Evolution of Nutrient Sensing in the mTORC1 Pathway

by

Grace Y. Liu

B.S. and M.S. Molecular Biochemistry and Biophysics
Yale University, 2016

SUBMTITED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

FEBRUARY 2023

Signature of Author: _____
Department of Biology
November 15, 2022

Certified by: _____
Iain Cheeseman
Member, Whitehead Institute
Professor of Biology
Thesis Supervisor

Accepted by: _____
Mary Gehring
Member, Whitehead Institute
Associate Professor of Biology
Director, Biology Graduate Committee

Evolution of nutrient sensing in the mTORC1 pathway

by

Grace Y. Liu

Submitted to the Department of Biology on December 15, 2022 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

ABSTRACT

The mTORC1 pathway regulates growth and metabolism in response to nutrient availability. In mammals, the mTORC1 pathway monitors the concentration of certain amino acids using dedicated nutrient sensors, which bind directly to their cognate metabolites. Unlike other components of the mTORC1 pathway, which are present from yeast to human, the known nutrient sensors are poorly conserved in lower eukaryotes and may have emerged as specialized innovations. A goal of mTORC1 biology is to understand the evolutionary mechanisms that allow a highly conserved core pathway to adapt to the diverse nutritional niches that animals occupy. How does the mTORC1 pathway add new layers of regulatory sophistication to accommodate animals with divergent diets and lifestyles? Do organisms acquire novel nutrient sensors under environmental pressure? If so, where do those sensors come from?

In this thesis, we discover a new species-specific *S*-adenosylmethionione (SAM) sensor and use its evolutionary history to pry open the structural logic of the mTORC1 pathway. We show that the sensor, the *Drosophila melanogaster* protein Unmet expectations (Unmet, formerly CG11596), is an "evolutionary intermediate," caught between its ancestral enzymatic function and a recently acquired role in the mTORC1 pathway. Unmet interacts with the fly GATOR2 (dGATOR2) complex, a core component of the pathway, to inhibit dTORC1 during methionine starvation. This inhibition is directly relieved by SAM, a proxy for methionine availability. Unmet expression is elevated in the ovary, a methionine-sensitive niche, and flies lacking Unmet fail to maintain the integrity of the female germline under methionine restriction. By tracing Unmet's incorporation into the mTORC1 pathway, we show that Unmet was an independent methyltransferase before it was captured by flexible loops on the GATOR2 complex. These data suggest a general mechanism in which the mTORC1 pathway assimilates new sensors by using evolvable modules on core complexes to co-opt proteins with ligand-binding capabilities. We discuss how similar principles can be used to build artificial sensors for the mTORC1 pathway and explore how repurposing ancient enzymes enables the mTORC1 pathway to rapidly adapt to metabolic niches across evolution.

Thesis supervisor: David M. Sabatini
Title: Formerly Professor of Biology

Acknowledgements

This thesis is dedicated to my advisors, Drs. David Sabatini and Norbert Perrimon. I "met" David through his papers—dazzling tours de force spanning mTOR, mitochondria, and metabolism—years before I encountered him in person. Meeting your scientific heroes is usually more punishment than reward, but in David the scientist, I saw the same qualities that set his work apart: an astonishing knack for asking the right questions, a critical eye, and an audacity and clarity of thought bent toward the pursuit of something *new*. If I am a scientist today, it is because David created opportunities for me and committed himself to every step of my training and development. I will always be in his debt.

I have been lucky enough to train with not one but two of the towering figures in modern biology. At a critical juncture in my PhD, Norbert offered me shelter and put his insight and resources at my disposal. Norbert's kindness to me encapsulates, in miniature, his broader generosity as a pillar of the fly community. In his lab, I've seen how new tools empower biological discovery, especially when shared freely with collaborators around the world.

I am grateful to the mentors who served on my thesis committee for their patience and support. Drs. Iain Cheeseman, Bob Sauer, and Harvey Lodish have gone far beyond the usual call of committee member duty, advocating for my best interests with the same conviction that they would bring to bear for their own trainees. I would also like to thank Dr. Brendan Manning for serving as the external examiner on my thesis committee.

Over the past two decades, my colleagues in the Sabatini lab have mapped the mTOR pathway from the kinase at its heart to the sensors at its outer periphery. In doing so, they have redefined our understanding of growth control and identified a murderers' row of therapeutic targets. To keep company with these labmates—and to add my own contribution to their body of work—has been the privilege of a lifetime. I am indebted to Kendall Condon and Justin Roberts, my beloved baymates, for their advice and encouragement. For six years, they have inspired me with their rigor, experimental savvy, and encyclopedic Spongebob knowledge. In their friendship, I found a scientific home.

I am also grateful to Jose Orozco, who taught me to be skeptical but fearless, and to Kuang Shen and Max Valenstein, whose scientific advice shaped this thesis. There's no better whetstone for dull scientific instincts than spending time with the sharpest, most dedicated researchers in town, and in that spirit, I owe a debt of gratitude to all of my labmates. For the good times, the bad times, and the times of uncertain valence, thank you: Jordan Ray, Jibril Kedir, Hank Adelmann, Jess Spinelli, Andrew Cangelosi, Ania Puszynska, Genya Frenkel, Raghu Chivukula, Xin Gu, Paul Rosen, Julian Roessler, Bobby Saxton, Anna Traunbauer, Monther Abu-Remaileh, Nora Kory, and Tong Zhang. Although we never overlapped in the lab, Shuyu Wang is my favorite member of the alumni corps, and I admire her wisdom and curiosity more than I can say. The Sabatini lab was an extraordinary environment because of its extraordinary people. I will always cherish this chance to be part of a legacy of discovery, standing at the edge of the map with all of you and stepping out into the unknown.

This thesis would not exist without contributions from my collaborator, Patrick Jouandin, and Raymond Bahng, an MIT undergraduate who worked with me. Additional thanks go to Rich Binari, who welcomed me to the Perrimon lab, and Jacob Paiano, who helped me right the ship and steer toward the future.

Outside of the lab, I have been so fortunate in my classmates and my friends. I am grateful to the brave, brilliant Olivia Murton, who taught me that overthrowing the tyranny of man may take many meetings, but it's worth doing nonetheless. My life is richer (and my waistline thicker) thanks to Sophia Xu, Thy Pham, Julie Takagi, and Chantal Guegler, among many others. If my graduate career had done nothing more than put me in the path of Priya Budde, the marvelous development officer at the Whitehead, it would still have been a triumph. I adore my teammates on the High Tech, Yale, and MIT quizbowl teams, and although I've forgotten so many clues, I'll always remember our wonderful adventures together. I may never win ACF Nationals, but in submitting this thesis, I've finally lost my quizbowl eligibility, which is the greatest victory of all.

Finally, I would like to thank my family—my parents, Yulan Tan and Xufei Liu, and my brother, Eric—for everything. My parents came to the United States as graduate students, carried across the ocean by their scientific gifts at a time when America seemed as distant as the moon. For their very ordinary daughter, they have built a life filled with extraordinary freedoms and opportunities. This thesis is fashioned from the masonry of my parents' love, glued together by years of nightly phone calls and all those Marie Curie biographies they forced on me back in the early 2000s. I am the luckiest person in the world.

Table of Contents

Chapter 1: Introduction

This chapter was first published as:

Liu, G.Y. and Sabatini, D.M. mTOR at the nexus of nutrition, growth, ageing, and disease. (2020). *Nat Rev Mol Cell Biol.* 21(4):183-203.

mTOR at the nexus of nutrition, growth, ageing and disease

The mTOR pathway integrates a diverse set of environmental cues, such as growth factor signals and nutritional status, to direct eukaryotic cell growth. Over the past two and a half decades, mapping of the mTOR signalling landscape has revealed that mTOR controls biomass accumulation and metabolism by modulating key cellular processes, including protein synthesis and autophagy. Given the pathway's central role in maintaining cellular and physiological homeostasis, dysregulation of mTOR signalling has been implicated in metabolic disorders, neurodegeneration, cancer, and ageing. In this Review, we highlight recent advances in our understanding of the complex regulation of the mTOR pathway and discuss its function in the context of physiology, human disease and pharmacological intervention.

Introduction

In 1964, a team of pharmaceutical prospectors from Ayerst Research Laboratories struck microbial gold in a soil sample from the island of Rapa Nui (Easter Island). From a *Streptomyces hygroscopicus* soil bacterium, Sehgal and colleagues isolated a novel macrolide with potent antifungal activity, which they named "rapamycin" in deference to its place of origin[1]. Subsequent studies of rapamycin elaborated on its immunosuppressive, antitumor, and neuroprotective properties, generating significant clinical excitement[2-4]. Nonetheless, its mechanism of action remained elusive for more than twenty years until a series of breakthroughs in the early 1990s cracked open both the mystery of rapamycin and one of the most important signaling networks in biology.

In 1990, Schreiber and colleagues demonstrated that rapamycin acts in part by binding the prolyl-isomerase FKBP12 to form a gain-of-function complex that broadly inhibits cell growth and proliferation[5,6]. Still, the full mechanism of action of rapamycin was only elucidated in 1994, when three groups used biochemical affinity purification of the FKBP12-rapamycin complex to identify a large kinase as the mechanistic (originally “mammalian”) target of rapamycin (mTOR) in mammals[7-9]. This discovery also revealed homology between mTOR and the yeast TOR/DRR proteins, which had previously emerged as rapamycin targets in genetic screens for rapamycin resistance[10-13].

As intimated by the profound effects of rapamycin treatment, we now know that the mTOR protein kinase lies at the nexus of many major signalling pathways and plays a key part in organizing the cellular and organismal physiology of all eukaryotes. In the two and a half decades since its discovery, mTOR has emerged as the central node in a network that controls cell growth. As such, it integrates information about the availability of energy and nutrients to coordinate the synthesis or breakdown of new cellular components. Dysregulation of this fundamental signalling pathway disrupts cellular homeostasis and may fuel the overgrowth of cancers and the pathologies associated with ageing and metabolic disease.

In this Review, we analyse the signalling landscape of the mTOR pathway, from the inputs that regulate mTOR activation to the downstream effectors that enact its pro-growth programs. In particular, we highlight how the intimate association between mTOR and the lysosome can facilitate rapid mobilization of nutrients upon stress or starvation. We then discuss how the mTOR pathway responds to metabolic signals in diverse organisms, cell types and tissues. Finally, drawing on recent advances in our understanding of mTOR pathway structure and function, we examine pharmacological approaches that target the pathway and evaluate their therapeutic potential in the treatment of metabolic disease, neurodegeneration, cancer, and ageing.

I. Architecture of the mTORC1 and mTORC2 complexes

mTOR is a 289 kDa serine/threonine protein kinase in the phosphoinositide 3-kinase (PI3K)-related protein kinases (PIKK) family[14]. In mammals, it constitutes the catalytic subunit of two distinct complexes known as mTOR Complex 1 (mTORC1) and mTORC2. These complexes are distinguished by their accessory proteins and their differential sensitivity to rapamycin, as well as by their unique substrates and functions (Fig. 1A).

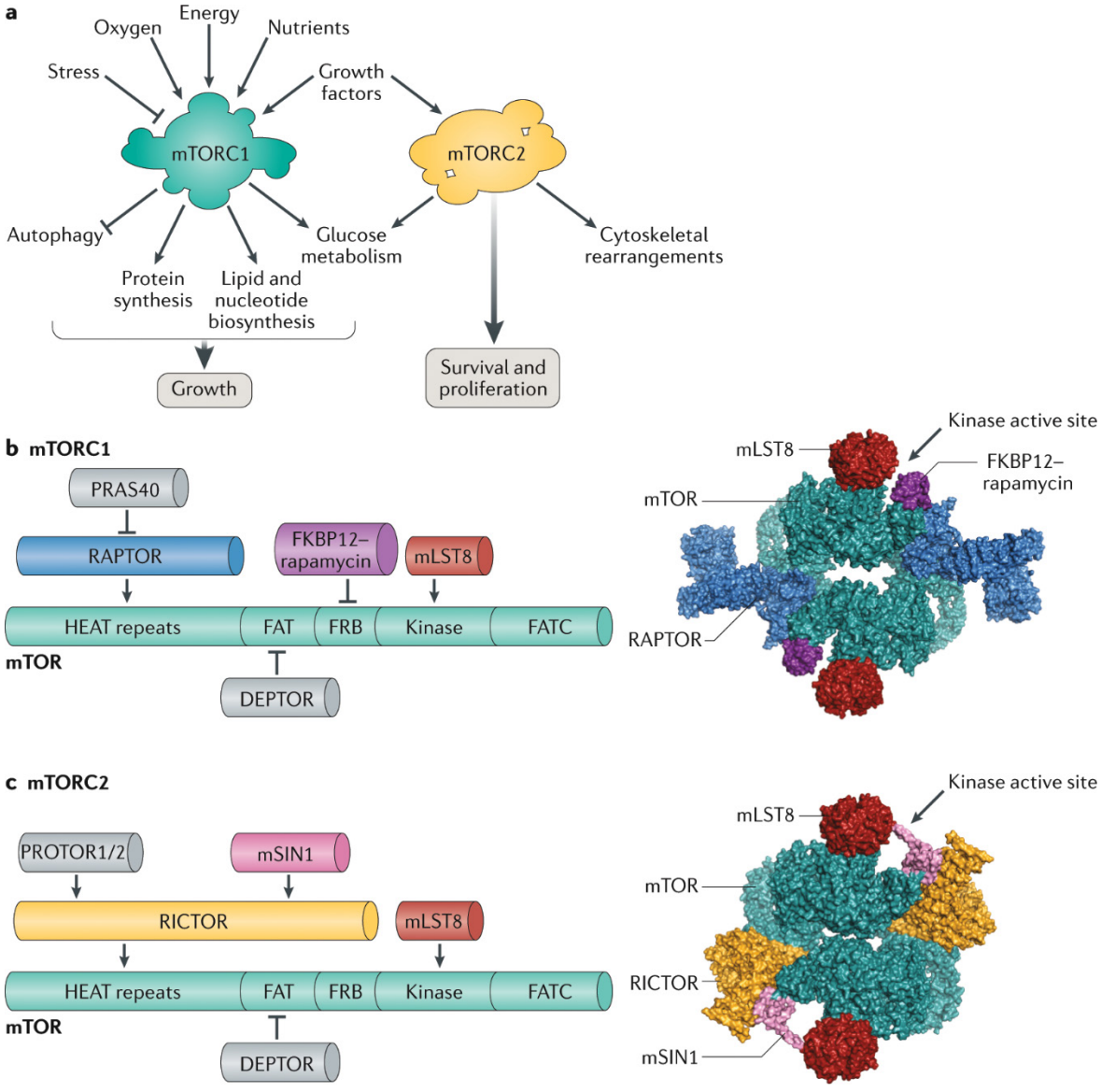


Figure 1: Structure and function of mTORC1 and mTORC2

(A) Molecular target of rapamycin complex 1 (mTORC1) and mTORC2 have distinct signalling roles in the cell. mTORC1 integrates information about nutritional abundance

and environmental status to tune the balance of anabolism and catabolism in the cell, while mTORC2 governs cytoskeletal behavior and activates several pro-survival pathways. Unlike mTORC1, which is acutely inhibited by rapamycin, mTORC2 responds only to chronic rapamycin treatment.

(B) Components of mTORC1 (left). The domain structure of the mTOR kinase (green) is annotated with binding sites for the other mTORC1 subunits. The N-terminus of mTOR contains clusters of huntingtin, elongation factor 3, a subunit of protein phosphatase 2A, and TOR1 (HEAT) repeats, followed by a FRAP, ATM, and TRRAP (FAT) domain; the FKBP12-rapamycin binding (FRB) domain; the catalytic kinase domain; and the C-terminal FATC domain. mTOR binds mLST8, a core component of the complex, and DEPTOR, an endogenous inhibitor of mTORC1 activity. Raptor, the defining subunit of mTORC1, binds mTOR with its own HEAT repeats and is required for lysosomal localization of the complex. Raptor also recruits PRAS40, an insulin-regulated inhibitor of mTORC1 activity. A 5.9-angstrom reconstruction of mTORC1 (without PRAS40 and DEPTOR) complexed with FKBP12-rapamycin is shown as a surface representation (PDB: 5FLC) (right).

(C) Components of mTORC2 (left). The mTOR kinase (green) is annotated with the binding sites for the other constituent subunits of mTORC2. These subunits include mLST8, DEPTOR, and RICTOR, the defining component of mTORC2. As a scaffolding protein, RICTOR recruits PROTOR1 or PROTOR2 to the complex, along with mSIN1, which contains a pleckstrin-homology domain. A 4.9-angstrom reconstruction of mTORC2 (without DEPTOR and PROTOR) is shown as a surface representation (PDB: 5ZCS) (right).

mTORC1 is nucleated by three core components: mTOR, mammalian lethal with SEC13 protein 8 (mLST8, also known as GβL)[15], and its unique defining subunit, the scaffold protein regulatory-associated protein of mTOR (RAPTOR)[16,17] (Fig. 1B). While structural data suggest that mLST8 may stabilize the kinase domain of mTOR[18], ablation of this protein does not affect phosphorylation of known mTORC1 substrates *in vivo*[19]. Meanwhile, RAPTOR is essential for proper subcellular localization of mTORC1 and can recruit substrates of mTORC1 by binding the TOR signalling (TOS) motifs that are present on a number of canonical mTOR substrates[20,21]. In addition, RAPTOR forms a scaffold for the mTORC1 accessory factor proline-rich AKT substrate 40 kDa (PRAS40)[22,23], which acts as an endogenous inhibitor of mTORC1 activity alongside DEP-domain-containing mTOR-interacting protein (DEPTOR)[24].

In the last decade, structural studies have shed new light on the assembly and catalysis of mTORC1. Cryo-EM and crystallographic analyses have revealed that mTORC1 dimerizes to form a megaDalton “lozenge,” with dimerization occurring along the mTOR HEAT repeats and

the mTOR-RAPTOR interface[25,26]. In isolation, this complex is relatively inactive; a recent structure suggests that key residues in the kinase domain of mTOR may only shift into catalytic position after the complex binds its essential activator, the small-GTPase Rheb[27]. Similar co-crystallization approaches have also established the basis of mTORC1 inhibition by FKBP12-rapamycin and PRAS40, both of which bind the FRB domain of mTOR to partially occlude substrate entry into the kinase active site[18,27]. Further structural analysis of mTORC1 in the presence of its substrates and regulators may offer additional insights into mTORC1 mechanism and function.

In contrast to mTORC1, mTORC2 retains the ability to phosphorylate its substrates upon acute rapamycin treatment. As with mTORC1, the core of mTORC2 is formed by mTOR and mLST8, the latter of which is required for mTORC2 stability and function[19,28] (Fig. 1C). In lieu of RAPTOR, however, mTORC2 is defined by the unrelated scaffolding protein RICTOR[29,30], which binds MAPK-interacting protein 1 (mSIN1)[31-33], DEPTOR (as in mTORC1)[24], and protein associated with rictor 1 or 2 (PROTOR1/2) to form the complex[34,35]. Of note, mSIN1 has a phospholipid-binding pleckstrin homology domain, which may help mTORC2 assemble on the plasma membrane[36]. Recent cryo-EM reconstructions of mTOR bound to mLST8, RICTOR and mSin1 show that mTORC2 also dimerizes to adopt a “lozenge” shape[37,38]. These structures further suggest that RICTOR blocks the FKBP12-rapamycin complex binding site on the FRB of mTOR, thereby rendering mTORC2 insensitive to acute inhibition by rapamycin. Nonetheless, prolonged rapamycin treatment can inhibit mTORC2 signalling by sequestering the cellular pool of mTOR into rapamycin-bound complexes that cannot nucleate new mTORC2[39,40].

II. Functions of the mTOR signaling pathway

Activation of mTOR marks cellular entry into a “growth” regime characterized by increases in both cell size and number. To keep pace with metabolic demand in these growing

cells, mTORC1 and mTORC2 initiate biosynthetic cascades to support anabolism and cell proliferation.

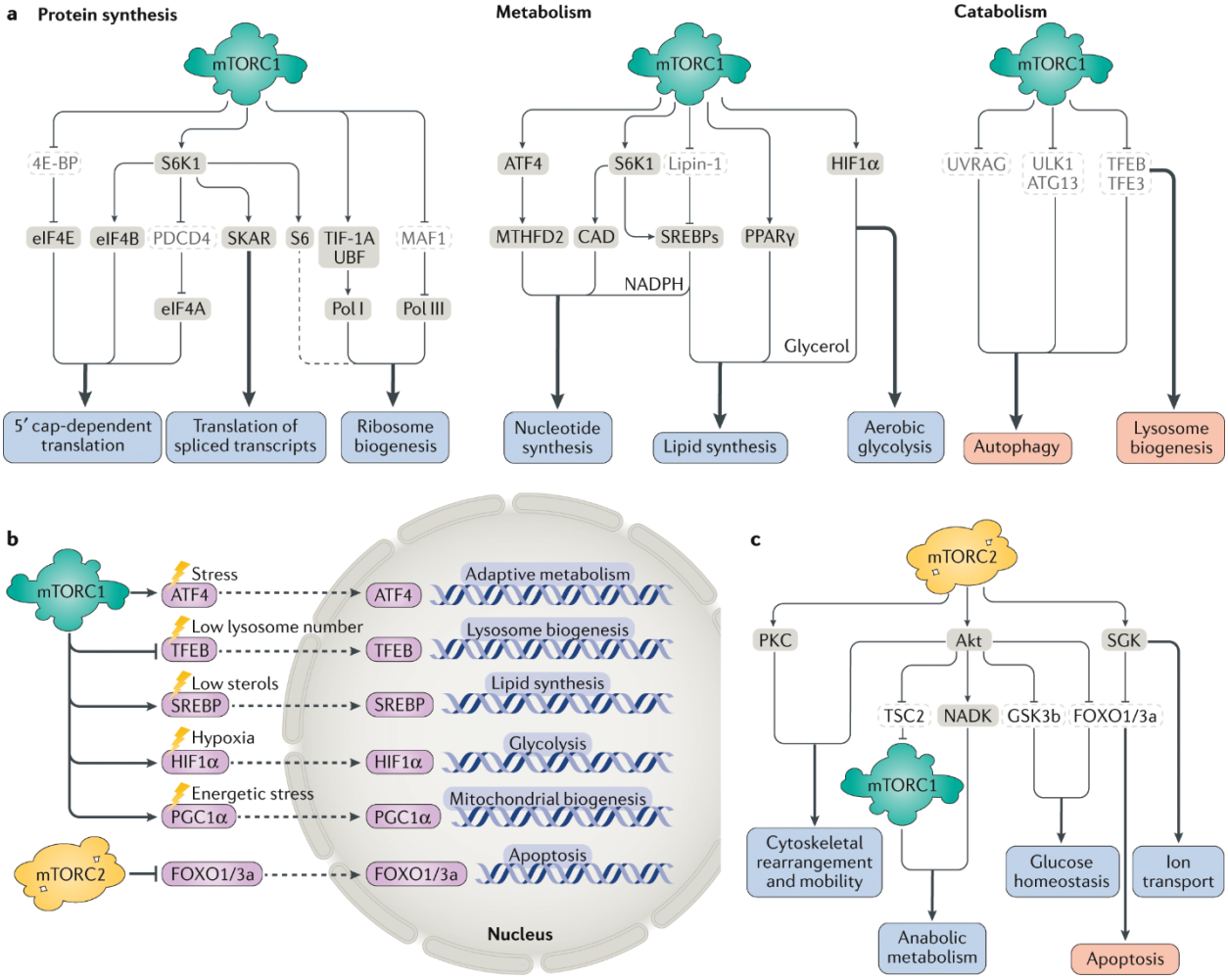


Figure 2: Targets of mTORC1 and mTORC2 signalling

(A) mTORC1 activation initiates a downstream anabolic program that enhances the production of proteins, lipids, nucleotides, and other macromolecules while inhibiting catabolic processes, such as autophagy and lysosome biogenesis.

(B) By regulating the expression or nuclear localization of transcription factors, mTORC1 and mTORC2 control the expression of genes that promote organelle biogenesis or alter metabolic flux through biosynthetic pathways. Although these transcription factors can be independently activated by specific, acute cellular stress signals (e.g. HIF1α can be directly activated by hypoxia and ATF4 can be directly activated by ER stress), mTORC1 and mTORC2 toggle the activation of these factors in a coordinated manner to support growth and proliferation. Thus, activation of mTORC1 can *simultaneously* activate ATF4, the SREBPs, HIF1α, and PGC1α to drive diverse processes involved in cellular growth, all while blocking lysosomal biogenesis through TFEB.

(C) mTORC2 activates the AGC family kinases PKC, Akt, and SGK to regulate the cytoskeleton, metabolism, ion transport and promote cell survival.

A. Roles of mTORC1

mTORC1 phosphorylates substrates that increase the production of proteins, lipids, nucleotides and ATP while limiting autophagic breakdown of cellular components. Here, we review the major substrates and effectors downstream of mTORC1 (Fig. 2a). Many of these effectors were first identified through phosphoproteomic analyses in rapamycin-treated mammalian cell lines. However, this approach is far from comprehensive: mTORC1 function is exquisitely sensitive to physiological and pharmacological context, and certain mTORC1 substrates are resistant to inhibition by rapamycin[41-43]. We posit that future studies using novel and specific mTORC1 inhibitors may uncover additional substrates and mTORC1-dependent processes.

Activation of protein synthesis

Protein synthesis is the most energy- and resource-intensive process in growing cells[44]. It is therefore tightly regulated by mTORC1, which promotes protein synthesis by phosphorylating the eukaryotic initiation factor 4E-binding proteins (4E-BPs) and p70 S6 kinase 1 (S6K1) (Fig. 2a). In its unphosphorylated state, 4E-BP1 suppresses translation by binding and sequestering eukaryotic translation initiation factor 4E (eIF4E), an essential component of the eIF4F cap-binding complex. Upon phosphorylation by mTORC1, 4E-BP1 releases eIF4E and enhances 5' cap-dependent translation of mRNAs[45-47].

In concert with PDK, which phosphorylates the activation loop (T229), mTORC1 phosphorylates S6K1 on its hydrophobic motif (T389) to stimulate kinase activity[48,49] (Fig. 2a). S6K1 subsequently phosphorylates its namesake target, ribosomal protein S6, a component of the 40S subunit. The function of S6 phosphorylation remains ambiguous: ablation of all five phosphorylation-target serine residues on S6 does not impair organismal viability or translation efficiency[50], although some evidence suggests that S6 phosphorylation may promote transcription of genes involved in ribosomal biogenesis[51]. More directly, S6K1 and mTORC1

upregulate transcription of rRNA, the dominant component of newly-assembled ribosomes, by enhancing the activity of RNA polymerase I and RNA polymerase III through phosphorylation of the regulatory factors upstream binding factor (UBF)[52], transcription initiation factor-1A (TIF-1A)[53], and MAF1[54,55]. S6K1 also enhances protein synthesis by activating eIF4B[56], a positive regulator of cap-dependent translation, and by degrading the eIF4A inhibitor programmed cell death 4 (PDCD4)[57]. In addition, S6K1 associates with SKAR at exon junction complexes to boost the rate of translation elongation in spliced transcripts[58] (Fig. 2a).

Although 4E-BP1 and S6K1 both contribute to the regulation of global translation, recent evidence indicates that 4E-BP1 has a more prominent role. Deletion of S6K1 in mouse liver and muscle cells does not reduce global translation[59,60]; likewise, rapamycin treatment, which preferentially inhibits S6K1 over 4E-BP1, produces only a weak effect on global translation. By contrast, transcriptome-scale ribosome profiling reveals that mTOR inhibition dramatically suppresses translation of mRNAs carrying 5' terminal oligopyrimidine (TOP) motifs in a 4E-BP-dependent manner[61,62]. These TOP transcripts encode much of the translation machinery, including ribosomal proteins, suggesting yet another route by which mTORC1 may modulate protein synthesis.

Biomass accumulation: lipid and nucleotide synthesis and energetic homeostasis

As cells increase in size, they must generate lipids to sustain biogenesis of new membranes. Accordingly, mTORC1 drives lipid synthesis through two axes centred on the transcription factors sterol regulatory element binding protein 1/2 (SREBP1/2) and peroxisome proliferator-activated receptor-γ (PPARγ) (Fig. 2a). When sterol levels are low, the SREBPs translocate from the ER membrane to the nucleus, where they upregulate genes for *de novo* lipid and cholesterol synthesis[63]. Activated mTORC1 promotes this SREBP transcriptional program by phosphorylating the SREBP inhibitor lipin-1 to exclude it from the nucleus[64]. Although the mechanism remains unclear, mTORC1 may also enhance the nuclear

translocation and processing of the SREBPs in an S6K1-dependent manner (Fig. 2b)[65,66]. In addition, inhibition of mTORC1 has been shown to impair the expression of lipid homeostasis genes controlled by the nuclear receptor PPARγ[67].

To maintain DNA replication and rRNA synthesis in proliferating cells, mTORC1 regulates the supply of one-carbon units for nucleotide biosynthesis. Recent work has shown that mTORC1 activates the transcription factor ATF4 and its downstream target, mitochondrial tetrohydrofolate cycle enzyme methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), to drive *de novo* purine synthesis[68]. Through its effector S6K1, mTORC1 also promotes phosphorylation and activation of carbamoyl-phosphate synthetase 2, apartate transcarbamoylase, dihydroorotase (CAD), the rate-limiting enzyme in pyrimidine biosynthesis[69,70]. This mTORC1-dependent tuning of the nucleotide pool is crucial for anabolic balance and homeostasis. Indeed, in cells where mTORC1 is hyperactive, uncoupling nucleotide biogenesis from nucleotide demand with a guanylate synthesis inhibitor leads to DNA damage, as limiting nucleotides are preferentially funnelled into rRNA to sustain high rates of ribosomal biogenesis and protein synthesis[71]. Because mTORC1 dysregulation is a signature of many cancers, inhibition of nucleotide synthesis may allow us to selectively target a metabolic vulnerability in transformed cells.

Besides its direct effects on biosynthetic enzymes, mTORC1 also potentiates growth by dictating large-scale changes in the metabolic fate of glucose. To generate energy and carbon units, mTORC1 upregulates the transcription factor hypoxia inducible factor (HIF1α), which increases expression of glycolytic enzymes and favours glycolysis over oxidative phosphorylation[66,72] (Fig. 2a,b). mTORC1-dependent activation of the SREBPs also increases flux through the pentose phosphate pathway, providing NADPH and carbon-rich precursors for lipid and nucleotide synthesis[66]. Finally, because biomass accumulation demands vast reserves of energetic currency, mTORC1 enhances translation of nuclear-encoded mitochondrial

transcripts through 4E-BP1 to expand the ATP production capacity of the cell[73]. mTORC1 may additionally stimulate mitochondrial biogenesis by driving formation of the yin-yang 1 (YY1) –PPARγ coactivator 1 (PGC1a) transcriptional complex[74].

Repression of catabolism and autophagy

In order to prevent a futile cycle in which newly synthesized cellular building blocks are prematurely broken down again, mTORC1 suppresses catabolic autophagy (Fig. 2a). To that end, mTORC1 applies inhibitory phosphorylation marks to unc-51-like autophagy-activating kinase 1 (ULK1) and ATG13, two key early effectors in the induction of autophagy[75-77]. In complex with 200 kDa FAK family kinase-interacting protein (FIP200) and ATG101, ULK1 and ATG13 drive formation of the autophagosome[78]. mTORC1 phosphorylation of ULK1 and ATG13 blocks this process, allowing proteins and organelles—including some that may be redundant or damaged—to accumulate in the cell rather than being degraded and recycled. Under nutrient-replete conditions, mTORC1 also phosphorylates UVRAG, which normally associates with the HOPS complex to assist in trafficking and fusion, as well as Rab7 activation. By disrupting this interaction, mTORC1 inhibits autophagosome maturation and the conversion of endosomes into lysosomes, thereby acting as a check on both early and late stages of autophagy[79].

Inhibition of mTORC1 by nutrient deprivation or rapamycin treatment flips the cell into a “starvation” regime, shunting resources away from biosynthesis and toward autophagy. In interphase cells, turning off the mTORC1 molecular switch restores autophagosome initiation and permits nuclear translocation of both the transcription factor EB (TFEB) and the related transcription factor E3 (TFE3), which activate genes for lysosomal biogenesis in a coordinated fashion[81-83] (Fig. 2a,b). Newly-formed lysosomes then break down proteins and release constituent monomers back to the cytoplasm to regenerate the pool of cellular amino acids, enabling reactivation of the mTORC1 pathway after prolonged starvation[84]. Importantly, this coupling between nutrient status and autophagy is disrupted during mitosis, when CDK1 inhibits

both mTORC1 and autophagosome formation to protect the genome from degradation after the dissolution of the nuclear envelope[80].

Recent studies demonstrate that the feedback loop between the lysosome and mTORC1 is crucial for cell survival in nutritionally sparse environments. For example, pancreatic cancer cell lines that rely on macropinocytosis for nutrients stop proliferating when ablation of the transporter SLC38A9 traps essential amino acids inside the lysosome, impairing autophagic reactivation of mTORC1[85]. Strikingly, a similar fitness defect is observed in nutrient-deprived cells that lack the autophagy receptor nuclear fragile X mental retardation-interacting protein 1 (NUFIP1), which recruits ribosomes to the autophagosome upon mTORC1 inhibition[86]. Defects in ribosome degradation appear to block reactivation of the mTORC1 pathway, while supplementation of exogenous nucleotides can restore growth[87]. These data suggest that ribosomes may serve as a major storage depot for amino acids and ribonucleotides and thus imply that mTORC1 may trigger selective 'ribophagy' to maintain cell viability under nutritional stress[88]. How mTORC1 balances bulk versus selective autophagy[89], how it exerts control over the kinetics of its own reactivation in starved cells, and the functional importance of this reactivation are not fully understood. As lysosome-mTORC1 communication is essential in certain conditions in several tumor models, addressing these questions may shed light on the lysosome as a signalling organelle and guide new approaches for the treatment of cancer and metabolic disease.

B. Roles of mTORC2

The first direct substrate of mTORC2 was discovered serendipitously. While immunoblotting for T389 phosphorylation of the mTORC1 target S6K1 in RICTOR-depleted cells, researchers observed that mTORC2 knockdown did not affect S6K1 phosphorylation; instead, it suppressed a cross-reacting background band, which they identified as a homologous phosphorylation site on PKCα[29] (Fig. 2c). A member of the AGC (PKA/PKG/PKC)

family of protein kinases, PKCα is thought to act as a cytoskeletal regulator, although the mechanistic basis of this process remains unclear[90]. Accordingly, knockdown of RICTOR, mTOR, or mLST8, but not RAPTOR, impairs the reorganization of the actin cytoskeleton network and inhibits chemotaxis and migration[30,91]; this phenotype, in turn, may account in part for the well-documented role of mTORC2 in the mobility and metastasis of cancer cells[92,93].

Subsequent studies have revealed that mTORC2 also collaborates with PDK1 to activate other AGC family kinases, including several classes of PKCs[94,95], the ion transport regulator serum- and glucocorticoid-induced protein kinase 1 (SGK1)[96], and the oncogene Akt[97]. Akt is a central early effector in the PI3K pathway, where it mediates the cellular response to insulin and promotes proliferation. In that capacity, Akt rewires metabolism to resist stressors through the forkhead-box FOXO1/3a transcription factors[98] and NAD kinase[99] (Fig. 2b,c). As one of the most frequently-mutated signaling nodes in cancer cells, Akt also governs the activity of glycogen synthase kinase 3b (GSK-3b) to suppress apoptosis and modulate glucose homeostasis. In addition, Akt may mediate crosstalk between the mTORC1 and mTORC2 complexes by inactivating tuberous sclerosis complex 2 (TSC2), a strong inhibitor of mTORC1 activity[100], and phosphorylating mSin1, an obligate component of mTORC2[101].

