvation and RagA/B DKO (Fig. 2.5). 4E-BP1, which is rapidly dephosphorylated upon AA starvation (Fig. 2.2A), was upregulated according to our proteomics data (Fig. 2.5). This might hint at the presence of a feedback loop whereby lower phosphorylation levels are compensated by an increase in total protein levels, though this idea remains to be tested.

Other proteins that have been previously linked to mTOR also displayed dramatic changes in total protein levels. Consistently with its ability to inhibit mTORC2's activity, Torin1 upregulated the cytoskeleton-related Ras homolog family member B (RHOB) (Fig. 2.5). RHOB was also upregulated, albeit to a lesser extent, in RagA/B DKO cells, which is consistent with the high enrichment of cytoskeleton-related GO Terms in RagA/B DKO cells which will be discussed later. Notably, the endogenous mTOR inhibitors DEPTOR and sestrin 2 (SESN2) were upregulated by AA starvation, but were left largely unchanged, if not slightly downregulated, by Rapamycin and Torin1 (Fig. 2.5).

Stress response-related proteins such as heme oxygenase 1 (HMOX1), sequestosome 1 (SQSTM1), interferon-related developmental regulator 1 (IFRD1) and activating transcription factor 3 (ATF3) were also strongly affected by all four treatments (Fig. 2.5). HMOX1 was strongly downregulated by Rapamycin, Torin1 and AA starvation, but was found to be strongly upregulated in RagA/B DKO cells (Fig. 2.5). SQSTM1 was similarly downregulated by all acute treatments (Fig. 2.5), which suggests an increase in autophagic flux. IFRD1 showed a more variable pattern being strongly upregulated in AA starvation and RagA/B DKO cells while exhibiting strong downregulation in Rapamycin (Fig. 2.5). ATF3 levels sharply increased in AA starvation and Torin1, but were only slightly increased by RagA/B DKO and remained unaffected by Rapamycin (Fig. 2.5), suggesting that Torin1 may induce a transcriptional program partially resemblant to the one induced by AA starvation. In fact, the closely-related ATF4 is a very well-characterized player in the response to AA starvation and might share redundant functions with ATF3.

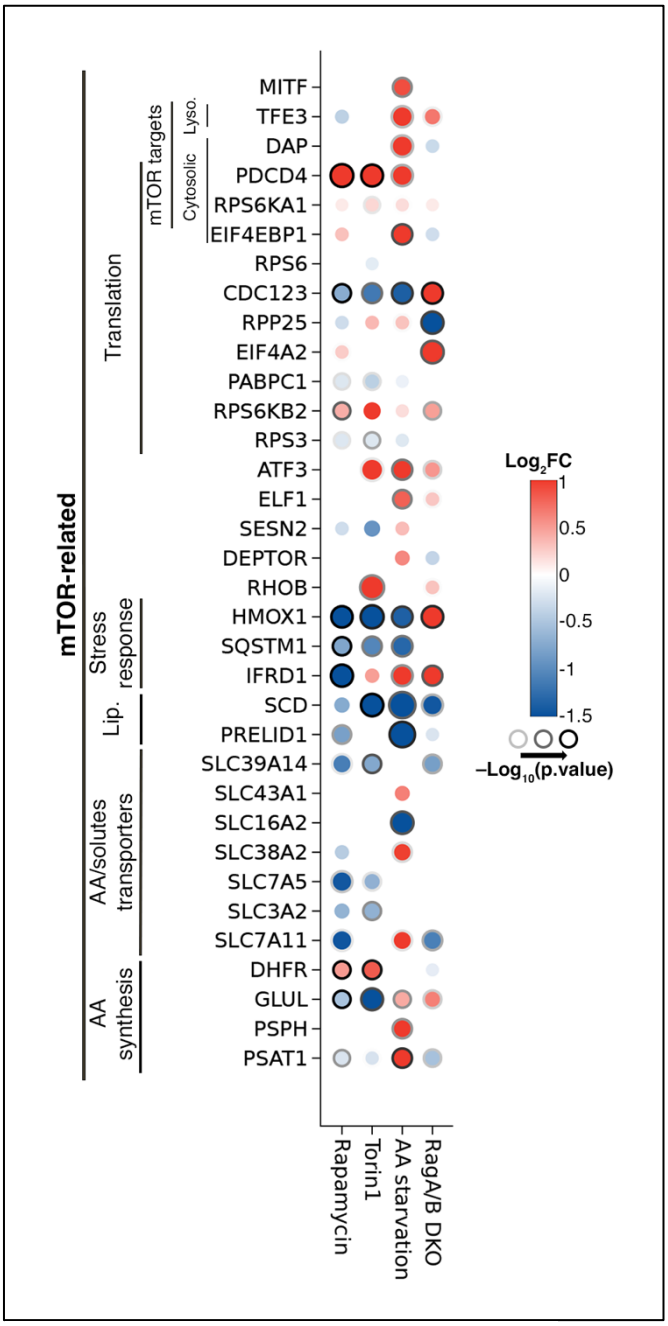


Figure 2.5 Differential regulation of mTOR-related targets across interventions.
Scatter plot (dot plot) representing changes in total levels of mTOR-related proteins grouped by functional categories. Dot size represents magnitude of log_2FC values. Dot color represents magnitude and directionality of log_2FC values. Dot edge color represents $-log_{10}$(p.value) with darker edges representing more significant hits. “Lyso.” = lysosomal; “Lip.” = lipids.

Additional classes of proteins known to be regulated by mTOR were affected by our treatments. The lipid metabolism-related, mitochondrial PRELI domain containing 1 (PRELID1) and lipid-related stearoyl-coA desaturase (SCD) were universally, albeit to different extents, downregulated by all treatments (Fig. 2.5). Total levels of many AA and solute transporters were affected by the treatments. The cystine/glutamate antiporter solute carrier family 7 member 11 (SLC7A11) was upregulated by AA starvation, but downregulated by Rapamycin and RagA/B DKO (Fig. 2.5). The SLC7A5/SLC3A2 transporter system was downregulated by the two inhibitors Rapamycin and Torin1 while remaining unchanged in AA starvation and RagA/B DKO cells (Fig. 2.5). Additional AA and solutes’ transporters such as SLC39A14, SLC43A1, SLC16A2 and SLC38A2 were also up- and downregulated by the treatments to varying degrees (Fig. 2.5). Additionally, enzymes involved in AA synthesis such as phosphoserine aminotransferase 1 (PSAT1), phosphoserine phosphatase (PSPH), glutamate-ammonia ligase (GLUL) and dihydrofolate reductase (DHFR) were also strongly affected, but likewise did not follow a particular pattern, with the possible

exception of PSAT1 and PSPH which were both upregulated by AA starvation (Fig. 2.5). Interestingly, DHFR and GLUL seemed to be affected in the same way by both Rapamycin and Torin1, but in opposite directions with DHFR being strongly up- and GLUL being strongly downregulated, respectively (Fig. 2.5).

Finally, proteins controlling translation were also heavily regulated by the four treatments. Ribosomal protein 6 kinase B2 (RPS6KB2), a close homolog of RPS6KA1, was upregulated by all conditions, especially by Torin1 (Fig. 2.5). Eukaryotic translation initiation factor 4A2 (EIF4A2) and ribonuclease P and MRP subunit p25 (RPP25), both crucial effectors of proper ribosomal function, were respectively up- and downregulated in RagA/B DKO cells (Fig. 2.5). Lastly, cell division cycle 123 (CDC123), a protein required for S phase entry and predicted to be involved in translation initiation, was downregulated by all acute interventions, but strongly upregulated in RagA/B DKO cells (Fig. 2.5). This last example is particularly interesting because it underscores how, even though all four treatments have common targets, a subset of these are regulated in opposite directions depending on whether the treatment is acute or chronic like in the case of RagA/B DKO.

2.2| Rapamycin vs Torin1: comparing two pharmacological mTOR inhibitors

After focusing on proteins that were shared by our treatments, we next sought to study what made each treatment “unique”. We thus decided to focus our analysis on proteins whose levels changed most dramatically in each intervention with the aim of identifying unique targets. To isolate the most strongly-affected proteins within each treatment, we ordered all statistically-significant changes from highest to lowest and plotted their log_2FC values on a scatter plot. This resulted into S-shaped curves with two tails, each representing the top up- and downregulated proteins (Fig. 2.6). We then picked log_2FC cut off values for both strongly up- and downregulated proteins by looking at where the slope of the curves became the steepest.

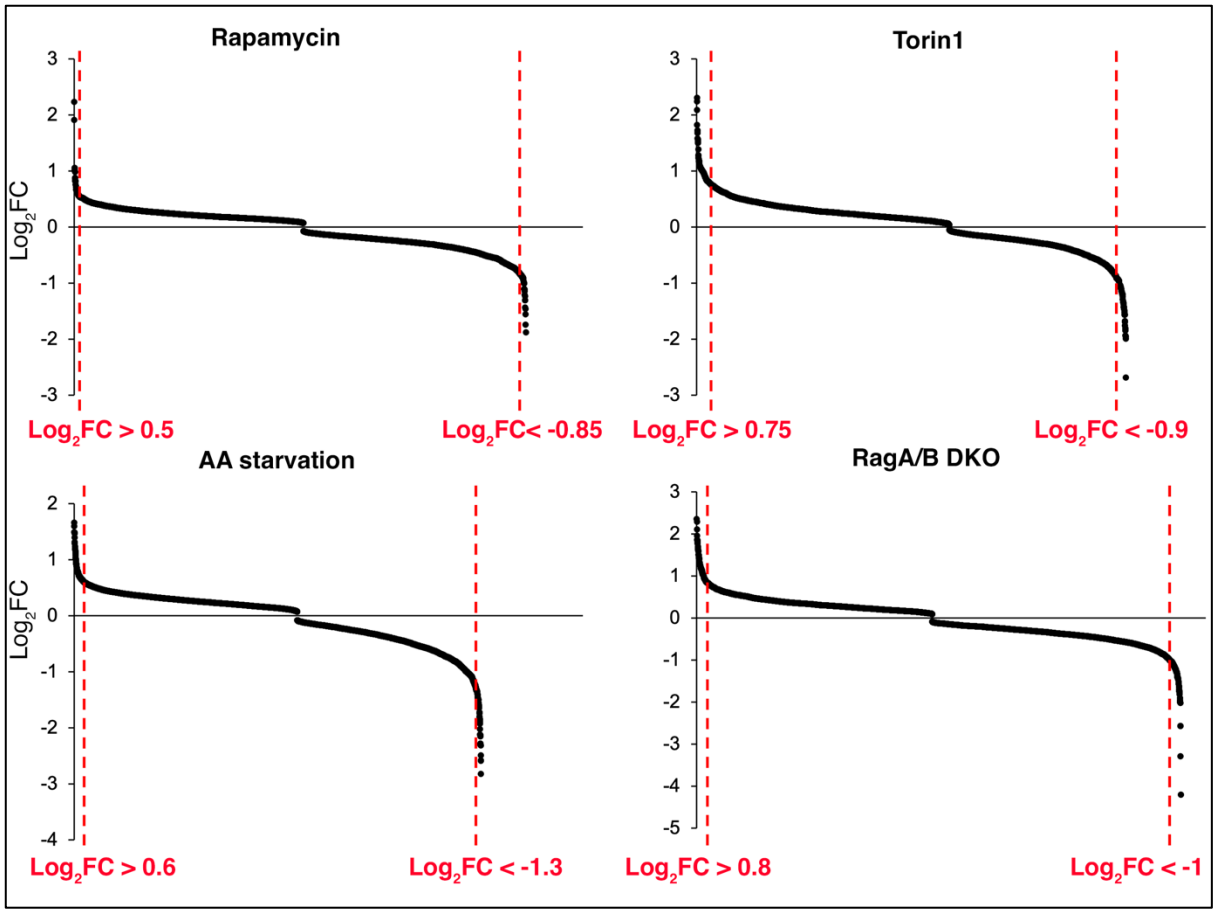


Figure 2.6 Identification of strongly up- and downregulated proteins (top hits). Scatter plots showing the distribution of log_2FC values for all statistically-significant ($p<0.05$) protein level changes, ordered from largest to smallest. Chosen log_2FC cut offs for each treatment are marked with dashed red lines. Proteins that met cut offs were considered strongly-affected by their respective intervention.

Venn diagram comparisons across treatments using only strongly-affected proteins revealed a remarkable lack of overlap across treatments with only one strongly-affected protein (HMOX1) being commonly downregulated across all four conditions (Fig. 2.7). Being interested in what made each treatment “unique”, we sought to use only strongly-changing proteins for further comparisons whenever possible. Unfortunately, GO Term-enrichment analysis, which we used as one of our main methods for comparing different treatments, requires a relatively-large number of proteins to give meaningful results, so we had to revert to using all statistically-significant changes for some comparisons. Whether all statistical changes or only strongly-affected proteins were used will be clearly stated for each comparison.

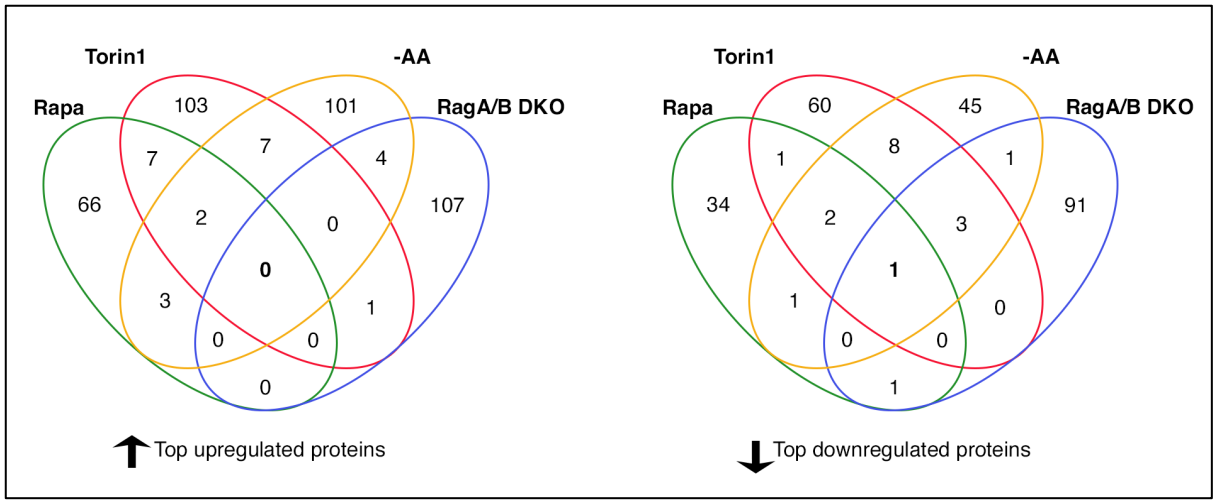


Figure 2.7 Venn diagram comparisons of top up- and downregulated proteins across different modes of mTOR inhibition.
Venn diagrams indicating number of top up-(left) and downregulated (right) proteins shared by Rapamycin, Torin1, -AA and RagA/B DKO cells. Only one downregulated protein (HMOX1) is common to all modes.