As yet, the relationship between mTORC2 and Akt is incompletely understood. Emerging evidence suggests that mTORC2 and Akt engage in mutually-reinforcing layers of feedback phosphorylation that regulate localization and activity, though the effect of these marks—individually and cumulatively—is still unclear[102]. Moreover, unlike SGK1, Akt may not require mTORC2 for basal activation. Although mTORC2 kinase activity is necessary for phosphorylation of certain Akt substrates, such as FOXO1/3a, it is dispensable for others, including TSC2 and GSK-3b[19]. Given that the FOXO proteins are regulated by both SGK1 and Akt, it is possible that SGK is in this context the more important mTORC2 effector, while Akt plays a subtler modulatory role.

III. Regulation of mTOR function

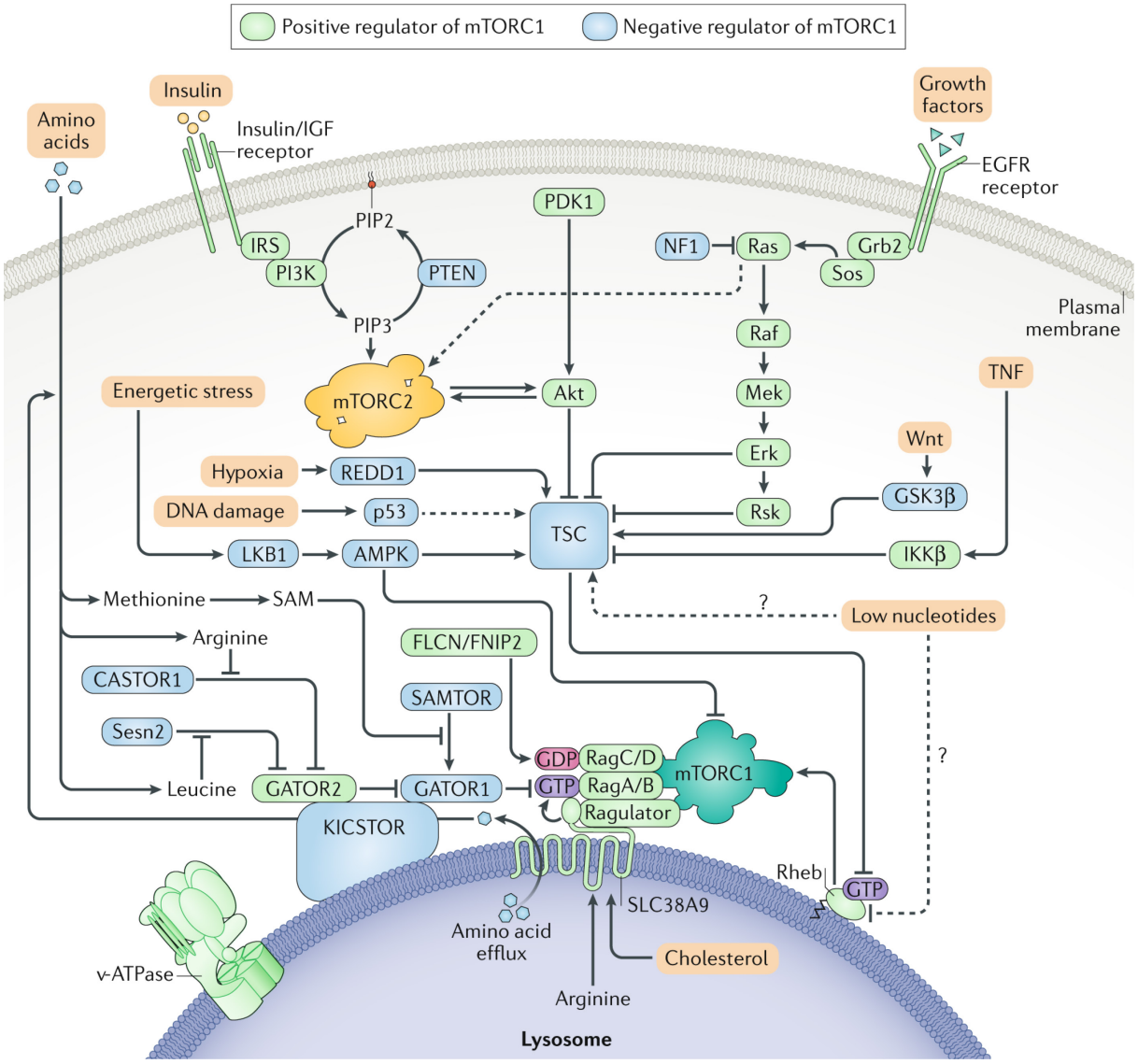


Figure 3: Upstream regulators of the mTOR signalling pathway
mTORC1 and mTORC2 integrate upstream environmental information to gate their own activation. Because mTORC1 controls cellular entry into an anabolic state that requires copious amounts of energy and macromolecules, activation of the complex should only occur when amino acids, insulin/growth factors, ATP and oxygen are all readily available. To ensure that all of these requirements are satisfied, mTORC1 must translocate to the lysosome by anchoring onto the Rag GTPases, which are only competent to recruit mTORC1 in the presence of amino acids. Once localized to lysosomal surface, mTORC1 can be then be activated by the small GTPase Rheb in its GTP-bound state. Importantly, GTP-loading of Rheb is promoted by growth factors and opposed by energetic stress or hypoxia. All of these inputs converge on the TSC complex, which acts as a GAP for Rheb. mTORC2 is thought to be primarily regulated by growth factors. Although it is not clear where mTORC2 activation occurs, the pleckstrin homology domain on mSIN1 may recruit mTORC2 to the plasma membrane. Positive regulators

of the mTORC1 pathway are shown in green, while negative regulators of mTORC1 are shown in red.

Abbreviations: Grb2, growth factor receptor-bound protein 2; IKKβ, inhibitor of nuclear factor kappa-B kinase β; LKB1, liver kinase B 1; Mek, MAPK/ERK kinase; NF1, neurofibromatosis type 1; PIP2, phosphatidylinositol (4,5)-biphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PTEN, phosphatase and tensin homolog; Sos, son of sevenless

To mediate between cellular behaviour and the cellular environment, mTORC1 and mTORC2 integrate upstream signals, including nutrient levels, growth factor availability, energy and stress, to gate their own activation (Fig 3). While the inputs and modes of regulation differ for each complex, we now recognize that mTORC1 and mTORC2 engage in substantial crosstalk—giving rise to signaling feedback loops with important consequences for health and disease.

A. Regulators of mTORC1

Cells must toggle mTORC1 activity in response to nutrient oscillations and other environmental changes stimulated by feeding or fasting. Because mTORC1 initiates a resource-intensive anabolic program, it should only turn “on” when energy, growth factors, and macromolecular building blocks are all plentiful. To monitor and integrate these inputs, the mTORC1 pathway collects upstream signals at two sets of small G proteins, termed the Rheb and Rag GTPases (Fig. 3). Biochemical studies over the last decade have led to a model in which the nucleotide-loading state of the Rheb and Rag GTPases modulate, respectively, mTOR kinase activity[103,104] and intracellular localization[105,106] to promote cell growth. When the cellular environment is rich in cytokines, endocrine signals and ATP, Rheb maintains its active GTP-bound state on the surface of the lysosome and is competent to stimulate mTORC1 kinase activity[107]. However, mTORC1 can only colocalize with this population of GTP-Rheb when amino acids, glucose, and other nutrients are readily available to activate the Rag heterodimer, which recruits mTORC1 from the cytoplasm to the lysosome. By funnelling all major

environmental cues through this spatial 'AND gate', cells ensure that mTORC1 only potentiates anabolism when intra- and extracellular conditions can support sustained growth.

Growth factors

mTORC1 acts as a downstream effector for growth factors and other mitogens, which often serve as proxies for broader paracrine and endocrine status. To regulate the mTORC1 pathway, these signals converge upon the tuberous sclerosis complex (TSC), a heterotrimeric signalling node upstream of Rheb that is.composed of TSC1, TSC2 and TBC1D7[108]. TSC acts as a GTPase-activating protein (GAP) for lysosomal Rheb, catalysing the conversion from the active Rheb-GTP state to the inactive GDP-bound state[103,109]. As a key "molecular brake" for mTORC1 activation[110], TSC is subject to many levels of regulation. Upon exposure to insulin, the insulin/insulin-like growth factor (IGF-1) activates Akt, which phosphorylates TSC2 at multiple sites to dissociate TSC from the lysosomal surface and relieve inhibition of Rheb and mTORC1[111-114]. To tune the extent and duration of mTORC1 activation and restore TSC regulation after this stimulus, the mTORC1 substrate S6K1 then directly phosphorylates insulin receptor substrate 1 (IRS-1) as part of a negative feedback loop, blocking further insulin-mediated activation of the PI3K-Akt pathway[115,116]. Wnt and tumor necrosis factor-alpha (TNF-α) signalling also repress TSC activity, although the precise mechanism of this regulation is unclear[117,118]. In addition, TSC is subject to inhibitory phosphorylation from ERK[119] and p90 ribosomal S6 kinase (RSK)[120], two downstream substrates of the Ras receptor tyrosine kinase signaling pathway. Because mutations that activate the Ras and PI3K-Akt pathways occur in many cancers, TSC regulation of mTORC1 is often lost in oncogenic contexts, resulting in constitutive mTORC1 activity even in the absence of appropriate growth factor signals.

Independently of TSC and Rheb, growth factors can also modulate mTORC1 activity through PRAS40, an endogenous inhibitor of the mTORC1 complex. A substrate and component of mTORC1, PRAS40 associates with Raptor to abolish Rheb-driven mTORC1

activation *in vitro*[22,23]. However, in the presence of insulin, Akt phosphorylates PRAS40, leading to its sequestration by a cellular 14-3-3 scaffold protein and restoring mTORC1 kinase activity. How growth factor signals are coordinated through PRAS40 and Rheb and the relative importance of each branch in different cellular contexts remains an area of active study.

Energy and oxygen availability, and other cellular stresses

Under conditions of energy or oxygen scarcity, several factors work together to activate the TSC axis and suppress mTORC1 signalling. Periods of intense metabolic exertion or glucose withdrawal can deplete cellular stores of ATP, triggering the AMP-activated protein kinase (AMPK) complex, a master regulator of cellular energy charge. As an antagonist of most major ATP-consumptive processes, AMPK inhibits mTORC1 directly, by phosphorylating Raptor, and indirectly, by activating TSC2[121-123]. At the same time, by reprogramming metabolism away from anabolic pathways, AMPK relieves the pressure on mitochondrial respiration and reduces the chances of cellular damage from the generation of reactive oxygen species[124].

Independently of AMPK, oxidative stress can also inhibit mTORC1 by upregulating REDD1, a small protein that activates TSC[125,126]. Other signs of cellular stress—ranging from organelle dysfunction to DNA replication stress—can further oppose mTORC1 activation[127]. For example, the endoplasmic reticulum unfolded protein response (UPR) can inhibit mTORC1 by increasing transcription of the Sestrin proteins, key negative regulators that will be discussed in greater detail below[128]. Likewise, DNA damage induces various p53 target genes, including an AMPK subunit (AMPKβ), PTEN, and TSC2, all of which can dampen mTORC1 activity to slow proliferation and protect genome integrity[129].

Amino acids and other nutrients

Besides spurring growth factor release, feeding also replenishes the pool of intracellular nutrients. These nutrients, which constitute the basic molecular substrates for biology, include

amino acids, nucleotides, and vitamins, all of which may be partially or wholly derived from the diet. Among the major nutrients, amino acids play a dominant role in regulating the mTORC1 pathway[130]; indeed, Avruch and colleagues observed as early as 1998 that the amino acids leucine and arginine, in particular, are absolutely required for mTORC1 activation in mammalian cells[131]. How these amino acids communicate their availability to mTORC1, however, remained a complete mystery until 2008, when two groups independently reported the discovery of the Rag-GTPases as essential components of the nutrient sensing machinery[105,106].

Unlike all other known small GTPases, the Rags are obligate heterodimers, configured such that RagA or RagB is bound to RagC or RagD. Anchored to the lysosome by the pentameric Ragulator complex (comprising p18, p14, MP1, C7orf59, and HBXIP, otherwise known as LAMTOR1-5)[132-134], the Rags can be found in one of two stable conformations: an "on" state, in which RagA/B is bound to GTP and RagC/D to GDP; and an "off" state, in which the reverse is true. These stable nucleotide-loading states are maintained by intersubunit crosstalk between the Rags[135], but they can be modulated by amino acid and nutrient status through a series of upstream factors with GAP or GTP exchange factor (GEF) activity towards the Rags. Emerging structural evidence shows that under amino-acid-replete conditions, Raptor grasps the "on-state" Rags via a protruding "claw"[136]. This interaction recruits mTORC1 from the cytosol to the lysosome, allowing lysosomal Rheb to stimulate mTORC1 kinase activity. Thus, the Rags and Rheb define the two independent arms that converge to license the mTORC1 pathway (Fig. 3).

Drawing on work by several groups over the last decade, we now recognize that mTORC1 senses cytosolic and lysosomal amino acid concentrations through distinct mechanisms. Of the "nutrient sensing complexes" that transmit cytosolic amino acid signals to the Rags, the most direct regulator of Rag status is the GAP activity towards the Rags 1 (GATOR1) complex[137]. GATOR1 is composed of three subunits—DEP domain-containing 5

(DEPDC5), nitrogen permease related-like 2 (NPRL2) and NPRL3—with GAP activity residing in the NPRL2 subunit[138]. When cytosolic amino acid levels fall, GATOR1 experiences a poorly understood regulatory event that enables it to hydrolyze the GTP bound to RagA/B and inhibit the mTORC1 pathway[139]. In turn, GATOR1 is itself regulated by other upstream factors. The large KICSTOR complex, consisting of the proteins KPTN, ITFG2, C12orf66, and SZT2, tethers GATOR1 to the lysosome and is required for cellular sensitivity to amino acid deprivation[140,141]. Meanwhile, GATOR1 also physically interacts with GAP activity towards the Rags 2 (GATOR2), a pentameric complex of WDR59, WDR24, MIOS, SEH1L, and SEC13[137]. Through unknown molecular mechanisms, the GATOR2 complex antagonizes GATOR1 function and acts as a potent positive regulator of mTORC1. Elucidating the link between GATOR2 and GATOR1 activity remains one of the most intriguing challenges in basic mTOR biology.

Recently, the question of GATOR2 function has attracted special attention because of the identification of two novel “amino acid sensors,” which relay the cytosolic availability of leucine and arginine to the mTORC1 pathway through interactions with GATOR2. Upon acute leucine starvation, the cytosolic leucine sensor Sestrin2 binds and inhibits GATOR2, preventing lysosomal recruitment of mTORC1[142]. Refeeding restores leucine levels and allows the amino acid to bind a pocket on Sestrin2, dissociating the protein from GATOR2 to relieve mTORC1 inhibition[142,143]. Although the leucine-binding affinity of Sestrin2 dictates mTORC1 sensitivity to leucine deprivation in cell culture, Sestrin2 and its relatives Sestrins1/3 may also be effectors of leucine-independent stress pathways. In support of this hypothesis, the Sestrins are transcriptionally upregulated by ATF4 and the ER unfolded protein response[128,144], and Sestrin overexpression alone is sufficient to suppress mTORC1 signaling *in vitro*[145,146]. By contrast, the Cellular Arginine Sensor for mTORC1 (CASTOR1) appears to be exquisitely sensitive to cytosolic arginine alone[147,148]. A protein that can exist either as a homodimer or as a

heterodimer with CASTOR2, CASTOR1 also inhibits GATOR2 in the absence of arginine and dissociates from the complex when arginine is bound.

A second arginine sensor, SLC38A9, monitors amino acid levels inside the lysosomal lumen and defines the lysosomal branch of the nutrient sensing machinery[149,150]. SLC38A9 resides on the lysosomal membrane and transports neutral amino acids out of the organelle in an arginine-gated fashion[85]. This efflux activity may enable the products of autophagic protein degradation to reactivate the mTORC1 pathway after prolonged starvation. Synthesizing structural and biochemical evidence, we posit that the binding of lysosomal arginine to the first transmembrane helix of SLC38A9 frees the N-terminus of the protein from the central pore[151]. This domain can then collaborate with Ragulator to push the Rags into the active state by promoting GTP-loading of RagA/B[152]. Through a separate mechanism, the lysosomal v-ATPase, which maintains the pH gradient of the lysosome, has also been reported to interact with the Rag-Ragulator complex to influence the nucleotide-loading state of the Rags[153]. Finally, the folliculin (FLCN)-FNIP2 complex acts as a GAP for RagC/D to sustain mTORC1 activation in the presence of amino acids[154,155]. By modulating the status of RagC/D, FLCN-FNIP2 may also recruit and enhance the phosphorylation of the transcription factors TFEB/TFE3, although it is unclear whether this process is mTORC1-independent[156,157]. If FLCN-RagC/D-TFEB/TFE3 do indeed constitute a distinct axis, loss of FLCN could amplify the TFEB/TFE3 transcriptional program, an oncogenic signature in some cancers[158,159], allowing us to reconcile FLCN's status as a tumor suppressor *in vivo* with its activating role in the mTORC1 pathway.

The recent discovery of an *S*-adenosylmethionine (SAM) sensor, named SAMTOR, has shown that mTORC1 responds not only to amino acids (e.g. leucine and arginine) but also to their metabolic byproducts—in this case, a key methyl donor derived from methionine. Unlike Sestrin2 and CASTOR1, which oppose GATOR2 signaling when their cognate amino acids are absent, SAMTOR negatively regulates mTORC1 by binding GATOR1 and KICSTOR under

methionine or SAM deprivation[160]. Restoration of SAM levels dissociates SAMTOR from these complexes and stimulates mTORC1 activity.

At present, we do not know how other amino acids impact mTORC1 activation, nor do we understand what role, if any, the general amino acid sensors GCN2 and ATF4 play in acute mTORC1 signalling cascades. While longer-term amino acid deprivation is thought to feed from GCN2 back to mTORC1 through transcriptional upregulation of ATF4 and the sestrins, it is not clear whether GCN2 and ATF4 regulate mTORC1 in transiently starved cells. Moreover, we still lack mechanistic explanations for how several known metabolic inputs impinge on the pathway. For example, although acute withdrawal of glucose inhibits mTORC1 at least partially through activation of AMPK, a study in AMPK-null cells has demonstrated that glucose deprivation also signals through the Rag-GTPases[161], reinforcing earlier evidence that glucose can signal independently of both AMPK and TSC[162]. Similarly, depletion of purine nucleotides inhibits mTORC1, perhaps as an indicator of replication stress, but it is not clear whether this inhibition is driven by TSC or by degradation of Rheb[163,164]. One recent study suggests that phosphatidic acid may activate mTOR signaling as a proxy for fatty acid availability[165], while another implicates glutamine in Rag-independent reactivation of mTORC1[166]. Cholesterol has also been shown to activate mTORC1 through a complex composed of SLC38A9 and the Niemann–Pick C1 protein[282].It remains an open question whether mTORC1 senses other metabolites essential for cell growth, such as vitamins or lipid components; equally unclear is how these nutritional requirements might diverge in cell types or organisms with different dietary and metabolic needs.

B. Regulators of mTORC2

In part because it has been difficult to tease apart the regulation of mTORC1 and mTORC2 with pharmacological agents, the activators of mTORC2 are still poorly defined. Even so, it is clear that mTORC2 is primarily regulated by growth factors through the PI3K pathway,

with the unique mTORC2 component mSin1 acting as a key signal integrator (Fig. 3). Like other PI3K effectors, mSin1 possesses a pleckstrin homology domain, which autoinhibits mTORC2 kinase activity in the absence of insulin[36]. This inhibition is relieved by the binding of PIP3, a product of insulin- or serum-induced PI3K activation[36,167]. PIP3 may recruit mTORC2 and Akt to the plasma membrane, where reciprocal phosphorylations between the two kinases modulate their localization and activation[102]. In several model systems, including *D. discoideum*, this localization and activation is also regulated by the small GTPases Rac1, Rap1 and Ras, which bind to mTORC2 to direct chemotaxis and growth[168-170]. A recent study extends this paradigm to human cells by showing that mSin1 can recruit oncogenic Ras to directly catalyse mTORC2 kinase activity at the plasma membrane[171]. This finding connects mTORC2 to a major cancer pathway and reinforces its role in driving survival and proliferation.

Because mTORC1 downregulates insulin/PI3K/Akt signaling through feedback inhibition, it also engages in negative crosstalk with mTORC2[172]. As previously described, mTORC1 can disrupt PI3K-Akt signaling through S6K1-dependent degradation of IRS1[115,116]; alternatively, mTORC1 can activate Grb10, a negative regulator of the insulin/IGF-1 receptor[173,174]. Both of these mechanisms have downstream implications for mTORC2 activity and may account for some of the paradoxical metabolic phenotypes associated with chronic rapamycin treatment (Fig. 4). Unexpectedly, mTORC2 is also activated by AMPK under energetic stress, suggesting that it may mediate cellular adaptation to oxygen- or nutrient-poor tumor environments *in vivo*[175].

IV. mTOR in physiology and pathophysiology

Characterization of mTOR signalling nodes is a work in progress at the cellular scale, but the functional regulation of the pathway becomes exponentially more complex at the organismal level, as mTOR must coordinate the storage and mobilization of nutrients and energy across different tissues. Unlike cells in culture, which are bathed in growth factors and nutrients and consequently maintain high mTOR activity, cells *in vivo* tend to display lower

baseline activity and experience sharper fluctuations in mTOR activity upon fasting or feeding. Coordinating physiological responses with nutrient status requires the mTOR pathway to sense conditions within specialized niches and to enact tissue-specific anabolic or catabolic cascades. Appropriate regulation of mTOR is crucial for homeostasis and organismal health; conversely, imbalances in mTOR activity in various tissues can lead to metabolic dysregulation and disease.

A. mTOR in metabolic syndrome

As a critical regulator of glucose metabolism and lipogenesis across a variety of tissues, the mTOR pathway is readily hyperactivated by overfeeding and underwrites many diseases of constitutive growth, including obesity and type 2 diabetes.

Insulin sensitivity and glucose homeostasis

To prevent the accumulation of nutrients in the blood, animals have evolved mechanisms to sequester macromolecules and energy after feeding. These processes are coordinated across different tissues by the release of insulin from the pancreas, which co-activates mTORC1 and mTORC2 to promote hypertrophy and growth (Fig. 4A). In skeletal muscle, insulin induces the uptake of glucose and enhances its storage as glycogen by stimulating the mTORC2–Akt axis[176]; at the same time, circulating amino acids are incorporated into new muscle biomass in an mTORC1-dependent manner.

By contrast, low levels of insulin following fasting induce autophagy in ‘dispensable tissues’ (i.e. muscle and liver, as opposed to the brain), which break down protein stores to fuel gluconeogenesis in the liver. This catabolic program has profound effects on metabolic organs: one study found that livers from mice fasted for 24 hours decreased in weight by nearly 25%, with the difference arising not from changes in cell number but from reductions in cell size[177]. Strikingly, this fasting-induced shrinkage was abolished in mice with liver-specific knockouts of TSC1, Raptor, or the autophagy gene ATG7, suggesting that the switch from anabolism to catabolism is primarily regulated by mTORC1[177,178].

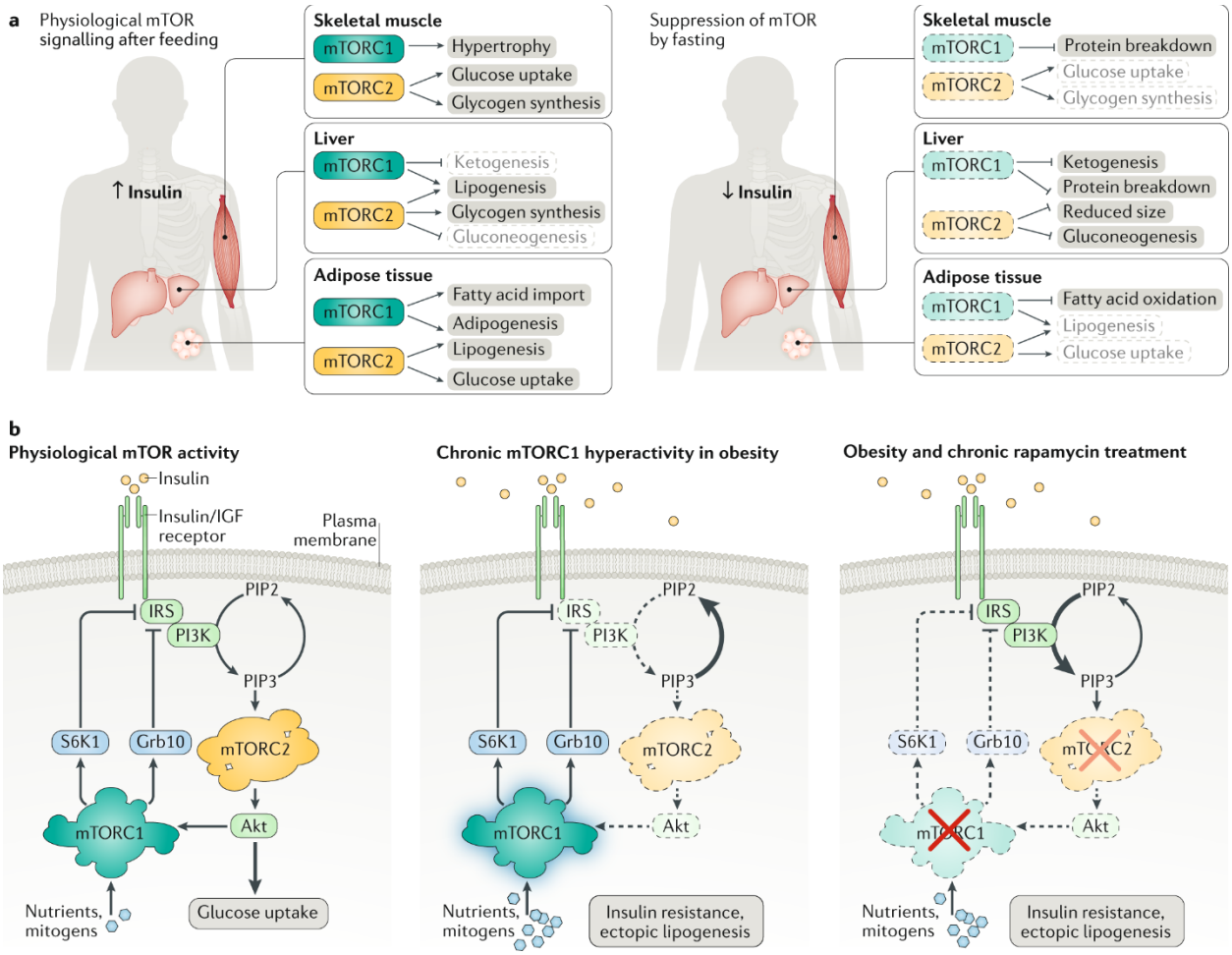


Figure 4: mTOR signalling in metabolism

(A) mTOR coordinates feeding and fasting with nutrient storage and mobilization. In the liver, skeletal muscle, and adipose tissue, rising insulin levels after feeding activate both mTORC1 and mTORC2, promoting lipogenesis, glycogen synthesis, and protein synthesis (left). During fasting, nutrient, growth factor and insulin levels drop precipitously, tilting the metabolic balance in favour of gluconeogenesis, ketogenesis and lipolysis (right).

(B) Dysregulation of mTOR signalling in metabolic syndrome. Although the negative feedback loop between mTORC1 and mTORC2 is carefully balanced under physiological conditions (left), chronic hyperactivation of mTORC1 by excessive nutrients and mitogens can shut off PI3K/mTORC2 signalling, leading to insulin resistance, ectopic accumulation of lipids in muscle and liver, and type 2 diabetes (middle). Rapamycin-based therapies have not been effective in diabetes patients with hyperactive mTORC1 signalling because prolonged rapamycin treatment also inhibits mTORC2 (right).

Substantial evidence now points to mTORC1 as a central mediator of organismal survival during nutrient restriction. For mice that cannot tune mTORC1 signalling, prolonged

fasting—like the postnatal fast caused by disruption of the placental nutrient stream—can pose an insurmountable challenge. Unlike wild-type neonates, which rapidly inhibit mTORC1 after an initial drop in circulating glucose, mice expressing a constitutively active allele of RagA (RagA-GTP) are unable to suppress mTORC1 signaling during the perinatal fasting period[161]. Because these mutant mice fail to restrict their energy expenditure or trigger autophagy to supply free amino acids for gluconeogenesis, their plasma glucose levels plummet, leading to fatal hypoglycaemia within one day of birth. A similar perinatal lethality occurs in mice lacking the sestrin proteins (upstream negative regulators of mTORC1)[179] and in mice with defects in the autophagy machinery (downstream targets of mTORC1)[180], demonstrating that mTORC1 activity must be tightly coupled to diet to maintain glucose homeostasis *in vivo*.

Adipocyte formation and lipid synthesis

Postprandial mTOR activation also promotes longer-term energy storage by increasing the synthesis and deposition of triglycerides in white adipose tissue (WAT). As the largest repository of energy in the body, WAT serves as a metabolic hub, tailoring its biosynthetic activity to fluctuations in mTOR signalling. In these cells, the mTORC1–S6K1–SREBP axis drives *de novo* lipogenesis[64-66], while mTORC1 activation of PPARγ helps pre-adipocytes differentiate into mature tissue[67,181] (Fig. 4A). S6K1 may also increase fatty acid import into adipocytes through a complex mechanism involving the glutamyl-prolyl-tRNA synthetase (EPRS)[182]. Consistent with the importance of mTORC1 in WAT, adipocyte-specific deletion of Raptor reduces WAT tissue mass and enhances lipolysis in mouse models[183]. Tantalizingly, Adi-Raptor KO mice are also resistant to diet-induced obesity[184]. Unfortunately, these defects in adipocyte expansion can drive fat deposits to accumulate in the liver instead, leading ultimately to hepatic steatosis and insulin resistance[183].

While regulation of adipose tissue exerts second-order effects on other organs, mTOR also directly modulates lipid metabolism in the liver. Several groups have found that hepatic

lipogenesis is impaired in both Raptor and Rictor-depleted mice, with mTORC2-dependent effects at least partially rescuable by constitutive activation of Akt[64,185,186]. In addition, mice with liver-specific hyperactivation of mTORC1 fail to fully stimulate the production of ketone bodies, which are synthesized from fatty acids to supply peripheral tissues with alternative energy packets during fasting[177]. Although the relationship between mTORC1 and ketogenesis is not entirely clear, insulin withdrawal likely inhibits mTORC1 phosphorylation of S6 kinase 2 (S6K2), which then enhances expression of ketogenic factors by freeing the transcription factor PPARα from its corepressor, nuclear receptor corepressor 1 (NCoR1). Similar ketogenic defects are also observed in aged mice, suggesting that long-term declines in liver function may stem from mTOR-driven dysregulation of lipid metabolism[177].

Pharmacological interventions for metabolic disease

Many diseases of overfeeding, among them obesity and type 2 diabetes, produce a major and detrimental energy imbalance in the body. By generating a constant surplus of hormones, cytokines, and nutrients, these diseases collapse the metabolic cycles that underwrite tissue homeostasis, forcing mTORC1 to remain in a persistent “on” state. Constitutive mTORC1 signaling activates S6K1 and Grb10 to decouple the insulin/IGF-1 receptor from downstream PI3K pathway effectors, dampening the physiological response to insulin[115,116,173,174] (Fig. 4B). Moreover, PI3K inhibition suppresses mTORC2/Akt to block glucose uptake and promote gluconeogenesis[185,187], thereby further elevating glycemic load and exacerbating the ectopic fat deposition and glucose intolerance that constitute hallmarks of metabolic syndrome.

Given that mTORC1 sits at the center of a web of dysregulated metabolic signaling, it is tempting to imagine that inhibition of this node might reverse both the symptoms and underlying causes of obesity and diabetes. Lending support to these hopes, metformin, a first-line treatment for type 2 diabetes, has been shown to potently suppress mTORC1 by activating

AMPK and TSC[188]; likewise, ablation of the mTORC1 effector S6K1 can protect against diet-induced obesity and enhance insulin sensitivity[172]. Unfortunately, direct pharmacological inhibition of mTORC1 yields more complex outcomes. Patients administered with rapamycin experience more severe insulin resistance, perhaps because chronic rapamycin treatment disrupts not only mTORC1 but also the integrity of the mTORC2 complex, blunting the Akt-dependent insulin response[40] (Fig. 4B). To bypass these adverse effects, it will be necessary to develop new, truly specific mTORC1 inhibitors, as well as tissue-specific modulators of mTORC1 function.

B. mTOR regulation of brain physiology and function

Within the brain, the mTOR pathway orchestrates a wide array of neuronal functions, temporally spanning every stage of development[189,190]. From framing basic cortical architecture to remodelling neuronal circuitry in response to experience, mTOR and its molecular accomplices shape both the signalling and the physical terrain of the brain (Fig. 5a,b). Not surprisingly, loss of mTOR regulation—through either genetic or chemical perturbations—has severe repercussions for neuronal function (Fig. 5a). Brain-specific knockouts of Raptor and Rictor display remarkably similar phenotypes, typified by microcephaly—via reductions in neuron size and number—and improper differentiation[191,192]. In addition, Raptor deletion in the brain also triggers early postnatal death[191], while Rictor deletion leads to aberrant brain foliation and impaired dendrite extension[192].

mTOR in neurodevelopmental disorders

Hyperactive mTOR signaling, as observed in neurodevelopmental “mTORopathies,” is associated with its own characteristic defects (Fig. 5a). As a class, the mTORopathies are caused by loss-of-function mutations in negative regulators of mTORC1, usually manifesting with some subset of the following symptoms: focal malformations in the brain, epileptic seizures, macrocephaly, autism spectrum disorder (ASD), and benign tumors or cystic growths[193].

Perhaps the best-studied such disease is TSC, which arises when loss of either TSC1 or TSC2 induces constitutive mTORC1 activity. Patients with TSC often grow lesions that disrupt the laminar organization of the cortex, nucleating epileptogenic foci; these patients may also have enlarged neurons and "giant" astrocytes[193]. Similar phenotypes are found in patients with inactivating mutations in the negative PI3K regulator PTEN[194], the AMPK activator STRADα[195], and the negative regulatory complexes GATOR1 and KICSTOR[196-200], as well as those with activating mutations in Rheb or mTOR[201-203]. Given that mTORC1 has many roles in defining the morphology of the developing brain, the epilepsy that clinically distinguishes these disorders is likely seeded by prenatal neuronal mis-wiring[204]. However, acute rapamycin treatment can nonetheless suppress seizures caused by TSC1 loss in adult mice[205], suggesting that mTOR hyperactivity can further stimulate "seizing" in established neural circuits. Consistent with a model in which mTORC1 participates in multiple stages of epileptogenesis, recent speculation contends that the ketogenic diet, a validated therapy for treatment-refractory epilepsy, may work by depriving the mTORC1 pathway of activating nutrients[206]. Other, more direct mTOR inhibitors are currently in clinical trials as anti-epileptic agents[207,208].

mTOR control of brain function via protein translation and autophagy regulation

Surprisingly little is known about the regulation of mTOR signaling in normal brain function and homeostasis. Unlike cell culture systems, the postnatal brain is mostly post-mitotic, such that environmental inputs are consolidated not to stimulate growth or proliferation but rather to enact changes in neuronal morphology and connectivity. Although it is not clear which inputs are actually relevant *in vivo*, given that the brain is 'nutritionally protected' from acute fasting (that is, brain biomass and function are generally left intact for as long as possible under starvation, with the brain having first use of available glucose and ketone bodies), brain-derived neurotropic factor (BDNF) has emerged as a major tissue-specific agonist of the neuronal mTOR pathway. As a PI3K activator, BDNF increases mTORC1 signaling near injured axons to

encourage wound healing and repair[209,210]; in turn, BDNF release may itself be regulated by a feed-forward loop downstream of S6K1[211].