Rapamycin and Torin1, despite having very different mechanisms of action, are both pharmacological inhibitors of mTOR and therefore warrant a thorough comparison. Given the relatively small number of strongly-affected proteins in both interventions, we opted to use all statistically-significant changes for this comparison. 3512 and 3335 proteins changed significantly in Rapamycin and Torin1, respectively (Fig. 2.8A). Out of these, 1690 (~33%) were shared between the two inhibitors. GO Term (CAT: KEGG pathway) enrichment analysis of rapamycin-induced protein changes revealed a striking enrichment for terms related to the ribosome (hsa03010:Ribosome) (Fig. 2.8B) with most associated proteins being downregulated (RPS3, RPL8, RPL26, RPL22L1, RPL36A, RPL27A, RPL28, RPL17, RPS15A, RPL18A). Likewise, dozens of ribosome-related proteins were downregulated by Torin1 treatment. Other GO terms that were commonly enriched in Rapamycin and Torin1 were related to amino acid degradation (hsa00280:Valine, leucine and isoleucine degradation), protein processing in the endoplasmic reticulum (ER) (has:04141:Protein processing in endoplasmic reticulum), endocytosis (hsa04144:Endocytosis) and diseases such as COVID-19 (hsa05171:Coronavirus disease – COVID-19) as well as ALS (has:05014: Amyotrophic lateral sclerosis) (Fig. 2.8B,C).

Most proteins belonging to common GO terms changed in the same direction with amino acid degradation-related proteins being highly upregulated (ACADS, MCCC1,

ALDH6A1, ACSF3, OXCT1, HMGCL, HIBADH, ACAD8, ALDH7A1, BCAT2) and COVID-19-related proteins being highly downregulated (RPS3, MAP3K7, ADAM17, RPL8, RPL26, RPL22L1, EGFR, RPL36A, RSL24D1, RPL27A) in both conditions. The remaining common terms exhibited a mixed pattern with proteins being both up- and downregulated. Notably, both rapamycin and Torin1 had GO terms that were either unique or much more enriched than the other condition. Superior enrichment is here defined by 1) a p-value that is lower by several orders of magnitude, 2) a higher count of underlying associated proteins and 3) a higher fold enrichment. For instance the GO term “hsa03040:Spliceosome” appears in both Rapamycin and Torin1, but is much more enriched in Torin1 with a p-value of 8.7137×10^{-15} (vs. 0.01 in Rapamycin), a protein count of 73 (vs. 41 in Rapamycin) and a fold enrichment of 2.41 (vs. 1.45 in Rapamycin) (Fig. 2.8B,C). Other GO terms that were preferentially enriched in Torin1 over rapamycin were related to DNA replication (hsa03030:DNA replication), fatty-acid metabolism (hsa01212:Fatty acid metabolism) and metabolism of AAs and nucleotides (hsa01230:Biosynthesis of amino acids, hsa01232:Nucleotide metabolism, hsa00520:Amino sugar and nucleotide sugar metabolism).

Even though GO term enrichment analysis gave meaningful results only when we included all statistically-significant changes, we also decided to compare rapamycin and Torin1 by looking at the top strongly-affected proteins in each intervention to gain a better understanding of what made each treatment unique. The two top upregulated proteins in Rapamycin were MAX dimerization protein 4 (MXD4) and PDCD4, while the top downregulated ones were acting gamma 1 (ACTG1), IFRD1 and HMOX1 (Fig. 2.9). Torin1 also upregulated PDCD4 in addition to increasing levels of MRN complex interacting protein (MRNIP), RHOB, JUN transcription factor (JUN) and ATF3 (Fig. 2.9). A decrease in HMOX1 levels was likewise observed in Torin1 in addition to a decrease in translocase of inner mitochondrial membrane 17A (TIMM17A) and fatty acid desaturase 2 (FADS2) (Fig. 2.9).

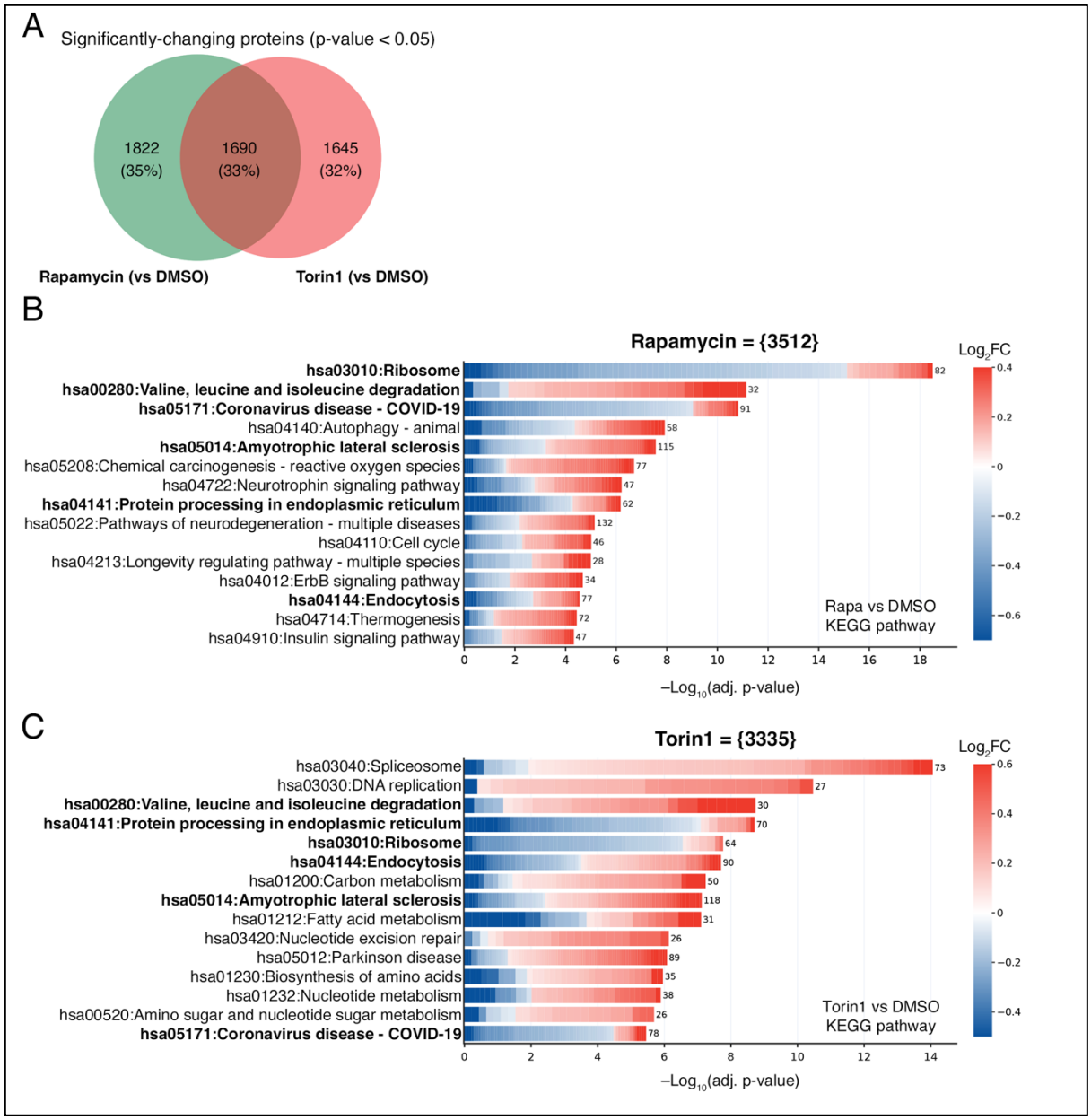


Figure 2.8 Rapamycin vs Torin1.
A) Venn diagram showing number of proteins commonly affected by rapamycin and Torin1. B) Cell plot representing GO term enrichment analysis (KEGG pathway) of all statistically-significant ($p<0.05$) changes induced by rapamycin. C) Cell plot representing GO term enrichment analysis (KEGG pathway) of all statistically-significant ($p<0.05$) changes induced by Torin1. GO terms shared by both treatments are bolded.

Overall, our comparison of rapamycin and Torin1 revealed that, while both inhibitors are likely to downregulate ribosomal function and upregulate amino acid degradation, Torin1 is likely to affect the spliceosome and thus mRNA splicing to a much greater extent than rapamycin (Fig. 2.8B,C). Both inhibitors are likely to affect the cytoskeleton as evidenced by rapamycin and Torin1's ability to upregulate ACTG1 and RHOB respectively. PDCD4 and HMOX1 levels were respectively up- and downregulated by

both treatments indicating that mTOR inhibitors' use might cause significant oxidative stress and even sensitize cells to apoptosis. Finally, Torin1 seemed to have a broader reach in terms of the cellular functions it affected given its ability to regulate JUN signaling, ATF3-driven transcription, mitochondrial function (TIMM17A) and fatty acid biosynthesis (FADS2).

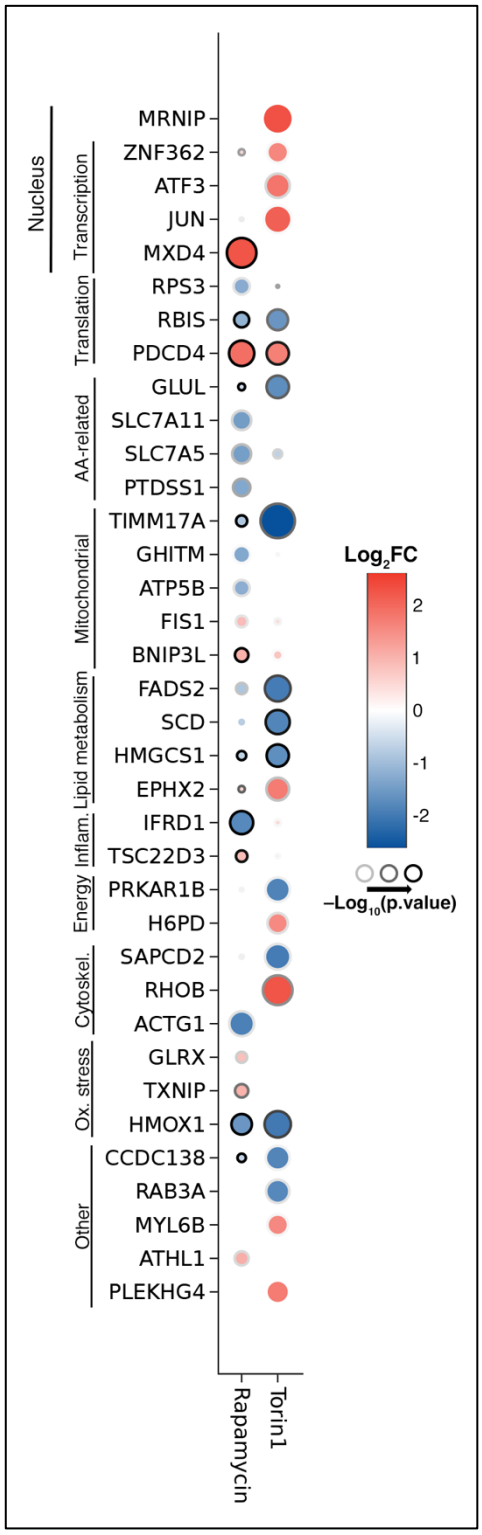


Figure 2.9 Top up- and downregulated proteins in rapamycin and Torin1.
Scatter plot (dot plot) representing changes in total levels of top up- and downregulated proteins in rapamycin and Torin1 grouped by functional categories. Dot size represents magnitude of log_2FC values. Dot color represents magnitude and directionality of log_2FC values. Dot edge color represents -log_{10}(p.value) with darker edges representing more significant hits. "Inflam." = inflammation; "Ox. stress" = oxidative stress.

2.3| AA starvation vs Torin1: investigating Torin1 as a pharmacological model of AA starvation

We next decided to compare AA starvation with Torin1 since the effects of AA starvation are commonly modeled with Torin1 treatment by researchers. Given the relatively-large number of strongly-affected proteins in each treatment (AA starvation = 178; Torin1 = 195) (Fig. 2.10), we were able to limit our GO term enrichment analysis to only strongly-affected proteins for this comparison. AA starvation was primarily associated with GO (MF, CC) terms related to the nucleus and regulation of gene expression (GO:0005654~nucleoplasm, GO:0003677~DNA binding, GO:0005694~chromosome, GO:0003676~nucleic acid binding, GO:0001067~regulatory region nucleic acid binding, GO:0043565~sequence-specific DNA binding, GO:0001159~core promoter proximal region DNA binding, GO:0000976~transcription regulatory region sequence-specific DNA binding, GO:0000977~RNA polymerase II regulatory region sequence-specific DNA binding) (Fig. 2.10).

The overwhelming majority of proteins enriched under nucleoplasm-related terms were upregulated (TFE3, CHTF8, ATF3, H1F0, MAFG, ZBTB2, ZFX, RAD9A, MNT, MITF) with the exception of a few including CCNDBP1, TAF8 and AKIRIN1. Other terms implicated mitochondrial changes (GO:0005759~mitochondrial matrix, GO:0005739~mitochondrion) (Fig. 2.10) with most proteins being upregulated (ALDH1L2, PCK2, TXNRD2, SDHAF4, CARKD, COQ5, COQ7, CLIC4, COQ6, TXNRD1), except for a handful including PRELID1, TIMM17A and MPV17L2.

While AA starvation-induced changes were most pronounced in the nucleus, Torin1 treatment led to dramatic changes in the levels of both cytosolic and nuclear proteins (GO:0005654~nucleoplasm, GO:0005829~cytosol) (Fig. 2.10). Cytosolic proteins exhibited a mixed expression pattern following Torin1 exposure with some being up- (RHOB, JUN, PLEKHG4, EPHX2, PDCD4, MYL6B, ATXN3, ZYX) and others being downregulated (HMOX1, SAPCD2, PRKAR1B, RAB3A, GLUL, HMGCS1). One of the top enriched GO terms referred to organic acid-binding proteins (GO:0043177~organic acid binding) (Fig. 2.10) with proteins being both up- (HBB, PCIF1, PPARD, DHFR, HBA2, NEDD4) and downregulated (SESN2, TYMS,

SLC19A1, SLC1A3, HMGCS1, GLUL). Interestingly, most downregulated proteins associated with this term, except for TYMS and HMGCS1, directly interact with amino acids. Another highly enriched term under Torin1 was a specific type of exosome (GO:0072562~blood microparticle) (Fig. 2.10) where most proteins where upregulated (HP, A2M, HBB, ITIH2, HBA2, TF, AHSG, PON1, C3). Many of these proteins are normally secreted in the bloodstream and are involved in hemoglobin, iron and redox metabolism. A subset of these proteins (HP, HBB, HBA2) together with SESN2 and GPX1 were also grouped under the term “GO:0016209~antioxidant activity”.