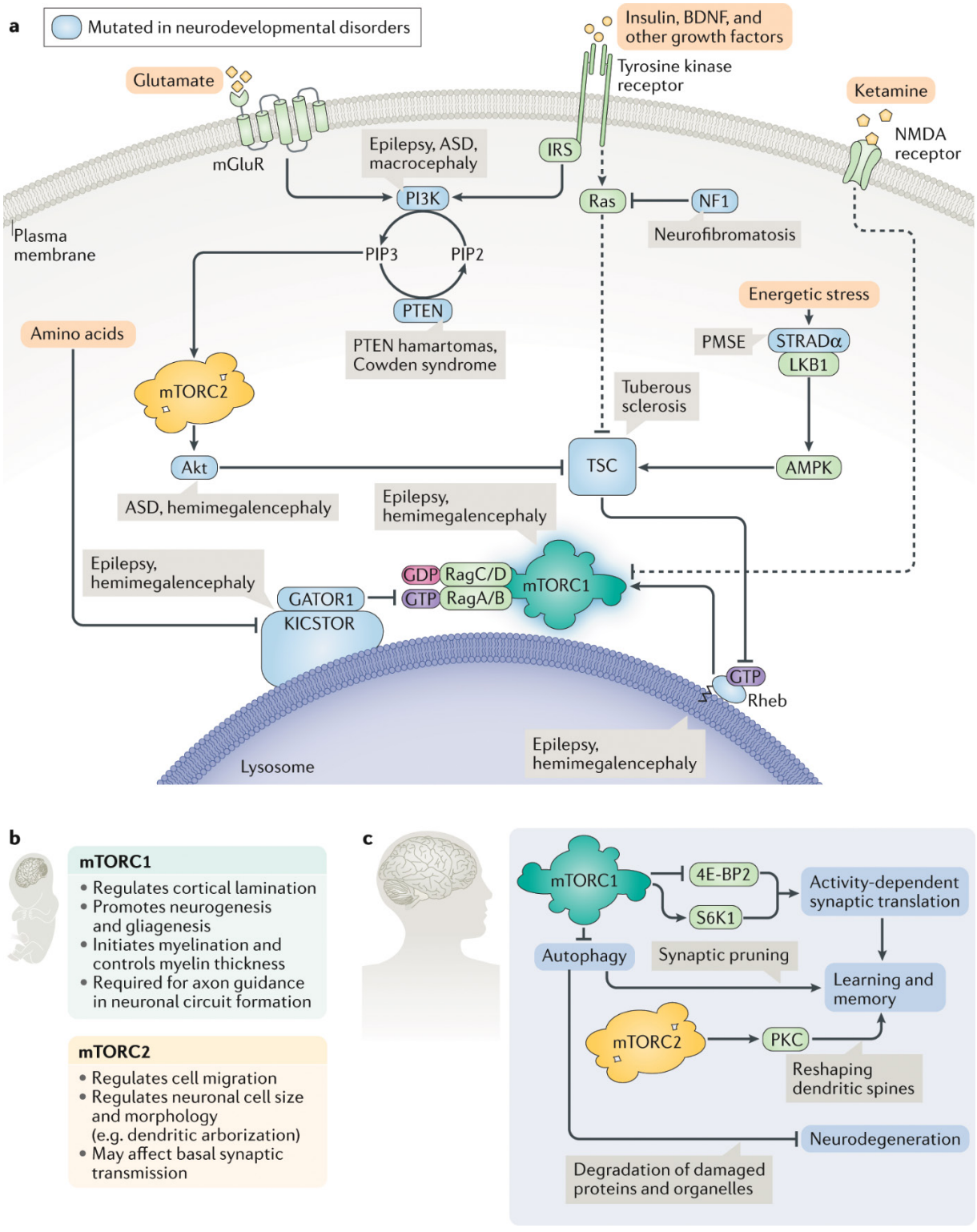


Figure 5: mTOR signalling in the brain

(A) In the brain, mTORC1 signalling is activated not just by nutrients and insulin but also by several tissue-specific inputs, including the neurotransmitter glutamate and the neurotrophic growth factor BDNF. Dysregulation of the mTORC1 pathway is associated with a set of characteristic neurodevelopmental diseases, collectively termed "mTORopathies." Patients with mTORopathies suffer from severe epilepsy and may also display focal cortical dysplasia, macro- or megalencephaly, cognitive and social defects, and benign tumors. Genes bearing mutations in neurodevelopmental diseases are shown in red. ASD, autism spectrum disorder; PMSE, polyhydramnios, megalencephaly, and symptomatic epilepsy; STRADα, STE20-related kinase adapter protein α

(B) Roles of mTORC1 and mTORC2 during neuronal development. Ablation of mTORC1 or mTORC2 in the nervous system perturbs cell and organ size and disrupts the cortical architecture of the brain. mTORC1 deletion also causes early postnatal lethality.

(C) Roles of mTORC1 and mTORC2 in postnatal maintenance of synaptic plasticity and homeostasis. mTORC1 regulates activity-dependent synaptic translation through its substrates 4E-BP2 and S6K1 to strengthen or weaken a given neuronal circuit; moreover, it also promotes synaptic plasticity by pruning obsolete synapses through autophagy. Autophagy may additionally play a neuroprotective role by degrading misfolded proteins and damaged organelles. mTORC2 remodels the actin cytoskeleton in response to neuronal signal transmission and helps convert transient excitatory events into long-term memory.

In collaboration with BDNF, mTOR regulates learning and memory by promoting translation at synapses through S6K1 and 4E-BP2[212] in a manner that is dependent on neuronal activity (Fig. 5c). This localized translation is rapamycin-sensitive and is crucial for the remodelling of dendritic spines that accompanies long-term potentiation[213]. Strikingly, animal models lacking TSC or 4E-BP2 recapitulate some of the social and cognitive abnormalities associated with ASD, suggesting that dysregulation of synaptic translation may affect higher-order brain functions[214,215]. In accordance with this paradigm, synaptic translation has also been linked to depression and psychiatric mood disorders. The NMDA receptor antagonist ketamine, a fast-acting antidepressant, has been shown to boost mTORC1 activity at the synapse, with psychiatric relief coinciding with an increase in synaptic protein, dendritic spine density, and synaptic function[211,216] . In animal models, the sestrin inhibitor NV-5138 appears to mediate similar improvements by directly activating mTORC1, independently of other upstream signals[217]. However, while these lines of evidence implicate mRNA translation in diverse aspects of synaptic plasticity and brain health, we still do not know which neuronal mRNAs are

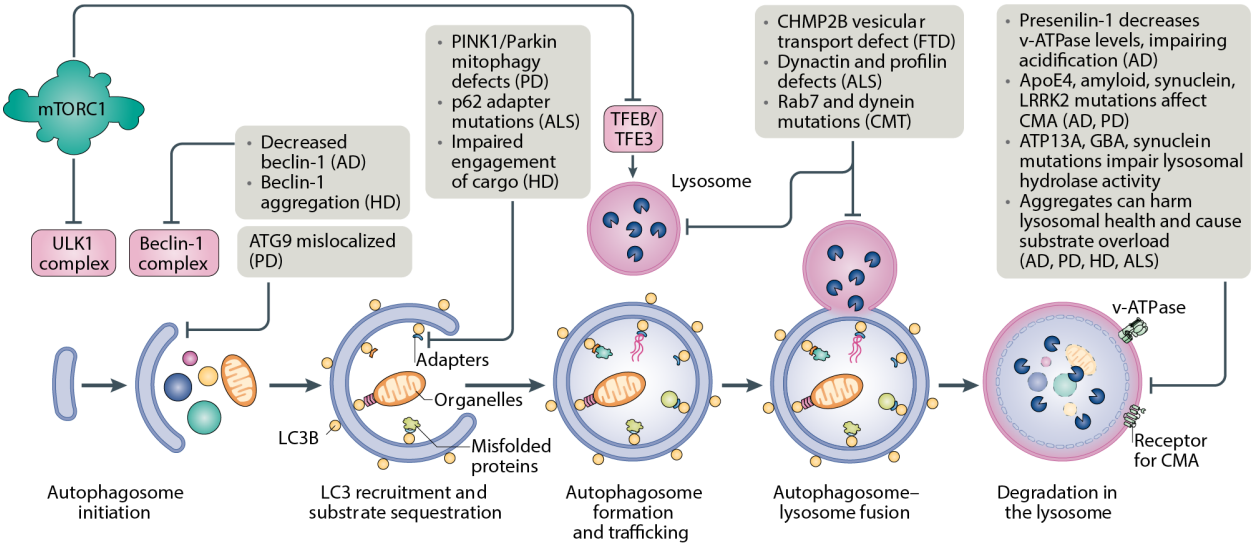
regulated by the mTORC1 pathway in response to specific stimuli, nor do we understand how mTORC1 and its substrates localize protein synthesis within individual neurons.

In recent years, it has become increasingly clear that translation is not the only mTORC1 output required for plasticity. In order to adjust the strength of a neuronal circuit, mTORC1 must simultaneously promote the building of new proteins at some synapses and the degradation of excess synaptic machinery at others. The latter process calls for local inhibition of the mTOR pathway, which triggers macroautophagy[218] (Fig.5c). Consistent with the apparent importance of autophagy in cognitive function, constitutive mTOR hyperactivity has been shown to compromise synaptic pruning and contribute to ASD-like social deficits in TSC-deficient mice[219]. Rapamycin treatment was sufficient to rescue these defects, but only when the autophagy pathway remained intact. While these findings are quite preliminary, taken in sum with the apparent efficacy of mTORC1 activators like ketamine, they suggest that modulation of the mTORC1 pathway in the brain may hold promise as a therapeutic strategy to improve cognitive performance and memory in certain disease states.

mTOR, autophagy and neurodegeneration

Genetic evidence implicates autophagy—and its major regulator, mTORC1—in a number of devastating neurodegenerative disorders[273] (see figure). These disorders, which include Alzheimer's disease, Parkinson disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), and frontotemporal dementia (FTD), lead to the progressive, permanent destruction of neurons, wreaking havoc on cognition and motor control. Although most cases of neurodegeneration arise sporadically, increasing in frequency with age, certain heritable mutations can boost disease incidence and severity within families, with many such mutations mapping to genes associated with proteostasis and lysosomal function. Indeed, failures in autophagic clearance have emerged as a key hallmark of neurotoxic cell death. In Alzheimer's disease, as in several of its pathological cousins, misfolded, ubiquitinylated proteins appear to

clog autophagic vacuoles, which subsequently accumulate in dystrophic neurites[274,275]. Because neuronal cells cannot divide to dilute unwanted macromolecules or organelles and must rely on autophagy, any jam in autolysosome clearance propagates through the entire endocytic machinery and may compound metabolic and immunological traumas that lie far afield from the initial amyloid stressor. Multiple groups have confirmed that deletion of essential autophagy genes in the brain is sufficient to induce neurodegeneration even in the absence of disease proteins[276,277], supporting a model that puts autophagy—and not amyloids—at the core of neurodegenerative disease.



AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CMA, chaperone-mediated autophagy; CMT, Charcot-Marie-Tooth disease; FTD, frontotemporal dementia; HD, Huntington's disease; PD, Parkinson's disease

The recent failure of Alzheimer's drugs targeting amyloid-beta and tau in clinical trials has demonstrated that reduction of protein aggregates alone has little effect on cognitive function. Given the massive financial and societal costs of neurodegeneration, there is an urgent need for new therapies that delay or reverse disease progression through alternative mechanisms. Based on preclinical evidence, rapamycin may be a promising lead. Induction of autophagy through rapamycin treatment has been shown to eliminate aggregates and improve

memory and behaviour in six different mouse models of Alzheimer's disease, as well as several models of Parkinson's disease[278-280]. Moreover, as we will discuss below, rapamycin-mediated inhibition of mTOR may also reverse some of the cellular effects of aging, the most important risk factor for neurodegeneration[237]. It should be noted, however, that rapamycin does not penetrate the blood-brain barrier with ease and only partially blocks mTORC1 phosphorylation of autophagy regulator ULK1[42]; in addition, chronic application of rapamycin for neuroprotection would likely disrupt major pathways inside and outside the brain. These caveats suggest that future therapeutic strategies may need to establish a precise balance of neuronal mTOR activity to maintain homeostasis—a goal that will require us to develop a more nuanced understanding of when, why, where, and how mTOR acts in the brain.

C. mTOR in cancer

Although the mTOR kinase itself is rarely mutated in cancer, it is readily hijacked by upstream oncogenic nodes, including those in the PI3K/Akt pathway and the Ras-driven MAPK pathway. As a result, mTOR signaling is hyperactive in up to 80 percent of human cancers[220], in which context it plays a pivotal role in sustaining cancer cell growth and survival (Fig 6A). Because tumor microenvironments are poorly vascularized and subject to severe nutritional restrictions, loss of the mTORC1 nutrient sensing machinery may help cancer cells evade metabolic checks on anabolism and proliferation. Thus, mutations in all three components of the GATOR1 complex have been implicated in glioblastomas[137], while RagC and folliculin mutations have been found in follicular lymphoma and Birt-Hogg-Dubé syndrome respectively[221,222]. Meanwhile, hyperactivation of mTORC2 can aggravate negative cancer prognoses by activating Akt and by supporting the cytoskeletal transformations that underlie metastasis[92,93].

To date, mTOR inhibitors have met with limited success as chemotherapeutic agents. The first generation of clinical rapamycin derivatives, known as "rapalogs," were approved for advanced renal cell carcinomas in the late 2000s. Outside of certain exceptional contexts[223],

these rapalogs have proven to be more cytostatic than cytotoxic, perhaps because they only partially block 4E-BP-dependent translation and fail to inhibit the pro-survival pathways regulated by mTORC2/Akt[61,224]. Inhibition of mTORC1 also drives autophagy, which has been shown to nourish cells in nutrient-poor tumor microenvironments[225]. A second generation of catalytic mTOR inhibitors (e.g. Torin1, PP242, Ku-0063794) competes with ATP to occupy the kinase active site and side-steps many of these issues by inhibiting all known substrates of mTORC1 and mTORC2[41,226,227]. Despite some concerns about tissue toxicity because of their broad effects, early clinical data suggest that catalytic mTOR inhibitors can be tolerated at effective doses[228]. However, prolonged treatment with these inhibitors can lead to a metabolic retrenchment that allows cancer cells to reactivate Akt without positive input from mTORC2, highlighting resistance as a key problem that must be tackled by next-generation therapies[229-231].

V. mTOR in ageing

In line with a growing body of genetic and pharmacological evidence, mTOR activity is now recognized as a major driver of aging—a process defined here as a progressive decline in physiological function that increases vulnerability to disease and death (Fig. 6B). Genetic inhibition of the mTORC1 pathway through depletion of mTOR or Raptor has been shown to extend lifespan in organisms as diverse as yeast[232], nematodes[233,234], flies[235], and mammals[236]; in a similar vein, rapamycin treatment also promotes longevity across a wide swath of the evolutionary tree[237-240]. Tantalizingly, rapamycin appears to prolong not just lifespan but also healthspan—the length of time that an organism enjoys efficient biological performance, free of disease or disability—suggesting that mTORC1 inhibition may slow ageing by reversing molecular changes associated with cellular deterioration[241].

What are these mTORC1-sensitive molecular changes that affect ageing? One clue may come from dietary restriction (DR), the only other intervention that produces a comparable and

conserved increase in lifespan. DR reduces nutrient intake without incurring malnutrition, pushing mTORC1 toward a catabolic regime. Indeed, DR is thought to counter ageing by acting through the mTORC1 pathway, as DR on top of chemical or genetic inhibition of mTORC1 fails to confer any additional longevity benefit in flies, worms, and yeast[232,235,242]. Intriguingly, DR of a single amino acid, methionine, is sufficient to increase lifespan in flies[243], implying that restrictions on protein synthesis may have a particularly important anti-ageing effect. Consistent with this observation, loss of the translation effector S6K1 extends lifespan in worms and mice, perhaps by halting the production of misfolded or aggregated proteins[242,244]. By reducing the energetic burden of translation, mTOR inhibition also relieves oxidative stress and prevents the accumulation of harmful metabolic byproducts, leading to broad improvements in cellular function[73].

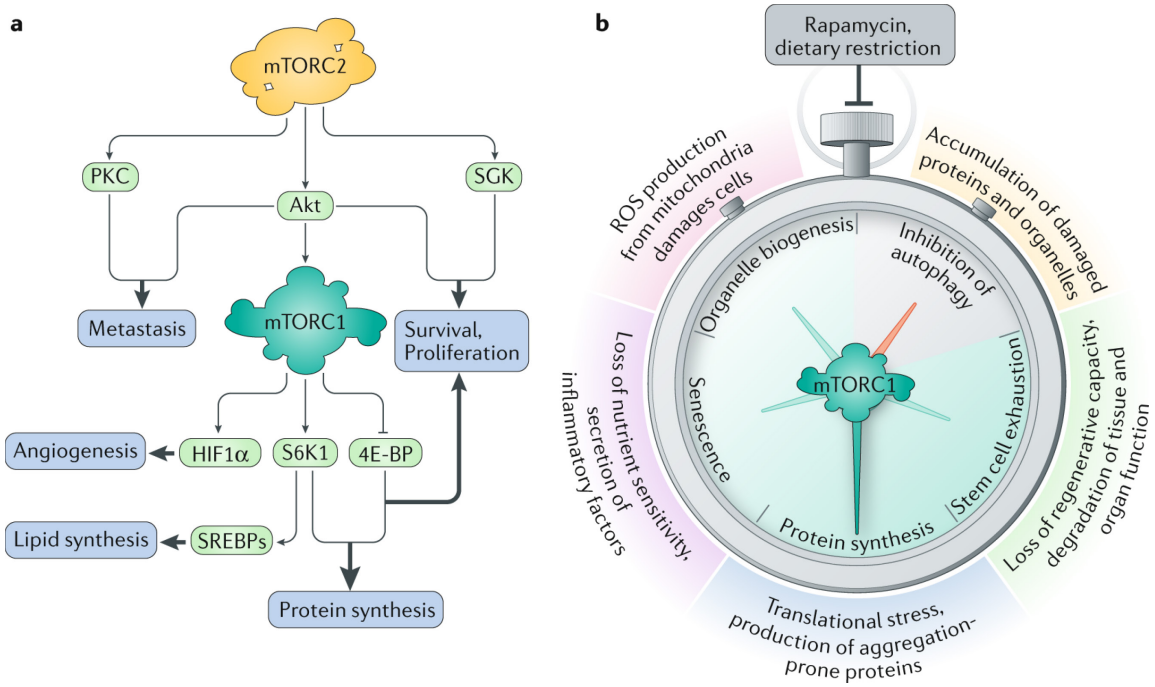


Figure 6: mTOR in cancer and aging

(A) mTORC1 and mTORC2 participate in cancer pathogenesis by underwriting biosynthetic programs and promoting proliferation and survival. Emerging evidence also implicates mTORC2 activity in metastatic transformations.

(B) Modulation of mTORC1 signalling in aging cells may enable us to slow the molecular clock. mTORC1 activates processes that may accelerate cellular and tissue aging, including protein synthesis, mitochondrial energy production, and entry into senescence.

Chronic mTORC1 activation also blocks autophagic clearance of damaged cellular components. Inhibition of this pathway—either by rapamycin treatment, genetic inactivation of mTORC1, or dietary restriction—has been shown to extend lifespan and improve physiological performance across a range of model organisms.

In parallel with its downregulation of translation, mTOR inhibition restores autophagic capacity, which undergoes an age-related decline in many tissues[245]. Autophagy degrades obsolete or damaged cellular components and salvages them for "spare macromolecular parts." Through this process, aged cells refresh their molecular equipment and clear damaged proteins and organelles, which have been implicated in age-related diseases from cardiomyopathy to neurodegeneration. Underscoring the importance of autophagy in healthy ageing, direct activation of autophagic flux can significantly increase lifespan and healthspan in mice[246]. Conversely, mTOR inhibition fails to extend lifespan in ATG-deficient worms, indicating that mTOR modulates longevity at least partly through autophagy-dependent mechanisms[247,248].

mTORC1 has also been implicated in ageing at the tissue level. Several groups have shown that persistent mTORC1 signaling contributes to the exhaustion of stem cell pools, hindering tissue self-renewal in aged organisms[249,250]. mTORC1 hyperactivity is also a distinctive feature of senescent cells, which permanently arrest in the G0 phase of the cell cycle and undergo morphological alterations that eliminate sensitivity to amino acid and growth factor deprivation[251]. Exploiting translational programs downstream of mTORC1, senescent cells synthesize and secrete pro-inflammatory cytokines to exacerbate ageing-related declines in fitness and tissue function[252,253]. Rapamycin treatment attenuates this inflammatory phenotype, although it is unclear whether mTORC1 inhibition can rescue cell cycle arrest or aid in the clearance of senescent cells.

Even though mTOR inhibitors are well-validated as geroprotective agents in animal models, the potential side effects of chronic dosing (particularly insulin resistance and immunosuppression) have thus far precluded their use in healthy elderly humans. Recent

studies, however, suggest that these concerns are not insurmountable. Because side effect profiles for mTOR inhibitors have largely been inferred from patients undergoing cancer therapy or organ transplantation, they tend to reflect intense, high-dose regimens. Far lower doses are needed for anti-ageing benefits. Taking advantage of this distinction, one group found that intermittent dosing of rapamycin in mice could extend lifespan without inciting glucose intolerance[254,255]. Another reported that low doses of mTOR inhibitors could actually improve immune function in elderly patients[256]. Efforts to harness mTOR inhibition as an anti-ageing strategy will have to build on these studies to define safe and effective doses in human cohorts.

VI. Conclusions and perspectives

Perched at the interface between organisms and their environments, the mTOR pathway toggles the balance of anabolism and catabolism in response to contextual signals and guides nearly every aspect of metabolic function. Recent work has clarified the logical structure of the pathway and drawn the lysosome into renewed focus; structural advances have also allowed us to see, mechanistically, how key mTOR signaling nodes transduce nutritional information into molecular action. Building on careful *in vivo* studies, we have made remarkable progress in cataloguing the inputs and effectors of the mTOR pathway across a variety of tissues and metabolic states, enhancing our understanding of mTOR signaling in health and disease.

Nonetheless, certain open questions remain stubbornly unresolved. Given that mTORC1 activation occurs at the lysosomal surface, how does the complex capture and phosphorylate its downstream substrates, which, with the exception of TFEB/TFE3, do not maintain lysosomal subpopulations? It is possible that lysosomal interactions with the ER, the Golgi, and the plasma membrane may help bring mTORC1 into contact with some of its substrates[257-259]. Alternatively, while efforts to visualize the dynamics of the substrate search have not been conclusive[260,261], we speculate that activated mTORC1 could exit the lysosome to phosphorylate targets at other loci in the cell, perhaps carrying Rheb in tow. Moving forward, we also seek an integrated

understanding of mTOR signalling in specific tissues. Although many components of the mTOR pathway have been identified, it is not clear which regulatory inputs are dominant in any particular physiological milieu. In order to develop new therapeutics that evade some of the metabolic side effects of existing mTOR inhibitors, we hope to identify complex- or tissue-specific modulators of mTOR activity and establish them as targets for rational drug development.

One emerging theme from the study of mTOR dysregulation in human disease is that these pathologies are not just linked by a common etiological basis—they also intersect with each other in mutually-reinforcing ways. Just as excessive mTOR activity can lead to metabolic syndrome, obesity accelerates molecular ageing, which in turn amplifies the risk of neurodegenerative disease and cancer. Thus, even though the complexity and breadth of the mTOR signaling network increases the risk of toxicity, the unique spectrum of mTOR-dependent processes is also one of its most powerful advantages as a therapeutic target. More so than other strategies to delay ageing or counter disease, mTOR inhibition disrupts a wide variety of degenerative processes with a single intervention. Further insights into this fundamental pathway may ultimately lead to new treatments for currently intractable diseases and transform our ability to regulate health and homeostasis.

References:

1 Vezina, C., Kudelski, A. & Sehgal, S. N. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J Antibiot* **28**, 721-726, doi:10.7164/antibiotics.28.721 (1975).

2 Martel, R. R., Klicius, J. & Galet, S. Inhibition of the immune response by rapamycin, a new antifungal antibiotic. *Can J Physiol Pharmacol* **55**, 48-51, doi:10.1139/y77-007 (1977).

3 Eng, C. P., Sehgal, S. N. & Vezina, C. Activity of rapamycin (AY-22,989) against transplanted tumors. *The J. Antibiot* **37**, 1231-1237, doi:10.7164/antibiotics.37.1231 (1984).

4 Houchens, D. P., Ovejera, A. A., Riblet, S. M. & Slagel, D. E. Human brain tumor xenografts in nude mice as a chemotherapy model. *Eur J Cancer Clin Oncol* **19**, 799-805 (1983).

5 Bierer, B. E. *et al.* Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin. *Proc Natl Acad Sci U S A* **87**, 9231-9235, doi:10.1073/pnas.87.23.9231 (1990).

6 Chung, J., Kuo, C. J., Crabtree, G. R. & Blenis, J. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* **69**, 1227-1236, doi:10.1016/0092-8674(92)90643-q (1992).

7 Brown, E. J. *et al.* A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* **369**, 756-758, doi:10.1038/369756a0 (1994).

8 Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P. & Snyder, S. H. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **78**, 35-43, doi:10.1016/0092-8674(94)90570-3 (1994).

9 Sabers, C. J. *et al.* Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J Biol Chem* **270**, 815-822, doi:10.1074/jbc.270.2.815 (1995).

10 Heitman, J., Movva, N. R. & Hall, M. N. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253**, 905-909, doi:10.1126/science.1715094 (1991).

11 Cafferkey, R. *et al.* Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. *Mol Cell Biol* **13**, 6012-6023, doi:10.1128/mcb.13.10.6012 (1993).

12 Kunz, J. *et al.* Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell* **73**, 585-596, doi:10.1016/0092-8674(93)90144-f (1993).

13 Helliwell, S. B. *et al.* TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol Biol Cell* **5**, 105-118, doi:10.1091/mbc.5.1.105 (1994).

14 Keith, C. T. & Schreiber, S. L. PIK-Related kinases: DNA repair, recombination, and cell cycle checkpoints. *Science* **270**, 50, doi:10.1126/science.270.5233.50 (1995).

15 Kim, D. H. *et al.* GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol Cell* **11**, 895-904 (2003).

16 Kim, D. H. *et al.* mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell* **110**, 163-175 (2002).

17 Hara, K. *et al.* Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell* **110**, 177-189 (2002).

18 Yang, H. *et al.* mTOR kinase structure, mechanism and regulation. *Nature* **497**, 217-223, doi:10.1038/nature12122 (2013).
19 Guertin, D. A. *et al.* Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev Cell* **11**, 859-871, doi:10.1016/j.devcel.2006.10.007 (2006).
20 Schalm, S. S., Fingar, D. C., Sabatini, D. M. & Blenis, J. TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. *Curr Biol* **13**, 797-806, doi:10.1016/s0960-9822(03)00329-4 (2003).
21 Nojima, H. *et al.* The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. *J Biol Chem* **278**, 15461-15464, doi:10.1074/jbc.C200665200 (2003).
22 Sancak, Y. *et al.* PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. *Mol Cell* **25**, 903-915, doi:10.1016/j.molcel.2007.03.003 (2007).
23 Vander Haar, E., Lee, S.-I., Bandhakavi, S., Griffin, T. J. & Kim, D.-H. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat Cell Biol* **9**, 316-323, doi:10.1038/ncb1547 (2007).
24 Peterson, T. R. *et al.* DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell* **137**, 873-886, doi:10.1016/j.cell.2009.03.046 (2009).
25 Yip, C. K., Murata, K., Walz, T., Sabatini, D. M. & Kang, S. A. Structure of the human mTOR complex I and its implications for rapamycin inhibition. *Mol Cell* **38**, 768-774, doi:10.1016/j.molcel.2010.05.017 (2010).
26 Aylett, C. H. *et al.* Architecture of human mTOR complex 1. *Science* **351**, 48-52, doi:10.1126/science.aaa3870 (2016).
27 Yang, H. *et al.* Mechanisms of mTORC1 activation by RHEB and inhibition by PRAS40. *Nature* **552**, 368-373, doi:10.1038/nature25023 (2017).
28 Hwang, Y. *et al.* Disruption of the scaffolding function of mLST8 selectively inhibits mTORC2 assembly and function and suppresses mTORC2-dependent tumor growth *in vivo*. *Cancer Res* **79**, 3178, doi:10.1158/0008-5472.CAN-18-3658 (2019).
29 Sarbassov, D. D. *et al.* Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol* **14**, 1296-1302, doi:10.1016/j.cub.2004.06.054 (2004).
30 Jacinto, E. *et al.* Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol* **6**, 1122-1128, doi:10.1038/ncb1183 (2004).
31 Frias, M. A. *et al.* mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr Biol* **16**, 1865-1870, doi:10.1016/j.cub.2006.08.001 (2006).
32 Jacinto, E. *et al.* SIN1/MIP1 Maintains rictor-mTOR Complex Integrity and Regulates Akt Phosphorylation and Substrate Specificity. *Cell* **127**, 125-137, doi:10.1016/j.cell.2006.08.033 (2006).
33 Yang, Q., Inoki, K., Ikenoue, T. & Guan, K. L. Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. *Genes Dev* **20**, 2820-2832, doi:10.1101/gad.1461206 (2006).
34 Pearce, L. R. *et al.* Identification of Protor as a novel Rictor-binding component of mTOR complex-2. *Biochem J* **405**, 513-522, doi:10.1042/bj20070540 (2007).
35 Woo, S. Y. *et al.* PRR5, a novel component of mTOR complex 2, regulates platelet-derived growth factor receptor beta expression and signaling. *J Biol Chem* **282**, 25604-25612, doi:10.1074/jbc.M704343200 (2007).

36 Yuan, H.-X. & Guan, K.-L. The SIN1-PH domain connects mTORC2 to PI3K. *Cancer Discov* **5**, 1127-1129, doi:10.1158/2159-8290.CD-15-1125 (2015).
37 Chen, X. *et al.* Cryo-EM structure of human mTOR complex 2. *Cell research* **28**, 518-528, doi:10.1038/s41422-018-0029-3 (2018).
38 Stuttfeld, E. *et al.* Architecture of the human mTORC2 core complex. *Elife* **7**, e33101, doi:10.7554/eLife.33101 (2018).
39 Sarbassov, D. D. *et al.* Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* **22**, 159-168, doi:10.1016/j.molcel.2006.03.029 (2006).
40 Lamming, D. W. *et al.* Rapamycin-induced insulin resistance is mediated by mTORC2 loss and uncoupled from longevity. *Science* **335**, 1638-1643, doi:10.1126/science.1215135 (2012).
41 Thoreen, C. C. *et al.* An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J Biol Chem* **284**, 8023-8032, doi:10.1074/jbc.M900301200 (2009).
42 Kang, S. A. *et al.* mTORC1 Phosphorylation Sites Encode Their Sensitivity to Starvation and Rapamycin. *Science* **341**, 1236566, doi:10.1126/science.1236566 (2013).
43 Choo, A. Y., Yoon, S.-O., Kim, S. G., Roux, P. P. & Blenis, J. Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation. *Proc Natl Acad Sci U S A* **105**, 17414-17419, doi:10.1073/pnas.0809136105 (2008).
44 Buttgereit, F. & Brand, M. D. A hierarchy of ATP-consuming processes in mammalian cells. *Biochem J* **312 (Pt 1)**, 163-167, doi:10.1042/bj3120163 (1995).
45 Brunn, G. J. *et al.* Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science* **277**, 99-101, doi:10.1126/science.277.5322.99 (1997).
46 Gingras, A. C. *et al.* Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev* **13**, 1422-1437, doi:10.1101/gad.13.11.1422 (1999).
47 Hara, K. *et al.* Regulation of eIF-4E BP1 phosphorylation by mTOR. *J Biol Chem* **272**, 26457-26463, doi:10.1074/jbc.272.42.26457 (1997).
48 Pullen, N. *et al.* Phosphorylation and activation of p70s6k by PDK1. *Science* **279**, 707, doi:10.1126/science.279.5351.707 (1998).
49 Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H. & Sabatini, D. M. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A* **95**, 1432-1437, doi:10.1073/pnas.95.4.1432 (1998).
50 Ruvinsky, I. *et al.* Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev* **19**, 2199-2211, doi:10.1101/gad.351605 (2005).
51 Chauvin, C. *et al.* Ribosomal protein S6 kinase activity controls the ribosome biogenesis transcriptional program. *Oncogene* **33**, 474, doi:10.1038/onc.2012.606 (2013).
52 Hannan, K. M. *et al.* mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. *Mol Cell Biol* **23**, 8862-8877, doi:10.1128/mcb.23.23.8862-8877.2003 (2003).
53 Mayer, C., Zhao, J., Yuan, X. & Grummt, I. mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev* **18**, 423-434, doi:10.1101/gad.285504 (2004).
54 Michels, A. A. *et al.* mTORC1 Directly Phosphorylates and Regulates Human MAF1. *Mol Cell Biol* **30**, 3749, doi:10.1128/MCB.00319-10 (2010).
55 Shor, B. *et al.* Requirement of the mTOR kinase for the regulation of Maf1 phosphorylation and control of RNA polymerase III-dependent transcription in cancer cells. *J Biol Chem* **285**, 15380-15392, doi:10.1074/jbc.M109.071639 (2010).