Another predominant cellular compartment affected by Torin1 treatment was the mitochondrion (GO:0044429~mitochondrial part, GO:0005739~mitochondrion, GO:0005740~mitochondrial envelope) (Fig. 2.10). Mitochondrial proteins were both up- and downregulated in response to Torin1 suggesting complex regulation of cellular respiration. For instance, the levels of both COQ proteins (COQ5, COQ7, COQ9) and the ATP-synthase subunit ATP5S increased in response to Torin1. Other major effectors of the electron transport chain (ETC) such as UQCRB and NDUFA6/7 were also found to be upregulated. Several other proteins involved in mitochondrial morphology and function were found to be either up- (APOO, MFF, MRPS36) or downregulated (CHCHD2, RPUSD4, TIMM17A). Moreover, proteins in charge of mitochondrial quality control and antioxidant defense were found to be regulated by Torin1 (H6PD, ATXN3, SQSTM1, HMOX1, SESN2, HSPA1L, GPX1). Interestingly, levels of the mitochondrial apoptosis effector DIABLO, which promotes caspase activation, were increased by Torin1.

More proteins belonging to other cellular compartments and with different molecular functions were affected by Torin1 (GO:0070062~extracellular exosome, GO:0061135~endopeptidase regulator activity, GO:0090079~translation regulator activity, nucleic acid binding, GO:0016597~amino acid binding, GO:0044389~ubiquitin-like protein ligase binding) (Fig. 2.10). Interestingly, the GO

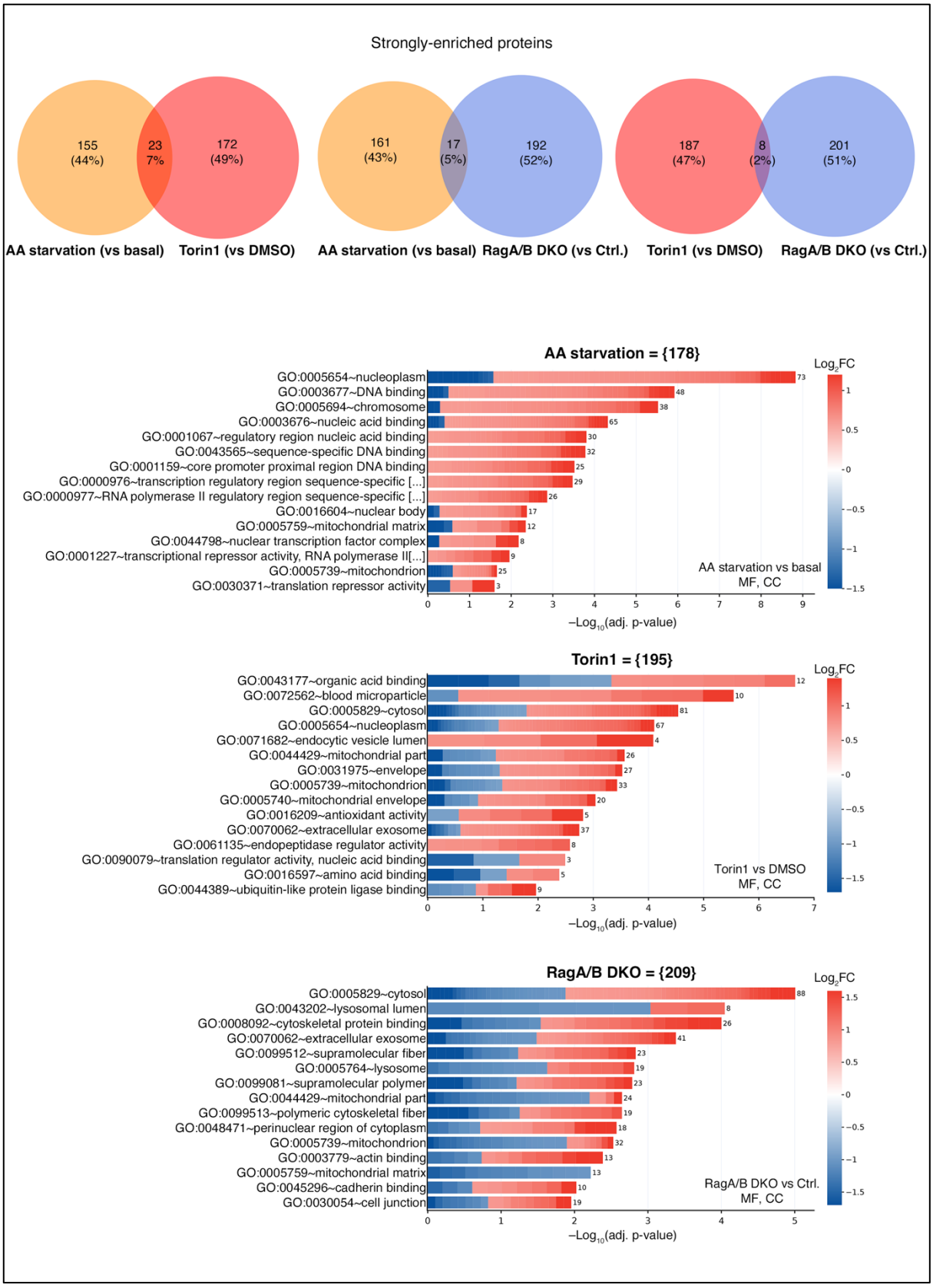
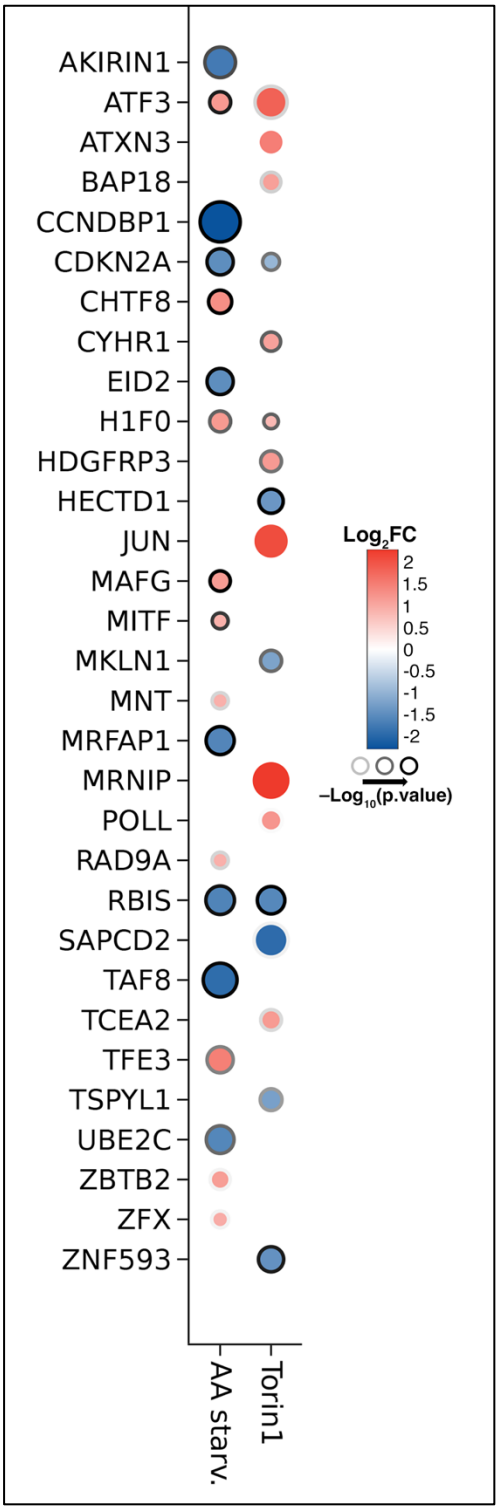

Strongly-enriched proteins
155
(44%)
23
7%
172
(49%)
AA starvation (vs basal)
Torin1 (vs DMSO)
161
(43%)
17
(5%)
192
(52%)
AA starvation (vs basal)
RagA/B DKO (vs Ctrl.)
187
(47%)
8
(2%)
201
(51%)
Torin1 (vs DMSO)
RagA/B DKO (vs Ctrl.)
AA starvation = {178}
GO:0005654~nucleoplasm
GO:0003677~DNA binding
GO:0005694~chromosome
GO:0003676~nucleic acid binding
GO:0001067~regulatory region nucleic acid binding
GO:0043565~sequence-specific DNA binding
GO:0001159~core promoter proximal region DNA binding
GO:0000976~transcription regulatory region sequence-specific [...]
GO:0000977~RNA polymerase II regulatory region sequence-specific [...]
GO:0016604~nuclear body
GO:0005759~mitochondrial matrix
GO:0044798~nuclear transcription factor complex
GO:0001227~transcriptional repressor activity, RNA polymerase II[...]
GO:0005739~mitochondrion
GO:0030371~translation repressor activity
73
48
38
65
30
32
25
29
26
17
12
8
9
25
3
AA starvation vs basal
MF, CC
Log2FC
1
0.5
0
−0.5
−1
−1.5
0 1 2 3 4 5 6 7 8 9
−Log10(adj. p-value)
Torin1 = {195}
GO:0043177~organic acid binding
GO:0072562~blood microparticle
GO:0005829~cytosol
GO:0005654~nucleoplasm
GO:0071682~endocytic vesicle lumen
GO:0044429~mitochondrial part
GO:0031975~envelope
GO:0005739~mitochondrion
GO:0005740~mitochondrial envelope
GO:0016209~antioxidant activity
GO:0070062~extracellular exosome
GO:0061135~endopeptidase regulator activity
GO:0090079~translation regulator activity, nucleic acid binding
GO:0016597~amino acid binding
GO:0044389~ubiquitin-like protein ligase binding
12
10
81
67
4
26
27
33
20
5
37
8
3
5
9
Torin1 vs DMSO
MF, CC
Log2FC
1
0.5
0
−0.5
−1
−1.5
0 1 2 3 4 5 6 7
−Log10(adj. p-value)
RagA/B DKO = {209}
GO:0005829~cytosol
GO:0043202~lysosomal lumen
GO:0008092~cytoskeletal protein binding
GO:0070062~extracellular exosome
GO:0099512~supramolecular fiber
GO:0005764~lysosome
GO:0099081~supramolecular polymer
GO:0044429~mitochondrial part
GO:0099513~polymeric cytoskeletal fiber
GO:0048471~perinuclear region of cytoplasm
GO:0005739~mitochondrion
GO:0003779~actin binding
GO:0005759~mitochondrial matrix
GO:0045296~cadherin binding
GO:0030054~cell junction
88
8
26
41
23
19
23
24
19
18
32
13
13
10
19
RagA/B DKO vs Ctrl.
MF, CC
Log2FC
1.5
1
0.5
0
−0.5
−1
−1.5
0 1 2 3 4 5
−Log10(adj. p-value)

Figure 2.10 AA starvation vs Torin1 vs RagA/B DKO (strongly-regulated proteins).
Venn diagram comparisons of strongly up- and downregulated proteins in AA starvation, Torin1 and RagA/B DKO cells (top). Cell plots representing GO-term (MF: molecular function; CC: cellular compartment) enrichment analysis performed on strongly-regulated proteins in AA starvation (top), Torin1 (middle) and RagA/B DKO (bottom).

term associated with the highest number of proteins, after GO:0005829~cytosol (n=81) and GO:0005654~nucleoplasm (n=67), was GO:0070062~extracellular exosome (n=37) (Fig. 2.10). These included a diverse set of proteins involved in multitude cellular functions. The top up- (RHOB, EPHX2, MYL6B, HP, ITIH3) and downregulated (GLUL, PABPC1, MDH1, TTC38, SLC3A2) proteins belonging to this term are involved in cytoskeletal dynamics, lipid metabolism, antioxidant defenses, inflammation, extracellular matrix remodeling and more. Unfortunately, due to the limited nature of GO term annotation, it is impossible to establish whether these proteins are actual cargo, structural components of exosomes or rather just simply linked with exosomes in the literature. Further experimental evidence is required to establish whether Torin1 treatment induces secretion of extracellular exosomes with particular cargo proteins.

While many of the changes induced by Torin1 were in the cytosol, a large number of nuclear proteins (GO:0005654~nucleoplasm) were affected as well. Some of the top up- (MRNIP, JUN, ATF3, ATXN3, POLL, RPS6KB2, HDGFRP3, TCEA2, C17orf49, CYHR1) and downregulated (TIMM17A, HMOX1, SAPCD2, C8orf59, ZNF593, HECTD1, AMOT, TSPYL1, SLC3A2, MKLN1) proteins were directly or indirectly involved in regulating gene expression and chromatin remodeling. Since some of the greatest changes induced by AA starvation were directed to nuclear proteins, we decided to compare the top up- and downregulated nuclear proteins from AA starvation and Torin1 to get a comprehensive picture of the different nuclear responses induced by these two interventions.

Out of the 31 nuclear proteins plotted in Fig. 2.11, only 4 were commonly up- (ATF3, H1F0) and downregulated (CDKN2A, RBIS) by both AA starvation and Torin1. All 4 common proteins were regulated in the same direction by both interventions, albeit to different extents (Fig. 2.11), and belonged to different protein families including transcription factors (ATF3), histones (H1F0), cyclin-dependent kinases (CDKN2A)



and ribosome biogenesis factors (RBIS). However, the remaining 27 proteins were uniquely affected by only one of the two interventions with AA starvation upregulating several transcription factors such as TFE3, MNT, MITF and MAFG and downregulating other transcription-related proteins such as TAF8, AKIRIN1 and CCNDBP1, which were not affected by Torin1 (Fig. 2.11). Conversely, Torin1 was found to up- (MRNIP, JUN) and downregulate (SAPCD2, ZNF593) proteins that were left unchanged by AA starvation (Fig. 2.11). Thus, even though 4 key nuclear proteins were regulated in the same direction by both interventions, AA starvation and Torin1 led to vastly different nuclear responses.

Overall, AA starvation and Torin1 were distinguished by unique proteomic profiles with AA starvation mainly affecting nuclear proteins and Torin1 impacting a wider range of both cytosolic and nuclear proteins. Both interventions affected the mitochondrial proteome although in different ways and with Torin1 seemingly inducing more oxidative stress-related proteins than AA starvation. Torin1 also seemed to uniquely affect exosome-associated proteins, which was not

Figure 2.11 Nuclear responses in AA starvation and Torin1.
Scatter plot (dot plot) representing changes in total levels of top up- and downregulated nuclear proteins in AA starvation and Torin1. Dot size represents magnitude of log_2FC values. Dot color represents magnitude and directionality of log_2FC values. Dot edge color represents $-log_{10}$(p.value) with darker edges representing more significant hits.

seen in AA starvation. Finally, although both interventions led to extensive changes in the nucleus, it is clear that nuclear proteins respond vastly differently to AA starvation and Torin1, presumably suggesting wide discrepancies in downstream gene expression between the two interventions.