56 Holz, M. K., Ballif, B. A., Gygi, S. P. & Blenis, J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* **123**, 569-580, doi:10.1016/j.cell.2005.10.024 (2005).
57 Dorrello, N. V. *et al.* S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science* **314**, 467-471, doi:10.1126/science.1130276 (2006).
58 Ma, X. M., Yoon, S. O., Richardson, C. J., Julich, K. & Blenis, J. SKAR links pre-mRNA splicing to mTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs. *Cell* **133**, 303-313, doi:10.1016/j.cell.2008.02.031 (2008).
59 Mieulet, V. *et al.* S6 kinase inactivation impairs growth and translational target phosphorylation in muscle cells maintaining proper regulation of protein turnover. *Am J Physiol Cell Physiol* **293**, C712-722, doi:10.1152/ajpcell.00499.2006 (2007).
60 Pende, M. *et al.* S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol* **24**, 3112-3124, doi:10.1128/mcb.24.8.3112-3124.2004 (2004).
61 Hsieh, A. C. *et al.* The translational landscape of mTOR signalling steers cancer initiation and metastasis. *Nature* **485**, 55, doi:10.1038/nature10912 (2012).
62 Thoreen, C. C. *et al.* A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature* **485**, 109-113, doi:10.1038/nature11083 (2012).
63 Horton, J. D., Goldstein, J. L. & Brown, M. S. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* **109**, 1125-1131, doi:10.1172/JCI15593 (2002).
64 Peterson, T. R. *et al.* mTOR complex 1 regulates lipin 1 localization to control the SREBP pathway. *Cell* **146**, 408-420, doi:10.1016/j.cell.2011.06.034 (2011).
65 Porstmann, T. *et al.* SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. *Cell Metab* **8**, 224-236, doi:10.1016/j.cmet.2008.07.007 (2008).
66 Duvel, K. *et al.* Activation of a metabolic gene regulatory network downstream of mTOR complex 1. *Mol Cell* **39**, 171-183, doi:10.1016/j.molcel.2010.06.022 (2010).
67 Kim, J. E. & Chen, J. Regulation of peroxisome proliferator-activated receptor-gamma activity by mammalian target of rapamycin and amino acids in adipogenesis. *Diabetes* **53**, 2748-2756, doi:10.2337/diabetes.53.11.2748 (2004).
68 Ben-Sahra, I., Hoxhaj, G., Ricoult, S. J. H., Asara, J. M. & Manning, B. D. mTORC1 induces purine synthesis through control of the mitochondrial tetrahydrofolate cycle. *Science* **351**, 728-733, doi:10.1126/science.aad0489 (2016).
69 Ben-Sahra, I., Howell, J. J., Asara, J. M. & Manning, B. D. Stimulation of de novo pyrimidine synthesis by growth signaling through mTOR and S6K1. *Science* **339**, 1323-1328, doi:10.1126/science.1228792 (2013).
70 Robitaille, A. M. *et al.* Quantitative phosphoproteomics reveal mTORC1 activates de novo pyrimidine synthesis. *Science* **339**, 1320-1323, doi:10.1126/science.1228771 (2013).
71 Valvezan, A. J. *et al.* mTORC1 Couples nucleotide synthesis to nucleotide demand resulting in a targetable metabolic vulnerability. *Cancer Cell* **32**, 624-638.e625, doi:10.1016/j.ccell.2017.09.013 (2017).
72 He, L. *et al.* mTORC1 promotes metabolic reprogramming by the suppression of GSK3-dependent Foxk1 phosphorylation. *Mol Cell* **70**, 949-960.e944, doi:10.1016/j.molcel.2018.04.024 (2018).

73 Zid, B. M. *et al.* 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila. *Cell* **139**, 149-160, doi:10.1016/j.cell.2009.07.034 (2009).
74 Cunningham, J. T. *et al.* mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* **450**, 736-740, doi:10.1038/nature06322 (2007).
75 Kim, J., Kundu, M., Viollet, B. & Guan, K. L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* **13**, 132-141, doi:10.1038/ncb2152 (2011).
76 Hosokawa, N. *et al.* Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy. *Mol Biol Cell* **20**, 1981-1991, doi:10.1091/mbc.E08-12-1248 (2009).
77 Ganley, I. G. *et al.* ULK1·ATG13·FIP200 complex mediates mTOR signaling and is essential for autophagy. *J Biol Chem* **284**, 12297-12305, doi:10.1074/jbc.M900573200 (2009).
78 Dikic, I. & Elazar, Z. Mechanism and medical implications of mammalian autophagy. *Nat Rev Mol Cell Biol* **19**, 349-364, doi:10.1038/s41580-018-0003-4 (2018).
79 Kim, Y. M. *et al.* mTORC1 phosphorylates UVRAG to negatively regulate autophagosome and endosome maturation. *Mol Cell* **57**, 207-218, doi:10.1016/j.molcel.2014.11.013 (2015).
80 Odle, R. I. *et al.* An mTORC1-to-CDK1 switch maintains autophagy suppression during mitosis. *Mol Cell*, doi:https://doi.org/10.1016/j.molcel.2019.10.016 (2019).
81 Settembre, C. *et al.* A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. *EMBO J* **31**, 1095-1108, doi:10.1038/emboj.2012.32 (2012).
82 Martina, J. A., Chen, Y., Gucek, M. & Puertollano, R. MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. *Autophagy* **8**, 903-914, doi:10.4161/auto.19653 (2012).
83 Roczniak-Ferguson, A. *et al.* The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis. *Sci Signal* **5**, ra42, doi:10.1126/scisignal.2002790 (2012).
84 Yu, L. *et al.* Termination of autophagy and reformation of lysosomes regulated by mTOR. *Nature* **465**, 942, doi:10.1038/nature09076 (2010).
85 Wyant, G. A. *et al.* mTORC1 activator SLC38A9 is required to efflux essential amino acids from lysosomes and use protein as a nutrient. *Cell* **171**, 642-654.e612, doi:10.1016/j.cell.2017.09.046 (2017).
86 Wyant, G. A. *et al.* NUFIP1 is a ribosome receptor for starvation-induced ribophagy. *Science* **360**, 751-758, doi:10.1126/science.aar2663 (2018).
87 Guo, J. Y. *et al.* Autophagy provides metabolic substrates to maintain energy charge and nucleotide pools in Ras-driven lung cancer cells. *Genes Dev* **30**, 1704-1717, doi:10.1101/gad.283416.116 (2016).
88 Kraft, C., Deplazes, A., Sohrmann, M. & Peter, M. Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. *Nat Cell Biol* **10**, 602-610, doi:10.1038/ncb1723 (2008).
89 An, H. & Harper, J. W. Systematic analysis of ribophagy in human cells reveals bystander flux during selective autophagy. *Nat Cell Biol* **20**, 135-143, doi:10.1038/s41556-017-0007-x (2018).
90 Larsson, C. Protein kinase C and the regulation of the actin cytoskeleton. *Cell Signal* **18**, 276-284, doi:10.1016/j.cellsig.2005.07.010 (2006).

91 Liu, L., Das, S., Losert, W. & Parent, C. A. mTORC2 regulates neutrophil chemotaxis in a cAMP- and RhoA-dependent fashion. *Dev Cell* **19**, 845-857, doi:10.1016/j.devcel.2010.11.004 (2010).
92 Schmidt, K. M. *et al.* Inhibition of mTORC2/RICTOR impairs melanoma hepatic metastasis. *Neoplasia* **20**, 1198-1208, doi:10.1016/j.neo.2018.10.001 (2018).
93 Morrison Joly, M. *et al.* Two distinct mTORC2-dependent pathways converge on Rac1 to drive breast cancer metastasis. *Breast Cancer Res* **19**, 74, doi:10.1186/s13058-017-0868-8 (2017).
94 Ikenoue, T., Inoki, K., Yang, Q., Zhou, X. & Guan, K. L. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. *EMBO J* **27**, 1919-1931, doi:10.1038/emboj.2008.119 (2008).
95 Li, X. & Gao, T. mTORC2 phosphorylates protein kinase Cζ to regulate its stability and activity. *EMBO Rep* **15**, 191-198, doi:10.1002/embr.201338119 (2014).
96 García-Martínez, Juan M. & Alessi, Dario R. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). *Biochem J* **416**, 375, doi:10.1042/BJ20081668 (2008).
97 Sarbassov, D. D., Guertin, D. A., Ali, S. M. & Sabatini, D. M. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098-1101, doi:10.1126/science.1106148 (2005).
98 Webb, A. E. & Brunet, A. FOXO transcription factors: key regulators of cellular quality control. *Trends Biochem Sci* **39**, 159-169, doi:10.1016/j.tibs.2014.02.003 (2014).
99 Hoxhaj, G. *et al.* Direct stimulation of NADP+ synthesis through Akt-mediated phosphorylation of NAD kinase. *Science* **363**, 1088-1092, doi:10.1126/science.aau3903 (2019).
100 Inoki, K., Li, Y., Zhu, T., Wu, J. & Guan, K. L. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* **4**, 648-657, doi:10.1038/ncb839 (2002).
101 Humphrey, S. J. *et al.* Dynamic adipocyte phosphoproteome reveals that Akt directly regulates mTORC2. *Cell Metab* **17**, 1009-1020, doi:10.1016/j.cmet.2013.04.010 (2013).
102 Ebner, M., Sinkovics, B., Szczygieł, M., Ribeiro, D. W. & Yudushkin, I. Localization of mTORC2 activity inside cells. *J Cell Biol* **216**, 343, doi:10.1083/jcb.201610060 (2017).
103 Inoki, K., Li, Y., Xu, T. & Guan, K. L. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev* **17**, 1829-1834, doi:10.1101/gad.1110003 (2003).
104 Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K. & Avruch, J. Rheb binds and regulates the mTOR kinase. *Curr Biol* **15**, 702-713, doi:10.1016/j.cub.2005.02.053 (2005).
105 Sancak, Y. *et al.* The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science* **320**, 1496-1501, doi:10.1126/science.1157535 (2008).
106 Kim, E., Goraksha-Hicks, P., Li, L., Neufeld, T. P. & Guan, K. L. Regulation of TORC1 by Rag GTPases in nutrient response. *Nat Cell Biol* **10**, 935-945, doi:10.1038/ncb1753 (2008).
107 Dibble, C. C. & Manning, B. D. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat Cell Biol* **15**, 555-564, doi:10.1038/ncb2763 (2013).
108 Dibble, C. C. *et al.* TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1. *Mol Cell* **47**, 535-546, doi:10.1016/j.molcel.2012.06.009 (2012).
109 Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C. & Blenis, J. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr Biol* **13**, 1259-1268, doi:10.1016/s0960-9822(03)00506-2 (2003).

110 Valvezan, A. J. & Manning, B. D. Molecular logic of mTORC1 signalling as a metabolic rheostat. *Nat Metab* **1**, 321-333, doi:10.1038/s42255-019-0038-7 (2019).
111 Garami, A. *et al.* Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell* **11**, 1457-1466 (2003).
112 Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J. & Cantley, L. C. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol Cell* **10**, 151-162 (2002).
113 Menon, S. *et al.* Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome. *Cell* **156**, 771-785, doi:10.1016/j.cell.2013.11.049 (2014).
114 Demetriades, C., Doumpas, N. & Teleman, A. A. Regulation of TORC1 in response to amino acid starvation via lysosomal recruitment of TSC2. *Cell* **156**, 786-799, doi:10.1016/j.cell.2014.01.024 (2014).
115 Harrington, L. S. *et al.* The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J Cell Biol* **166**, 213-223, doi:10.1083/jcb.200403069 (2004).
116 Shah, O. J., Wang, Z. & Hunter, T. Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. *Curr Biol* **14**, 1650-1656, doi:10.1016/j.cub.2004.08.026 (2004).
117 Inoki, K. *et al.* TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* **126**, 955-968, doi:10.1016/j.cell.2006.06.055 (2006).
118 Lee, D. F. *et al.* IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell* **130**, 440-455, doi:10.1016/j.cell.2007.05.058 (2007).
119 Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P. & Pandolfi, P. P. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. *Cell* **121**, 179-193, doi:10.1016/j.cell.2005.02.031 (2005).
120 Roux, P. P., Ballif, B. A., Anjum, R., Gygi, S. P. & Blenis, J. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proc Natl Acad Sci U S A* **101**, 13489-13494, doi:10.1073/pnas.0405659101 (2004).
121 Gwinn, D. M. *et al.* AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell* **30**, 214-226, doi:10.1016/j.molcel.2008.03.003 (2008).
122 Shaw, R. J. *et al.* The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell* **6**, 91-99, doi:10.1016/j.ccr.2004.06.007 (2004).
123 Inoki, K., Zhu, T. & Guan, K. L. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115**, 577-590, doi:10.1016/s0092-8674(03)00929-2 (2003).
124 Herzig, S. & Shaw, R. J. AMPK: guardian of metabolism and mitochondrial homeostasis. *Nat Rev Mol Cell Biol* **19**, 121-135, doi:10.1038/nrm.2017.95 (2018).
125 Brugarolas, J. *et al.* Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev* **18**, 2893-2904, doi:10.1101/gad.1256804 (2004).
126 DeYoung, M. P., Horak, P., Sofer, A., Sgroi, D. & Ellisen, L. W. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. *Genes Dev* **22**, 239-251, doi:10.1101/gad.1617608 (2008).
127 Demetriades, C., Plescher, M. & Teleman, A. A. Lysosomal recruitment of TSC2 is a universal response to cellular stress. *Nat Commun* **7**, 10662, doi:10.1038/ncomms10662 (2016).

128 Saveljeva, S. *et al.* Endoplasmic reticulum stress-mediated induction of SESTRIN 2 potentiates cell survival. *Oncotarget* **7**, 12254-12266, doi:10.18632/oncotarget.7601 (2016).
129 Feng, Z. *et al.* The regulation of AMPK beta1, TSC2, and PTEN expression by p53: stress, cell and tissue specificity, and the role of these gene products in modulating the IGF-1-AKT-mTOR pathways. *Cancer Res* **67**, 3043-3053, doi:10.1158/0008-5472.Can-06-4149 (2007).
130 Blommaart, E. F., Luiken, J. J., Blommaart, P. J., van Woerkom, G. M. & Meijer, A. J. Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. *J Biol Chem* **270**, 2320-2326, doi:10.1074/jbc.270.5.2320 (1995).
131 Hara, K. *et al.* Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J Biol Chem* **273**, 14484-14494, doi:10.1074/jbc.273.23.14484 (1998).
132 Sancak, Y. *et al.* Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell* **141**, 290-303, doi:10.1016/j.cell.2010.02.024 (2010).
133 Bar-Peled, L., Schweitzer, L. D., Zoncu, R. & Sabatini, D. M. Ragulator is a GEF for the Rag GTPases that signal amino acid levels to mTORC1. *Cell* **150**, 1196-1208, doi:10.1016/j.cell.2012.07.032 (2012).
134 Su, M. Y. *et al.* Hybrid structure of the RagA/C-Ragulator mTORC1 activation complex. *Mol Cell* **68**, 835-846.e833, doi:10.1016/j.molcel.2017.10.016 (2017).
135 Shen, K., Choe, A. & Sabatini, D. M. Intersubunit crosstalk in the Rag GTPase heterodimer enables mTORC1 to respond rapidly to amino acid availability. *Mol Cell* **68**, 821, doi:10.1016/j.molcel.2017.10.031 (2017).
136 Rogala, K. B. *et al.* Structural basis for the docking of mTORC1 on the lysosomal surface. *Science*, eaay0166, doi:10.1126/science.aay0166 (2019).
137 Bar-Peled, L. *et al.* A Tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. *Science* **340**, 1100-1106, doi:10.1126/science.1232044 (2013).
138 Shen, K., Valenstein, M. L., Gu, X. & Sabatini, D. M. Arg-78 of Nprl2 catalyzes GATOR1-stimulated GTP hydrolysis by the Rag GTPases. *J Biol Chem* **294**, 2970-2975, doi:10.1074/jbc.AC119.007382 (2019).
139 Shen, K. *et al.* Architecture of the human GATOR1 and GATOR1-Rag GTPases complexes. *Nature* **556**, 64-69, doi:10.1038/nature26158 (2018).
140 Peng, M., Yin, N. & Li, M. O. SZT2 dictates GATOR control of mTORC1 signalling. *Nature* **543**, 433-437, doi:10.1038/nature21378 (2017).
141 Wolfson, R. L. *et al.* KICSTOR recruits GATOR1 to the lysosome and is necessary for nutrients to regulate mTORC1. *Nature* **543**, 438-442, doi:10.1038/nature21423 (2017).
142 Wolfson, R. L. *et al.* Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science* **351**, 43-48, doi:10.1126/science.aab2674 (2016).
143 Saxton, R. A. *et al.* Structural basis for leucine sensing by the Sestrin2-mTORC1 pathway. *Science* **351**, 53-58, doi:10.1126/science.aad2087 (2016).
144 Ye, J. *et al.* GCN2 sustains mTORC1 suppression upon amino acid deprivation by inducing Sestrin2. *Genes Dev* **29**, 2331-2336, doi:10.1101/gad.269324.115 (2015).
145 Chantranupong, L. *et al.* The Sestrins interact with GATOR2 to negatively regulate the amino-acid-sensing pathway upstream of mTORC1. *Cell Rep* **9**, 1-8, doi:10.1016/j.celrep.2014.09.014 (2014).
146 Parmigiani, A. *et al.* Sestrins inhibit mTORC1 kinase activation through the GATOR complex. *Cell Rep* **9**, 1281-1291, doi:10.1016/j.celrep.2014.10.019 (2014).

147 Chantranupong, L. *et al.* The CASTOR proteins are arginine sensors for the mTORC1 pathway. *Cell* **165**, 153-164, doi:10.1016/j.cell.2016.02.035 (2016).
148 Saxton, R. A., Chantranupong, L., Knockenhauer, K. E., Schwartz, T. U. & Sabatini, D. M. Mechanism of arginine sensing by CASTOR1 upstream of mTORC1. *Nature* **536**, 229-233, doi:10.1038/nature19079 (2016).
149 Rebsamen, M. *et al.* SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1. *Nature* **519**, 477-481, doi:10.1038/nature14107 (2015).
150 Wang, S. *et al.* Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. *Science* **347**, 188-194, doi:10.1126/science.1257132 (2015).
151 Lei, H. T., Ma, J., Sanchez Martinez, S. & Gonen, T. Crystal structure of arginine-bound lysosomal transporter SLC38A9 in the cytosol-open state. *Nat Struct Mol Biol* **25**, 522-527, doi:10.1038/s41594-018-0072-2 (2018).
152 Shen, K. & Sabatini, D. M. Ragulator and SLC38A9 activate the Rag GTPases through noncanonical GEF mechanisms. *Proc Natl Acad Sci U S A* **115**, 9545-9550, doi:10.1073/pnas.1811727115 (2018).
153 Zoncu, R. *et al.* mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. *Science* **334**, 678-683, doi:10.1126/science.1207056 (2011).
154 Petit, C. S., Roczniak-Ferguson, A. & Ferguson, S. M. Recruitment of folliculin to lysosomes supports the amino acid-dependent activation of Rag GTPases. *J Cell Biol* **202**, 1107-1122, doi:10.1083/jcb.201307084 (2013).
155 Tsun, Z. Y. *et al.* The folliculin tumor suppressor is a GAP for the RagC/D GTPases that signal amino acid levels to mTORC1. *Mol Cell* **52**, 495-505, doi:10.1016/j.molcel.2013.09.016 (2013).
156 Wada, S. *et al.* The tumor suppressor FLCN mediates an alternate mTOR pathway to regulate browning of adipose tissue. *Genes Dev* **30**, 2551-2564, doi:10.1101/gad.287953.116 (2016).
157 Villegas, F. *et al.* Lysosomal signaling licenses embryonic stem cell differentiation via onactivation of Tfe3. *Cell Stem Cell* **24**, 257-270.e258, doi:10.1016/j.stem.2018.11.021 (2019).
158 Perera, R. M. *et al.* Transcriptional control of autophagy-lysosome function drives pancreatic cancer metabolism. *Nature* **524**, 361-365, doi:10.1038/nature14587 (2015).
159 Di Malta, C. *et al.* Transcriptional activation of RagD GTPase controls mTORC1 and promotes cancer growth. *Science* **356**, 1188-1192, doi:10.1126/science.aag2553 (2017).
160 Gu, X. *et al.* SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. *Science* **358**, 813-818, doi:10.1126/science.aao3265 (2017).
161 Efeyan, A. *et al.* Regulation of mTORC1 by the Rag GTPases is necessary for neonatal autophagy and survival. *Nature* **493**, 679-683, doi:10.1038/nature11745 (2013).
162 Kalender, A. *et al.* Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell Metab* **11**, 390-401, doi:10.1016/j.cmet.2010.03.014 (2010).
163 Hoxhaj, G. *et al.* The mTORC1 signaling network senses changes in cellular purine nucleotide levels. *Cell Rep* **21**, 1331-1346, doi:10.1016/j.celrep.2017.10.029 (2017).
164 Emmanuel, N. *et al.* Purine nucleotide availability regulates mTORC1 activity through the Rheb GTPase. *Cell Rep* **19**, 2665-2680, doi:10.1016/j.celrep.2017.05.043 (2017).
165 Menon, D. *et al.* Lipid sensing by mTOR via de novo synthesis of phosphatidic acid. *J Biol Chem* **292**, 6303-6311, doi:10.1074/jbc.M116.772988 (2017).
166 Jewell, J. L. *et al.* Differential regulation of mTORC1 by leucine and glutamine. *Science* **347**, 194, doi:10.1126/science.1259472 (2015).

167 Gan, X., Wang, J., Su, B. & Wu, D. Evidence for direct activation of mTORC2 kinase activity by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **286**, 10998-11002, doi:10.1074/jbc.M110.195016 (2011).
168 Khanna, A. *et al.* The small GTPases Ras and Rap1 bind to and control TORC2 activity. *Sci Rep* **6**, 25823, doi:10.1038/srep25823 (2016).
169 Saci, A., Cantley, Lewis C. & Carpenter, Christopher L. Rac1 regulates the activity of mTORC1 and mTORC2 and controls cellular size. *Mol Cell* **42**, 50-61, doi:10.1016/j.molcel.2011.03.017 (2011).
170 Senoo, H. *et al.* Phosphorylated Rho–GDP directly activates mTORC2 kinase towards AKT through dimerization with Ras–GTP to regulate cell migration. *Nat Cell Biol* **21**, 867-878, doi:10.1038/s41556-019-0348-8 (2019).
171 Kovalski, J. R. *et al.* The functional proximal proteome of oncogenic Ras includes mTORC2. *Mol Cell* **73**, 830-844.e812, doi:10.1016/j.molcel.2018.12.001 (2019).
172 Um, S. H. *et al.* Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* **431**, 200-205, doi:10.1038/nature02866 (2004).
173 Hsu, P. P. *et al.* The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. *Science* **332**, 1317-1322, doi:10.1126/science.1199498 (2011).
174 Yu, Y. *et al.* Phosphoproteomic analysis identifies Grb10 as an mTORC1 substrate that negatively regulates insulin signaling. *Science* **332**, 1322-1326, doi:10.1126/science.1199484 (2011).
175 Kazyken, D. *et al.* AMPK directly activates mTORC2 to promote cell survival during acute energetic stress. *Sci Signal* **12**, eaav3249, doi:10.1126/scisignal.aav3249 (2019).
176 Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. & Hemmings, B. A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-789, doi:10.1038/378785a0 (1995).
177 Sengupta, S., Peterson, T. R., Laplante, M., Oh, S. & Sabatini, D. M. mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. *Nature* **468**, 1100-1104, doi:10.1038/nature09584 (2010).
178 Komatsu, M. *et al.* Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. *J Cell Biol* **169**, 425, doi:10.1083/jcb.200412022 (2005).
179 Peng, M., Yin, N. & Li, M. O. Sestrins function as guanine nucleotide dissociation inhibitors for Rag GTPases to control mTORC1 signaling. *Cell* **159**, 122-133, doi:10.1016/j.cell.2014.08.038 (2014).
180 Kuma, A. *et al.* The role of autophagy during the early neonatal starvation period. *Nature* **432**, 1032-1036, doi:10.1038/nature03029 (2004).
181 Tontonoz, P., Hu, E. & Spiegelman, B. M. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* **79**, 1147-1156, doi:10.1016/0092-8674(94)90006-x (1994).
182 Arif, A. *et al.* EPRS is a critical mTORC1-S6K1 effector that influences adiposity in mice. *Nature* **542**, 357-361, doi:10.1038/nature21380 (2017).
183 Lee, P. L., Tang, Y., Li, H. & Guertin, D. A. Raptor/mTORC1 loss in adipocytes causes progressive lipodystrophy and fatty liver disease. *Mol Metab* **5**, 422-432, doi:10.1016/j.molmet.2016.04.001 (2016).
184 Polak, P. *et al.* Adipose-specific knockout of raptor results in lean mice with enhanced mitochondrial respiration. *Cell Metab* **8**, 399-410, doi:10.1016/j.cmet.2008.09.003 (2008).

185 Hagiwara, A. *et al.* Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. *Cell Metab* **15**, 725-738, doi:10.1016/j.cmet.2012.03.015 (2012).
186 Yuan, M., Pino, E., Wu, L., Kacergis, M. & Soukas, A. A. Identification of Akt-independent regulation of hepatic lipogenesis by mammalian target of rapamycin (mTOR) complex 2. *J Biol Chem* **287**, 29579-29588, doi:10.1074/jbc.M112.386854 (2012).
187 Puigserver, P. *et al.* Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature* **423**, 550-555, doi:10.1038/nature01667 (2003).
188 Howell, J. J. *et al.* Metformin Inhibits Hepatic mTORC1 Signaling via Dose-Dependent Mechanisms Involving AMPK and the TSC Complex. *Cell Metab* **25**, 463-471, doi:10.1016/j.cmet.2016.12.009 (2017).
189 Lipton, J. O. & Sahin, M. The neurology of mTOR. *Neuron* **84**, 275-291, doi:10.1016/j.neuron.2014.09.034 (2014).
190 Graber, T. E., McCamphill, P. K. & Sossin, W. S. A recollection of mTOR signaling in learning and memory. *Learn Mem* **20**, 518-530, doi:10.1101/lm.027664.112 (2013).
191 Cloetta, D. *et al.* Inactivation of mTORC1 in the developing brain causes microcephaly and affects gliogenesis. *J Neurosci* **33**, 7799-7810, doi:10.1523/jneurosci.3294-12.2013 (2013).
192 Thomanetz, V. *et al.* Ablation of the mTORC2 component rictor in brain or Purkinje cells affects size and neuron morphology. *J Cell Biol* **201**, 293-308, doi:10.1083/jcb.201205030 (2013).
193 Crino, P. B. mTOR signaling in epilepsy: insights from malformations of cortical development. *Cold Spring Harb Perspect Med* **5**, a022442, doi:10.1101/cshperspect.a022442 (2015).
194 Pilarski, R. *et al.* Cowden syndrome and the PTEN hamartoma tumor syndrome: systematic review and revised diagnostic criteria. *J Natl Cancer Inst* **105**, 1607-1616, doi:10.1093/jnci/djt277 (2013).
195 Puffenberger, E. G. *et al.* Polyhydramnios, megalencephaly and symptomatic epilepsy caused by a homozygous 7-kilobase deletion in LYK5. *Brain* **130**, 1929-1941, doi:10.1093/brain/awm100 (2007).
196 Dibbens, L. M. *et al.* Mutations in DEPDC5 cause familial focal epilepsy with variable foci. *Nat Genet* **45**, 546, doi:10.1038/ng.2599 (2013).
197 Weckhuysen, S. *et al.* Involvement of GATOR complex genes in familial focal epilepsies and focal cortical dysplasia. *Epilepsia* **57**, 994-1003, doi:10.1111/epi.13391 (2016).
198 Yuskaitis, C. J. *et al.* A mouse model of DEPDC5-related epilepsy: Neuronal loss of Depdc5 causes dysplastic and ectopic neurons, increased mTOR signaling, and seizure susceptibility. *Neurobiol Dis* **111**, 91-101, doi:10.1016/j.nbd.2017.12.010 (2018).
199 Basel-Vanagaite, L. *et al.* Biallelic SZT2 mutations cause infantile encephalopathy with epilepsy and dysmorphic corpus callosum. *Am J Hum Genet* **93**, 524-529, doi:10.1016/j.ajhg.2013.07.005 (2013).
200 Nakamura, Y. *et al.* Constitutive activation of mTORC1 signaling induced by biallelic loss-of-function mutations in SZT2 underlies a discernible neurodevelopmental disease. *PLOS ONE* **14**, e0221482, doi:10.1371/journal.pone.0221482 (2019).
201 Reijnders, M. R. F. *et al.* Variation in a range of mTOR-related genes associates with intracranial volume and intellectual disability. *Nature Commun* **8**, 1052-1052, doi:10.1038/s41467-017-00933-6 (2017).
202 Allen, A. S. *et al.* De novo mutations in epileptic encephalopathies. *Nature* **501**, 217-221, doi:10.1038/nature12439 (2013).

203 D'Gama, A. M. *et al.* Somatic mutations activating the mTOR pathway in dorsal telencephalic progenitors cause a continuum of cortical dysplasias. *Cell Rep* **21**, 3754-3766, doi:10.1016/j.celrep.2017.11.106 (2017).

204 Gallent, E. A. & Steward, O. Neuronal PTEN deletion in adult cortical neurons triggers progressive growth of cell bodies, dendrites, and axons. *Exp Neurol* **303**, 12-28, doi:10.1016/j.expneurol.2018.01.005 (2018).

205 Abs, E. *et al.* TORC1-dependent epilepsy caused by acute biallelic Tsc1 deletion in adult mice. *Ann Neurol* **74**, 569-579, doi:10.1002/ana.23943 (2013).

206 McDaniel, S. S., Rensing, N. R., Thio, L. L., Yamada, K. A. & Wong, M. The ketogenic diet inhibits the mammalian target of rapamycin (mTOR) pathway. *Epilepsia* **52**, e7-e11, doi:10.1111/j.1528-1167.2011.02981.x (2011).

207 US National Library of Medicine. *ClinicalTrials.gov*, https://ClinicalTrials.gov/show/NCT01713946 (2017).

208 Brandt, C. *et al.* The novel, catalytic mTORC1/2 inhibitor PQR620 and the PI3K/mTORC1/2 inhibitor PQR530 effectively cross the blood-brain barrier and increase seizure threshold in a mouse model of chronic epilepsy. *Neuropharmacology* **140**, 107-120, doi:10.1016/j.neuropharm.2018.08.002 (2018).

209 Hsu, W.-L. *et al.* Glutamate stimulates local protein synthesis in the axons of rat cortical neurons by activating α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) receptors and metabotropic glutamate receptors. *J Biol Chem* **290**, 20748-20760 (2015).

210 Park, K. K. *et al.* Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science* **322**, 963, doi:10.1126/science.1161566 (2008).

211 Autry, A. E. *et al.* NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. *Nature* **475**, 91-95, doi:10.1038/nature10130 (2011).

212 Takei, N. *et al.* Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. *J Neurosci* **24**, 9760-9769, doi:10.1523/jneurosci.1427-04.2004 (2004).

213 Henry, F. E., Hockeimer, W., Chen, A., Mysore, S. P. & Sutton, M. A. Mechanistic target of rapamycin is necessary for changes in dendritic spine morphology associated with long-term potentiation. *Mol Brain* **10**, 50-50, doi:10.1186/s13041-017-0330-y (2017).

214 Tsai, P. T. *et al.* Autistic-like behaviour and cerebellar dysfunction in Purkinje cell Tsc1 mutant mice. *Nature* **488**, 647-651, doi:10.1038/nature11310 (2012).

215 Gkogkas, C. G. *et al.* Autism-related deficits via dysregulated eIF4E-dependent translational control. *Nature* **493**, 371-377, doi:10.1038/nature11628 (2013).

216 Li, N. *et al.* mTOR-Dependent Synapse Formation Underlies the Rapid Antidepressant Effects of NMDA Antagonists. *Science* **329**, 959, doi:10.1126/science.1190287 (2010).

217 Kato, T. *et al.* Sestrin modulator NV-5138 produces rapid antidepressant effects via direct mTORC1 activation. *J Clin Invest* **129**, 2542-2554, doi:10.1172/JCI126859 (2019).

218 Nikoletopoulou, V., Sidiropoulou, K., Kallergi, E., Dalezios, Y. & Tavernarakis, N. Modulation of Autophagy by BDNF Underlies Synaptic Plasticity. *Cell Metab* **26**, 230-242.e235, doi:10.1016/j.cmet.2017.06.005 (2017).

219 Tang, G. *et al.* Loss of mTOR-dependent macroautophagy causes autistic-like synaptic pruning deficits. *Neuron* **83**, 1131-1143, doi:10.1016/j.neuron.2014.07.040 (2014).

220 Menon, S. & Manning, B. D. Common corruption of the mTOR signaling network in human tumors. *Oncogene* **27 Suppl 2**, S43-51, doi:10.1038/onc.2009.352 (2008).

221 Okosun, J. *et al.* Recurrent mTORC1-activating RRAGC mutations in follicular lymphoma. *Nat Genet* **48**, 183-188, doi:10.1038/ng.3473 (2016).

222 Nickerson, M. L. *et al.* Mutations in a novel gene lead to kidney tumors, lung wall defects, and benign tumors of the hair follicle in patients with the Birt-Hogg-Dube syndrome. *Cancer Cell* **2**, 157-164 (2002).
223 Wagle, N. *et al.* Activating mTOR mutations in a patient with an extraordinary response on a phase I trial of everolimus and pazopanib. *Cancer Discov* **4**, 546-553, doi:10.1158/2159-8290.Cd-13-0353 (2014).
224 Tabernero, J. *et al.* Dose- and schedule-dependent inhibition of the mammalian target of rapamycin pathway with everolimus: a phase I tumor pharmacodynamic study in patients with advanced solid tumors. *J Clin Oncol* **26**, 1603-1610, doi:10.1200/jco.2007.14.5482 (2008).
225 Palm, W. *et al.* The utilization of extracellular proteins as nutrients is suppressed by mTORC1. *Cell* **162**, 259-270, doi:10.1016/j.cell.2015.06.017 (2015).
226 Feldman, M. E. *et al.* Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. *PLOS Biol* **7**, e1000038, doi:10.1371/journal.pbio.1000038 (2009).
227 Garcia-Martinez, J. M. *et al.* Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). *Biochem J* **421**, 29-42, doi:10.1042/bj20090489 (2009).
228 Ghobrial, I. M. *et al.* TAK-228 (formerly MLN0128), an investigational oral dual TORC1/2 inhibitor: A phase I dose escalation study in patients with relapsed or refractory multiple myeloma, non-Hodgkin lymphoma, or Waldenström's macroglobulinemia. *Am J Hematol* **91**, 400-405, doi:10.1002/ajh.24300 (2016).
229 Rodrik-Outmezguine, V. S. *et al.* mTOR kinase inhibition causes feedback-dependent biphasic regulation of AKT signaling. *Cancer Discov* **1**, 248-259, doi:10.1158/2159-8290.CD-11-0085 (2011).
230 Rodrik-Outmezguine, V. S. *et al.* Overcoming mTOR resistance mutations with a new-generation mTOR inhibitor. *Nature* **534**, 272-276, doi:10.1038/nature17963 (2016).
231 Fan, Q. *et al.* A kinase inhibitor targeted to mTORC1 drives regression in glioblastoma. *Cancer Cell* **31**, 424-435, doi:10.1016/j.ccell.2017.01.014 (2017).
232 Kaeberlein, M. *et al.* Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* **310**, 1193-1196, doi:10.1126/science.1115535 (2005).
233 Vellai, T. *et al.* Genetics: influence of TOR kinase on lifespan in C. elegans. *Nature* **426**, 620, doi:10.1038/426620a (2003).
234 Jia, K., Chen, D. & Riddle, D. L. The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. *Development* **131**, 3897-3906, doi:10.1242/dev.01255 (2004).
235 Kapahi, P. *et al.* Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. *Curr Biol* **14**, 885-890, doi:10.1016/j.cub.2004.03.059 (2004).
236 Wu, J. J. *et al.* Increased mammalian lifespan and a segmental and tissue-specific slowing of aging after genetic reduction of mTOR expression. *Cell Rep* **4**, 913-920, doi:10.1016/j.celrep.2013.07.030 (2013).
237 Harrison, D. E. *et al.* Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature* **460**, 392-395, doi:10.1038/nature08221 (2009).
238 Powers, R. W., 3rd, Kaeberlein, M., Caldwell, S. D., Kennedy, B. K. & Fields, S. Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes Dev* **20**, 174-184, doi:10.1101/gad.1381406 (2006).
239 Robida-Stubbs, S. *et al.* TOR signaling and rapamycin influence longevity by regulating SKN-1/Nrf and DAF-16/FoxO. *Cell Metab* **15**, 713-724, doi:10.1016/j.cmet.2012.04.007 (2012).