2.4| Torin1 vs RagA/B DKO

Unlike Torin1, RagA/B DKO mainly led to changes in lysosomal (GO:0043202~lysosomal lumen, GO:0005764~lysosome) and cytoskeletal proteins (GO:0008092~cytoskeletal protein binding, GO:0099513~polymeric cytoskeletal fiber, GO:0003779~actin binding), in addition to mitochondrial proteins (GO:0005739~mitochondrion, GO:0005759~mitochondrial matrix) (Fig. 2.10).

Lysosomal enzymes involved in the breaking down of proteins, lipids and RNA (CTSB, LIPA, GM2A, ASAH1, RNASET2) were downregulated in RagA/B DKO cells. Proteins involved in membrane trafficking and vesicular transport (RAB9A/B, TMEM59, CHMP1B) were also significantly affected suggesting changes in endosomal and lysosomal dynamics. Some of the other top affected proteins were related to the cytoskeleton with proteins being both up- (SCIN, FLNA, CNTRL, TMSB4X, MTUS1) and downregulated (NEFM, DPYSL3/4, SNTB1, SHROOM2). Of note, SCIN and NEFM, two proteins heavily involved in cytoskeletal rearrangements, were the number one up- (log2FC: 2.353) and downregulated (log2FC: -4.209) proteins, respectively, across the entire RagA/B DKO proteome. Torin1 too showed a statistically significant enrichment for some terms related to the cytoskeleton, but none of these were among the top 20 terms ranked by p-value.

Torin1 and RagA/B DKO cells were uniquely characterized by an enrichment of proteins implicated in extracellular exosome (EEs) (GO:0070062~extracellular exosome) formation, trafficking, and cargo, most of which were both upregulated in Torin1 (Fig. 2.10) (RHOB, EPXH2, MYL6B, HP, ITIH3, PA2G4, A2M, HBB, SERPIND1, SRI) and RagA/B DKO cells (Fig. 2.10) (SCIN, FLNA, RAB9A, RENBP, IQCG, SLC2A3, TMEM59, FTL, TPM4, ASS1). Notably, none of the top upregulated proteins associated with EEs were shared by Torin1 and RagA/B DKO cells. In Torin1, a subspecialized type of EE, the blood microparticle (GO:0072562~blood microparticle),

was one of the top enriched GO terms (Fig. 2.10). Proteins enriched under the blood microparticle term (ITIH2, PON1, HP, HBB, AHSG, TF, HBA2, HSPA1L, C3, A2M) were closely related to the ones associated with EEs suggesting that blood microparticles may make up a significant fraction of EEs induced by Torin1. By contrast, RagA/B DKO cells did not feature blood microparticle-related GO terms at all suggesting different EE-related changes between the two interventions.

Several changes in mitochondrial proteins were induced by both Torin1 and RagA/B DKO, which will be extensively discussed later in the context of mitochondrial-specific changes induced by all modes of mTOR inhibition. Finally, Torin1 and RagA/B DKO were distinguished by unique GO term which appeared exclusively in only one of the two interventions. Torin1 showed an enrichment for translation, amino acid and ubiquitin-related proteins (GO:0090079~translation regulator activity, nucleic acid binding, GO:0016597~amino acid binding, GO:0044389~ubiquitin-like protein ligase binding) (Fig. 2.10). On the other hand, RagA/B DKO cells were characterized by an enrichment of cadherin and cell junction-related proteins (GO:0045296~cadherin binding, GO:0030054~cell junction) (Fig. 2.10). Overall, both interventions showed unique proteomic profiles with Torin1 exerting its strongest effects on nuclear, mitochondrial and EEs-related proteins and RagA/B DKO cells affecting lysosomal and cytoskeletal proteins the most.

2.5| Mitochondrial proteome

Over the course of our study, we uncovered that a large portion of proteins affected by all modes of mTOR inhibition belonged to mitochondria. This was evident by an enrichment of GO terms related to mitochondria (GO:0005739~mitochondrion), the mitochondrial matrix (GO:0005759~mitochondrial matrix) and other parts of mitochondria (GO:0044429~mitochondrial part) (Fig. 2.10). To estimate the amount of mitochondrial proteins present in our datasets, we identified all proteins associated with the GO term “GO:0005739~mitochondrion” and color coded them in newly generated scatter plots (Fig. 2.12A). We then ranked all statistically-significant changes in mitochondrial protein levels by log_2FC across interventions and selected the top 20 up- and downregulated proteins. We further confirmed these were mitochondrial proteins by consulting Human MitoCarta 3.0 and excluded 3 entries for

a total of 37 proteins. Next, we clustered proteins based on their location within the mitochondrion (OMM: outer mitochondrial membrane; IMS: intermembrane space; IMM: inner mitochondrial membrane; ETC: electron transport chain; Matrix: mitochondrial matrix; Unknown: unclear, mixed or unknown location) and generated a dot plot to visualize how the levels of individual proteins changed across interventions (Fig. 2.12B).

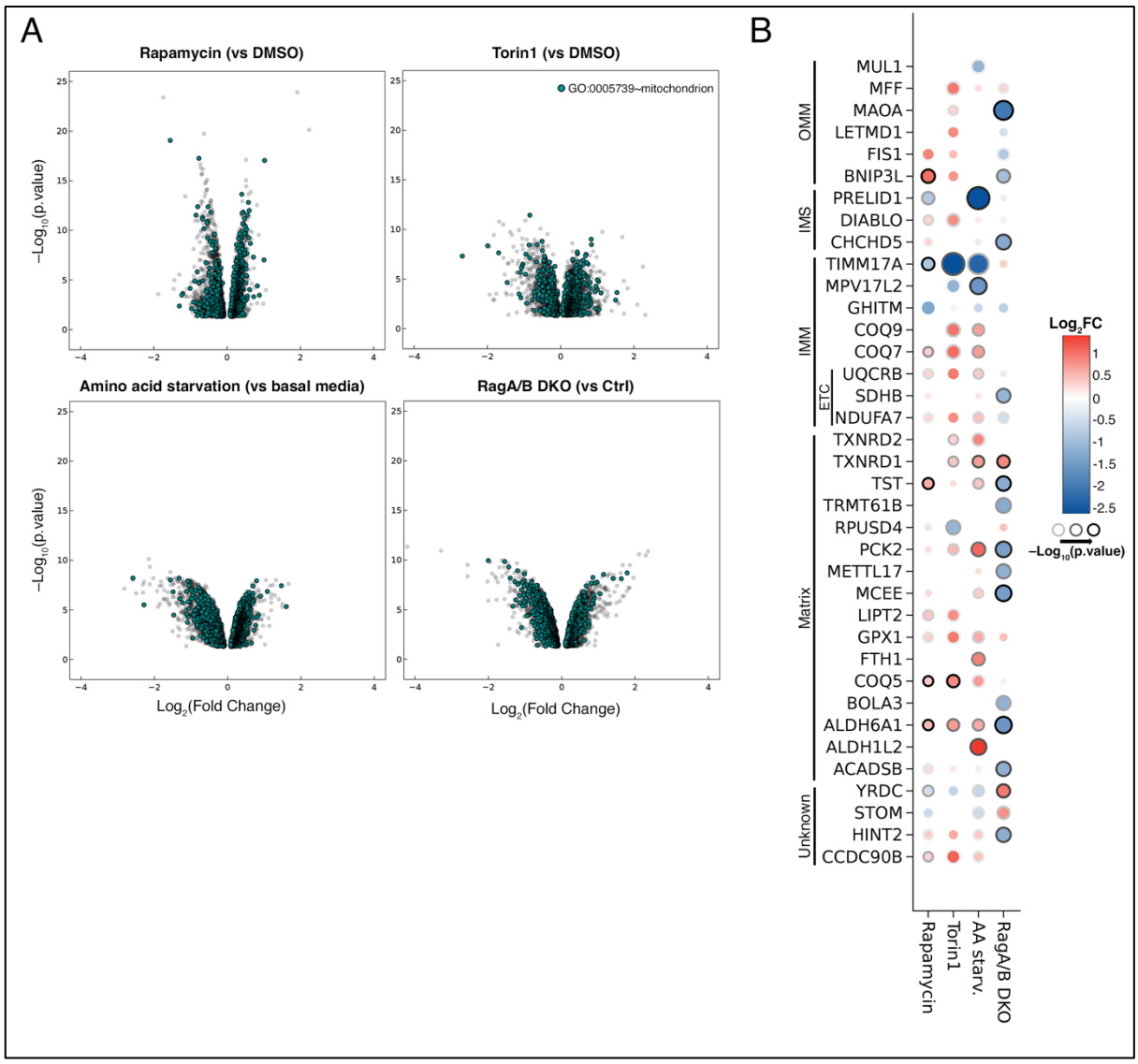


Figure 2.12 Modes of mTOR inhibition remodel the mitochondrial proteome. A) Scatter plots representing all statistically-significant proteins ($p<0.05$) changing in response to labeled interventions. Proteins associated with the GO term "GO:0005739~mitochondrion" are colored in teal. B) Dot plot of top up- and downregulated mitochondrial proteins affected by labeled interventions. Dot size represents magnitude of log_2FC values. Dot color represents magnitude and directionality of log_2FC values. Dot edge color represents $-log_{10}$(p.value) with darker edges representing more significant hits.

Our analysis revealed that Rapamycin, Torin1 and AA starvation upregulated the majority of outer mitochondrial membrane (OMM) and matrix proteins (Fig. 2.12B).

COQ (coenzyme Q) proteins COQ5, COQ7 and COQ9 were also all upregulated by Rapamycin, Torin1 and AA starvation (Fig. 2.12B). By contrast, RagA/B DKO downregulated the majority of mitochondrial proteins, including ETC-component succinate dehydrogenase complex iron sulfur subunit B (SDHB), regardless of their location within the mitochondrion (Fig. 2.12B). TIMM17A, a key component of the TIM23 protein import complex, was strongly downregulated by Torin1, AA starvation and, albeit to a lesser extent, Rapamycin (Fig. 2.12B). It was left unchanged, if not slightly upregulated by RagA/B DKO cells (Fig. 2.12B).

Other proteins were uniquely affected by only one of the four interventions like PRELID1, a IMM protein responsible for cardiolipin accumulation and proper ETC functioning, which was strongly downregulated only by AA starvation (Fig. 2.12B). Likewise, monoamine oxidase A (MAOA) was only strongly downregulated by RagA/B DKO, while aldehyde dehydrogenase 1 family member L2 (ALDH1L2) was uniquely upregulated by AA starvation (Fig. 2.12B). Overall, although a clear pattern of downregulation of mitochondrial proteins was observed for RagA/B DKO cells, each intervention affected the proteome in distinct ways. Additionally, major effectors of mitochondrial function such as TIMM17A and PRELID1 were strongly downregulated, suggesting potential changes in mitochondrial morphology and/or functioning. Further experiments are required to ascertain whether these proteomic changes lead to functional consequences such as changes in cellular respiration rates.

2.6| Rag-independent and dependent responses to AA starvation

Given that amino acids are sensed by mTOR via Rag GTPases, we saw a unique opportunity to use our RagA/B DKO model to uncover potential Rag-independent and Rag-dependent responses to AA starvation. To this end, we performed proteomics on WT and RagA/B DKO cells which were amino-acid starved for 24 hours and ran the same types of analysis we conducted for the other treatments. Out of the 6663 proteins that changed in a statistically-significant manner in AA-starved WT and RagA/B DKO cells, 3111 (~47%) were shared by both WT and RagA/B DKO cells (Fig. 2.13A). This subset of changes can be conceptualized as Rag-independent responses to amino acid starvation since they are observed regardless of the presence of RagA/B.

On the other hand, 1610 (~24%) statistically-significant changes occurred exclusively in WT AA-starved cells (Fig. 2.13A). These can be conceptualized as Rag-dependent responses to amino acid starvation since they are not observed in the absence of RagA/B. Additionally, 1942 (~29%) changes were observed exclusively in RagA/B DKO (Fig. 2.13A). These are more difficult to interpret and could reflect both compensatory changes in response to chronic absence of RagA/B DKO and/or changes due to clonal variability. Since these changes are more difficult to rationalize, we did not include them in our analysis of Rag-independent responses, but rather focused on changes observed in either both genotypes (Rag-independent) or exclusively in WT cells (Rag-dependent).

2.6.1 Rag-independent responses to AA starvation

First, we started with Rag-independent changes in response to AA starvation – the ones observed both in WT and RagA/B DKO cells. Reassuringly, the overwhelming majority of top up- and downregulated proteins in this category changed in the same direction both in WT and RagA/B DKO cells (Fig. 2.13B). This shows that knocking out RagA/B doesn't interfere with the normal Rag-independent responses observed in wild type cells, further validating RagA/B DKO cells as a reliable model to identify physiologically-relevant Rag-independent and -dependent responses to AA starvation.