240 Bjedov, I. *et al.* Mechanisms of life span extension by rapamycin in the fruit fly Drosophila melanogaster. *Cell Metab* **11**, 35-46, doi:10.1016/j.cmet.2009.11.010 (2010).
241 Bitto, A. *et al.* Transient rapamycin treatment can increase lifespan and healthspan in middle-aged mice. *Elife* **5**, doi:10.7554/eLife.16351 (2016).
242 Hansen, M. *et al.* Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans. *Aging Cell* **6**, 95-110, doi:10.1111/j.1474-9726.2006.00267.x (2007).
243 Grandison, R. C., Piper, M. D. W. & Partridge, L. Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. *Nature* **462**, 1061-1064, doi:10.1038/nature08619 (2009).
244 Selman, C. *et al.* Ribosomal protein S6 kinase 1 signaling regulates mammalian life span. *Science* **326**, 140-144, doi:10.1126/science.1177221 (2009).
245 Hansen, M., Rubinsztein, D. C. & Walker, D. W. Autophagy as a promoter of longevity: insights from model organisms. *Nat Rev Mol Cell Biol* **19**, 579-593, doi:10.1038/s41580-018-0033-y (2018).
246 Fernandez, A. F. *et al.* Disruption of the beclin 1-BCL2 autophagy regulatory complex promotes longevity in mice. *Nature* **558**, 136-140, doi:10.1038/s41586-018-0162-7 (2018).
247 Toth, M. L. *et al.* Longevity pathways converge on autophagy genes to regulate life span in Caenorhabditis elegans. *Autophagy* **4**, 330-338, doi:10.4161/auto.5618 (2008).
248 Hansen, M. *et al.* A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. *PLOS Genet* **4**, e24, doi:10.1371/journal.pgen.0040024 (2008).
249 Chen, C., Liu, Y., Liu, Y. & Zheng, P. mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells. *Sci Signal* **2**, ra75, doi:10.1126/scisignal.2000559 (2009).
250 Yilmaz, O. H. *et al.* mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. *Nature* **486**, 490-495, doi:10.1038/nature11163 (2012).
251 Carroll, B. *et al.* Persistent mTORC1 signaling in cell senescence results from defects in amino acid and growth factor sensing. *J Cell Biol* **216**, 1949, doi:10.1083/jcb.201610113 (2017).
252 Laberge, R. M. *et al.* MTOR regulates the pro-tumorigenic senescence-associated secretory phenotype by promoting IL1A translation. *Nat Cell Biol* **17**, 1049-1061, doi:10.1038/ncb3195 (2015).
253 Herranz, N. *et al.* mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype. *Nat Cell Biol* **17**, 1205-1217, doi:10.1038/ncb3225 (2015).
254 Arriola Apelo, S. I. *et al.* Alternative rapamycin treatment regimens mitigate the impact of rapamycin on glucose homeostasis and the immune system. *Aging Cell* **15**, 28-38, doi:10.1111/acel.12405 (2016).
255 Arriola Apelo, S. I., Pumper, C. P., Baar, E. L., Cummings, N. E. & Lamming, D. W. Intermittent administration of rapamycin extends the life span of female C57BL/6J mice. *J Gerontol A Biol Sci Med Sci* **71**, 876-881, doi:10.1093/gerona/glw064 (2016).
256 Mannick, J. B. *et al.* TORC1 inhibition enhances immune function and reduces infections in the elderly. *Sci Transl Med* **10**, eaaq1564, doi:10.1126/scitranslmed.aaq1564 (2018).
257 Lim, C.-Y. *et al.* ER–lysosome contacts enable cholesterol sensing by mTORC1 and drive aberrant growth signalling in Niemann–Pick type C. *Nat Cell Biol* **21**, 1206-1218, doi:10.1038/s41556-019-0391-5 (2019).

258 Hao, F. *et al.* Rheb localized on the Golgi membrane activates lysosome-localized mTORC1 at the Golgi-lysosome contact site. *J Cell Sci* **131**, doi:10.1242/jcs.208017 (2018).
259 Korolchuk, V. I. *et al.* Lysosomal positioning coordinates cellular nutrient responses. *Nat Cell Biol* **13**, 453-460, doi:10.1038/ncb2204 (2011).
260 Manifava, M. *et al.* Dynamics of mTORC1 activation in response to amino acids. *Elife* **5**, e19960, doi:10.7554/eLife.19960 (2016).
261 Ahmed, A. R. *et al.* Direct imaging of the recruitment and phosphorylation of S6K1 in the mTORC1 pathway in living cells. *Sci Rep* **9**, 3408, doi:10.1038/s41598-019-39410-z (2019).
262 Naito, T., Kuma, A. & Mizushima, N. Differential contribution of insulin and amino acids to the mTORC1-autophagy pathway in the liver and muscle. *J Biol Chem* **288**, 21074-21081, doi:10.1074/jbc.M113.456228 (2013).
263 Frey, J. W., Jacobs, B. L., Goodman, C. A. & Hornberger, T. A. A role for Raptor phosphorylation in the mechanical activation of mTOR signaling. *Cell Signal* **26**, 313-322, doi:10.1016/j.cellsig.2013.11.009 (2014).
264 You, J. S. *et al.* The role of raptor in the mechanical load-induced regulation of mTOR signaling, protein synthesis, and skeletal muscle hypertrophy. *FASEB J* **33**, 4021-4034, doi:10.1096/fj.201801653RR (2019).
265 Jacobs, B. L. *et al.* Eccentric contractions increase the phosphorylation of tuberous sclerosis complex-2 (TSC2) and alter the targeting of TSC2 and the mechanistic target of rapamycin to the lysosome. *J Physiol* **591**, 4611-4620, doi:10.1113/jphysiol.2013.256339 (2013).
266 Wang, A. *et al.* Activity-independent targeting of mTOR to lysosomes in primary osteoclasts. *Sci Rep* **7**, 3005, doi:10.1038/s41598-017-03494-2 (2017).
267 Stracka, D., Jozefczuk, S., Rudroff, F., Sauer, U. & Hall, M. N. Nitrogen source activates TOR (target of rapamycin) complex 1 via glutamine and independently of Gtr/Rag proteins. *J Biol Chem* **289**, 25010-25020, doi:10.1074/jbc.M114.574335 (2014).
268 Urano, J., Tabancay, A. P., Yang, W. & Tamanoi, F. The Saccharomyces cerevisiae Rheb G-protein is involved in regulating canavanine resistance and arginine uptake. *J Biol Chem* **275**, 11198-11206, doi:10.1074/jbc.275.15.11198 (2000).
269 Otsubo, Y. & Yamamato, M. TOR signaling in fission yeast. *Crit Rev Biochem Mol Biol* **43**, 277-283, doi:10.1080/10409230802254911 (2008).
270 Wolfson, R. L. & Sabatini, D. M. The dawn of the age of amino acid sensors for the mTORC1 pathway. *Cell Metab* **26**, 301-309, doi:10.1016/j.cmet.2017.07.001 (2017).
271 Piyankarage, S. C., Augustin, H., Grosjean, Y., Featherstone, D. E. & Shippy, S. A. Hemolymph amino acid analysis of individual Drosophila larvae. *Anal Chem* **80**, 1201-1207, doi:10.1021/ac701785z (2008).
272 Algret, R. *et al.* Molecular architecture and function of the SEA complex, a modulator of the TORC1 pathway. *Mol Cell Proteom* **13**, 2855, doi:10.1074/mcp.M114.039388 (2014).
273 Nixon, R. A. The role of autophagy in neurodegenerative disease. *Nat Med* **19**, 983, doi:10.1038/nm.3232 (2013).
274 Nixon, R. A. *et al.* Extensive involvement of autophagy in Alzheimer disease: an immuno-electron microscopy study. *J Neuropathol Exp Neurol* **64**, 113-122, doi:10.1093/jnen/64.2.113 (2005).
275 Tanik, S. A., Schultheiss, C. E., Volpicelli-Daley, L. A., Brunden, K. R. & Lee, V. M. Y. Lewy body-like α-Synuclein aggregates resist degradation and impair macroautophagy. *J Biol Chem* **288**, 15194-15210, doi:10.1074/jbc.M113.457408 (2013).

276 Komatsu, M. *et al.* Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature* **441**, 880-884, doi:10.1038/nature04723 (2006).
277 Hara, T. *et al.* Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature* **441**, 885-889, doi:10.1038/nature04724 (2006).
278 Kaeberlein, M. & Galvan, V. Rapamycin and Alzheimer's disease: time for a clinical trial? *Sci Transl Med* **11**, eaar4289, doi:10.1126/scitranslmed.aar4289 (2019).
279 Dehay, B. *et al.* Pathogenic lysosomal depletion in Parkinson's disease. *J Neurosci* **30**, 12535, doi:10.1523/JNEUROSCI.1920-10.2010 (2010).
280 Santini, E., Heiman, M., Greengard, P., Valjent, E. & Fisone, G. Inhibition of mTOR signaling in Parkinson's disease prevents L-DOPA–induced dyskinesia. *Sci Signal* **2**, ra36, doi:10.1126/scisignal.2000308 (2009).
281 Huang, W. *et al.* mTORC2 controls actin polymerization required for consolidation of long-term memory. *Nat Neurosci* **16**, 441, doi:10.1038/nn.3351 (2013).
282 Castellano, B. M. *et al.* Lysosomal cholesterol activates mTORC1 via an SLC38A9-Niemann-Pick C1 signaling complex. *Science* **355**, 1306-1311, doi:10.1126/science.aag1417 (2017).

Acknowledgements

We thank all the members of the Sabatini Lab for helpful discussions, with particular gratitude to K.J.C. and J.M.O for their insightful comments on this manuscript and K.S. for his assistance with Figure 1. This work was supported by grants from the NIH (R01 CA103866, R01 CA129105, and R37 AI047389) and the Lustgarten Foundation to D.M.S and fellowship funding from the NIH (T32 GM007287 and F31 CA232340) to G.Y.L. D.M.S. is an Investigator of the Howard Hughes Medical Institute and an American Cancer Society Research Professor.

Author contributions

G.Y.L. researched the data, discussed content and wrote the first full draft of the manuscript; D.M.S discussed article content and was involved in editing and revising the manuscript.

Competing interests

D.M.S. is a founder and a member of the scientific advisory board for Navitor Pharmaceuticals, which targets the mTORC1 pathway for therapeutic benefit.

Publisher's note

Peer review information

Nature Reviews Molecular Cell Biology thanks the reviewers for their contribution to the peer review of this work.

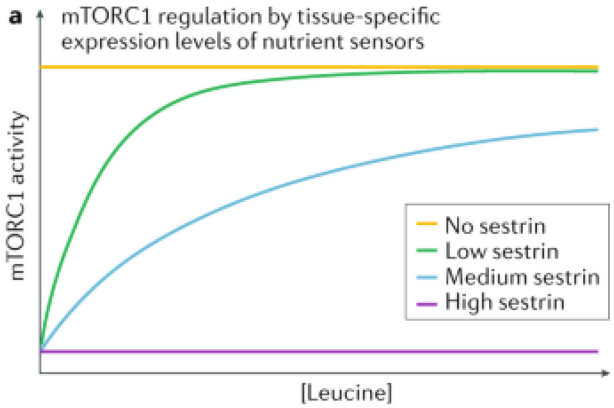
Preface

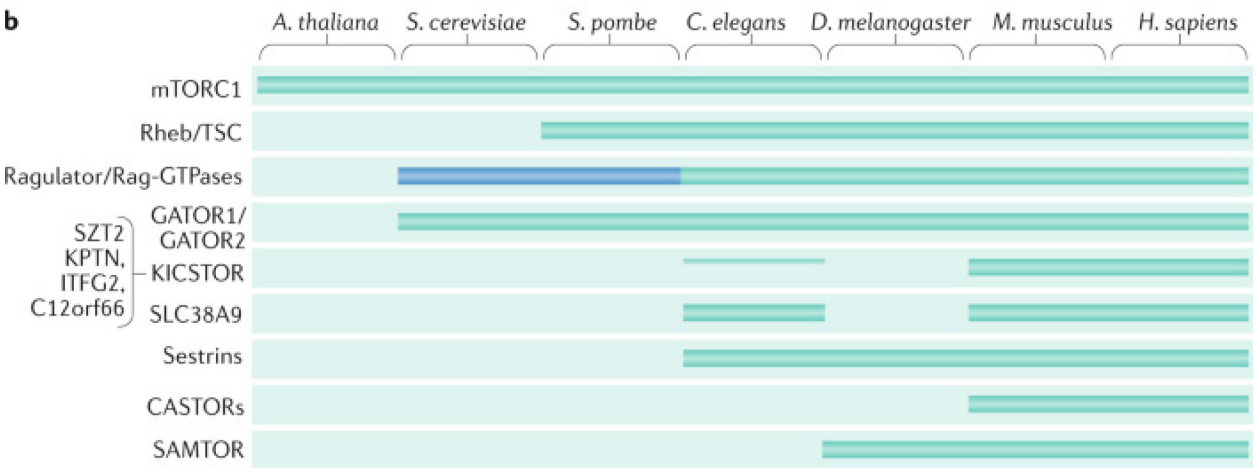
Parts of this chapter were first published as:

Liu, G.Y. and Sabatini, D.M. mTOR at the nexus of nutrition, growth, ageing, and disease. (2020). *Nat Rev Mol Cell Biol.* 21(4):183-203.

Cell- and organism-specific regulatory mechanisms across evolution in mTORC1 signaling

In order to align mTORC1 activity with tissue function, some specialized human cells may respond to unique inputs, adjust the weighting of upstream signals[1], or regulate mTORC1 through noncanonical mechanisms. In muscle cells, mechanical stimuli have been shown to activate mTORC1[2-4], whereas in primary osteoclasts, amino acid deprivation can abrogate mTORC1 signaling without dissociating the complex from the lysosome[5]. We postulate that specialized cells can also adapt to their niches by tuning expression of nutrient sensors. For example, in tissues where physiologically relevant leucine concentrations are relatively high, cells might selectively increase expression of Sestrin2 to raise the leucine threshold for mTORC1 activation. Conversely, cells that are protected from leucine fluctuations might abolish Sestrin2 expression altogether to render mTORC1 insensitive to leucine deprivation. Thus, differential expression of sestrin could modulate mTORC1 sensitivity to leucine levels in a tissue-specific manner (see the figure, part a).





Although the core components of the nutrient sensing machinery—the Rag GTPases, Ragulator, and the GATORs—are conserved in metazoans*, some of the direct amino acid sensors are absent in non-vertebrate lineages (the conservation of mTORC1 pathway components in common model organisms is shown in the figure, part b). Based on sequence homology, *Drosophila melanogaster* retains Sestrin and SAMTOR but lacks both the lysosomal and cytosolic arginine sensors; meanwhile, *C. elegans* possesses SLC38A9 and Sestrin homologs but does not have a clear SAMTOR equivalent. The irregular pattern of conservation of the sensors may be linked to the distinct nutritional needs of each organism. In support of this idea, computational searches suggest that *S. cerevisiae*, a model organism capable of synthesizing all twenty amino acids *de novo*, does not have any amino acid sensors and consequently does not require any individual amino acid for TORC1 activation. Instead, the *S. cerevisiae* TORC1 pathway may respond to the general availability of nitrogen and carbon sources[6]. Puzzlingly, *S. cerevisiae* also does not seem to require a Rheb homolog to activate TORC1[7], suggesting that its molecular circuitry may diverge sharply from that of other model organisms, including *S. pombe*[8]. The green box (see the figure, part b) indicates that the EGO complex in yeast shares little sequence homology with Ragulator, although it appears to serve an analogous function.

Even nutrient sensors with recognizable homology may display functional differences in divergent species. Binding assays with radioactive leucine reveal that the *D. melanogater* homolog of Sestrin (dSesn) has five-fold lower affinity for leucine than the human protein[9,10]. We speculate that this molecular difference may allow dSesn to sense physiological leucine fluctuations in the *D. melanogaster* hemolymph, which has about a five- to ten-fold higher amino acid concentration than human plasma[10,11]. Taken together with conservation patterns, these data also suggest an attractive hypothesis: perhaps organisms evolved or retained specific nutrient sensors to enable the TORC1 pathway to respond to limiting nutrients in their metabolic niches. However, because no unique sensors have yet been identified in non-human systems and the evolutionary lineage of the sensors is not well understood, it is difficult to draw correlations between evolutionary pressures and the functional architecture of the TORC1 nutrient sensing pathway. The discovery of novel nutrient sensors outside of higher eukaryotes would clarify the evolutionary logic of the nutrient sensing axis and define new inputs into the TORC1 pathway. Moreover, sensors initially characterized in other species could be conserved in human cell types with specialized metabolic environments.

*For reasons that remain unclear, the KICSTOR complex is the sole exception to this generalization. KPTN, ITFG2, and C12orf66 seem to drop out of the evolutionary tree in organisms more distal than mammals; SZT2, the largest component of the complex, may have a putative homolog in *C. elegans* but is not retained in flies or yeast. If KICSTOR serves as a molecular glue that holds human GATOR1 and GATOR2 together in a supercomplex, as one study has argued[12], it is possible it is dispensable in lower organisms where GATOR1/2 are more constitutively bound to each other. Consistent with this hypothesis, the *S. cerevisiae* homologs for the GATORs, the SEACIT and SEACAT complexes, are indeed more tightly

associated than their human counterparts and have been reported to form a supercomplex without any mediating proteins[13].

References

1 Naito, T., Kuma, A. & Mizushima, N. Differential contribution of insulin and amino acids to the mTORC1-autophagy pathway in the liver and muscle. *J Biol Chem* **288**, 21074-21081, doi:10.1074/jbc.M113.456228 (2013).

2 Frey, J. W., Jacobs, B. L., Goodman, C. A. & Hornberger, T. A. A role for Raptor phosphorylation in the mechanical activation of mTOR signaling. *Cell Signal* **26**, 313-322, doi:10.1016/j.cellsig.2013.11.009 (2014).

3 You, J. S. *et al.* The role of raptor in the mechanical load-induced regulation of mTOR signaling, protein synthesis, and skeletal muscle hypertrophy. *FASEB J* **33**, 4021-4034, doi:10.1096/fj.201801653RR (2019).

4 Jacobs, B. L. *et al.* Eccentric contractions increase the phosphorylation of tuberous sclerosis complex-2 (TSC2) and alter the targeting of TSC2 and the mechanistic target of rapamycin to the lysosome. *J Physiol* **591**, 4611-4620, doi:10.1113/jphysiol.2013.256339 (2013).

5 Wang, A. *et al.* Activity-independent targeting of mTOR to lysosomes in primary osteoclasts. *Sci Rep* **7**, 3005, doi:10.1038/s41598-017-03494-2 (2017).

6 Stracka, D., Jozefczuk, S., Rudroff, F., Sauer, U. & Hall, M. N. Nitrogen source activates TOR (target of rapamycin) complex 1 via glutamine and independently of Gtr/Rag proteins. *J Biol Chem* **289**, 25010-25020, doi:10.1074/jbc.M114.574335 (2014).

7 Urano, J., Tabancay, A. P., Yang, W. & Tamanoi, F. The Saccharomyces cerevisiae Rheb G-protein is involved in regulating canavanine resistance and arginine uptake. *J Biol Chem* **275**, 11198-11206, doi:10.1074/jbc.275.15.11198 (2000).

8 Otsubo, Y. & Yamamato, M. TOR signaling in fission yeast. *Crit Rev Biochem Mol Biol* **43**, 277-283, doi:10.1080/10409230802254911 (2008).

9 Wolfson, R. L. *et al.* Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science* **351**, 43-48, doi:10.1126/science.aab2674 (2016).

10 Wolfson, R. L. & Sabatini, D. M. The dawn of the age of amino acid sensors for the mTORC1 pathway. *Cell Metab* **26**, 301-309, doi:10.1016/j.cmet.2017.07.001 (2017).

11 Piyankarage, S. C., Augustin, H., Grosjean, Y., Featherstone, D. E. & Shippy, S. A. Hemolymph amino acid analysis of individual Drosophila larvae. *Anal Chem* **80**, 1201-1207, doi:10.1021/ac701785z (2008).

12 Peng, M., Yin, N. & Li, M. O. SZT2 dictates GATOR control of mTORC1 signalling. *Nature* **543**, 433-437, doi:10.1038/nature21378 (2017).

13 Algret, R. *et al.* Molecular architecture and function of the SEA complex, a modulator of the TORC1 pathway. *Mol Cell Proteom* **13**, 2855, doi:10.1074/mcp.M114.039388 (2014).

Chapter 2

An evolutionary mechanism to assimilate new nutrient sensors into the mTORC1 pathway

Authors: Grace Y. Liu[1,2,3]*, Patrick Jouandin[4,5], Raymond E. Bahng[1,2,3], Norbert Perrimon[4,5]*, David M. Sabatini

Affiliations:

[1] Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology, Department of Biology; 455 Main Street, Cambridge, Massachusetts 02142, USA.

[2] Department of Biology, Massachusetts Institute of Technology; 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA.

[3] Koch Institute for Integrative Cancer Research and Massachusetts Institute of Technology, Department of Biology; 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA.

[4] Department of Genetics, Blavatnik Institute, Harvard Medical School; Boston, MA 02115, USA.

[5]Howard Hughes Medical Institute, Harvard Medical School; Boston, MA 02115, USA.

*Corresponding author. Email: gyliu@mit.edu, perrimon@genetics.med.harvard.edu

Experiments in Figure 1A and 1B were performed by G.Y.L.

Experiments in Figure 2A-F were performed by G.Y.L. with assistance by R.E.B. on 2C.

Experiment in Figure 3 was performed by G.Y.L.

Experiments in Figure 4A-D were performed by G.Y.L.

Experiments in Figure 5A-F were performed by G.Y.L.

Experiments in Figure 6A-E were performed by G.Y.L.

Experiment in Figure 6F was performed by P.J.

Experiments in Figure 7A-F were performed by G.Y.L.

Experiments in Figure 8A-H were performed by G.Y.L. with assistance from P.J. on 8A.

Experiments in Figure 9A-E were performed by G.Y.L.

Experiments in Figure 10A-C were performed by G.Y.L.

Experiments in Figure 11A-C were performed by G.Y.L.

Experiment in Figure 12 was performed by G.Y.L.

Experiments in Figure 13A-C were performed by G.Y.L.

Abstract

Animals must sense and respond to nutrient availability in their local niche. This task is coordinated in part by the mTOR complex 1 (mTORC1) pathway, which regulates growth and metabolism in response to nutrients. In mammals, mTORC1 senses specific amino acids through specialized sensors that act through the upstream GATOR1/2 signaling hub. To reconcile the conserved architecture of the mTORC1 pathway with the diversity of environments that animals can occupy, we hypothesized that the pathway maintains plasticity by evolving distinct nutrient sensors in different metazoan phyla. How this customization occurs—and how the mTORC1 pathway captures new nutrient inputs—is not known. Here, we identify the *Drosophila melanogaster* protein Unmet expectations (Unmet, formerly CG11596) as a species-specific nutrient sensor and trace its incorporation into the mTORC1 pathway. Unmet interacts with the fly GATOR2 complex to inhibit dTORC1 during methionine starvation. This inhibition is directly relieved by *S*-adenosylmethionine (SAM), a proxy for methionine availability. Unmet expression is elevated in the ovary, a methionine-sensitive niche, and flies lacking Unmet fail to maintain the integrity of the female germline under methionine restriction. Through the evolutionary history of the Unmet-GATOR2 interaction, we show that the GATOR2 complex evolved rapidly in Dipterans to recruit and repurpose an independent methyltransferase as a SAM sensor. These findings demonstrate that mTORC1 co-opts preexisting enzymes to expand its nutrient sensing capabilities and reveal a modular architecture that builds evolvability into a highly conserved metabolic pathway.

Introduction

Eukaryotic sensory systems detect environmental signals that confer advantages for survival and reproduction. These signals are often specialized, diverging to accommodate the biochemical and biophysical properties of each organism's niche. To capture new signals over the course of evolution, sensory systems must acquire novel receptors and link those receptors to the ancient pathways that actuate behavioral or metabolic changes. With few exceptions, the mechanisms that enable conserved signaling networks to rapidly evolve new inputs are poorly understood (Julius and Nathans, 2012; Nei and Rooney, 2005; Oteiza and Baldwin, 2021).

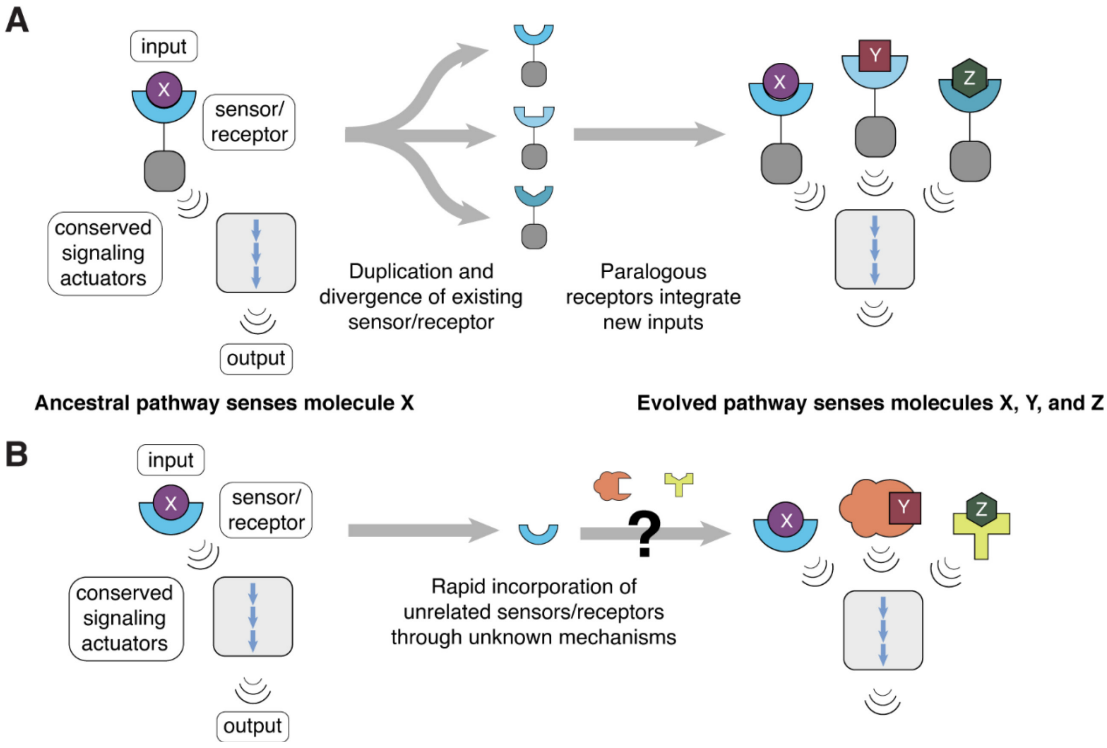


Figure 1: mTORC1 nutrient sensing is a model system for interrogating how conserved signaling pathways evolve sensory inputs through novel molecular partnerships.

(**A**) Classical sensory systems evolve new functional inputs by altering the ligand-binding capabilities of an existing sensor or receptor, often after duplication of the receptor. This evolutionary strategy gives rise to families of paralogous receptors that signal to conserved downstream actuators through shared domains.

(**B**) Some non-canonical sensory systems, such as the mTORC1 pathway, use sets of unrelated proteins as sensors/receptors. It is unclear whether these receptors evolved from nonsensory precursors, and it is not known how they forged new molecular interactions with conserved components of the pathway.

In some sensory systems, new features arise through duplication of existing receptors, followed by modification of the paralogs to increase promiscuity or alter substrate preference (Figure 1A). For example, successive expansion and mutation of certain receptor classes—including some hormone receptors, olfactory receptors, Toll-like receptors, and TRP ion channels—has driven the complexity of chemosensation in different species (Bridgham et al., 2006; Gracheva et al., 2010; Julius and Nathans, 2012; Leulier and Lemaitre, 2008; Slavik et al., 2021). However, although this strategy expands the ligand or activity space for receptors that are already connected to a pathway, it is a poor model for receptors that emerge through novel molecular partnerships (Figure 1B). A key question, therefore, is how functional diversification occurs in the absence of paralogous duplication. What evolutionary strategies are employed by signaling networks that evolve multiple *unrelated* receptors to integrate new inputs?

The mechanistic target of rapamycin complex 1 (mTORC1) pathway is a model for this latter type of network. The mTORC1 pathway surveys concentrations of amino acids and related metabolites to regulate growth and metabolism (Condon and Sabatini, 2019; Kim and Guan, 2019; Liu and Sabatini, 2020; Melick and Jewell, 2020; Valvezan and Manning, 2019). Upon activation by nutrients, mTORC1 allocates cellular resources toward anabolism by promoting protein and lipid biosynthesis and inhibiting autophagy. Because organisms have a wide range of lifestyles and diets, we postulate that the mTORC1 pathway is under pressure to evolve receptors for the most important nutrients within a given niche. In mammals, these receptors take the form of specialized “nutrient sensors”—Sestrin2, CASTOR1, and SAMTOR—that bind, respectively, to leucine, arginine, and the methionine-derived methyl donor *S*-adenosylmethionine (SAM) (Chantranupong et al., 2016; Gu et al., 2017; Wolfson et al., 2016). When cells are starved of nutrients, the mammalian nutrient sensors interact with several conserved protein complexes that relay signals to control mTORC1 kinase activity. These

complexes, which comprise the “core” nutrient sensing machinery of the mTORC1 pathway, include the large GATOR1 and GATOR2 complexes, as well as KICSTOR, a vertebrate-specific partner of GATOR1 (Bar-Peled et al., 2013; Peng et al., 2017; Wolfson et al., 2017). Replenishing nutrient levels allows the nutrient sensors to bind to their cognate metabolites, releasing the sensors from the core complexes and reactivating mTORC1 (Saxton et al., 2016a; Saxton et al., 2016b).

Although the general architecture of the mTORC1 pathway is conserved across eukaryotes, the pathway must remain sufficiently flexible to accommodate organisms with distinct nutritional needs. Unlike most of the core components of the mTORC1 pathway, which are present from yeast to humans, the mammalian nutrient sensors are only sporadically conserved in metazoans and completely absent from yeast (González and Hall, 2017; Wolfson and Sabatini, 2017). Genomic analyses reveal that *D. melanogaster* lacks homologs of the mammalian arginine sensors but retains genes for both a full Sestrin protein and a substantially truncated SAMTOR protein; by contrast, *C. elegans* possesses homologs of Sestrin and the lysosomal arginine sensor SLC38A9 while lacking a clear SAMTOR equivalent (Wang et al., 2015). Despite their similar modes of action, the known mammalian nutrient sensors bear no homology to each other. Based on these observations, we propose that nutrient sensors comprise a plastic regulatory layer atop the conserved core of the mTORC1 pathway machinery—one that can be customized to detect limiting nutrients in different metazoan phyla (Brunkard, 2020; Liu and Sabatini, 2020).

To understand whether and how the mTORC1 pathway acquires “custom” nutrient sensors, we searched for novel sensors in *Drosophila melanogaster*, an organism that shares many pathway components with humans but consumes a divergent diet. We discover a new species-specific SAM sensor and use its evolutionary history to pry open the structural logic of the nutrient-sensing axis. We show that this sensor, Unmet expectations, is an “evolutionary

intermediate," caught between its ancestral enzymatic function and a recently acquired role in the mTORC1 pathway. By comparing SAM sensing in different clades, we find that flies and vertebrates independently evolved unrelated, mechanistically distinct sensors that converge upon the same metabolite. Unexpectedly, our results shed light on the origins of the nutrient sensors and reveal remarkable features of GATOR2, a core signaling hub for the mTORC1 pathway, that allow the pathway to rapidly co-opt ligand-binding proteins and adapt to metabolic niches across evolution.

Unmet binds to fly GATOR2 in an *S*-adenosylmethionine (SAM)-regulated fashion

The GATOR complexes have emerged as central integrators of metabolic information for the mTORC1 pathway. To identify novel nutrient sensors, we searched for GATOR-binding partners in *Drosophila melanogaster*. We generated anti-FLAG immunoprecipitates from *D. melanogaster* Schneider 2 (S2R+) cells expressing FLAG-tagged Mio, a core component of the *Drosophila* GATOR2 (dGATOR2) complex. Mass spectrometry analyses revealed that beyond capturing other components of the dGATOR complexes and the leucine sensor dSestrin, these immunoprecipitates also contained the previously uncharacterized fly protein CG11596, which we have renamed Unmet expectations (Unmet) for reasons described below (Figure 2A). When transiently expressed in S2R+ cells, HA-tagged Unmet robustly co-immunoprecipitated endogenous dGATOR2, as detected via its dWDR59 component, as well as the dGATOR1 complex, as detected via its Iml1 component (Figure 2B). Because the dGATOR1 and dGATOR2 complexes appear to be more tightly associated in flies than in mammalian systems, these data are consistent with Unmet binding to either or both of the dGATOR complexes. To differentiate between those possibilities, we transiently co-expressed the dGATOR1 and dGATOR2 complexes with Unmet in human embryonic kidney 293T (HEK-293T) cells (Figure 2C). Like dSestrin, which has been characterized as a GATOR2-binding protein, Unmet co-immunoprecipitated dGATOR2, but not dGATOR1, in this reconstituted system. Unmet

therefore binds to dGATOR2 without requiring any additional *Drosophila*-specific factors.