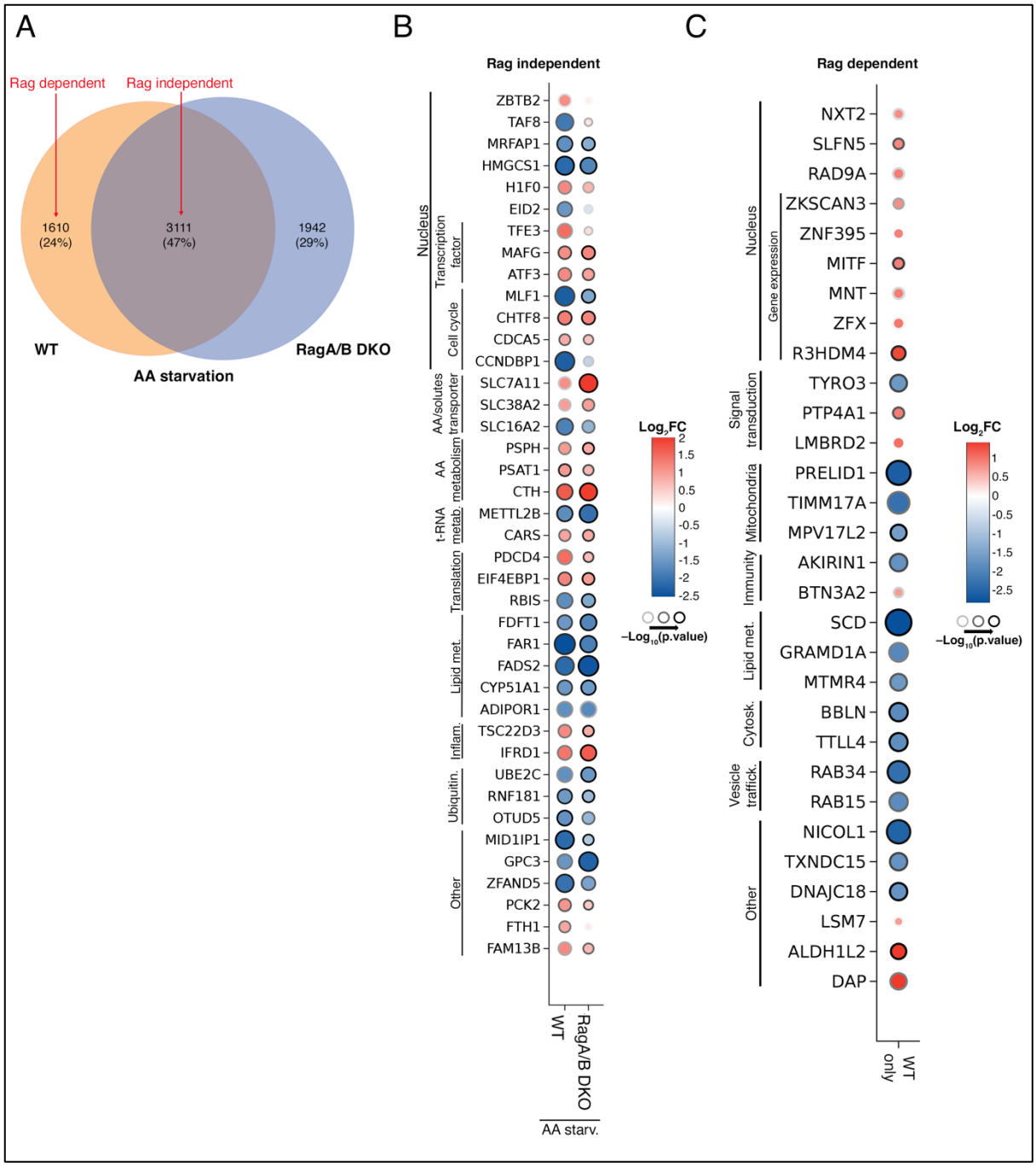


Figure 2.13 Rag-independent and -dependent responses to AA starvation. A) Venn diagram representing all statistically-significant ($p<0.05$) proteins changing in control (WT) and RagA/B DKO cells upon AA starvation. B) Dot plot representing top up- and downregulated proteins regulated in a Rag-independent manner (changing in both WT and RagA/B DKO cells) by AA starvation. C) Dot plot representing top up- and downregulated proteins regulated in a Rag-dependent manner (changing only in WT cells) by AA starvation. In dot plots, dot size represents magnitude of log_2FC values. Dot color represents magnitude and directionality of log_2FC values. Dot edge color represents $-log_{10}$(p.value) with darker edges representing more significant hits.

The top-differentially-regulated proteins that changed in a Rag-independent fashion were associated with a variety of biological processes including lipid metabolism

(FAR1, FADS2), cell cycle (CCNDBP1, MLF1) and AA transport/metabolism (SLC16A2, CTH) (Fig. 2.13B). This was partially confirmed by GO term analysis which validated an enrichment of cell cycle (GO:0042127~regulation of cell proliferation) and AA metabolism-related proteins (GO:0006520~cellular amino acid metabolic process, GO:0019219~regulation of nucleobase-containing compound metabolic process, GO:0051173~positive regulation of nitrogen compound metabolic process) (Fig. 2.14). Other top differentially-regulated proteins were involved in regulation of protein synthesis (PDCD4, EIF4EBP1, RBIS) and gene expression (MAFG, TFE3, ATF3, H1F0) (Fig. 2.13B). PDCD4 and EIF4EBP1, both negative regulators of protein synthesis, were upregulated, while RBIS, a positive regulator, was downregulated (Fig. 2.13B), suggesting overall negative regulation of protein synthesis. On the contrary, proteins involved in gene expression were all upregulated.

GO term analysis validated enrichment of proteins involved in regulation of protein synthesis (GO:0045182~translation regulator activity) and gene expression (GO:0006366~transcription from RNA polymerase II promoter, PF00538:linker histone H1 and H5 family, GO:0045893~positive regulation of transcription, DNA-templated, GO:0010628~positive regulation of gene expression) (Fig. 2.14). Overall, AA starvation induced Rag-independent changes in proteins involved in biological processes such as the cell cycle, AA metabolism, regulation of protein synthesis and gene expression.

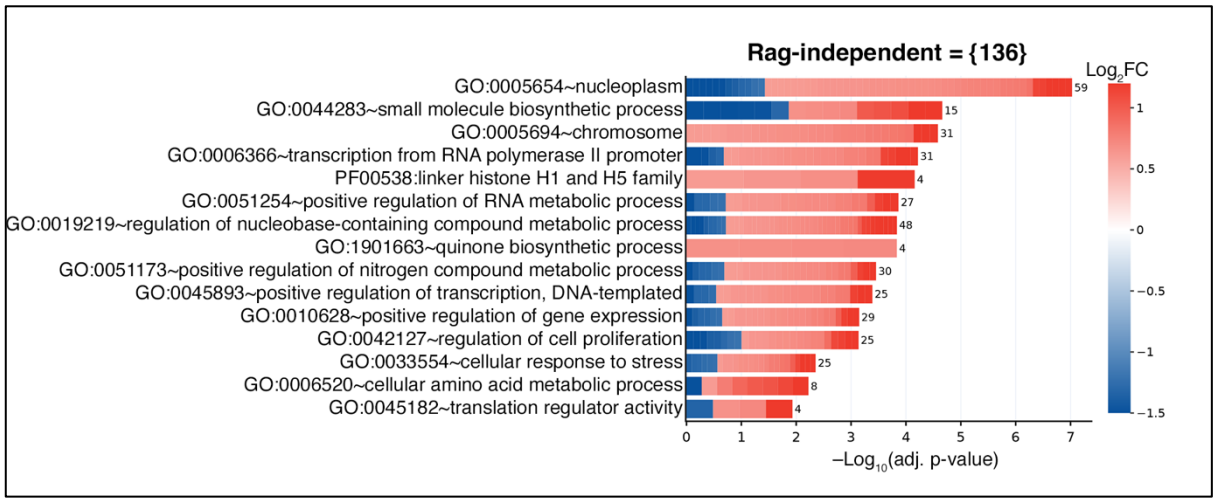


Figure 2.14 Rag-independent responses to AA starvation (strongly-regulated proteins).
Cell plot representing GO-term enrichment analysis performed on Rag-independent, strongly-regulated proteins (n=136) in response to AA starvation.

2.6.2 Rag-dependent responses to AA starvation

Next, we proceeded analyzing proteins that changed in a Rag-dependent way upon AA starvation, the ones observed in WT cells exclusively. The top up- and downregulated proteins belonging to this group were represented with a dot plot (Fig. 2.13C). Similarly to Rag-independent responses, some of the top upregulated proteins were related to gene expression (R3HDM4, MITF, MNT, ZFX, ZKSCAN3, ZNF395) (Fig. 2.13C). On the other hand, mitochondrial (PRELID1, TIMM17A, MPV17L2), lipid metabolism (SCD, GRAMD1A, MTMR4), cytoskeletal (BBLN, TTLL4) and vesicle trafficking-related proteins (RAB34, RAB15) were all downregulated (Fig. 2.13C).

GO term enrichment analysis of strongly-enriched proteins (n=59) did not provide highly-significant terms, thus we performed it on all statistically-significant changes. This was still able to validate an enrichment for mitochondrial (GO:0005739~mitochondrion, GO:0005759~mitochondrial matrix, GO:0005743~mitochondrial inner membrane) and lipid metabolism-related proteins (GO:0006635~fatty acid beta-oxidation) (Fig. 2.15). Among the top 15 GO terms, some were related to the nucleus and chromatin, but none specifically mentioned gene expression or transcription. However, “GO:0045893~positive regulation of transcription, DNA-templated” was nonetheless featured among relatively less enriched, but still highly-significant GO terms (p.value<0.001) (data not shown). Overall, AA starvation induced major Rag-dependent responses in nuclear, mitochondrial and lipid metabolism-related proteins.

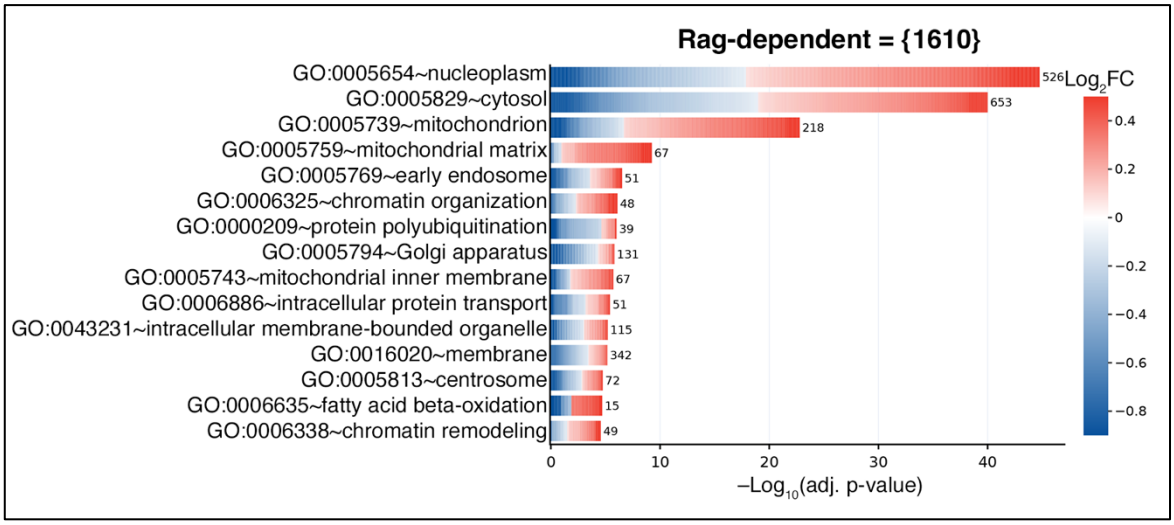


Figure 2.15 Rag-dependent responses to AA starvation.
Cell plot representing GO-term enrichment analysis performed on all statistically-significant ($p<0.05$) proteins (n=1610) that changed in a Rag-dependent manner in response to AA starvation.

In summary, although acting on distinct sets of proteins, Rag-independent and dependent responses to AA starvation converged on overlapping GO terms related to functions such as gene expression, lipid metabolism and the mitochondrion. This suggests that the proteomic changes induced by RagA/B upon AA starvation are not limited to specific classes of proteins or biological processes, but rather influence multiple cellular functions via a discrete set of Rag-dependent targets. Some of these are major regulators of transcriptional programs or metabolic pathways suggesting that more specialized biological processes within the more general categories identified above might be dependent on the presence of RagA/B. Further studies are required to investigate whether specific sub-pathways such as particular transcriptional programs and/or metabolic processes are solely regulated by RagA/B upon AA starvation.

2.7| Modes of mTOR inhibition produce unique proteomic signatures: results' summary

Our extensive study of the effects of different modes of mTOR inhibition on the cellular proteome has revealed unique proteomic signatures for each mode (Fig. 2.16). Our analysis uncovered that while Rapamycin, Torin1, AA starvation and RagA/B DKO act on a common set of targets (n=~800), most of these are differentially regulated across interventions. Among the four modes of mTOR inhibition included in this study, Rapamycin, Torin1 and AA starvation tended to regulate common targets in the same direction more frequently than RagA/B DKO cells. We were also able to show how rapamycin regulates these targets in a uniquely mTOR-dependent manner by generating a rapamycin-resistant cell line (**section 2.8**).

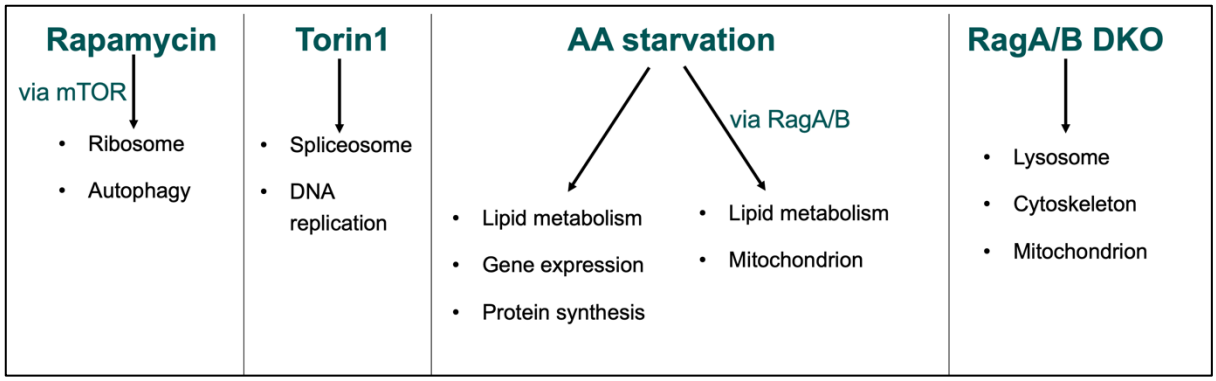


Figure 2.16 Different modes of mTOR inhibition produce unique proteomic signatures.
Schematic representation of functional categories belonging to proteins overrepresented, but not uniquely enriched, in each mode of mTOR inhibition.

Overall, GO term enrichment analysis of proteomics data from each intervention revealed large overlaps in proteins associated with similar biological processes and cellular compartments. However, two-way comparisons across interventions revealed that some GO terms were enriched to a much greater extent, although not always uniquely, in one intervention over the other. For instance, Torin1 was characterized by a high level of enrichment of terms related to mRNA splicing when compared to Rapamycin. Torin1 was also characterized by broader and stronger downregulation of lipogenesis-related proteins and did not induce the same changes in nuclear protein levels produced by AA starvation. Additionally, while rapamycin, Torin1 and AA starvation were all associated with an increase in oxidative stress-related proteins, differences in regulation of mitochondrial protein levels suggest that ROS production is likely to be induced via distinct mechanisms. RagA/B DKO cells exhibited a strong enrichment of lysosomal and cytoskeletal proteins, which was not observed in any of the other interventions. Finally, we were able to show how AA starvation affects proteins converging on a wide range of cellular functions via Rag-independent and dependent effectors. Taken together, these findings reveal important differences in the effects induced by different modes of mTOR inhibition, while providing a useful resource for researchers interested in probing different aspects of mTOR biology.