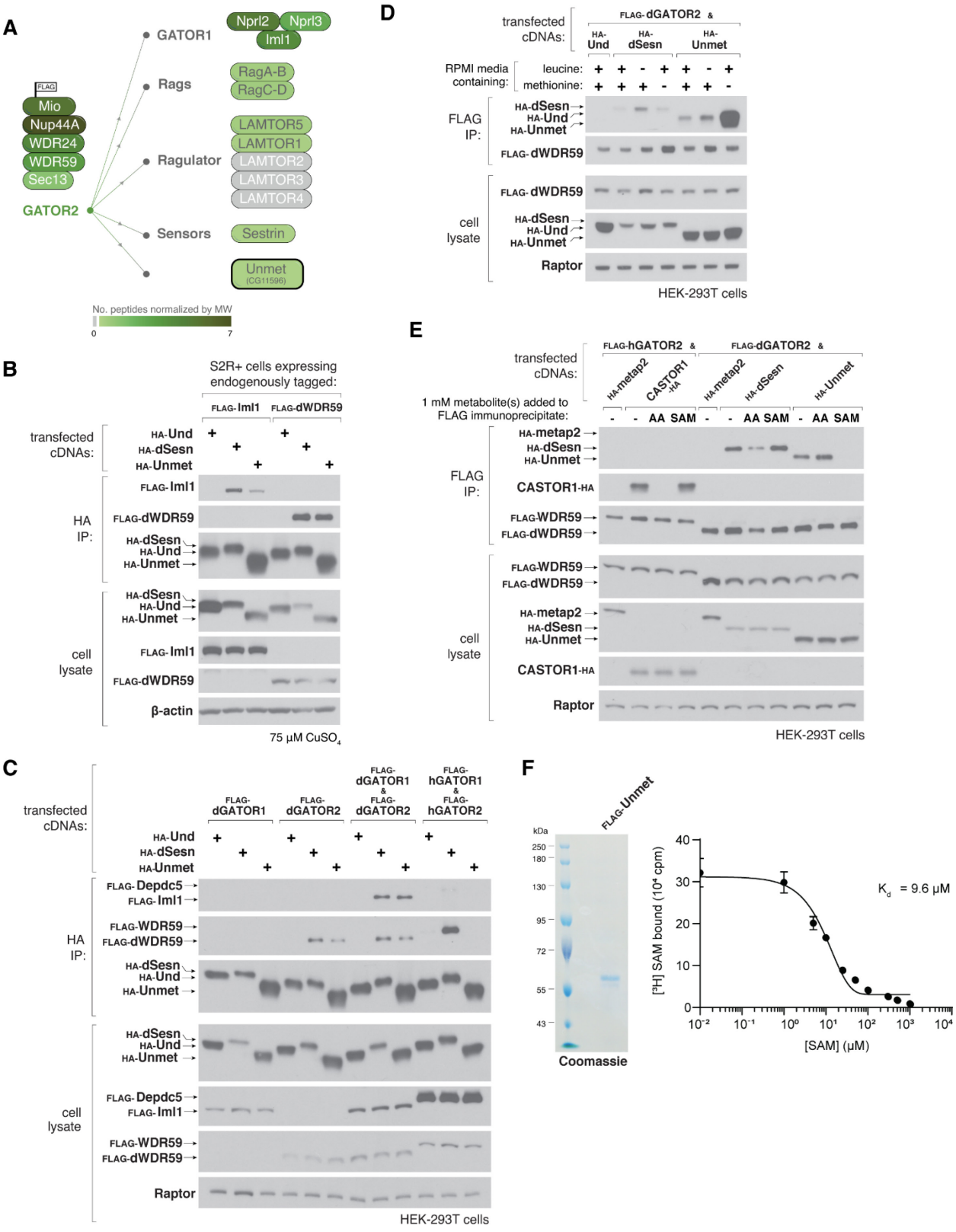


Figure 2: The interaction between the *D. melanogaster* protein Unmet expectations and the fly GATOR2 complex is regulated by SAM.

(**A**) Mass spectrometric analyses identify Unmet-derived peptides in immunoprecipitates from S2R+ cells expressing FLAG-tagged Mio, a component of the dGATOR2 complex. Unmet and known components of the mTORC1 pathway are colored by normalized peptide representation according to the scale below.

(**B**) Recombinant Unmet co-immunoprecipitates endogenous GATOR1 and GATOR2 components in S2R+ cells. Anti-HA immunoprecipitates were prepared from S2R+ cells bearing endogenous FLAG knock-in tags at either the Iml1 (dGATOR1) or the dWDR59 (dGATOR2) locus, transfected with the indicated cDNAs in copper-inducible metallothionein (MT) expression vectors. Following 48-hour induction with 75 μM $CuSO_4$, cell lysates and immunoprecipitates were analyzed by immunoblotting for levels of the relevant epitope tags. HA-Und served as a negative control.

(**C**) Recombinant Unmet interacts with dGATOR2, but not dGATOR1 or the corresponding human complexes. Anti-HA immunoprecipitates were collected from HEK-293T cells co-transfected with the indicated cDNAs in expression vectors and were analyzed alongside cell lysates as in (B).

(**D**) Deprivation of methionine, but not leucine, enhances the interaction between Unmet and dGATOR2. HEK-293T cells transiently expressing FLAG-tagged dGATOR2 and the indicated HA-tagged cDNAs were treated with full RPMI or RPMI lacking leucine or methionine for 1 hour. FLAG immunoprecipitates and cell lysates were analyzed by immunoblotting for levels of the relevant proteins.

(**e**) SAM, but not amino acids, disrupts the interaction between Unmet and dGATOR2 in vitro. FLAG immunoprecipitates were prepared from HEK-293T cells transfected with the indicated cDNAs. A mixture containing 1 mM of each amino acid or 1 mM of SAM was added directly to the immunoprecipitates. FLAG immunoprecipitates and cell lysates were analyzed as in (D).

(**F**) Unmet binds SAM with a K_d of 9.6 μM. Purified FLAG-Unmet protein was analyzed by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining. Binding assays were performed with 10 μg purified FLAG-Unmet incubated with 5 μM [^{3}H]SAM and the indicated concentrations of unlabeled SAM. Values for each point represent the means ± s.d. of three technical replicates from one representative experiment. Binding experiments were repeated three times.

The Unmet protein sequence possesses an N2227 domain, which defines homologs from yeast to human and may contain methyltransferase activity (Drozak et al., 2015). Indeed, recent work has shown that the human, rat, chicken, and *Saccharomyces cerevisiae* orthologs of Unmet are all capable of methylating the histidyl ring of the dipeptide L-carnosine to produce anserine, albeit at low catalytic efficiencies (Cao et al., 2018; Drozak et al., 2015). Despite strong sequence conservation at the putative small molecule binding sites (Figure 3), it is unknown whether Unmet retains this activity. Moreover, it is unclear whether such activity, even

if present, would be functionally relevant in flies, as carnosine and anserine are reported to be nearly absent from *Drosophila* tissues (Shiotani et al., 2013).

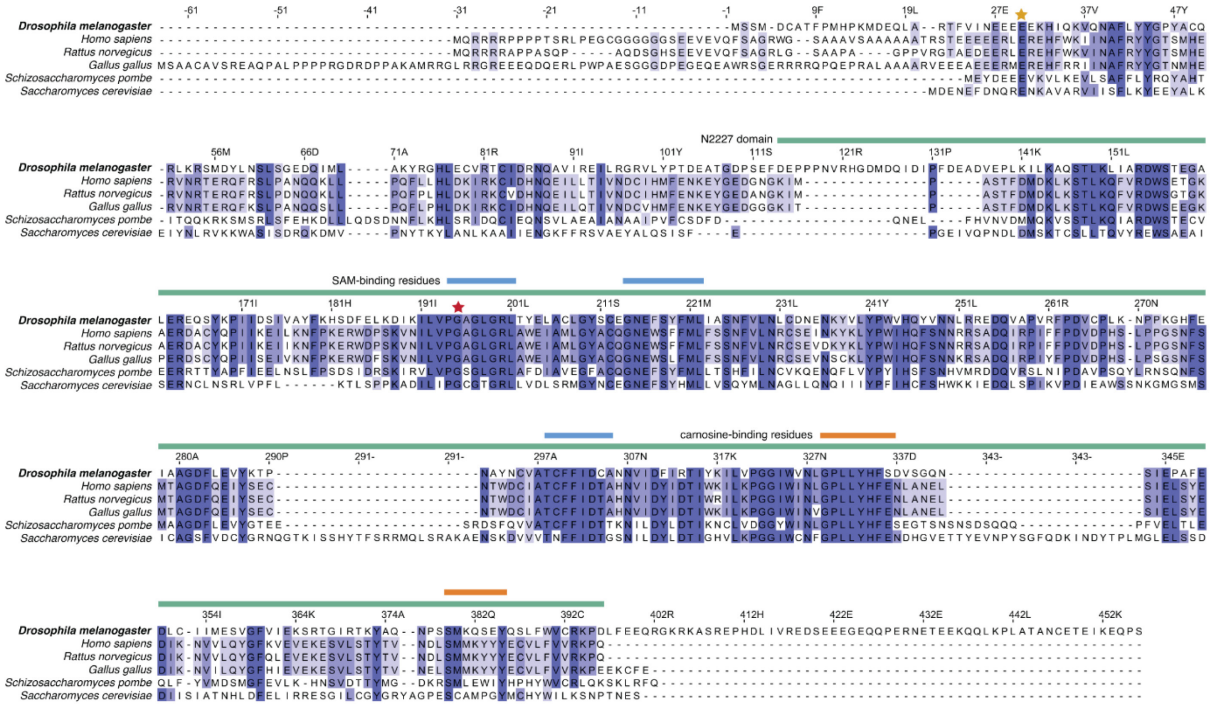


Figure 3: Multiple sequence alignment of Unmet homologs.

Sequence alignment of Unmet and homologs from various organisms. Residues are colored by percent identity. Numbering refers to the positions in the *Drosophila melanogaster* Unmet protein sequence. N2227 domain boundaries (green) were annotated based on the PFAM database; approximate metabolite-binding regions for SAM (blue) and carnosine (orange) were identified by finding residues within 3Å of each metabolite in a crystal structure of human CARNMT1 bound to sinefungin and carnosine (PDB: 5YF1). Residues important for the dGATOR2- and SAM-binding capacities of Unmet are marked with yellow and red stars, respectively, at E30 and G195.

Given the conservation of Unmet between flies and humans, we tested the capacity of Unmet to bind to the human GATOR2 complex. Unlike fly Sestrin, Unmet did not interact with transiently expressed or endogenous human GATOR2 (Figures 2C, 4A). These results indicate that the interaction between Unmet and dGATOR2 is not conserved in vertebrates and may instead be specific to the fly lineage.

Previous studies have shown that homologs of Unmet directly bind to the methionine-derived methyl donor SAM through their N2227 domains (Cao et al., 2018). By analogy to the

amino acid sensors Sestrin and CASTOR1, which contain small molecule binding sites and dissociate from GATOR2 in the presence of specific amino acids, we postulated that small molecules might also modulate the Unmet-dGATOR2 interaction. Consistent with this hypothesis, withdrawal of the amino acid methionine, but not leucine, from the culture medium enhanced the interaction of recombinant Unmet with dGATOR2 in both HEK-293T and S2R+ cells (Figures 2D, 4B).

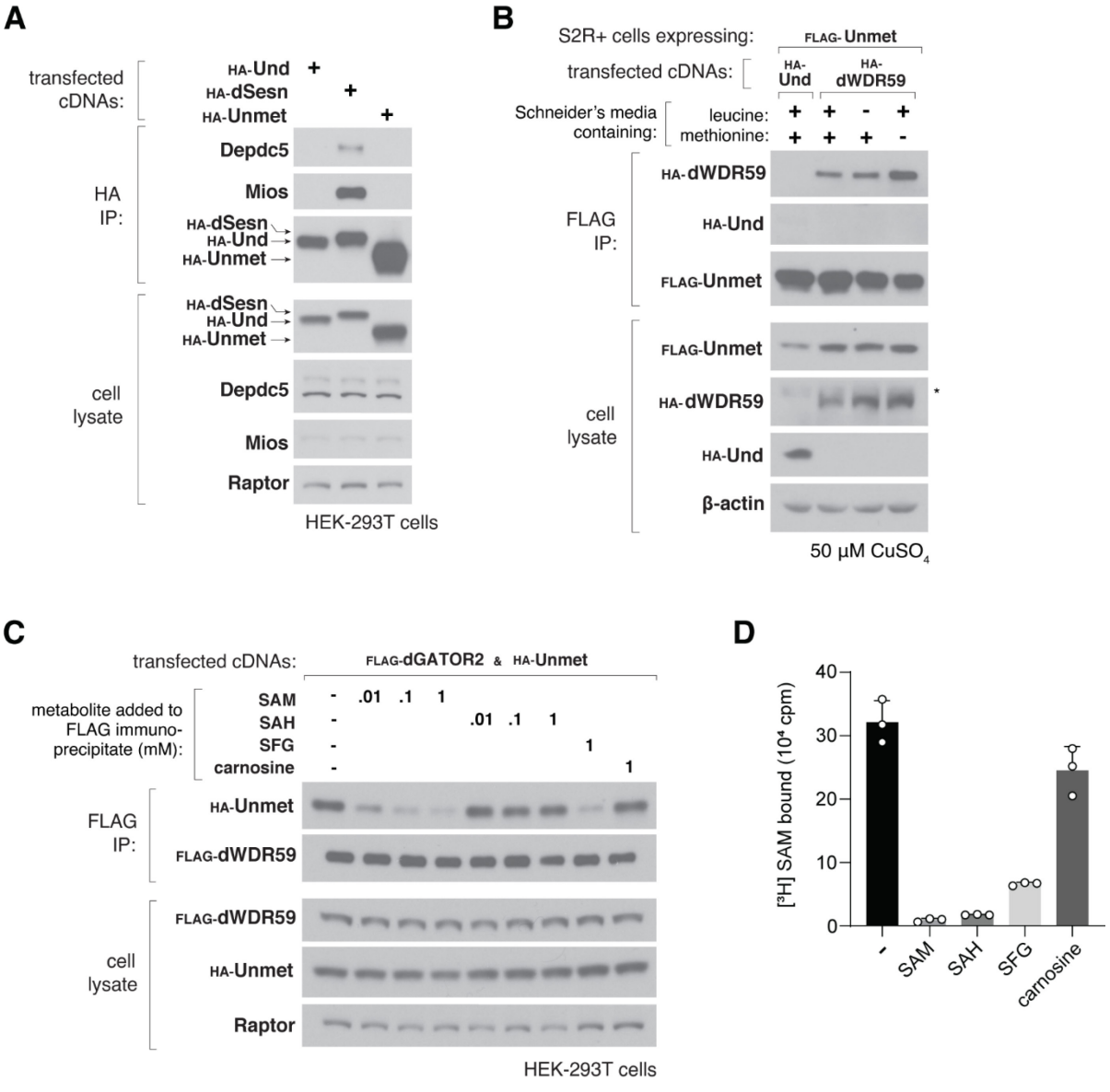


Figure 4: The fly-specific interaction between Unmet and dGATOR2 is regulated by SAM but not SAH or carnosine.

(**A**) Recombinant Unmet does not interact with the endogenous human GATOR1 or GATOR2 complexes. Anti-HA immunoprecipitates were collected from HEK-293T cells transfected with the indicated cDNAs in expression vectors and were analyzed alongside cell lysates by immunoblotting for levels of the indicated proteins and epitope tags. HA-Und served as a negative control. Depdc5 was used as a representative component of GATOR1 and Mios as a representative component of GATOR2.

(**B**) Methionine starvation enhances the interaction between Unmet and dGATOR2 in fly cells. S2R+ cells expressing copper-inducible FLAG-tagged Unmet from the endogenous locus were transfected with HA-Und or HA-dWDR59 in constitutive expression vectors and induced with 50 μM $CuSO_4$ for 72 hours. Cells were then treated with full, leucine-free, or methionine-free Schneider's media for 2 hours. FLAG immunoprecipitates and cell lysates were analyzed as in Fig. 2D.

(**C**) The Unmet-dGATOR2 complex is disrupted by 100 μM of SAM or 1 mM of SFG but not by 1 mM of SAH or carnosine. The experiment was performed and analyzed as in Fig. 2E.

(**D**) Unmet binds to SAM, SAH, and SFG. Binding assays were performed with 10 μg purified FLAG-Unmet incubated with 5 μM [^{3}H]SAM and 1 mM of unlabeled SAM, SAH, SFG, or carnosine. Values for each point represent the means ± s.d. from three independent replicates.

To determine whether methionine acts directly on Unmet—as leucine and arginine do on Sestrin2 and CASTOR1, respectively—or whether the interaction is mediated by a related metabolite, as with SAMTOR, we immunopurified the Unmet-dGATOR2 complex from amino acid-starved cells. Although the addition of a cocktail of amino acids to lysates disrupted the CASTOR1-human GATOR2 and dSestrin-dGATOR2 complexes, it did not release Unmet from dGATOR2. Instead, SAM, which had no effect on the CASTOR1 and dSestrin interactions with GATOR2, robustly dissociated Unmet from dGATOR2 (Figure 2E).

Because the human homolog of Unmet has been co-crystallized with carnosine and various derivatives of SAM (Cao et al., 2018), we tested whether these small molecules could perturb the interaction between Unmet and dGATOR2. Unlike SAM, which dissociated the Unmet-dGATOR2 complex in a dose-dependent manner, carnosine or *S*-adenosylhomocysteine (SAH), the demethylated form of SAM, had no effect (Figure 4C). Despite the discrepancy between the impact of SAM and SAH, SAM-dependent dissociation of Unmet from dGATOR2 is unlikely to require a methylation event, as the SAH analog sinefungin (SFG) is capable of breaking the Unmet-dGATOR2 interaction (Figure 4C).

Using an equilibrium binding assay similar to those previously used for analyses of Sestrin2 and SAMTOR, we found that radiolabeled SAM binds directly to purified Unmet. Excess cold SAM fully competed off the tritiated SAM, yielding a dissociation constant of 9.6 μM (Figure 2F). Although SAH does not disrupt the interaction between Unmet and dGATOR2, it readily competes with labeled SAM for binding to Unmet (Figure 4D). These results suggest that Unmet binds both SAM and SAH but undergoes a conformational change to evict dGATOR2 only when a methyl-like moiety occupies the metabolite-binding cleft. In line with that hypothesis, sinefungin, which replaces the sulfonium ($S\text{-}CH_3$) group of SAM with a primary amine, also competes with labeled SAM for binding to Unmet and, as described above, displaces dGATOR2, while carnosine does not (Figure 4D). How the Unmet-dGATOR2 complex discriminates between SAM/SFG and SAH remains an open question, as all three metabolites likely bind to the same site on Unmet.

Unmet confers methionine sensitivity on the fly TORC1 pathway

Given that SAM binds Unmet and regulates its interaction with dGATOR2, we reasoned that Unmet might affect the ability of the *Drosophila* TORC1 (dTORC1) pathway to respond to methionine deprivation. Indeed, depletion of *unmet* mRNA by double-stranded RNA (dsRNA)-mediated RNA interference rendered the dTORC1 pathway insensitive to methionine starvation in S2R+ cells (Figure 5A). Although dTORC1 responds to a different set of environmental amino acids than mammalian mTORC1, the effects of the *unmet* knockdown were remarkably specific. As detected by the phosphorylation of dS6K at residue Thr^{398}, the dsRNA targeting *unmet* prevented dTORC1 inhibition upon methionine starvation while leaving leucine (Figure 6A), threonine, glutamine, phenylalanine, and tryptophan sensitivity intact (Figure 5A).

We confirmed and extended this result using an orthogonal method for controlling *unmet* expression. To tune Unmet protein levels, we engineered an S2R+ cell line with a copper-inducible metallothionein (MT) promoter and a FLAG epitope tag knocked into the endogenous

unmet locus, such that FLAG-Unmet expression responded to the concentration of copper sulfate in the culture medium. In the absence of copper induction, *unmet* mRNA levels dropped more than 10-fold from the endogenous ones, mimicking an *unmet* knockdown (Figure 6B). Under those conditions, the dTORC1 pathway was wholly resistant to methionine starvation. Low induction (50 μM $CuSO_4$) of FLAG-Unmet restored the methionine responsiveness of the dTORC1 pathway, while substantial overexpression (500 μM $CuSO_4$) blunted dTORC1 activity (Figure 5B). These data show that Unmet inhibits dTORC1 signaling in the absence of methionine and, like CASTOR1 and Sestrin2 in human cells, suppresses the pathway when overexpressed.

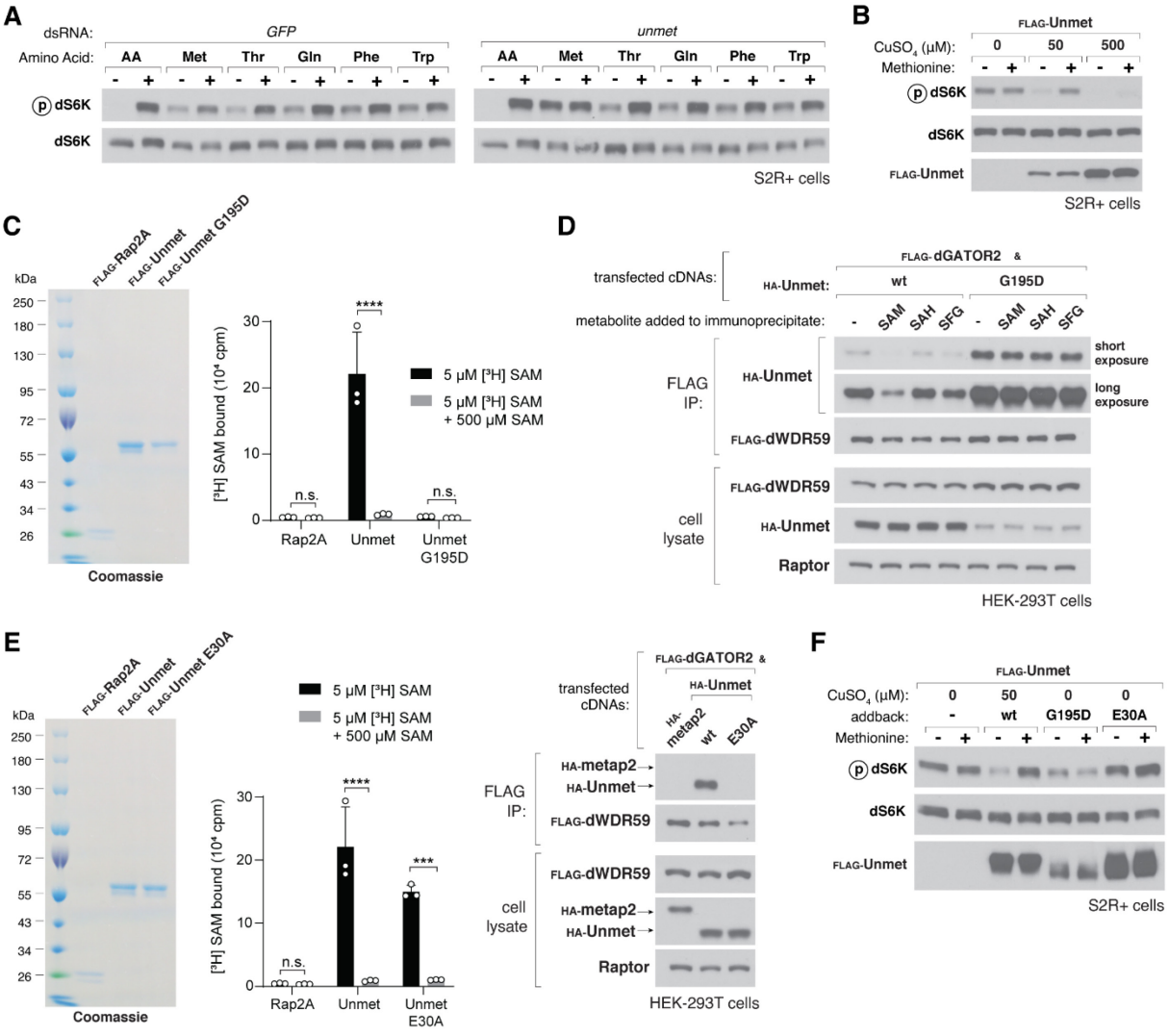


Figure 5: Unmet signals methionine sufficiency to dTORC1 by acting as a negative regulator of the pathway.

(**A**) In Unmet-depleted cells, the dTORC1 pathway is resistant to methionine starvation. S2R+ cells were transfected with dsRNAs targeting either a control mRNA (*GFP*) or *unmet* mRNA. dsRNA-treated cells were then starved of the indicated amino acids for 90 minutes or starved and restimulated for 15 min. Cell lysates were analyzed by immunoblotting for the phosphorylation states and the levels of dS6K.

(**B**) A decrease in Unmet expression abolishes dTORC1 sensitivity to methionine starvation, while its overexpression blunts dTORC1 activity. S2R+ cells expressing a copper-inducible FLAG-tagged Unmet from the endogenous locus were incubated with the indicated concentrations of $CuSO_4$ for 72 hours. Cells were then starved of methionine for 90 minutes or starved and restimulated for 15 min. Cell lysates were analyzed by immunoblotting for the phosphorylation states and levels of the indicated proteins.

(**C**) The G195D mutant of Unmet does not bind SAM. Binding assays were performed and immunoprecipitates were analyzed as in Fig. 2F. Two-way ANOVA followed by Tukey's multiple comparison test; from left to right: adjusted $P = 1.0$; ****$P < 0.0001$; $P = 1.0$; n.s., not significant. Error bars represent the s.d. of three independent samples. Binding data for Rap2A and wild-type Unmet shown again for clarity in (E).

(**D**) The interaction between Unmet G195D and dGATOR2 is insensitive to SAM and SFG. FLAG immunoprecipitates were prepared from HEK-293T cells transfected with the indicated cDNAs. 1 mM of the indicated metabolite was added directly to the immunoprecipitates. FLAG immunoprecipitates and cell lysates were analyzed as in Fig. 2D.

(**E**) The E30A mutant of Unmet has a decreased dGATOR2-binding capacity but maintains the ability to bind SAM. Binding assays were performed and immunoprecipitates were analyzed as in Fig. 1f; binding data for Rap2A and wild-type Unmet as previously shown in (C). Two-way ANOVA followed by Tukey's multiple comparison test; adjusted ***$P = 3.0 \times 10^{-4}$. For the Western blot, FLAG immunoprecipitates were prepared from HEK-293T cells transfected with the indicated cDNAs and analyzed as in (D).

(**F**) In *unmet*-null S2R+ cells, expression of the Unmet G195D and E30A mutants fails to restore methionine sensitivity to the dTORC1 pathway. S2R+ cells expressing a copper-inducible FLAG-tagged Unmet from the endogenous locus were engineered to stably express the indicated FLAG-tagged proteins. Cells were then induced for 72 hours with no $CuSO_4$, to mimic an *unmet*-null cell, or 50 µM $CuSO_4$, to mimic wild-type expression of Unmet. Cells were starved of methionine for 90 minutes or starved and restimulated for 15 min. Lysates were analyzed by immunoblotting, as in (B). *unmet*-null cells expressing the SAM-binding-deficient Unmet G195D mutant fail to reactivate the dTORC1 pathway in the presence of methionine, while cells expressing the dGATOR2-binding-deficient E30A mutant fail to sense the absence of methionine.

Our finding that Unmet conveys methionine availability to the dTORC1 pathway led us to reevaluate the role of another putative fly SAM sensor (Tang et al., 2022). Although the fly homolog of SAMTOR (dSAMTOR) is about 12 kDa smaller and only loosely conserved from its mammalian counterpart, we previously reported that dsRNA-mediated knockdown of *dSAMTOR*

in fly cells abrogated dTORC1 inhibition upon withdrawal of methionine (Gu et al., 2017). To our surprise, however, attempts to reproduce this observation with the original dsRNA yielded inconsistent results, while a different dsRNA robustly lowered *dSAMTOR* mRNA levels without affecting methionine signaling (Figure 6C). To circumvent dsRNA-mediated artifacts, we introduced a copper-inducible promoter at the endogenous *dSAMTOR* locus in S2R+ cells. We then deprived the cells of copper to generate a *dSAMTOR*-null state. These cells showed no detectable *dSAMTOR* expression but remained sensitive to methionine (Figure 6C). Importantly, the methionine sensitivity of the dTORC1 pathway in *dSAMTOR*-null cells could be abolished by dsRNA-mediated knockdown of *unmet*. Overexpression of *dSAMTOR* failed to suppress dTORC1 activity or alter the methionine sensitivity of the pathway (Figure 6D), and consistent with the absence of the KICSTOR complex—an obligate binding partner of human SAMTOR—in flies, dSAMTOR does not interact with either endogenous dGATOR1 or dGATOR2 (Figure 6E). Finally, *dSAMTOR*$^{-/-}$ larvae fed a methionine-free diet retain the capacity to inhibit dTORC1 (Figure 6F). While these data do not preclude dSAMTOR from acting on dTORC1 through other mechanisms, they suggest that its initial proposal as a component of the dTORC1 pathway in flies may have been due to misleading off-target effects of the particular dsRNA used. We therefore conclude that Unmet, rather than dSAMTOR, is the relevant mediator of methionine sensing for the dTORC1 pathway. The function of dSAMTOR in fly cells, however, remains unknown.

If Unmet is required for dTORC1 to sense methionine, does its SAM-regulated interaction with dGATOR2 transduce that signal? To decouple the metabolite-binding capacity of Unmet from its ability to bind dGATOR2, we performed structure-guided mutagenesis of the protein. A glycine-to-aspartate replacement at the highly conserved G195 residue in the SAM-binding pocket of Unmet abolished its ability to bind SAM in vitro (Figure 5C). The G195D SAM-binding mutant interacted robustly with dGATOR2 in a constitutive fashion (Figure 5D). Using

alanine scanning mutagenesis of surface-exposed residues, as inferred from the crystal structure of the human homolog of Unmet, we also identified a mutation at residue E30 of Unmet that disrupted its interaction with dGATOR2 without impairing its SAM-binding capacity (Figure 5E).

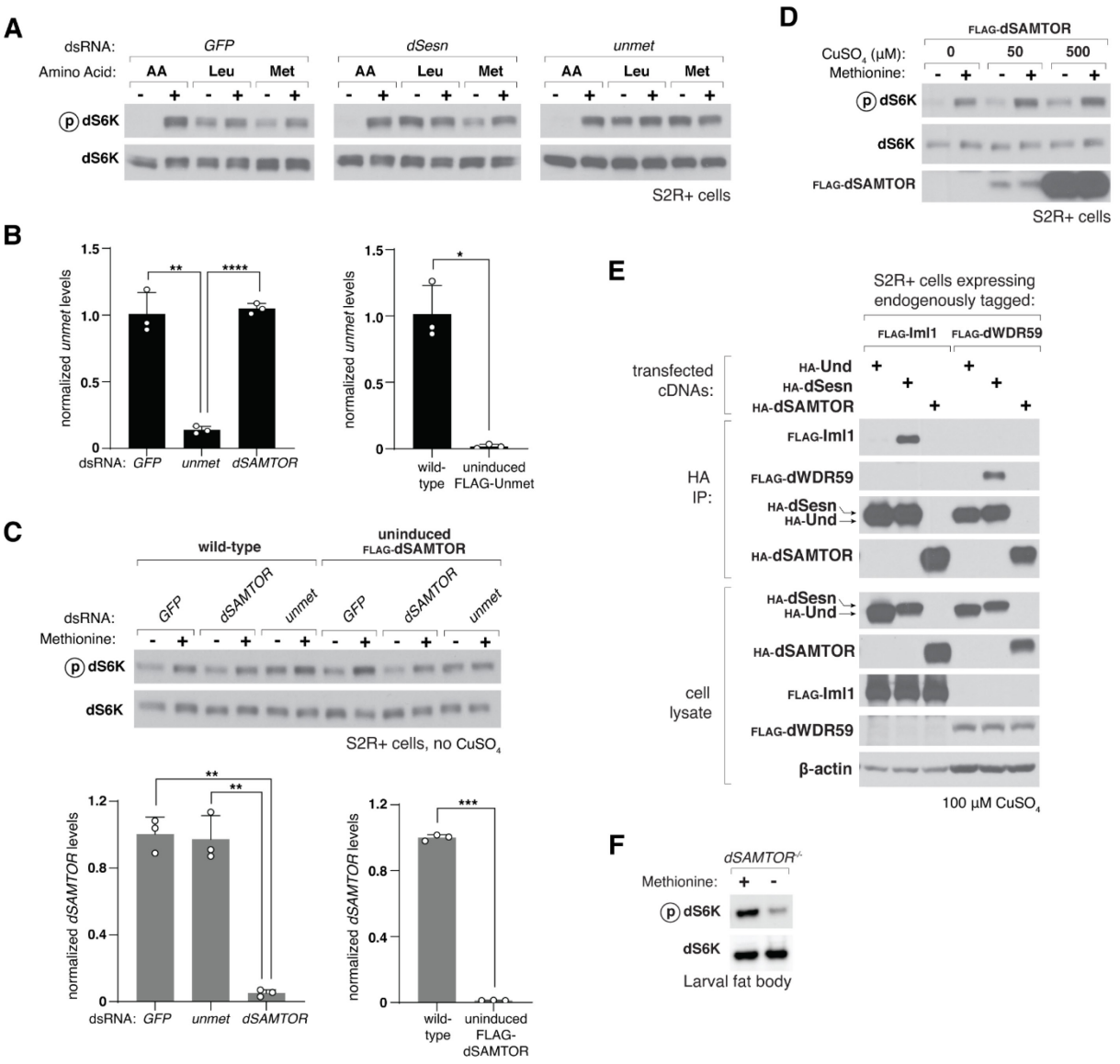


Figure 6: Unmet, not dSAMTOR, signals methionine sufficiency to the dTORC1 pathway.

(**A**) The dTORC1 pathway is resistant to methionine starvation in Unmet-depleted cells. S2R+ cells were transfected with dsRNAs targeting a control mRNA (*GFP*), *dSesn* mRNA, or *unmet* mRNA. dsRNA-treated cells were then starved of the indicated amino acids for 90 minutes or starved and restimulated for 15 min. Cell lysates were analyzed by immunoblotting for the phosphorylation states and the levels of dS6K.

(**B**) mRNA levels of *unmet* in S2R+ cells treated with the indicated dsRNAs. In the absence of copper induction, *unmet* mRNA levels in S2R+ cells expressing FLAG-Unmet from a metallothionein promoter at the endogenous locus are comparable to those of cells with knockdown of *unmet*. Reported values reflect the mean ± s.d. of three biological replicates of ΔΔCt values, using α-tubulin as an internal standard. Two-sided Student's t-test; from left to right: $^{**}P = 9.7 \times 10^{-3}$; $^{****}P < 1.4 \times 10^{-5}$; $^{*}P = 1.5 \times 10^{-2}$.

(**C**) Depleting *unmet* abolishes dTORC1 sensitivity to methionine starvation, while depleting *dSAMTOR* has no effect. Wild-type S2R+ cells or S2R+ cells expressing copper-inducible FLAG-tagged dSAMTOR from the endogenous locus were transfected with the indicated dsRNAs in the absence of copper. dsRNA-treated cells were starved of methionine as in (A), and cell lysates were analyzed by immunoblotting for the phosphorylation states and levels of dS6K. cDNA from transfected cells was synthesized and analyzed by qPCR. Two-sided Student's t-test; from left to right: $^{**}P = 2.8 \times 10^{-3}$; $^{**}P = 7.0 \times 10^{-3}$; $^{***}P = 1.1 \times 10^{-4}$.

(**D**) dSAMTOR expression does not modulate dTORC1 activity. S2R+ cells expressing a copper-inducible FLAG-tagged dSAMTOR from the endogenous locus were incubated with the indicated concentrations of $CuSO_4$ for 72 hours. Cells were then starved of methionine, and lysates were analyzed as in (A).

(**E**) dSAMTOR does not interact with the dGATOR1 or dGATOR2 complexes. Anti-HA immunoprecipitates were prepared from S2R+ cells bearing endogenous FLAG knock-in tags at either the Iml1 (dGATOR1) or the dWDR59 (dGATOR2) locus, transfected with the indicated cDNAs in copper-inducible metallothionein expression vectors. Following 48-hour induction with 100 μM $CuSO_4$, cell lysates and immunoprecipitates were analyzed by immunoblotting for levels of the relevant epitope tags.

(**F**) In the larval fat bodies of *dSAMTOR*$^{-/-}$ flies, the dTORC1 pathway remains sensitive to methionine starvation. *dSAMTOR*$^{-/-}$ L3 larvae were transferred to either full or methionine-free holidic diets for 24 hours. Dissected fat bodies were crushed and analyzed by immunoblotting for the phosphorylation states and the levels of dS6K.