2.8 Expansion: Rapamycin's specificity as an mTOR inhibitor

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RESEARCH ARTICLE

Unbiased evaluation of rapamycin's specificity as an mTOR inhibitor

Filippo Artoni[1,2] | Nina Grützmacher[1] | Constantinos Demetriades[1,2,3]

[1]Max Planck Institute for Biology of Ageing (MPI-AGE), Cologne, Germany

[2]Cologne Graduate School of Ageing Research (CGA), Cologne, Germany

[3]Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany

Correspondence
Constantinos Demetriades, Max Planck Institute for Biology of Ageing (MPI-AGE), 50931 Cologne, Germany.
Email: demetriades@age.mpg.de

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Abstract

Rapamycin is a macrolide antibiotic that functions as an immunosuppressive and anti-cancer agent, and displays robust anti-ageing effects in multiple organisms including humans. Importantly, rapamycin analogues (rapalogs) are of clinical importance against certain cancer types and neurodevelopmental diseases. Although rapamycin is widely perceived as an allosteric inhibitor of mTOR (mechanistic target of rapamycin), the master regulator of cellular and organismal physiology, its specificity has not been thoroughly evaluated so far. In fact, previous studies in cells and in mice hinted that rapamycin may be also acting independently from mTOR to influence various cellular processes. Here, we generated a gene-edited cell line that expresses a rapamycin-resistant mTOR mutant ($mTOR^{RR}$) and assessed the effects of rapamycin treatment on the transcriptome and proteome of control or $mTOR^{RR}$-expressing cells. Our data reveal a striking specificity of rapamycin towards mTOR, demonstrated by virtually no changes in mRNA or protein levels in rapamycin-treated $mTOR^{RR}$ cells, even following prolonged drug treatment. Overall, this study provides the first unbiased and conclusive assessment of rapamycin's specificity, with potential implications for ageing research and human therapeutics.

KEYWORDS
ageing, mTORC1, proteomics, rapamycin, sirolimus

This page is meant as a place holder for the results section of the following paper: Artoni, F., Grutzmacher, N. & Demetriades, C. Unbiased evaluation of rapamycin's specificity as an mTOR inhibitor. *Aging Cell* **22**, e13888 (2023). Available at: **https://doi.org/10.1111/acel.13888**

III. Discussion

3.1| Implications of research findings

The following discussion presents a curated selection of this study's most interesting findings. It is impossible to discuss at length all findings and observations given the large omics datasets at the center of this thesis. The reader might already find this discussion lengthy, however some of the value of descriptive studies like this one resides in novel hypothesis generation and discussion. The numerous hypotheses outlined in this section should not be in any way be interpreted as definitive findings, but rather as potential explanations that align with the data and current literature.

3.1.1 Rapamycin vs Torin1: a bird's eye view

Rapamycin and Torin1 are both pharmacological inhibitors of mTOR signaling with Rapamycin primarily affecting the phosphorylation levels of mTORC1 substrate S6K and Torin1 having a much broader impact on both mTORC1 and mTORC2 substrates including 4E-BP1, TFEB and TFE3 (Fig. 1.7). In line with what is known about mTOR signaling and rapamycin's mechanism of action [27,29,152-154], rapamycin was found to affect ribosomal and autophagy-related proteins to a great extent (Fig. 2.8B). Most ribosomal proteins were downregulated which suggests a reduction in protein synthesis, a phenomenon that has already been well documented[155-157]. Autophagy-related proteins were both up- and downregulated by rapamycin (Fig. 2.8B) suggesting changes in autophagic flux that are consistent with the increased rates of autophagy observed in rapamycin-treated cells[158-160]. This finding is further supported by strong downregulation of SQSTM1 (p62), an autophagy receptor protein whose levels are inversely proportional to rates of autophagy[161]. This was observed in the context of this study, but also in our previous proteomics experiments exploring rapamycin's specificity to mTOR where SQSTM1 levels were found to be strongly downregulated both at 24 and 48 hours post rapamycin treatment[152] (see **section 2.8**).

Rapamycin was also found to upregulate proteins related to oxidative stress, which although supported by previous findings in the literature[162], is puzzling given the anti-aging properties of this inhibitor. Studies have shown that rapamycin protects mitochondria against oxidative stress[163], so it is possible that enrichment of oxidative stress-related proteins reflects a particularly robust anti-oxidant response. In fact, rapamycin has been shown to enhance mitochondrial efficiency[164], rather than

increasing oxidative stress. This effect is thought to be partly mediated by rapamycin's ability to modulate the expression levels of key mTOR-regulated ETC genes[165,166], which we have observed changing in response to rapamycin in our proteomics data (Fig. 2.9).

Perhaps unsurprisingly, rapamycin exhibited an enrichment of longevity-related GO term "hsa04213:Longevity regulating pathway – multiple species" (p-value: $6.23234x10^{-5}$) (Fig. 2.8B), which was also enriched by Torin1, but to a much weaker extent (p-value: 0.0098). This highlights the predictive power of GO-term enrichment analysis as a discovery tool since quantitative measures like the longevity-related GO term's p-value seem, at least in this case, to correlate with real-life, empirical findings such as rapamycin-mediated lifespan extension. This suggests that, in the near future, proteomics experiments may be used to predict particular properties of an investigational drug bypassing the need for time-consuming screening assays. As an additional piece of evidence supporting this theory, researchers are currently trying to repurpose rapamycin for COVID-19 treatment[167], which was one of the top-enriched GO terms associated with rapamycin (hsa05171:Coronavirus disease – COVID-19) (Fig. 2.8B).

Torin1 was likewise characterized by an enrichment of terms related to the ribosome and degradation of amino acids via autophagy (Fig. 2.8C). What distinguished Torin1 from rapamycin was a highly-significant enrichment of terms related to the spliceosome and DNA replication. This is consistent with a study that uncovered mTORC2-driven phosphorylation of mRNA-binding protein IMP1[168], which was found to be inhibited by Torin1. Additional evidence supporting a potential connection between Torin1 and inhibition of mRNA splicing was offered by another study that showed how Torin1 inhibited de novo lipid biogenesis via repression of mTORC1-S6K1-SRPK2 signaling, which ensures proper splicing of lipogenic mRNAs[169]. Some of the top proteins downregulated by Torin1 were indeed lipid-related and will be discussed later in more detail. Nonetheless, even GO-term enrichment analysis identified fatty-acid metabolism as a highly-regulated biological process in Torin1 (Fig. 2.8C).

Overall, our comparison of rapamycin and Torin1 confirmed previously known properties of the two mTOR inhibitors such as their effects on processes like ribosomal biogenesis and autophagy. Additionally, GO-term enrichment analysis suggested interesting new effects produced by Torin1 such as its influence on spliceosome-related protein levels, which is hinted at by a couple of studies. Finally, enrichment of previously validated pathways (longevity pathways) and diseases (COVID-19) associated with rapamycin, hints at the possibility that other diseases featured in our GO-term analysis such as amyotrophic lateral sclerosis (ALS) and Parkinson disease (PD) might be responsive to mTOR inhibitors or have underlying pathophysiological changes in mTOR-regulated targets.

3.1.2 Rapamycin vs Torin1: zooming in on top up- and downregulated hits

One of the most informative findings of this study is the identification of top up- and downregulated proteins in each mode of mTOR inhibition. By focusing on top-differentially regulated proteins within each condition, we can gain a better understanding of what makes each intervention “unique”. This approach is particularly useful when known major regulators of specific biochemical or signaling pathways are listed among top-enriched proteins. For instance, by looking at how total levels of major transcription factors and signaling proteins change in response to different interventions, we can make educated guesses on how a certain pathway will be affected. This is a valuable tool to both shed more light into the biology of mTOR inhibition, but also for generation of new, testable hypotheses.

By comparing the top differentially-regulated proteins in rapamycin and Torin1, we can clearly notice an enrichment of proteins associated with different functional categories (Fig. 2.9). For instance, rapamycin’s top hits are related to processes such as translation, AA transport/metabolism, inflammation, the cytoskeleton and oxidative stress. Rapamycin was found to downregulate translation as evidenced by downregulation of important ribosomal proteins (RBIS, -1.14; RPS3, -1.24) and upregulation of negative regulator of protein synthesis PDCD4 (+1.9). This is consistent with Rapamycin’s known effects on rates of total translation and mTOR’s role in positive regulation of protein synthesis[2,170-173]. Upregulation of PDCD4 could also indicate an increase in apoptosis, which has also been observed in response to

rapamycin treatment in a context and cell type-specific manner[174-176]. However, rapamycin's connection with apoptosis is multi-faceted with studies reporting both pro- and anti-apoptotic effects[163,177-180]. Additionally, no significant amount of cell detachment indicating apoptosis was observed with prolonged (24 hrs) rapamycin treatment in our experiments (data not shown), further complicating the interpretation of PDCD4 upregulation. Torin1 also upregulated PDCD4 (+1.68) while downregulating RBIS (-1.57) suggesting that Torin1 downregulated translation too, a finding confirmed in the literature[173,181,182].

AA transporters (SLC7A11, -1.46; SLC7A5 -1.42) and phosphatidylserine synthase 1 (PTDSS1, -1.3) were mainly downregulated by Rapamycin, with Torin1 primarily downregulating glutamine synthetase (GLUL, -1.69) instead. SLC7A11 is a cysteine/glutamate antiporter, which imports extracellular cysteine in exchange for intracellular glutamate, and is essential for maintaining the proper intracellular levels of glutathione, a key antioxidant molecule. The first and rate-limiting step of glutathione synthesis involves converting L-glutamate and cysteine into γ-glutamylcysteine. By either downregulating SLC7A11 levels, thus reducing glutamate export, or by downregulating GLUL, which consumes L-glutamate in the synthesis of glutamine, the cell could be attempting to preserve a sufficiently large pool of L-glutamate required for glutathione synthesis. This raises the intriguing possibility that rapamycin and Torin1, which have been both reported to cause oxidative stress[183], might induce distinct yet functionally-equivalent antioxidant responses by downregulating SLC7A11 or GLUL respectively.

This hypothesis is further supported by the fact that some of the top-enriched proteins in rapamycin and Torin1 were related to oxidative stress with rapamycin upregulating glutaredoxin (GLRX, +0.83) and thioredoxin-interacting protein 1 (TXNIP, +1). GLRX upregulation in particular supports the notion that oxidative stress might pressure the cell to conserve glutamate for glutathione synthesis since GLRX uses glutathione as a cofactor. Both rapamycin and Torin1 strongly regulated other anti-oxidant proteins including heme oxygenase 1 (HMOX1, Rapa: -1.55, Torin1: -1.99). Even though downregulation of total HMOX1 levels is difficult to rationalize in the context of oxidative stress, when one would expect increased HMOX1 activity, regulation of HMOX1 in either direction suggests potential changes in redox species (ROS)

metabolism. Ultimately, it is possible that compensatory and/or feedback loops might lead to HMOX1 total protein levels downregulation under high levels of oxidative stress.

Mitochondrial proteins were also among the top-enriched proteins regulated by rapamycin and Torin1. Interestingly, rapamycin exhibited a subtler yet broader regulation of mitochondrial proteins with proteins associated with apoptosis and mitochondrial fission being up- (BNIP3L, +1; FIS1, +0.88) and others being downregulated (TIMM17A, -0.85; GHITM, -1.3; ATP5B, -1.21). Notably, GHITM, an inner mitochondrial membrane negative regulator of apoptosis was downregulated, which, combined with upregulation of BNIP3L, a pro-apoptotic protein, suggests that rapamycin might lead to increased levels of apoptosis. Additionally, rapamycin-induced downregulation of ATP5B, a subunit of the mitochondrial ATPase, suggests overall reduced ETC functioning. Torin1 treatment, on the other hand, primarily led to dramatic downregulation of TIMM17A (-2.68) implying changes in mitochondrial matrix protein import. In the future, oxygen consumption rate (OCR) and extra-cellular acidification rate (ECAR) assays will be performed to correlate these *in silico* observations with functional readouts.

Other categories enriched among rapamycin and Torin1's top-regulated proteins were related to lipid metabolism, inflammation, energy sensing/metabolism and the cytoskeleton. Interestingly, some of these categories were primarily affected by only one of the two inhibitors with lipid metabolism and energy/sensing metabolism being strongly affected by Torin1 and inflammation being mainly affected by rapamycin. Lipid metabolism, specifically fatty acid (FADS2 -1.95; SCD, -1.82) and cholesterol metabolism-related proteins (HMGCS1, -1.68) were all strongly downregulated by Torin1 implying a decrease in the biosynthesis of specific lipid types. This is consistent with mTORC1's ability to regulate lipin1 localization and control the SREBP pathway[31], a master regulator of a lipo- and sterol-genic transcription program. Though the exact mechanism underlying Torin1's ability to affect lipid metabolism to a much greater extent than rapamycin remains elusive, the original study that revealed SREBP to be under mTORC1 control showed that Torin1, but not rapamycin was able to fully abolish mTORC1-driven lipin1 phosphorylation, thus leading to full inhibition of the SREBP pathway[31]. mTORC2, which is inhibited only by Torin1 and thus is an obvious

candidate to explain rapamycin and Torin1's differential effects on the SREBP pathway, does not seem to be involved as RAPTOR knockdown, an essential component of mTORC1, recapitulates Torin1's effects on the SREBP pathway[31]. Interestingly, Torin1 led to significant upregulation of epoxide hydrolase 2 (EPHX2, +1.72) suggesting a possible increase in breakdown of fatty acid epoxides such as epoxyeicosatrienoic acids (EETs), which have been implicated in anti-inflammatory processes[184].

Inflammation-related proteins such as IFRD1 (-1.75) and TSC22D3 domain family member 3 (TSC22D3, +0.87) were respectively down- and upregulated by Rapamycin. IFRD1 and TSC22D3 are both anti-inflammatory proteins that can repress NF-kB activity. It is difficult to deduce the net effect produced by simultaneous IFRD1 down- and TSC22D3 upregulation on inflammation levels, however enrichment of both of these regulators suggests that rapamycin might induce changes in the NF-kB pathway.

Torin1 did not induce meaningful changes in either of these last two regulators, but seemed to uniquely affect energy sensing/metabolism-related proteins instead. Protein kinase cAMP-dependent type 1 regulatory subunit beta (PRKAR1B, -1.84) and hexose-6-phosphate dehydrogenase/glucose 1-dehydrogenase (H6PD, +1.54) were respectively down- and upregulated. PRKAR1B is an inhibitory regulatory subunit of PKA, which is in charge of sensing the cellular energy status via the presence of cAMP. H6PD is a key, initial step of the oxidative branch of the pentose phosphate pathway (PPP), which provides energy for biosynthetic processes. Regulation of these proteins could reflect an adaptive rewiring of cellular metabolism whereby PKA sensitivity to cAMP is adjusted to a lower, Torin1-induced energy state, while PPP is primed for increased metabolic demand. The cause underlying this speculative Torin1-induced lower energy state, whether attributable to mitochondrial dysfunction or other metabolic deficits remains to be determined.

Another protein category primarily affected by Torin1 was related to regulation of transcription with some key transcription factors being strongly upregulated (JUN, +2.09; ATF3, +1.83; ZNF362, +1.58). MRNIP, a nuclear protein involved in DNA-damage and cell cycle progression, was also strongly upregulated (+2.31).

Upregulation of MRNIP, ATF3 and JUN seems to reflect a general stress response, specifically a starvation (ATF3) and an oxidative-stress (MRNIP and JUN) response. JUN transcription factor can be induced by inflammatory cytokines, UV exposure and oxidative stress further supporting the presence of oxidative stress in Torin1-treated cells. None of these transcription factors were observed among rapamycin's top enriched proteins, however MXD4, a tumor suppressor which antagonizes MYC, was strongly upregulated exclusively by rapamycin. Interestingly, rapamycin was previously reported to control MYC translation[185] and signaling[186].

Both rapamycin and Torin1 are known for their anti-proliferative effects, however the exact mechanisms underlying this phenomenon are only partially understood. Rapamycin is thought to inhibit cell proliferation by inhibiting ribosomal biogenesis and protein synthesis in addition to affecting cell cycle-related proteins[152]. These findings suggest a novel mechanism through which rapamycin might inhibit cell proliferation, namely repression of MYC signaling via upregulation of MXD4. Torin1 is also thought to inhibit cell proliferation via inhibition of protein synthesis in addition to affecting synthesis of other essential biomolecules such as lipids. Our data suggests that Torin1 might also induce cell cycle arrest via upregulation of MRNIP, a mitotic G2 DNA damage checkpoint effector, and upregulation of stress-related transcription factors such as ATF3 and JUN. Further studies will be required to ascertain whether upregulation of total levels of the aforementioned nuclear proteins and transcription factors leads to increased transcription of their targets and/or cell cycle arrest.

The last protein category that was enriched among rapamycin and Torin1's top hits was cytoskeletal proteins with rapamycin strongly downregulating actin gamma 1 (ACTG1, -1.88) and Torin1 downregulating suppressor APC domain containing 2 (SAPCD2, -1.94), while strongly upregulating ras homlog family member B (RHOB, +2.24). Actin gamma proteins are essential components of the cellular cytoskeleton which primarily mediate intracellular motility, while SAPCD2 and RHOB are, respectively, a proto-oncogene and a tumor suppressor. While ACTG1 downregulation is more difficult to interpret, the simultaneous downregulation of proto-oncogene SAPCD2 and upregulation of tumor suppressor RHOB in Torin1 suggests a possible cytoskeleton-mediated mechanism for repression of cell proliferation. Additionally, RHOB's proclivity to be induced upon oxidative stress could further suggest that

oxidative stress plays a major role in Torin1-induced cell cycle arrest by inducing a multi-layer response including transcriptional, mitochondrial and cytoskeletal changes. However, RHOB-dependent effects might not be unique to Torin1 as RHOB upregulation was detected by a separate proteomics experiment which involved rapamycin treatment[152].

In summary, our analysis of rapamycin and Torin1's top up- and downregulated proteins suggests that both inhibitors are likely to affect multiple cellular functions as evidenced by changes in the total levels of both common (PDCD4, RBIS, TIMM17A, HMOX1) and unique effectors (Rapa: MXD4, SLC7A11, IFRD1, ACTG1, PTDSS1; Torin1: MRNIP, RHOB, JUN, FADS2, SAPCD2). Moreover, these findings suggest the presence of new anti-proliferative mechanisms for each inhibitor including inhibition of MYC signaling for rapamycin and oxidative stress-dependent transcriptional and cytoskeletal changes for Torin1. Importantly, many of the top differentially-regulated proteins in rapamycin were confirmed in a separate proteomics experiment generated for a different project[152]. Further studies should be conducted to experimentally test the role that these key effector proteins play in shaping the pharmacology of rapamycin and Torin1.

3.1.3 AA starvation vs Torin1: differential effects on mitochondrial proteins

Amino acid starvation had profound effects on total levels of nuclear (discussed in **section 3.1.4**) and mitochondrial proteins. In addition to inducing major transcriptional responses such as the ATF4-mediated starvation response, changes in total levels of multiple mitochondrial proteins suggest regulation of mitochondrial activity and, possibly, oxidative phosphorylation (OXPHOS). AA starvation has been reported to increase mitochondrial fusion[187], respiration and membrane potential in human cells[188]. At first glance, this seems counterintuitive since, in the absence of important metabolites like AAs, one would expect downregulation of cellular respiration as an adaptive, energy-conserving mechanism. However, it has been speculated that upregulation of OXPHOS might serve as a mechanism to inhibit nutrient-demanding anabolic processes such as cytosolic protein synthesis by redirecting AAs to the TCA cycle[188]. This is consistent with our proteomics data which show that AA starvation specifically induces upregulation of mitochondrial phosphoenolpyruvate

carboxykinase 2 (PCK2, +1.1) and aldehyde dehydrogenase 1 family member L2 (ALDH1L2, +1.47) (Fig. 2.12B), two key enzymes involved in the TCA cycle.

Increased rates of cellular respiration could also explain the upregulation of many mitochondrial oxidative stress-related proteins, which we observed in response to AA starvation (Fig. 2.12B). Arginine starvation has been shown to damage mitochondria via asparagine synthetase (ASNS) upregulation, which in turn leads to aspartate depletion and dysfunction of the mitochondrial malate-aspartate shuttle[189]. Our proteomics data did show mild, yet statistically-significant ($p<0.05$), upregulation of total ASNS levels (+0.154) following AA starvation, which further supports the idea that AA depletion, in particular arginine depletion, damages mitochondria. Notably, ASNS was strongly downregulated by both Torin1 (-1.18) and rapamycin (-0.61), suggesting that the upregulation of oxidative stress-related proteins observed after treatment with these two inhibitors is likely caused via different mechanisms. Rapamycin-induced ASNS downregulation was also observed in our study of rapamycin's specificity to mTOR both at 24 (-0.82) and 48 (-1.8) hours[152] (see **section 2.8**). In the future, it would be interesting to test whether aspartate supplementation, Torin1 or rapamycin treatment could rescue AA starvation-induced mitochondrial dysfunction by replenishing aspartate reserves or downregulating ASNS levels, respectively.

Torin1 has likewise been reported to induce changes in mitochondrial morphology and activity. Specifically, Torin1 promotes mitochondrial fusion[190], similarly to AA starvation, while increasing iron-induced mitochondrial redox species[183]. mTORC2 activity was shown to promote mitochondrial fission, as opposed to fusion, via a NDRG1-CDC42 axis[191]. This is interesting because Torin1 may then promote mitochondrial fusion in part by inhibiting the mTORC2-NDRG1-CDC42 signaling axis. This might also explain why non-ATP-competitive inhibitors like rapamycin don't fully recapitulate the Torin1-induced changes in mitochondrial morphology. The differential effects of rapamycin and Torin1 on mitochondrial morphology might be relevant for the treatment of mitochondrial diseases in which mitochondrial fusion and/or fission is affected. Ultimately, functional studies are needed to fully dissect the differential effects that AA starvation and Torin1 have on mitochondrial morphology and functioning. In the meantime, our proteomics analysis suggests that AA starvation and Torin1 may both

lead to increased mitochondrial fusion, while inducing oxidative stress via differential mechanisms.

3.1.4 AA starvation vs Torin1: differential nuclear responses

Another interesting contribution of this study is the comparison of nuclear proteins regulated by AA starvation and Torin1. AA starvation is frequently modeled with Torin1 treatment as Torin1 is considered a stronger mode of mTOR inhibition and thus more resemblant of the effects of AA starvation. Some top up- and downregulated nuclear proteins induced by Torin1 have already been discussed as part of our comparison between rapamycin and Torin1 and thus will not be repeated. AA starvation was found to upregulate stress-related transcription factors such as ATF3, MITF and TFE3 which were not affected, with the exception of ATF3, by Torin1 treatment. Several ATF4-dependent starvation responses under mTORC1 control have already been identified by researchers[192]. ATF3 is known to be one of the first genes to be upregulated by ATF4 upon AA starvation[193]. Although we did not observe an increase in total ATF4 levels, increased total levels of ATF3 suggest that an ATF4 stress response was initiated by both AA starvation and Torin1. This is valuable information for researchers seeking to study ATF4-dependent starvation responses with Torin1.

However, it is important to highlight how Torin1 treatment did not lead to upregulation of MITF and TFE3 making Torin1 a less valid option for researchers interested in studying starvation-induced changes in survival and lysosome-related proteins, respectively. Reassuringly, linker histone H1.0 (H1F0), a histone currently under investigation, which seems to be upregulated by starvation and even RagA/B DKO (data not shown), was upregulated by both AA starvation and Torin1. ChIP-Seq experiments should be conducted in the future to identify genes associated with histone H1.0 in order to expand our understanding of starvation-induced chromatin changes and transcriptional responses.

Notably several proteins with transcription coregulatory (AKIRIN1, EID2, TAF8, ZBTB2, ZFX) and cyclin-interacting activity (CCNDBP1, UBE2C) were exclusively affected by AA starvation highlighting the challenge in modeling AA starvation with Torin1. Likewise, other proteins involved in DNA replication (CHTF8), DNA damage

(RAD9A) and transcription (MNT) were only affected by AA starvation. Finally, Torin1 treatment led to unique changes in nuclear proteins that, among other functions, were involved in transcription (JUN, ZNF593, TCEA2), ubiquitination (HECTD1, ATXN3) and DNA damage (POLL, MRNIP), once again highlighting the limits of Torin1 treatment as a reliable starvation model.

3.1.5 Remodeling of mitochondrial proteome across interventions: a look at the top hits

Mitochondrial proteins were universally enriched across all four modes of mTOR inhibition as evidenced by GO term enrichment analysis. As mentioned in the results section, Rapamycin, Torin1 and AA starvation upregulated the vast majority of OMM and matrix proteins. Within matrix proteins, COQ proteins (COQ5, COQ7, COQ9), which are responsible for the synthesis of coenzyme Q10, were virtually all upregulated by the “acute” interventions. Coenzyme Q10 (CoQ10) is an essential electron carrier that transports electrons from ETC complexes I and II to complex III thereby allowing aerobic respiration to take place. Upregulation of COQ5/7/9 suggests an increased demand for CoQ10, which in turn could reflect a compensatory response to mitochondrial dysfunction or increased ATP demand. Alternatively, increased demand for CoQ10 could be due to oxidative stress since CoQ10 has been shown to reduce lipid peroxidation and oxidative damage of mitochondrial membrane proteins and mitochondrial DNA (mtDNA)[194,195]. More recently, CoQ10 supplementation has been suggested as a possible strategy to replenish aging-induced drops in CoQ10 levels[196]. In the future, it would be interesting to study whether the healthspan benefits afforded by rapamycin could be partially mediated by upregulation of CoQ10 biosynthesis.

Thioredoxin reductase 1/2 (TXNRD1/2), two oxidoreductases with potent anti-oxidant activity, were upregulated by Torin1 and AA starvation suggesting that the increased demand for CoQ10 might be partly due to increased oxidative stress, at least in these two settings. On the other hand, TXNRD1 was also upregulated in RagA/B DKO cells, which did not upregulate any COQ protein suggesting again that different modes of mTOR inhibition have unique ways of inducing and coping with oxidative stress. Remarkably, CoQ10-dependent ETC components ubiquinol-cytochrome c reductase

binding protein (UQCRB) and NADH:ubiquinone oxidoreductase subunit A7 (NDUFA7) which belong, respectively, to complex III and I, were upregulated, albeit to different extents, by Rapamycin, Torin1 and AA starvation. This could suggest an attempt to increase ETC activity in response to these interventions. Whether oxidative stress is the cause or the product of mitochondrial dysfunction and whether upregulation of ETC components is a cause or a response to such dysfunction cannot be determined by our methods. This is one of our study's main limitations since our proteomics data only provides us with a snapshot of the cellular proteome at a given time representing the net effect of both direct and compensatory changes.

An important mitochondrial protein which exhibits an interesting pattern of regulation across our interventions and is thus worth discussing more in depth is PRELID domain containing 1 (PRELID1), which is strongly downregulated by AA starvation (-2.58) and only mildly downregulated (-0.81) by Rapamycin. PRELID1 is located in the intermembrane space (IMS) and inhibits the mitochondrial apoptotic pathway by enabling cardiolipin accumulation within mitochondrial membranes[197]. This suggests that AA starvation may sensitize cells to apoptosis by downregulating the cytoprotectant PRELID1 or that more indirect mechanisms might trigger apoptosis, which in turn would lead to lower total PRELID1 levels. This could be tested by measuring whether levels of apoptosis are rescued by PRELID1 overexpression in AA-starved cells. The lack of PRELID1 downregulation in Torin1 may also suggest that AA starvation and Torin1 induce apoptosis via different mechanisms. Finally, Diablo IAP-binding mitochondrial protein (DIABLO) upregulation across Rapamycin, Torin1 and AA starvation also suggests increased levels of apoptosis or at least initiation of the apoptotic process which, especially in the case of 24-hour treatment with Torin1 and AA-depleted media, is consistent with the minimal levels of cell detachment observed in cell culture (data not shown).

The final mitochondrial protein worth of a more comprehensive discussion is TIMM17A, which sits at the top of all downregulated mitochondrial proteins together with PRELID1. TIMM17A is an essential component of the inner mitochondrial membrane (IMM) TIM23 transport complex. Although the exact function of TIMM17A (also known as TIM17) is not fully characterized, a recent structural study of TIM23 transport complex showed that a cavity within TIM17 forms the protein translocation

pathway[198], indicating that TIM17 plays a fundamental role within the complex. No additional information regarding TIM17 and its function, beyond its association with breast cancer when overexpressed[199], is available. However, we can speculate that strong downregulation of TIMM17A would likely lead to impaired protein transport, alterations in the mitochondrial proteome and possibly mitochondrial dysfunction. RagA/B DKO cells did not downregulate TIMM17A levels suggesting that this is likely to be a response to acute mTOR inhibition. In fact, restoring a baseline level of TIMM17A expression might have been essential for RagA/B DKO cells' long-term survival and might represent a compensatory mechanism. Additional, differential responses between RagA/B DKO cells and acute modes of mTOR inhibition can be observed in the levels of other important mitochondrial enzymes such as phosphoenolpyruvate carboxykinase 2 (PCK2) and aldehyde dehydrogenase 6 family member A1 (ALDH6A1) which possibly underscore the different demands of chronic vs acute mTOR inhibition.

Ultimately, the mitochondrial proteome was significantly changed by all modes of mTOR inhibition. Proteins related to the ETC, CoQ10 synthesis, oxidative stress and apoptosis were strongly affected suggesting mitochondrial stress. Key mitochondrial effectors such as PRELID1 and TIMM17A were downregulated in an intervention-dependent manner with PRELID1 being strongly downregulated only by AA starvation and TIMM17A being downregulated by all acute interventions. Future functional assays that measure rates of aerobic respiration, mitochondrial fragmentation and apoptosis will be needed to further elucidate the relationship between mTOR inhibition and mitochondrial function.