To assess the effect of these Unmet mutants on dTORC1 signaling, we expressed the SAM-binding (G195D) and dGATOR2-binding (E30A) mutants in S2R+ cells with copper-inducible expression of FLAG-Unmet (Figure 5F). In the absence of copper, which leads to an Unmet-null state, the dTORC1 pathway in these cells is insensitive to methionine deprivation. Although expression of wild-type Unmet restored the methionine sensitivity of the pathway, expression of the G195D mutant constitutively inhibited dTORC1 signaling, suggesting that SAM must be able to bind to Unmet in order to activate the pathway. Meanwhile, expression of Unmet E30A had no effect on dTORC1 activity, demonstrating that the interaction between Unmet and dGATOR2 is required for dTORC1 to sense the absence of methionine and SAM. Thus, we conclude that Unmet conveys methionine levels to dTORC1 in cells in culture.

Loss of Unmet in flies impairs organismal adaptation to methionine-restricted diets

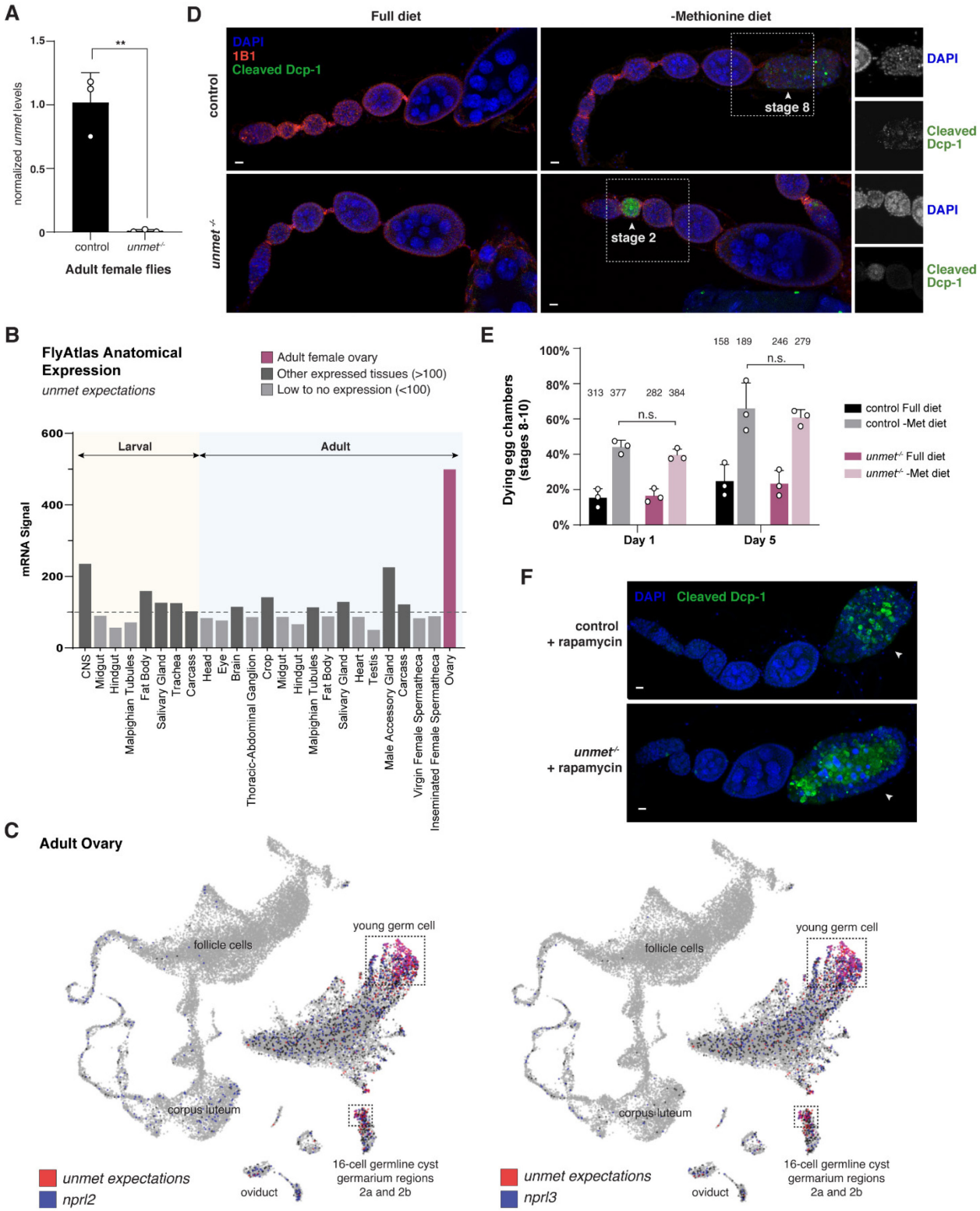


Figure 7: *unmet* is expressed in young germ cells and acts on early rather than vitellogenic egg chambers.

(**A**) *unmet*$^{-/-}$ flies with CRISPR-Cas9-mediated deletion of the gene locus do not express any detectable *unmet* mRNA. Reported values reflect the mean ± s.d. of three biological replicates of ΔΔCt values, using α-tubulin as an internal standard. Two-sided Student's t-test; $^{**}P = 8.5 \times 10^{-3}$.

(**B**) Anatomical expression of *unmet* based on the Fly Atlas.

(**C**) Single-cell expression map for GATOR1 components *nprl2* and *nprl3* (blue) in the adult ovary, plotted against the expression map for *unmet* (red). HVG UMAP display of single-cell RNA-seq expression data from the Fly Cell Atlas. *nprl2* and *nprl3* expression overlaps with expression of *unmet* in young germ cells and the germline cyst (purple).

(**D**) Ovarioles from female flies cultivated on the indicated diets for five days, labeled with DAPI (blue), the hu-li tai shao actin-associated antibody 1B1 (red), and cleaved *Drosophila* caspase 1 (cleaved Dcp-1 Asp216, green). Note that the degenerating egg chambers (white arrows) contain condensed DNA staining for pyknotic nuclei and are positive for cleaved Dcp-1. Scale bar, 10 μm.

(**E**) Mid-stage (8-10) vitellogenic egg chambers from flies starved of methionine undergo apoptosis at identical rates between *unmet*$^{-/-}$ and control flies. Percentage of stage 8-10 egg chambers undergoing cell death were recorded for each genotype and dietary condition. Two-way ANOVA followed by Tukey's multiple comparison test; from left to right: adjusted $P = 0.88$; $P = 0.83$; n.s., not significant. Error bars represent the s.d. of three independent experiments. Bars are labeled with number of stage 8-10 egg chambers analyzed for each condition.

(**F**) In both *unmet*$^{-/-}$ and control flies, rapamycin treatment induces degeneration of mid-stage (8-10) egg chambers, while early egg chambers (1-7) remain intact. Ovaries from flies cultured for five days on a full diet containing 10 μM rapamycin were labeled with DAPI (blue) and cleaved Dcp-1 Asp216 (green). Note that the degenerating stage 8-10 egg chambers (white arrows) are positive for cleaved Dcp-1. Scale bar, 10 μm.

To determine whether Unmet serves a corresponding function in vivo, we generated an *unmet*$^{-/-}$ mutant fly strain using CRISPR-Cas9-mediated deletion of the gene locus. *unmet*$^{-/-}$ flies had no detectable *unmet* mRNA (Figure 7A) but remained fully viable. However, unlike wild-type larvae, which showed blunted dTORC1 activity in the fat body after 24 hours on a methionine-free diet, *unmet*$^{-/-}$ larvae failed to inhibit the dTORC1 pathway upon methionine starvation (Figure 8A). This phenotype in the larval fat body, a homogeneous tissue amenable to biochemical analysis, recapitulates the signaling defect seen in cultured *unmet* knockdown cells. Loss of *unmet* had no effect on dTORC1 signaling in larvae fed a full diet, indicating that the link between Unmet and dTORC1 is nutrient-dependent.

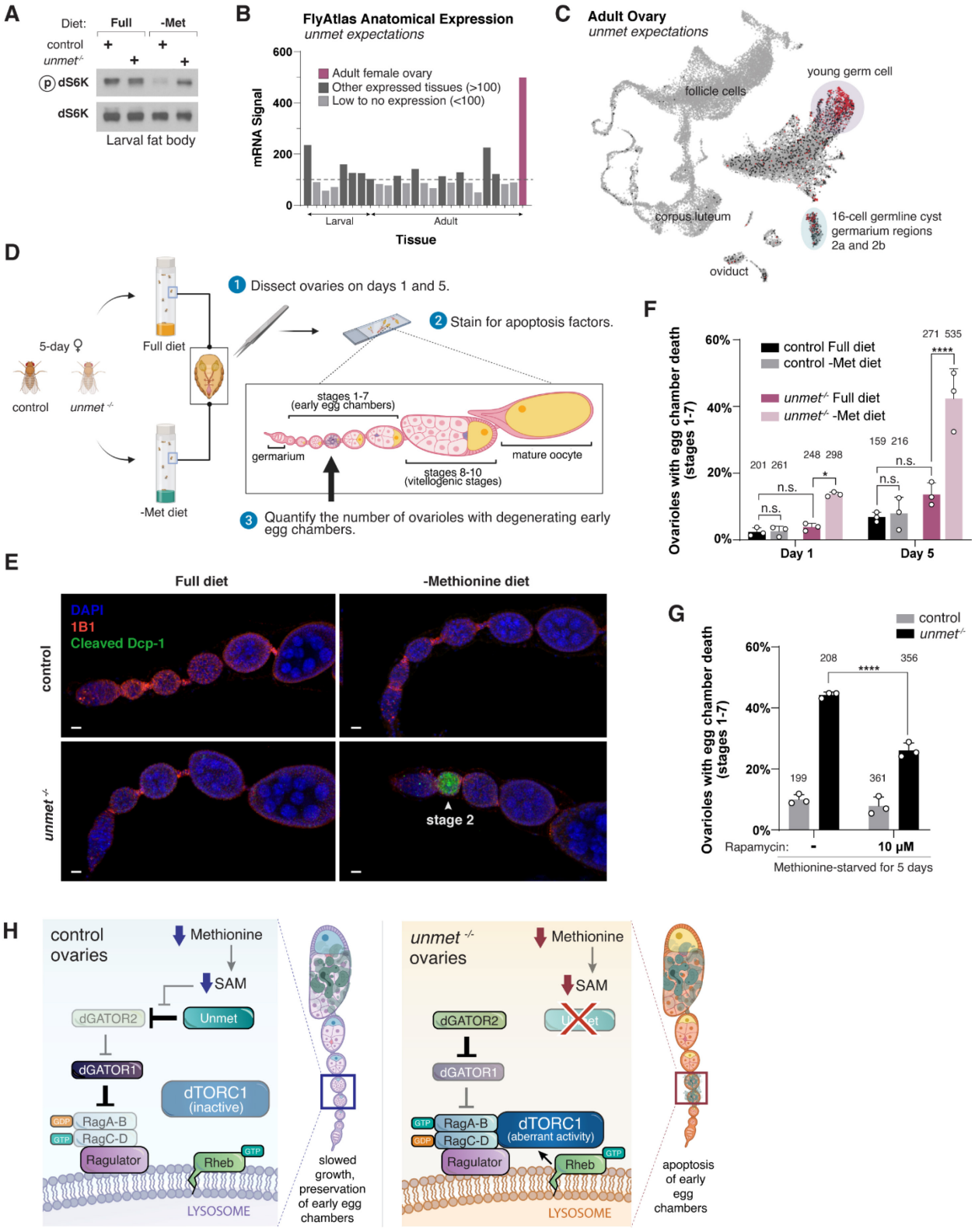


Figure 8: Unmet maintains germline integrity in the fly ovary by suppressing dTORC1 signaling upon methionine starvation.

(**A**) In the larval fat bodies of *unmet*$^{-/-}$ flies, the dTORC1 pathway is resistant to methionine starvation. Control and *unmet*$^{-/-}$ L3 larvae were transferred to either full or methionine-free holidic diets for 24 hours. Dissected fat bodies were crushed and analyzed by immunoblotting for the phosphorylation states and the levels of dS6K.

(**B**) Expression of *unmet* across tissues. Anatomical expression data from the Fly Atlas, with full labels displayed in Fig. 7B.

(**C**) Single-cell expression map for *unmet* in the adult ovary. HVG UMAP display of single-cell RNA-seq expression data from the Fly Cell Atlas. Cluster annotations derived from ref. (Li et al., 2021).

(**D**) Schematic of the experimental set up for quantifying apoptotic early-stage egg chambers in control or *unmet*$^{-/-}$ ovaries from flies fed full or methionine-deficient diets.

(**E**) Early egg chambers in *unmet*$^{-/-}$ ovaries undergo apoptosis upon methionine deprivation. As outlined in (d), ovaries from female flies cultivated on the indicated diets for five days were labeled with DAPI (blue), the hu-li tai shao actin-associated antibody 1B1 (red), and cleaved *Drosophila* caspase 1 (cleaved Dcp-1 Asp216, green). The degenerating egg chamber (white arrow) is positive for cleaved Dcp-1. Scale bar, 10 μm. Early egg chambers (stages 1-7) shown here; full ovarioles displayed in Fig. 7D.

(**F**) Percentage of ovarioles containing at least one dying early egg chamber (stages 1-7) for each genotype and dietary condition. Two-way ANOVA followed by Tukey's multiple comparison test; from left to right: adjusted $P = 1.0$; $P = 0.97$; $^{*}P = 3.4 \times 10^{-2}$; $P = 0.99$; $P = 0.19$; $^{****}P < 0.0001$; n.s., not significant. Error bars represent the s.d. of three independent experiments. Bars are labeled with number of ovarioles analyzed for each condition.

(**G**) Rapamycin, a dTORC1 inhibitor, substantially rescues the increased apoptosis of early egg chambers in the ovaries of methionine-starved *unmet*$^{-/-}$ flies. Two-way ANOVA followed by Tukey's multiple comparison test; adjusted $^{****}P < 0.0001$. Error bars represent the s.d. of three independent experiments. Bars are labeled with number of ovarioles analyzed for each condition.

(**H**) Model for how Unmet maintains the survival of early egg chambers during methionine starvation by detecting the absence of SAM and suppressing dTORC1 signaling. Loss of *unmet* expression permits inappropriately high dTORC1 activity during methionine starvation, activating a checkpoint that triggers apoptosis in early egg chambers.

Guided by the expression pattern of *unmet*, which showed it to be highly enriched in the adult female ovary (Figures 7B, 8B), we sought to define a physiological requirement for SAM-sensing by the dTORC1 pathway. The *Drosophila* ovary is a nutrient-responsive tissue comprised of ovarioles, strings of egg chambers that proceed from a germarium through progressively more mature stages of development. Because egg production is so energy- and resource-intensive, oogenesis halts under protein starvation or prolonged stress (Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005; Lebo and McCall,

2021). To avoid investments in eggs or progeny that will not be viable, vitellogenic (yolk-forming) mid-stage egg chambers (stages 8-10) and some germline cysts undergo apoptosis in the ovaries of starved flies (Drummond-Barbosa and Spradling, 2001). However, to ensure rapid reestablishment of egg production after permissive conditions are restored, early (stage 1-7) egg chambers are protected from apoptosis during starvation, slowing their growth but remaining intact and so preserving female fertility (Drummond-Barbosa and Spradling, 2001; Shimada et al., 2011; Wei and Lilly, 2014).

Survival of early egg chambers in starved flies requires finely-tuned control of the dTORC1 pathway. In flies fed an amino-acid-free diet, ovarian-specific knockdown of negative regulators of dTORC1, including the dGATOR1 substituents dNprl2 and dNprl3, produces a sharp increase in apoptotic early egg chambers, suggesting that failure to downregulate dTORC1 signaling during early oogenesis triggers cell death under amino acid limitation (Wei and Lilly, 2014). Interestingly, single-cell sequencing of the fly ovary shows that expression of *unmet* is concentrated in young germ cells within the germarium (Figure 8C), overlapping strongly with the cell populations that express *dNprl2* and *dNprl3* (Figure 7C) (Li et al., 2021). The fly ovary is also well-validated as a methionine-sensitive niche, with lifetime egg production tied to methionine availability (Grandison et al., 2009; Lee et al., 2014). Indeed, methionine supplementation alone is sufficient to restore fecundity in flies during dietary restriction, indicating that methionine may be a limiting nutrient for ovarian function (Grandison et al., 2009). We therefore hypothesized that Unmet contributes to the maintenance of early egg chambers under methionine and SAM restriction.

To test this model, we placed control or *unmet*$^{-/-}$ flies on either a full diet or a chemically-defined diet lacking methionine. After 1 or 5 days on this diet, ovaries were dissected and stained for the apoptosis factor cleaved *Drosophila* caspase 1 (Dcp-1) (Figure 7D, 8D). Methionine starvation increased the number of degenerating early egg chambers in *unmet*$^{-/-}$ flies

but not in the background-matched control, with the longer starvations enhancing the severity of the phenotype (Figures 8E, 8F). By contrast, methionine-starved mid-stage egg chambers underwent apoptosis at identical rates between *unmet*$^{-/-}$ and control flies (Figure 7E). Rapamycin treatment substantially rescued early egg chamber viability in methionine-starved *unmet*$^{-/-}$ flies, indicating that Unmet exerts a protective function under these conditions by suppressing dTORC1 signaling (Figures 7F, 8G). Following fly community convention, we have renamed *CG11596* as *unmet expectations*, because loss-of-function flies fail to sense and anticipate low-methionine (un-Met) conditions, leading to degradation in the female germline.

Taken in sum, these data converge upon a model in which Unmet detects drops in SAM levels within the germ cell environment and downregulates dTORC1 to prevent damage to early egg chambers. Loss of Unmet permits aberrant activation of dTORC1 under methionine restriction, triggering apoptosis in early egg chambers and compromising germline integrity and likely fertility (Figure 8H). The evolutionary acquisition of a SAM sensor may have conferred selective advantages by allowing flies to use a critical nutrient to gate reproductive investment (Alves et al., 2022).

GATOR2 guides evolution of the nutrient sensing capabilities of the mTORC1 pathway

But how does the mTORC1 pathway recruit new sensors like Unmet, especially on the relatively short time scales required for dietary adaptation? To understand how Unmet emerged as a nutrient sensor for the fly TORC1 pathway, we examined the interactions between Unmet and GATOR2 homologs in different species. Like Unmet itself (Figure 2C), the human homolog of Unmet, carnosine N-methyltransferase 1 (CARNMT1), co-immunoprecipitated dGATOR2 but failed to bind to human GATOR2 (Figure 9A). Similarly, the *Schizosaccharomyces pombe* homolog of Unmet interacted with dGATOR2 but not the apposite *S. pombe* SEA complex (Figure 9B). Together, these data show that the fly GATOR2 complex has diverged from other GATOR2 lineages to allow for binding of Unmet and its homologs (Figure 9C). Strikingly, they

also reveal that structural changes in the dGATOR2 complex, rather than fly-specific adaptations in Unmet, direct the capture and incorporation of Unmet into the dTORC1 pathway.

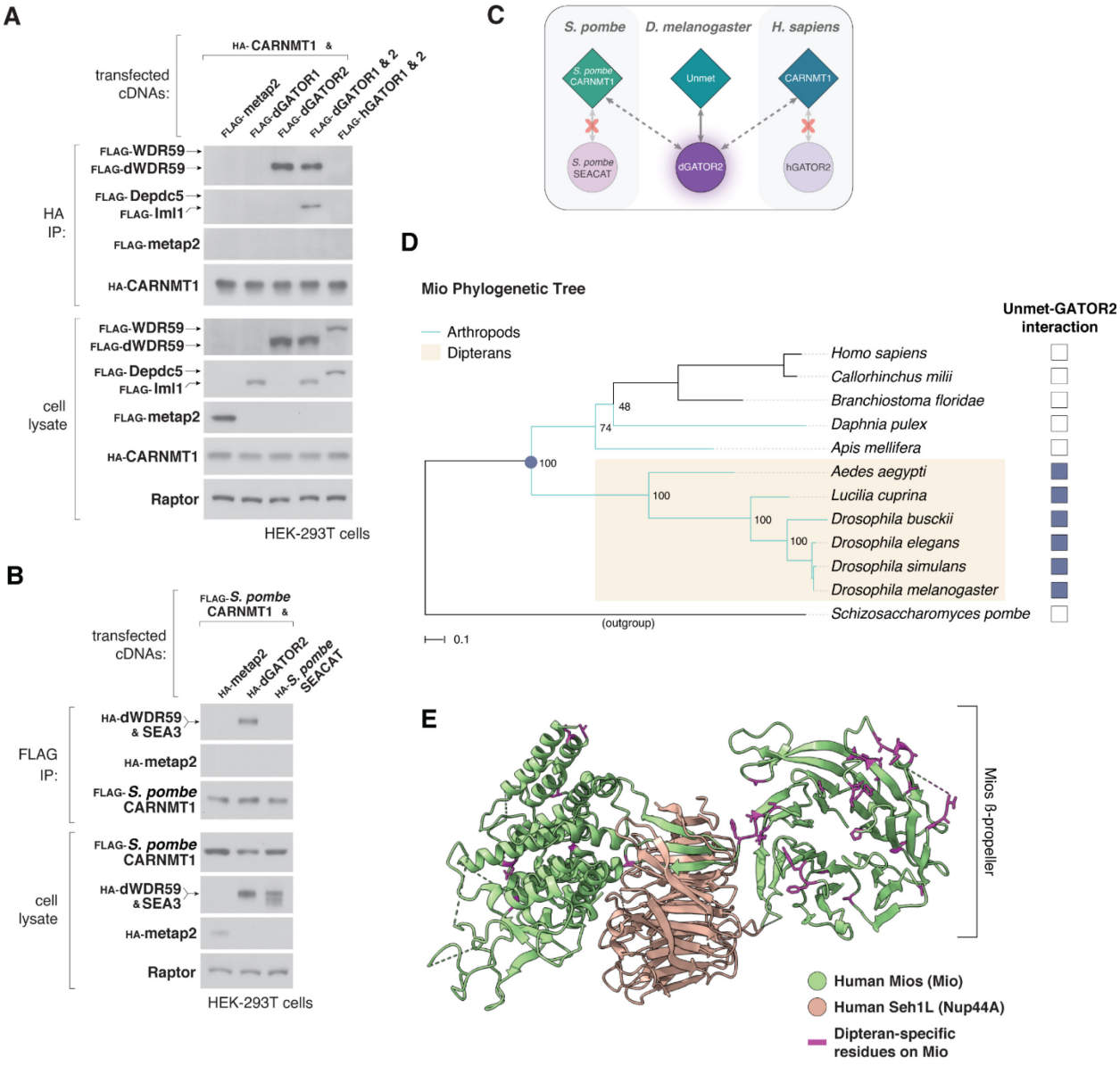


Figure 9: Evolutionary adaptations in the GATOR2 complex drive the incorporation of Unmet as a nutrient sensor for the dTORC1 pathway.

(**A**) Recombinant CARNMT1, the human homolog of Unmet, interacts with dGATOR2 but not its human counterpart. Anti-HA immunoprecipitates from HEK-293T cells expressing the indicated cDNAs were analyzed as in Fig. 2B. FLAG-metap2 served as a negative control.

(**B**) Recombinant *S. pombe* CARNMT1, the fission yeast homolog of Unmet, interacts with dGATOR2 but not the *S. pombe* GATOR2 complex (SEACAT). Anti-FLAG immunoprecipitates from HEK-293T cells expressing the indicated cDNAs were analyzed as in Fig. 2D.

(**C**) Schematic of the interactions between homologs of Unmet and GATOR2 in three species.

(**D**) Rapid evolution of the Mio sequence in Dipterans corresponds to the acquisition of Unmet binding. A maximum likelihood phylogenetic tree constructed using Mio protein sequences from 12 species was matched to the results of binding assays between Unmet and GATOR2 homologs, as assayed in Fig. 10B. Mio diverged so sharply in Dipterans that arthropod sequences from outside the order cluster with vertebrate sequences, in contrast to the topology of a classical species tree, shown in Fig. 10C. Node labels indicate bootstrap support values. Scale bar, 0.1 substitutions per site.

(**E**) Dipteran-specific residues on Mio (magenta) are surface-exposed and map to flexible loops on the beta-propeller of Mio. Green cartoon, human Mios; orange cartoon, human Seh1L; derived from the structure of the full human GATOR2 complex (PDB: 7UHY). Dipteran-specific residues are annotated on the alignment in Fig. 11A.

This strategy, in which a conserved “core” component of the pathway evolves to grab an allosteric regulator, is unusual. Other signaling pathways take the opposite approach: in the MAPK pathway, novel regulators establish a toehold in a pathway by targeting “latent” features on conserved node, followed by lengthy co-evolution (Coyle et al., 2013). To determine how the GATOR2 complex evolved a new binding surface for Unmet without compromising its existing signaling functions, we first assessed the ability of individual dGATOR2 subunits to co-immunoprecipitate Unmet (Figure 10A). The dWDR24, Mio, and Nup44A subcomplex was sufficient to recapitulate the interaction with Unmet; indeed, the remaining components of dGATOR2—dWDR59 and dSec13—were wholly dispensable for full binding. We therefore used the dWDR24-Mio-Nup44A subcomplex as a proxy for GATOR2 as a whole.

We then traced the evolutionary history of the Unmet-GATOR2 interaction across 11 species distributed between arthropods and vertebrates. We co-expressed homologs of Unmet and the GATOR2 tricomplex from these species in HEK293T cells and assayed for binding (Figures 9D, 10B). GATOR2 acquires the ability to bind Unmet late in insect evolution, at an evolutionary branch point situated between honeybee (*Apis mellifera*) and mosquito (*Aedes aegypti*). The location of this branch point corresponds to the emergence of the order Diptera. To understand how the GATOR2 tricomplex recruited Unmet, we examined GATOR2 protein

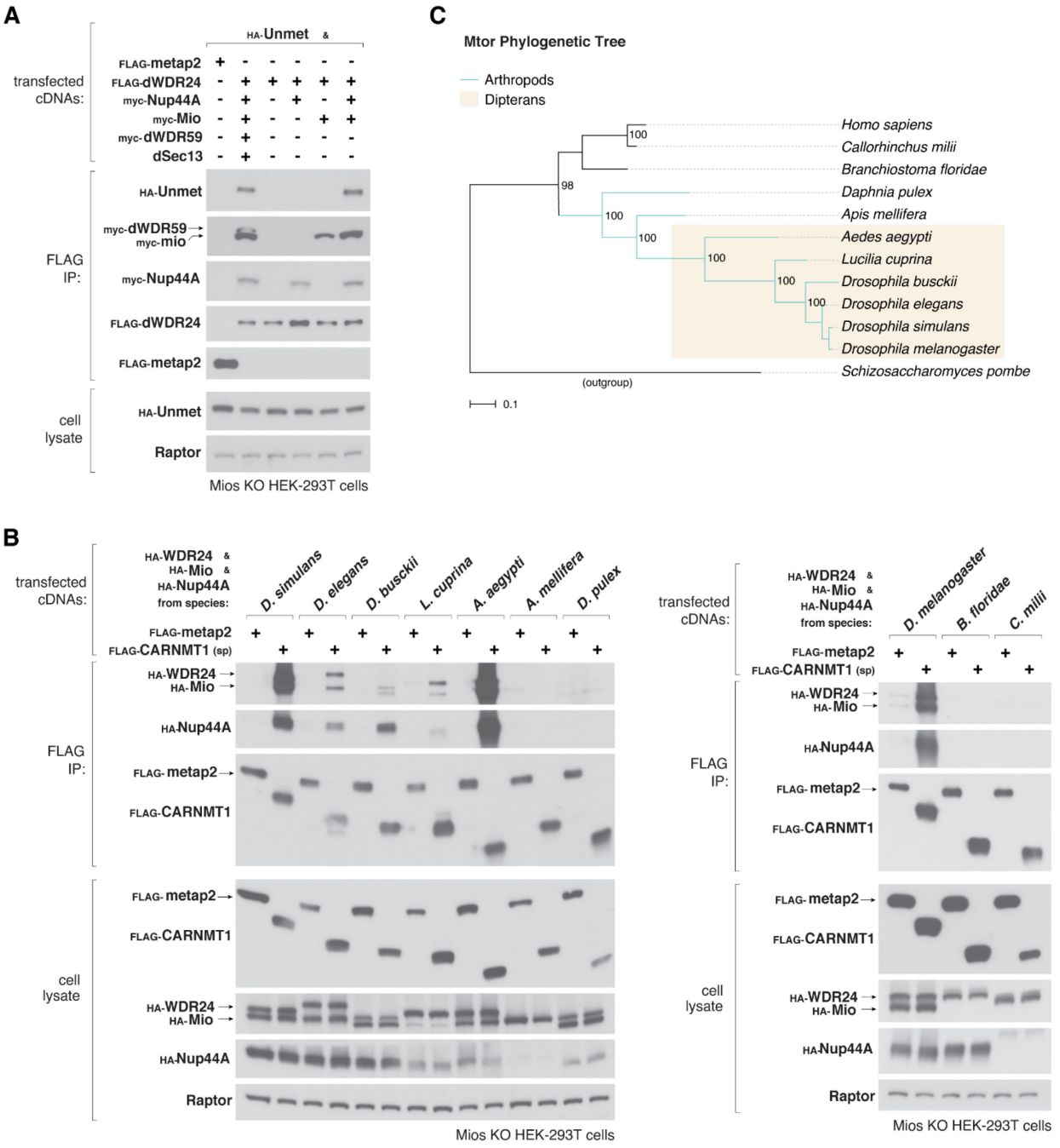


Figure 10: Dipteran GATOR2 acquired a novel interaction with Unmet.

(**A**) The dGATOR2 components dWDR24, Mio, and Nup44A form a minimal complex that is sufficient to co-immunoprecipitate Unmet. HEK-293T cells lacking the human GATOR2 complex protein Mios were co-transfected with the indicated cDNAs, and anti-FLAG immunoprecipitates and cell lysates were analyzed by immunoblotting for the indicated proteins and epitope tags.

(**B**) Dipteran GATOR2 complexes are capable of interacting with cognate CARNMT1 proteins from the same species. HEK-293T cells lacking human Mios were co-transfected with cDNAs encoding homologs of WDR24, Mio, and Nup44A from the indicated species, as well as either

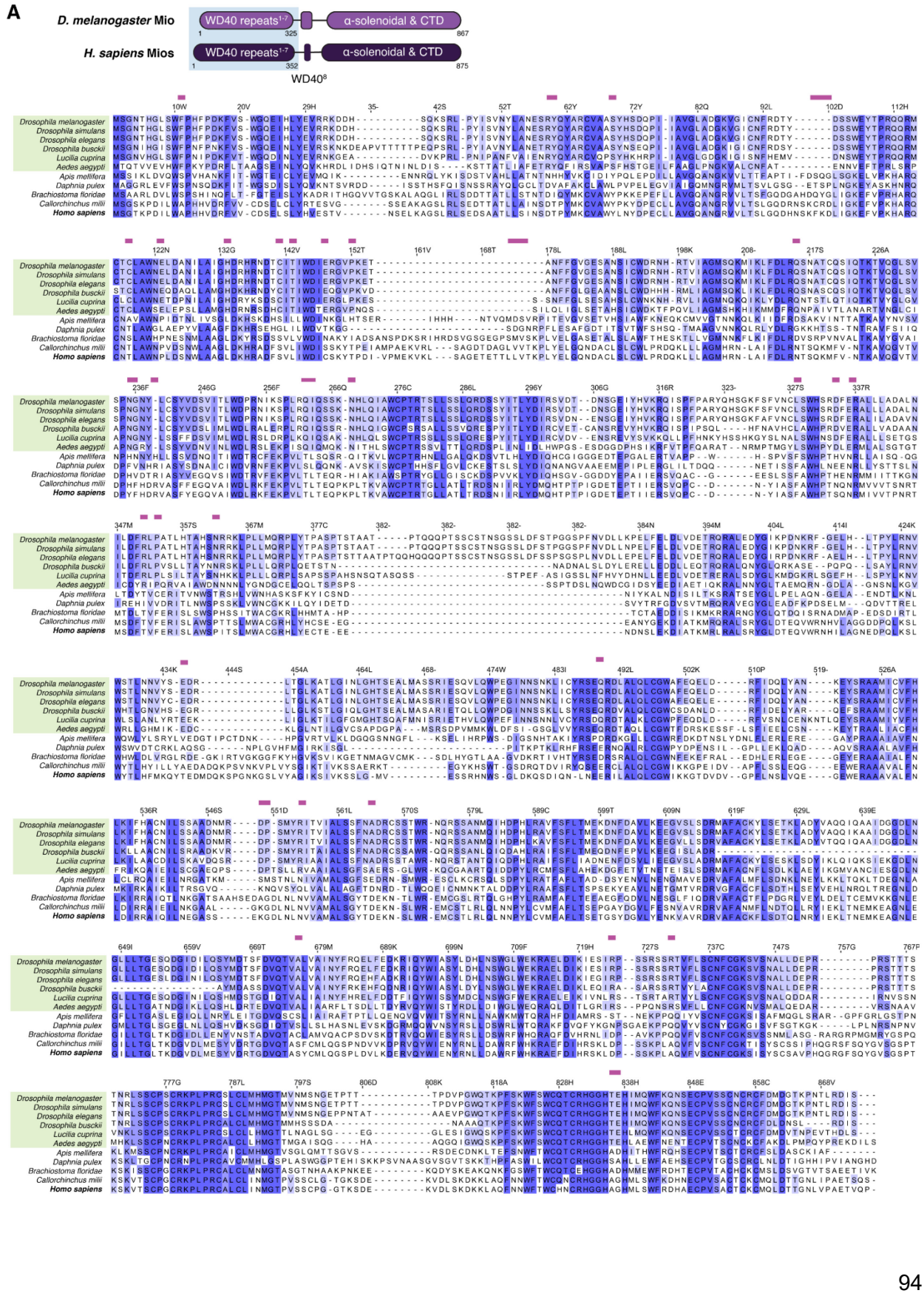
the negative control protein metap2 or a homolog of CARNMT1 from the indicated species. Anti-FLAG immunoprecipitates were analyzed as in (A).

(**C**) Species tree constructed using mTOR protein sequences from 12 species. Node labels indicate bootstrap support values. Scale bar, 0.1 substitutions per site.

sequences for signatures of rapid evolution across the Dipteran branch point. Of the two unique components of the GATOR2 tricomplex, WDR24 showed no such signatures; a phylogenetic tree constructed from WDR24 sequences followed the topology of a classic species tree, in which the arthropod phylum is monophyletic, descending from a single ancestor (Figure 10C). By contrast, in a phylogenetic tree constructed from Mio sequences, Mio diverges so profoundly in Dipterans that homologs from other arthropods (e.g. honeybee or the crustacean *D. pulex*) cluster more closely with human and vertebrate proteins (Figure 9D). Though WDR24 and Nup44A likely make additional contacts with Unmet, these data suggest that rapid evolution of Mio drove the gain-of-function in GATOR2.

To identify the molecular basis for sensor acquisition, we inspected Mio sequences for residues that are conserved in Dipterans but diverge in species that have not assimilated Unmet as a sensor. When mapped onto a recent structure of the human GATOR2 complex, these “variable residues” cluster on surface-exposed, flexible loops that decorate the N-terminal WD40 repeat (WDR) domain of Mio (Fig. 9E, Fig. 11A) (Valenstein et al., 2022). While the Mio WDR domain folds into a characteristic 7-bladed beta-propeller, very few of the variable residues are involved in generating the structural fold. Instead, these residues extend from the surface of the beta-propeller and are generally not constrained by intra-complex interactions. We infer that the divergent loops define the specificity of GATOR2’s protein-protein interactions. Consistent with this model, we find that swapping the fly Mio WDR domain for a WDR domain from human Mios is sufficient to abolish binding to Unmet without disrupting formation of the dGATOR2 complex (Figures 11B, 11C). Collectively, these data argue that exposed, evolutionarily divergent loops between the structural units of the GATOR2 beta-propellers direct

the fly-specific binding of Unmet.