3.1.7 Rag-independent and -dependent responses to AA starvation

Our study of AA starvation-induced proteomic changes in RagA/B DKO cells revealed a variety of Rag-independent and -dependent changes. Some of the top Rag-independent changes were upregulation of transcription factors ATF3, MAFG and TFE3. Increased ATF3 levels are consistent with mTORC1's ability to control ATF4's translation and mRNA stability[144], two mechanisms which do not involve RagA/B GTPases. mTORC1 controls the expression levels of AA transporters via ATF4[144], which is also consistent with our proteomics data which showed Rag-independent

changes in different AA transporters (SLC7A11, +1.11; SLC38A2, +0.98; SLC16A2, -1.8; WT log2FC values). These changes were also observed in the proteomics data obtained from our original AA starvation experiments, which were performed independently from later AA starvation experiments designed to identify Rag-independent and -dependent responses.

Activation of ATF4 has been shown to lead to increased glutathione synthesis[192], which could be one of the reasons underlying changes in levels of SLC7A11, the cysteine-glutamate antiporter, which was observed to change in response to multiple modes of mTOR inhibition. Interestingly, SLC7A11 was either up- or downregulated depending on the mode of mTOR inhibition with AA starvation upregulating it and Rapamycin as well as RagA/B DKO downregulating it. This differential regulation could reflect the different priorities that each mode of mTOR inhibition have in relation to maintaining balance between cysteine and glutamate. It is possible that in the absence of amino acids, the limiting factor for glutathione synthesis is cysteine rather than glutamate which would require upregulation of SLC7A11. Alternatively, glutamate could still be the limiting factor for glutathione synthesis in the absence of amino acids, but, given the total lack of all amino acids, the cell might decide to prioritize cysteine import in order to feed other critical biosynthetic pathways. However, AA-induced upregulation of cystathionine gamma-lyase (CTH, +1.66 in WT) could indicate a pressing need for glutathione synthesis even during AA starvation since CTH catalyzes the conversion of cystathionine into cysteine, an essential building block of glutathione.

Out of the three transcription factors (ATF3, MAFG, TFE3) that were upregulated by AA starvation in a Rag-independent manner, the presence of TFE3 was the most surprising since TFE3 relies on Rag GTPases, in particular RagA/D dimers, to properly respond to AA availability[34,150]. In the absence of RagA/B, RagA/D dimers cannot form and thus cannot phosphorylate TFE3 to deactivate it and sequester it in the cytoplasm in the presence of amino acids. Thus, we would expect TFE3 to be always active and localized to the nucleus regardless of the presence of amino acids in the absence of RagA/B. The fact that total TFE3 protein levels increased during AA starvation in RagA/B DKO cells (although to a lesser extent than in WT cells) suggests the presence of redundant, Rag-independent mechanisms through which TFE3 could be regulated.

It is possible that constitutively active TFE3 might lead, over time, to downregulation of TFE3-regulated lysosomal genes in order to prevent excessive lysosomal biogenesis in the presence of nutrients. TFE3 upregulation might then be needed in RagA/B DKO cells to re-activate transcription of lysosomal genes under conditions of actual nutrient scarcity. However, this hypothesis is undermined by the fact that WT cells also greatly increased TFE3 levels upon AA starvation, suggesting that upregulation of total TFE3 levels is likely a normal response to AA starvation. It is even possible that RagA/B might be partially responsible for increased total TFE3 levels given the disparity between levels of TFE3 upregulation seen in WT (+1.49) vs RagA/B DKO cells (+0.32). Ultimately, more experiments are needed to understand TFE3's behavior and regulation, but it is likely that both regulation of total protein levels, in addition to Rag-driven post-translational modifications (PTMs), are required for proper TFE3 functioning during AA starvation. Interestingly, other transcription factors were upregulated in a strictly Rag-dependent manner (MITF, MNT, ZFX), which suggests the existence of AA starvation-induced transcriptional responses that are both Rag-dependent and independent.

Additional proteins related to processes such as protein synthesis regulation (PDCD4, EIF4EBP1, RBIS), the cell cycle (MLF1, CHTF8, CDCA5, CCNDBP1), lipid metabolism (FDFT1, FAR1, FADS2, CYP51A1, ADIPOR1), inflammation (IFRD1, TSC22D3) and ubiquitination (UBE2C, RNF181, OTUD5) were regulated by AA starvation in a Rag-independent manner. Total levels of direct mTOR targets such as PDCD4 and 4E-BP1, which are phosphorylated by mTOR in a Rag-dependent manner, were nonetheless upregulated upon AA starvation in a Rag-independent manner. This reveals how, like in the case of TFE3, total protein levels might play an important role in the response to AA starvation. The stability of both PDCD4 and 4E-BP1 has been shown to be affected by their phosphorylation status[200-205], thus it is conceivable that these two negative regulators of protein synthesis might be stabilized via phosphorylation of Rag-independent phosphorylation sites. This would provide a mechanistic explanation for the increase in PDCD4 and 4E-BP1 total protein levels observed in both WT and RagA/B DKO cells.

Decreased ubiquitination levels could also offer an alternative explanation for the increased levels of PDCD4 and 4E-BP1 since both are subject to ubiquitination[205,206]

and significant changes in ubiquitination-related proteins (UBE2C, RNF181, OTUD5) were observed in AA starvation. Interestingly, ubiquitin-conjugating enzyme 2C (UBE2C) has been shown to affect mTOR signaling via ubiquitination of mTOR-inhibitor DEPTOR[207]. Thus, UBE2C could potentially affect PDCD4 and 4E-BP1 stability by regulating mTOR activity via DEPTOR levels. However, this hypothesis is complicated by the fact that DEPTOR was found to be upregulated (+0.6, data not shown) exclusively in a Rag-dependent manner, suggesting that UBE2C downregulation may not in fact affect DEPTOR levels in this setting.

Fatty acid-related proteins such as fatty acyl-coA reductase 1 (FAR1, -2.59 in WT) and fatty acid desaturase 2 (FADS2, -2.13 in WT) were strongly downregulated in a Rag-independent manner, while others like stearoyl-coA desaturase (SCD, -2.82), an enzyme catalyzing the rate-limiting step of monounsaturated fatty acids (MUFAs) biosynthesis, were downregulated in a Rag-dependent manner. Rate-limiting step enzymes are often tightly regulated since they often commit a substrate to a particular biochemical reaction, which cannot always be easily reversible. This is the case for SCD which catalyzes the insertion of a cis double bond at the Δ-9 position into palmitoyl-CoA and stearoyl-CoA. SCD expression levels have already been shown to be downregulated by rapamycin[208], which our study confirms at the protein level. However, to our knowledge, we are the first to show downregulation of SCD across multiple modes of mTOR inhibition and to observe that AA starvation-induced SCD downregulation is Rag-dependent. Further experiments including western blot analysis of starved WT and RagA/B DKO cells are required to confirm this finding and additional work is needed to determine the underlying mechanism. Nonetheless, our proteomics data suggests MUFAs biosynthesis as a potential sub-pathway within lipogenesis that is potentially regulated in Rag-dependent fashion under starvation.

Our analysis revealed that one of the strongest responses to AA starvation, which we have previously discussed, namely the downregulation of mitochondrial proteins PRELID1 and TIMM17A, were strictly Rag-dependent. This suggests that RagA/B might potentially signal the absence of amino acids to the mitochondria via unknown mechanisms. A previous study has already uncovered a connection between the mitochondrial threonyl-tRNA synthetase (TARS2) and RagA/C dimers, which are activated by TARS2 in the presence of threonine thereby influencing mTORC1

activity[142]. Given the presence of crosstalk between Rags and mitochondrial proteins, it is conceivable that Rag GTPases themselves might influence mitochondrial activity via uncharacterized feedback loops. At the very least, Rag GDP/GTP loading status is likely to influence mitochondrial activity via promotion or inhibition of mitophagy, a specialized form of autophagy regulated by mTORC1 activity.

An additional connection that emerged from our study of Rag-dependent responses to AA starvation, which could potentially provide a bridge between the Rags and mitochondrial function is that both PRELID1 and SCD are downregulated in a Rag-dependent fashion. As mentioned before, PRELID1 controls cardiolipin accumulation with the inner mitochondrial membrane, which ensures mitochondrial structural integrity and prevents unregulated cytochrome c release. Cardiolipin is a unique, dimeric phospholipid containing four acyl chains. SCD can alter the composition of fatty acids contained by various lipids including phospholipids and cardiolipin. SCD downregulation could have significant impacts on the saturation levels of fatty acids incorporated in cardiolipin thus changing the biophysical properties of mitochondrial membranes. Malfunctioning of mitochondrial membranes could then lead to regulation of PRELID1 levels in an attempt to “correct” mitochondrial membranes’ lipid composition. Mitochondrial dysfunction driven by membrane incorporation of defective cardiolipin molecules could also partially explain the presence of high levels of oxidative stress implied by the upregulation of multiple antioxidant proteins observed in AA starvation.

3.2| Limitations and future directions

Although this study provides useful information on how the levels of classes and/or individual proteins change in response to different modes of mTOR inhibition, we were unable to conclusively determine whether specific biological processes were affected by some interventions and not others. In certain cases, GO term enrichment analysis did produce terms that had a high degree of statistical significance only in one of the two interventions under comparison, but rarely there were terms that were highly significant in only one out of the four modes included in this study. Thus, it is difficult to assign particular biological processes or signaling pathways to specific modes of mTOR inhibition.

For instance, we cannot claim that Torin1 is the only intervention that leads to dramatic changes in mRNA splicing only because spliceosome was the top CC GO term for that intervention. Likewise, we cannot say that lipid metabolism wasn't affected in RagA/B DKO cells only because of a lack of highly enriched lipid-related GO terms. However, we can conclude, in the context of two-way comparisons, that some classes of proteins associated with particular functions, are more impacted in one of the two interventions. It is challenging to quantify the level of such impact, but we can at least use parameters such as the GO term's p-value, count (# of proteins) and fold enrichment to focus the direction of future studies on a shorter list of biological functions or organelles.

It would be simplistic to assume that some modes of mTOR inhibition would affect some biological processes in isolation without exerting any effects on other cellular functions. Our proteomics data suggest that different modes of mTOR inhibition affect multiple aspects of cellular physiology to different degrees and via different effectors. This would likely lead to subtle yet meaningful differences in how metabolic and signaling pathways are regulated. It is also difficult to predict whether a biological process as a whole is up- or downregulated by a specific intervention without integrating information regarding the function of individual proteins. For instance, downregulating the levels of a major, negative regulator of protein synthesis might result in increased total levels of translation. This should caution against assuming that a particular biological process is up- or downregulated solely on the basis of whether the majority of underlying proteins associated with the term are up- or downregulated.

Another issue to consider when trying to predict how a certain biological process will be affected based on protein level changes is the issue of weighing. Different proteins have varying levels of impact on a particular pathway, with major signaling nodes or rate-limiting steps having a greater impact than minor regulators. For instance, changes in the total levels of a transcription factor will have cascading effects on the expression levels of possibly hundreds or thousands of proteins, while changes in the levels of a downstream effector protein might have a much more limited impact. Thus, ideally each protein's contribution to a particular pathway should be weighed to make more accurate predictions.

More and more sophisticated systems biology tools surpassing GO term enrichment analysis are being developed to predict how different biological functions are affected based on omics data. For instance, a novel signaling pathway impact analysis (SPIA) tool, which attempts to take into account the magnitude of individual genes' expression changes as well as their position and interactions within a given pathway[209,210], is freely available as a R package: (https://bioconductor.org/packages/release/bioc/html/SPIA.html). Unfortunately, this tool requires more advanced bioinformatics skills and was developed for analysis of RNA-Seq data which is not applicable to our proteomics datasets.

Many more tools aimed at overcoming the limitations of classical ontological profiling, namely the reliance on over-representation analysis (ORA) and functional class scoring (FCS), have been developed since SPIA. Notably, the more recent causal interactions from molecular profiles (CausalPath) tool developed by Babur et al.[211], although designed primarily for phosphoproteomics, is compatible with total proteomics datasets and might represent a valuable tool for further analysis of our data. Ultimately, the advent of more powerful data-driven methodologies including new machine-learning models will allow us in the future to more accurately predict the effects of particular interventions on cellular physiology with proteomics and other high-throughput datasets.

Another addition that could benefit this study, besides the implementation of more powerful bioinformatics approaches, would be the inclusion of functional assays to quantify the effects of different modes of mTOR inhibition on cellular functions such as protein and mRNA synthesis as well as autophagy and mitochondrial respiration. Cell proliferation, survival and motility assays could also be included to quantify the effects of the changes observed in cell cycle, apoptosis and cytoskeletal proteins. Preliminary oxygen consumption rate (OCR) assays suggest that RagA/B DKO cells have lowered baseline OCR (data not shown), which might be explained by the general downregulation of top mitochondrial proteins, including ETC components, observed in our proteomics experiments. Future OCR assays will also include Rapamycin, Torin1 and possibly AA starvation as additional experimental conditions. AA starvation is difficult to test in Seahorse assays for technical reasons, namely the number of washes required to remove all AAs, which leads to significant cell detachment of HEK293FT

cells, however we will attempt to test it whenever possible. Ultimately, the inclusion of functional assays will allow us to test the predictive power of our *in silico* approach and draw more definitive conclusions regarding the different cellular functions affected by our treatments.

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Appendix A: a note on methods

All methods used for cell culture, drug treatments, western blot analysis, proteomics and bioinformatics analysis are described in the methods' section of the following paper:

Artoni, F., Grutzmacher, N. & Demetriades, C. Unbiased evaluation of rapamycin's specificity as an mTOR inhibitor. *Aging Cell* **22**, e13888 (2023). Available at: https://doi.org/10.1111/acel.13888

Upon publication of the work described in this thesis, all processed proteomics data will be provided in excel tables and raw proteomics data will be uploaded on Proteomics IDEntifications database (PRIDE). We will also include an interactive, browsable excel file for easy data navigation. All raw data for Artoni et al. (2023) is available at the URL addresses listed in the paper.

The following antibodies, not listed in Artoni et al. (2023) were used:

- Rabbit polyclonal anti-TFEB, 1:1000 WB, CST 4240
- Rabbit polyclonal anti-phospho-AKT (S473), 1:1000 WB, CST 9271
- Rabbit polyclonal anti-AKT, 1:1000 WB, CST 9272
- Rabbit polyclonal anti-phospho-4EBP1 (T37/46), 1:1000 WB, CST 9452
- Rabbit polyclonal anti-4E-BP1, 1:1000 WB, CST 9452

All information regarding proteins mentioned in the discussion was obtained by consulting appropriate databases such as UniProt.

Appendix B: Erklärung zur Dissertationgemäß der Promotionsordnung vom 12. März 2020

Erklärung zur Dissertation

gemäß der Promotionsordnung vom 12. März 2020

„Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen dieser Ordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht."

Teilpublikationen: Artoni, F., Grutzmacher, N. & Demetriades, C. Unbiased evaluation of rapamycin's specificity as an mTOR inhibitor. *Aging Cell* **22**, e13888 (2023). https://doi.org:10.1111/acel.13888

Datum, Name und Unterschrift

24/09/2023, Filippo Artoni