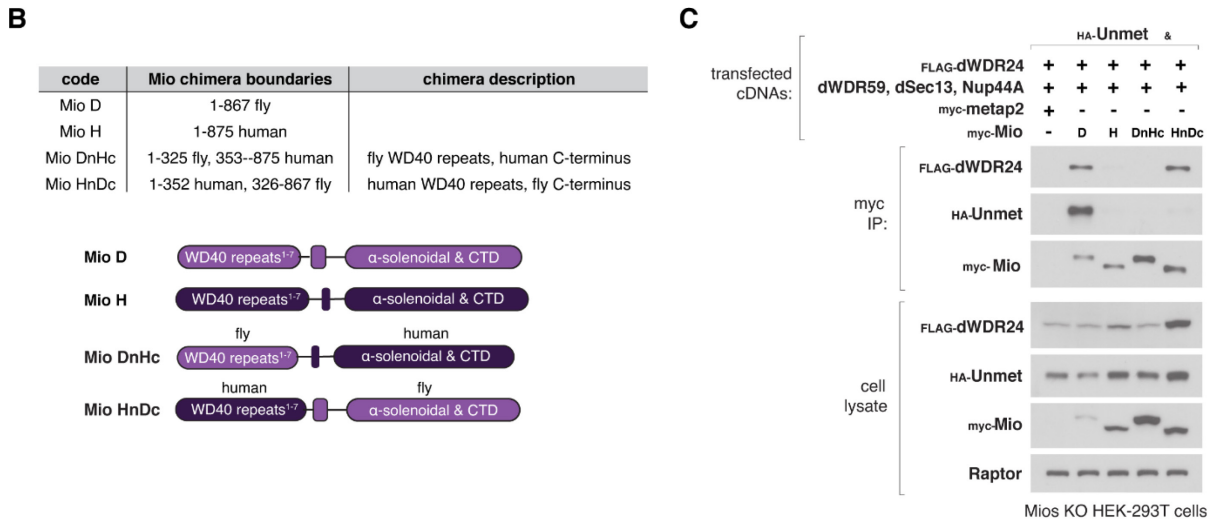


Figure 11: The N-terminal WD40 repeat domains of fly GATOR2 mediate species-restricted binding of Unmet.

(**A**) Schematic of the domain structure of Mios homologs in flies and humans. Sequence alignments of Mios from 11 species, with Dipteran species highlighted in green. Residues were colored by percent identity and numbered with reference to positions in the human protein sequence. Variable surface-exposed residues conserved in Dipterans and divergent in other species (magenta bars) were mapped onto a structure of human Mios in Fig. 9E.

(**B**) Description of Mio constructs, including fly Mio, human Mios, and Mio chimeras with WDR domain swaps between the human and fly homologs.

(**C**) Substitution of the human N-terminal WDR domain of Mios into the fly Mio protein maintains the integrity of the dGATOR2 complex but abolishes binding to Unmet. HEK-293T cells lacking human Mios were co-transfected with the indicated cDNAs, and anti-myc immunoprecipitates were analyzed by immunoblotting for the indicated proteins and epitope tags. Mio HnDc, containing the human WD40 repeat region, fails to interact with Unmet but maintains dGATOR2 formation by binding to dWDR24.

Indeed, GATOR2 is so critical for defining regulatory inputs into the mTORC1 pathway that we can engineer artificial inputs for the human mTORC1 pathway by changing its binding behavior. Because the human GATOR2 complex cannot bind to Unmet or its human homolog CARNMT1, CARNMT1 does not regulate mTORC1 signaling in HEK-293T cells (Figure 12). However, coercing a physical interaction between CARNMT1 and a core component of the mTORC1 machinery—for example, by replacing human GATOR2 with dGATOR2—allows CARNMT1 overexpression to suppress mTORC1 activity in human cells (Figure 13A). Altering the binding capabilities of GATOR2 can thus rewire the mTORC1 pathway to respond to an

enzyme that does not act as a nutrient sensor in its native cellular context. GATOR2 is therefore a flexible node that sustains regulatory complexity and innovation in the mTORC1 pathway.

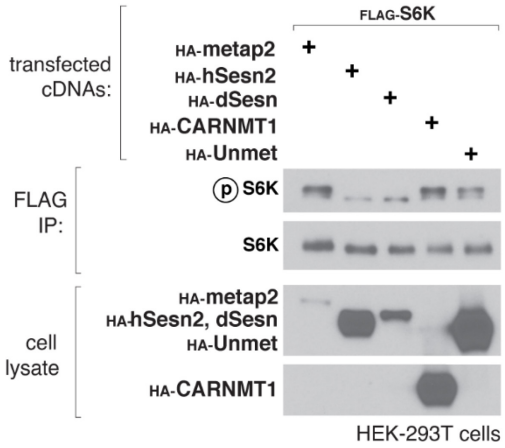


Figure 12: Overexpression of Unmet homologs fails to suppress mTORC1 in human cells.

Overexpression of Unmet and its human homolog, CARNMT1, in human cells fails to inhibit mTORC1 signaling. Anti-FLAG immunoprecipitates were analyzed by immunoblotting for the indicated proteins and epitope tags.

Together, these findings suggest a general mechanism for evolution of nutrient sensors without recourse to paralogous duplication (Figure 13B). GATOR2, a conserved signaling hub for the mTORC1 pathway, can generate new binding surfaces through rapid sequence divergence of flexible loops on the beta-propellers of Mios and WDR24. Because residues on these loops do not maintain the secondary or tertiary structure of the complex, they are highly evolvable. New binding surfaces recruit pre-existing proteins, such as Unmet. If opportunistic interactions confer a selective advantage, they can be embedded into the pathway through further refinement of the interface. Strikingly, the modular structure of the GATOR2 complex, with exposed beta-propellers distributed across five different proteins, allows sequential recruitment of new pathway components without compromising existing signaling interfaces (Tafur et al., 2022; Valenstein et al., 2022). Given that Unmet's methyltransferase activity is conserved from yeast to vertebrates, while its sensor role is restricted to Dipterans, we infer that Unmet is an ancestral enzyme co-opted by GATOR2 for its ligand-binding capabilities. The

known mammalian nutrient sensors Sestrin and CASTOR, which bind to the WDR domains of WDR24 and Mios, respectively, likely followed a similar evolutionary trajectory from enzyme to sensor (Figure 13C). This pathway design is particularly attractive because small molecule ligand-binding is fragile and difficult to evolve *de novo*, in contrast to the robust evolutionary landscape for gain-of-function in protein-protein interactions (Fowler et al., 2010; Tenthorey et al., 2020). By exploiting evolvable modules on GATOR2, the mTORC1 pathway can rapidly assimilate new sensors by repurposing proteins that already bind to a metabolite of interest while preserving information flow through the conserved core of the pathway.

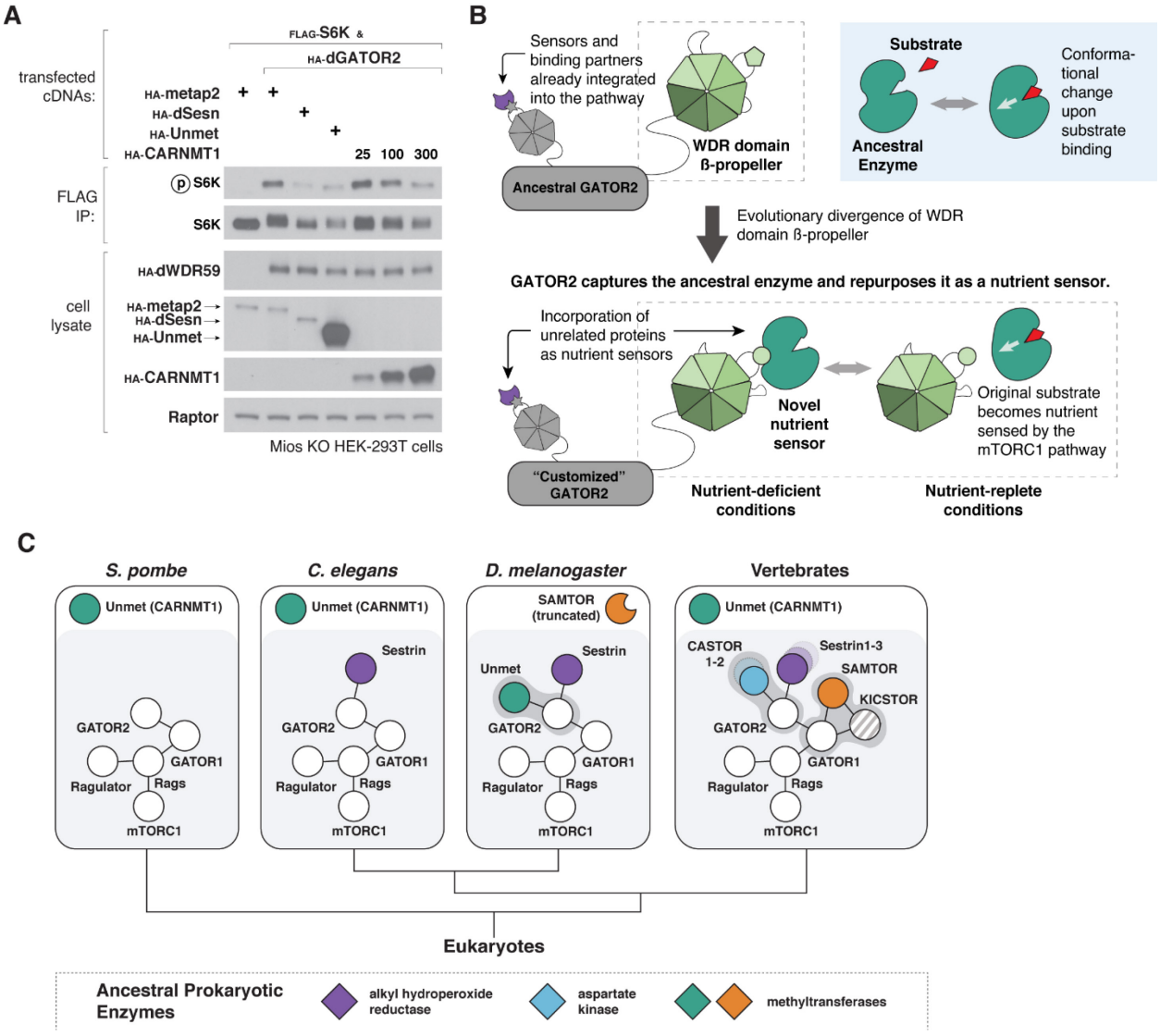


Figure 13: An evolutionary mechanism to assimilate novel nutrient sensors and rewire mTORC1 signaling.

(**A**) Human CARNMT1 can act as a negative regulator of mTORC1 signaling when human GATOR2 is replaced with the fly GATOR2 complex. Mios-deficient HEK-293T cells expressing the indicated cDNAs were starved in RPMI lacking amino acids for 1 hour and then restimulated with amino acids for 15 minutes. Anti-FLAG immunoprecipitates were analyzed as in Fig. 2D.

(**B**) Evolutionary model for co-option of ligand-binding proteins by GATOR2.

(**C**) Phylogenetic tree representing the evolution of nutrient sensing capabilities in the mTORC1 pathway. Conserved core components of the mTORC1 pathway are shown as white circles, connected by lines that represent protein-protein interactions. Orthologs share the same color. Dark grey blobs highlight species-restricted interactions between nutrient sensors and core components of the mTORC1 pathway. The eukaryotic nutrient sensors may ultimately share evolutionary origins with prokaryotic enzymes (shown as diamonds).

Discussion

We establish Unmet expectations as a SAM sensor for the fly TORC1 pathway. Unmet interacts with the fly GATOR2 complex in a SAM-dependent manner to regulate dTORC1 activity. Loss of Unmet renders the dTORC1 pathway insensitive to methionine deprivation, while expression of a mutant of Unmet that cannot bind SAM constitutively suppresses dTORC1 signaling in fly cells. Because they cannot couple SAM levels to dTORC1 activity, *unmet*$^{-/-}$ flies exhibit severe ovarian defects on methionine-free diets.

Unmet offers unique insights into the evolution of nutrient sensors in the mTORC1 pathway. Although the mammalian nutrient sensors bear structural similarities to some bacterial proteins, Sestrin, CASTOR1, and SAMTOR do not retain any known enzymatic activity, and their homologs have been lost in fungi and many metazoa (Saxton et al., 2016a; Saxton et al., 2016b). As a result, they appear to emerge in higher eukaryotes as fully assimilated nutrient sensors, with few clues about their ancestral functions or evolutionary origins. Our identification of Unmet bridges that gap by showing how an independent methyltransferase, conserved across Eukarya, can be specifically repurposed in flies as a nutrient sensor. By tracing the evolutionary history of Unmet, we find that variable loops in the beta-propellers of GATOR2 can act as adapters to grab sensors from a toolkit of preexisting small-molecule-binding proteins.

These data suggest an evolutionary mechanism in which ancestral enzymes are co-opted as nutrient sensors for the mTORC1 pathway (Figure 13C).

Differences between the fly and human mechanisms of SAM sensing offer additional evidence for this model. To monitor SAM levels, flies have repurposed Unmet to bind the dGATOR2 complex, while humans use SAMTOR and GATOR1-KICSTOR (Gu et al., 2017; Peng et al., 2017; Wolfson et al., 2017). Although both of these SAM sensors have homologs in the other species—that is, human Unmet and fly SAMTOR, respectively—those homologs are not components of the mTORC1 pathway. As neither Unmet nor SAMTOR acts as a nutrient sensor in yeast or in worms, the most parsimonious explanation for these data is that SAM sensing evolved twice—once in flies and once in the vertebrate lineage—with two independent co-option events involving different methyltransferases. While we have highlighted evolvable features on GATOR2, the emergence of KICSTOR as a “glue” between GATOR1 and GATOR2 in vertebrates may add additional surfaces for recruitment of new mTORC1 pathway components (Tafur et al., 2022). Indeed, the evolution of SAMTOR as a sensor in vertebrates coincides with both the retention of a full-length isoform of SAMTOR and the appearance of the KICSTOR complex, suggesting that the combined GATOR1-KICSTOR binding surface is required for co-option of SAMTOR as a nutrient sensor.

Why did Dipterans and vertebrates both converge upon SAM as a metabolic regulator of the mTORC1 pathway? It is not clear what environmental triggers promoted the evolution of Unmet in the fly lineage, but one possibility is a change in diet toward less proteinaceous food sources at the evolutionary branch point between honeybees and Dipterans. The transition from diets of microorganisms or pollen, which have consistently high levels of protein, to blood or rotting fruit, where protein content is lower or variable, may have made it beneficial for Dipterans to sense SAM as a proxy for carbon or methionine (Fink et al., 2011; Steck et al., 2018; Zheng et al., 2014). Another possibility, raised by the mechanism of mTORC1 sensor evolution, is that

SAM sensors may simply be easier to evolve than sensors for other nutrients. If core complexes in the mTORC1 pathway recruit sensors by developing ligand-regulated interactions with existing proteins, SAM sensors may arise more frequently because there are so many methyltransferases available for the pathway to co-opt.

Our work suggests that exaptation—repurposing existing proteins to enhance fitness in a new context—is an underappreciated theme in the evolution of sensory complexity (Brunkard, 2020). Co-option of metabolite-binding enzymes by conserved pathway components serves as an evolutionary shortcut, exchanging the difficult task of evolving a ligand-binding site for the simpler one of evolving a new protein-protein interaction (Tenthorey et al., 2020). In the mTORC1 pathway, this strategy is especially effective due to the modular architecture of GATOR2, which insulates core signaling functions from the fitness costs of evolutionary exploration by placing hotspots for sensor acquisition in separate domains. We speculate that co-option may play a role in other conserved pathways, such as innate immune systems, that evolve receptors for new targets over short evolutionary spans.

Although Unmet offers several tantalizing hints about how living systems customize the mTORC1 pathway, full resolution of the functional organization of the pathway likely awaits the discovery of additional nutrient sensors in diverse organisms. Exploiting evolutionary insights into the mTORC1 pathway may allow us to generate artificial switches or therapeutics that regulate mTORC1 signaling with greater precision. Moreover, sensors initially characterized in other species may even be conserved in humans, expressed in so-far poorly-characterized rare cell types that have specialized metabolic environments or needs.

Methods

Materials

Reagents were obtained from the following sources: HRP-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology; antibody against the FLAG M2 epitope (F1804) from Millipore Sigma; antibody against Raptor (09-217) from EMD Millipore; antibodies against β-actin (4967), phospho-T398 dS6K (9209), Mios (13557), cleaved *Drosophila* Dcp-1 Asp216 (9578), FLAG epitope tag (14793), HA epitope tag (3724), and myc epitope tag (2278) from Cell Signaling Technology; antibody against hu-li tai shao (1B1) from the Developmental Studies Hybridoma Bank (DSHB); antibody against Depdc5 (ab185565) from Abcam; Alexa 488 and 555-conjugated secondary antibodies from Thermo Fisher Scientific. The anti-dS6K antibody was a generous gift from Mary Stewart (North Dakota State University). InstantBlue Coomassie Protein Stain was obtained from Abcam; Anti-FLAG M2 affinity gels, amino acids, SAH, carnosine, sinefungin, thiamine, riboflavin, nicotinic acid, calcium pantothenate, pyridoxine (HCl), biotin, folic acid, choline chloride,myo-inositol, inosine, uridine, methyl 4-hydroxybenzoate, potassium phosphate monobasic, sodium bicarbonate, calcium chloride hexahydrate, copper sulfate pentahydrate, iron sulfate heptahydrate, magnesium sulfate, manganese chloride tetrahydrate, zinc sulfate heptahydrate, glacial acetic acid, sucrose, and propionic acid from Millipore Sigma; DMEM, RPMI, Schneider's Medium, FreeStyle 293 Expression Medium, inactivated fetal serum (IFS), UltraPure Salmon Sperm DNA Solution, Dynabeads M-270 Epoxy, anti-HA magnetic beads from Thermo Fisher Scientific; amino acid-free RPMI and Schneider's media lacking leucine, methionine, threonine, glutamine, phenylalanine, tryptophan from US Biologicals; [^{3}H]-labeled SAM in sterile water (0288) from American Radiolabeled Chemicals, Inc.; SAM (13956) from Cayman Chemical; Effectene transfection reagent from Qiagen; QuickExtract DNA Extraction solution from Lucigen; EDTA-free Complete Protease Cocktail from Roche; Micropropagation Agar-Type II from Caisson Laboratories; rapamycin from LC Laboratories; Vectashield with DAPI from Vector Laboratories.

Cell culture

HEK-293T cells obtained from ATCC (American Type Culture Collection) were cultured in Dulbecco modified Eagle's medium (Thermo Fisher Scientific) with 10% IFS (Thermo Fisher Scientific), 4.5 g/L glucose containing 2 mM GlutaMAX (Thermo Fisher Scientific), 100 IU/mL penicillin, and 100 µg/mL streptomycin. Adherent cell lines were maintained at 37°C and 5% CO2. Suspension-adapted HEK-293T cell lines were grown in FreeStyle 293 Expression Medium (Thermo Fisher Scientific) supplemented with 1% IFS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Suspension cells were grown in a Multitron Pro shaker operating at 37°C, 8% CO2, 80% humidity, and 125 rpm. *Drosophila* S2R+ cells obtained from the Perrimon lab were grown at 25°C in Schneider's medium (Thermo Fisher Scientific) supplemented with 10% IFS (Thermo Fisher Scientific), 100 IU/mL penicillin, and 100 µg/mL streptomycin. For single-cell isolation of S2R+ cells, conditioned Schneider's media was prepared as recommended by the DRSC/TRiP (https://fgr.hms.harvard.edu/single-cell-isolation).

Cell and tissue lysis and immunoprecipitation experiments

For lysis of S2R+ and adherent HEK-293T cells, cells were washed once with ice-cold PBS and then lysed with lysis buffer (1% Triton X-100, 40 mM HEPES pH7.4, 10 mM β-glycerol phosphate, 10 mM sodium pyrophosphate, 2.5 mM magnesium chloride) and 1 tablet of EDTA-free protease inhibitor (Roche) per 25 mL buffer. Lysates were clarified by centrifugation at 21,000 x g at 4°C for 10 min. Dissected *Drosophila* tissues and whole flies were crushed physically utilizing a bead beater in Triton lysis buffer and processed as above.

For anti-FLAG, anti-HA, or anti-myc immunoprecipitations leading to Western blot analyses, either anti-FLAG M2 agarose beads (Millipore Sigma) or anti-HA or anti-myc-coupled magnetic beads (Thermo Fisher Scientific) were used. Beads were washed three times prior to use with Triton lysis buffer and were then incubated with the supernatant of each clarified lysate for 2 hours at 4°C. Following immunoprecipitation, beads were washed three times with Triton lysis buffer supplemented to contain 300 mM NaCl. Immunoprecipitated proteins were denatured by addition of SDS-PAGE sample buffer and boiling at 95°C for 3 minutes and resolved by 8%, 10%, or 4-20% SDS-PAGE before analysis by immunoblotting.

Identification of Unmet by immunoprecipitation followed by mass spectrometry

S2R+ cells expressing FLAG-tagged Mio from a copper-inducible promoter at the endogenous locus were induced with 75 μM $CuSO_4$ treatment for 4 days. To generate anti-FLAG immunoprecipitates for proteomic analysis by mass spectrometry, magnetic beads bound to antibody recognizing the FLAG epitope tag were prepared in-house by coupling Dynabeads M-70 Epoxy (Thermo Fisher Scientific) to FLAG M2 antibody (Millipore Sigma), as previously described(Cristea and Chait, 2011). Cell lysates were prepared as described above and incubated with magnetic FLAG beads for 2 hours at 4°C. Following immunoprecipitation, beads were washed three times in lysis buffer supplemented to contain 300 mM NaCl. Proteins were eluted from the beads with the FLAG peptide (sequence: DYKDDDDK), resolved on 4-12% NuPAGE gels (Thermo Fisher Scientific), and stained with Instant Blue (Abcam). Each gel lane was sliced into 8 pieces, followed by digestion of gel slices overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described in (Sancak et al., 2008).

Transfections

For experiments requiring transfection of DNA into HEK-293T cells, 2 million cells were plated in 10 cm culture dishes. 24 hours later, cells were transfected with the appropriate pRK5-based cDNA expression plasmids using the polyethylenimine method, as previously described (Longo et al., 2013). The total amount of DNA in each transfection was normalized to 5 μg with UltraPure Salmon Sperm DNA solution (Thermo Fisher Scientific). 48 hours following transfection, cells were lysed as described above.

For experiments requiring transfection of DNA into S2R+ cells, 10 million cells were plated in 10 cm culture dishes. Cells were transfected with pGL1 or pGL2 cDNA expression plasmids using Effectene transfection reagent (Qiagen). In brief, cDNA expression plasmids added to 400 μL EC buffer were mixed with Effectene Enhancer (8 μL per 1 μg of cDNA), incubated for 5 minutes at RT, mixed with Effectene Reagent (10 μL per 1 μg cDNA), incubated for 10 minutes at RT, and then dispensed dropwise into culture dishes. 72 hours after transfection and $CuSO_4$ induction (if using a pGL1 MT expression system), cells were lysed as described above.

Amino acid starvation and restimulation of cells in culture

For experiments that required amino acid starvation, cells were washed twice with PBS and incubated in RPMI or Schneider's media lacking the designated amino acids for 90 minutes. To restimulate cells following starvation, an amino acid mixture prepared from individual powders of amino acids (Millipore Sigma) was added to cell culture media for 15 min.

RNAi in *Drosophila* S2R+ cells and analysis of knockdown by qPCR

dsRNA sequences were selected from cell-screening RNAi sequences used by the DRSC. The following primer sequences, including underlined 5' and 3' T7 promoter sequences, were used to amplify DNA templates for dsRNAs targeting GFP, dSesn, Unmet, and dSAMTOR:

F-dsGFP primer:

GAATTAATACGACTCACTATAGGGAGAAGCTGACCCTGAAGTTCATCTG
R-dsGFP primer:
GAATTAATACGACTCACTATAGGGAGATATAGACGTTGTGGCTGTTGTAGTT
F-dsdSesn primer:
GAATTAATACGACTCACTATAGGGAGAGACTACGACTATGGCGAAGTGAA
R-dsdSesn primer:
GAATTAATACGACTCACTATAGGGAGATCAAGTCATATAGCGCATTATCTCG
F-dsUnmet primer:
GAATTAATACGACTCACTATAGGGAGAGCCTCCAATTTTGTCCTCAA
R-dsUnmet primer:
GAATTAATACGACTCACTATAGGGAGAGGGTTCTGTGCGTACTTGGT
F-dsdSAMTOR primer:
GAATTAATACGACTCACTATAGGGAGAAAGAAACGGTAGCGAAATGG
F-dsdSAMTOR primer:
GAATTAATACGACTCACTATAGGGAGAGATGTAGTCGATGGCCCACT

dsRNAs were produced by in vitro transcription of DNA templates using a MEGAshortscript T7 kit (Thermo Fisher Scientific).

On day one, 2 million cells S2R+ were plated into 6-well culture dishes in 1.5 mL of Schneider's media. 24 hours later, cells were transfected with 2 µg of each dsRNA using an Effectene-based system (200 µL EC buffer mixed with 16 µL Effectene Enhancer and 10 µL Effectene reagent). On day four, a second round of dsRNA transfection was performed. On day five, 3 dsRNA-treated million cells were plated in 6-well culture dishes pre-coated with fibronectin. After 12 hours, cells were starved for the indicated amino acids as described above.

To validate knockdown of *unmet*, *dSAMTOR*, and *dSesn*, the following primer pairs were used in qPCR reactions due to the lack of available antibodies against these proteins. *α-tubulin* was used as an internal standard. The data were analyzed by the ΔΔCt method.

F-*α-tubulin*: CAACCAGATGGTCAAGTGCG
R-*α-tubulin*: ACGTCCTTGGGCACAACATC
F-*unmet*: CTCACCTACGAGCTTGCCTG
R-*unmet*: TTGTCGCAGAGGTTGAGGAC
F-*dSAMTOR*: GACCAACGATGGGAAGGTGG
R-*dSAMTOR*: GCTCTGTAGGATTCCAGGAGT
F-*dSesn*: TCCGCTGCCTAACGATTACAG
R-*dSesn*: TTCACCAGATACGGACACTGA

Generation of fly cells expressing endogenously FLAG-tagged proteins
To insert an N-terminal 3x-FLAG epitope tag into the *mio*, *dWDR59*, *Iml1*, *unmet*, and *dSAMTOR* genes in S2R+ cells, we adapted a method described in (Kunzelmann et al., 2016). Homologous recombination donor constructs were generated by PCR amplification of the following primer sequences flanking the template plasmid pRB33 (encoding a constitutively-expressed puromycin resistance marker, a copper-inducible MT promoter, and a 3x FLAG tag). Underlined sequences are complementary to the template plasmid.

mio HR sense:
TGCAAACTGATAACGCGACGCAATTTAGTCTGTAGTGAAAATTGttttttttttACATCGATGGAAAA
TCGGCCACGgaagttcctatactttctagagaataggaacttccatatg
mio HR antisense:

TTCCTGGCCCCAGGATACGAATTTGTCGGGAAAATGTGGAAACCAGCTGAGTCCGTGAGT
GTTGCCGCTCATaccgccgcttggagcagctggaga
dWDR59 HR sense:
TTGTTTGTTGCAAAAATGGTTTAAATTCGCAGTCTTTTGCTTTTTGAGCACTTATTAGAGTAG
GACAATgaagttcctatactttctagagaataggaacttccatatg
dWDR59 HR antisense:
CGGGTGCTCCTGCTCCCGGTCCACCGGCTGTTCCGCGTTCTCCCGGACGCAGAGTCTCC
GTCGGCGGCATaccgccgcttggagcagctggaga
lml1 HR sense:
GCAAATGGGCAAATGTTGGAATTGAGTAAATAATTGTCCGTTGGTTTTGCAACCACTAAGTC
AACgaagttcctatactttctagagaataggaacttccatatg
lml1 HR antisense:
GCAATATCCACTTTCGCTTACCGTAGGATTTGTTGCAGCCCCTCGTATGCGTGTTCAGCTT
GTACAGCTTCATaccgccgcttggagcagctggaga
unmet HR sense:
GATTACTCCCAGGATTTAAATAGCATAGATTATCGTTGAAACCGCTGACGACGCGCCCAGg
aagttcctatactttctagagaataggaacttccatatg
unmet HR antisense:
GGCCAGTTGCTCGTCCATTTTAGGATGCATTGGGAACGTGGCGCAGTCCATGCTGCTCATa
ccgccgcttggagcagctggaga
dSAMTOR HR sense:
TGTCTCATCCCTGCTGCACGCGACCCACCATTTTAGTAACACCGAAGAAACGGTAGCGAAg
aagttcctatactttctagagaataggaacttccatatg
dSAMTOR HR antisense:
CAGGCTTTCGTGGCAGCTCTTCACGATGCTGGCCAGGCGCTGGTGCTCTTCAGTGGCCAT
accgccgcttggagcagctggaga

U6-sgRNA fusion constructs were generated by annealing the following sequences to a U6 promoter and an optimized sgRNA scaffold as previously described (Kunzelmann et al., 2016):

mio: cctattttcaatttaacgtcgCGATGAGCGGCAATACACAgtttaagagctatgctg
dWDR59_01: cctattttcaatttaacgtcgTAGGACAATATGCCGCCCAgtttaagagctatgctg
dWDR59_02: cctattttcaatttaacgtcgGACGCAGTGTCTCCGTGGGgtttaagagctatgctg
lml1_01: cctattttcaatttaacgtcgAGCTGAACACGCATACGCGgtttaagagctatgctg
lml1_02: cctattttcaatttaacgtcgCAGCTTCATGTTGACTTAGgtttaagagctatgctg
Unmet_01: cctattttcaatttaacgtcgCGCGCCCAGATGAGCTCCAgtttaagagctatgctg
Unmet_02: cctattttcaatttaacgtcgGGGAACGTGGCGCAGTCCAgtttaagagctatgctg
dSAMTOR_01: cctattttcaatttaacgtcgGGTAGCGAAATGGCCACGGgtttaagagctatgctg
dSAMTOR_02: cctattttcaatttaacgtcgAACGGTAGCGAAATGGCCAgtttaagagctatgctg

S2R+ cells were transfected with dsRNAs targeting lig4 and mus308 (Kunzelmann et al., 2016) to reduce non-homologous end-joining. 600,000 dsRNA-treated S2R+ cells were then seeded in 24-well culture dishes in 400 µL of Schneider's media. 24 hours later, each well was transfected with the following constructs using the Effectene transfection system (100 µL EC buffer, 6 µL Effectene Enhancer, 7.5 µL Effectene reagent): 250 ng of the U6-sgRNA fusion, 250 ng pRB14 (encoding Cas9), and 250 ng of the homologous recombination donor construct.

24 hours after transfection, cells were induced with 100 µM $CuSO_4$. On day 3 after transfection, cells were split 1:5 and replated in a 6-well dish in fresh media containing 100 µM $CuSO_4$ and 4 µg/mL puromycin. Cells were passaged for up to 2 weeks in puromycin-containing media until control untransfected cells died. Puromycin-resistant cells were then single-cell-sorted into 96-

well plates with 200 µL conditioned media. Plates were sealed with parafilm to reduce evaporation.

After 1 month of culture, individual clones were expanded. To identify clones that had an MT promoter and a 3x-FLAG tag incorporated in the endogenous gene locus, genomic DNA was extracted from each clone using QuickExtract DNA solution (Lucigen) according to manufacturer instructions. The primers indicated below were used to amplify the genomic region surrounding the insertion site:

mio_F: GTGTTTTGCGCAGCATTTTAAGTGG
mio_R: CGACTTTGCCATCCGCCAGA
dWDR59_F: TACAAACTTTTGCGACAAAATATTAGGTACAATTTTT
dWDR59_R: GTACTCTTTGCGACTGGGACATATGG
Iml1_F: GCTGACAGGGAATGCAGATTAAGTTAG
Iml1_R: GAGTACGGACGCATTTTGAAGGCA
Unmet_F: GACCCTCTTACATCCCCGTTT
Unmet_R: ACTAGCCAGATTTGGCGTGATT
dSAMTOR_F: TTATGATAAAACCAGACGGCGGC
dSAMTOR_R: GATTCCAGGAGTCGCTGCTC

Clones were validated by sequencing and by immunoblotting for the FLAG epitope after $CuSO_4$ induction.

To restore endogenous expression of FLAG-dWDR59 and FLAG-Iml1, we transfected copper-inducible clones with 250 ng of FLP recombinase (pKF295) to flip out the puromycin resistance cassette and the MT promoter, which are flanked by FRT sites (Kunzelmann et al., 2016). Single-cell clones with tagged protein expression under the control of the endogenous promoter were validated by sequencing and by immunoblotting for the FLAG epitope in the absence of copper.

Generation of inducible and constitutive fly cell expression vectors

Copper-inducible pGL1 fly expression vectors for N-terminal FLAG- and HA-tagged cDNAs were generated by using EcoRI and XhoI restriction sites to insert the tag and SalI/NotI restriction sites from pRK5-FLAG or pRK5-HA vectors into a pMT-V5-His backbone (Life Technologies), followed by mutation of 2070C>A to remove a SalI site in the backbone. Constitutive pGL2 expression vectors for N-terminal FLAG- and HA- tagged cDNAs were generated by replacing the MT promoter in pGL1 with a copia promoter using Gibson assembly.

In vitro Unmet-dGATOR2 dissociation assay

HEK-293T cells were transiently co-transfected with the following pRK5-based cDNA expression vectors: 50 ng FLAG-dWDR59, 50 ng myc-dWDR24, 50 ng myc-Mio, 50 ng myc-Nup44A, 50 ng dSec13, and 5 ng HA-Unmet. 48 hours after transfection, cells were subjected to anti-FLAG immunoprecipitations as described above. The dGATOR2-Unmet complexes immobilized on FLAG beads were washed twice in lysis buffer containing 300 mM NaCl and then incubated for 30 min. in 300 µL of cytosolic buffer (0.1% Triton, 40 mM HEPES pH 7.4, 10 mM NaCl, 150 mM KCl, 2.5 mM $MgCl_2$) with the indicated concentrations of SAM, SAH, sinefungin, or carnosine at 4°C. The amount of Unmet that remained bound to dGATOR2 was assayed by SDS-PAGE and immunoblotting as described previously.

Unmet protein expression and purification

To purify Rap2A and Unmet for radiolabeled SAM-binding assays, suspension-adapted HEK-293T cells grown in FreeStyle 293 Expression Medium (Thermo Fisher) supplemented with 1% IFS were transiently transfected with cDNAs encoding FLAG-tagged Rap2A or FLAG-tagged wild-type, G195D mutant, or E30A mutant Unmet on the pRK5 vector. Cells were transfected at a density of 800,000 cells/mL using 600 µg cDNA and 1.8 µg polyethylenimine per 500 mL culture. 48 hours after transfection, cells were harvested, washed in ice cold PBS, and lysed in Triton lysis buffer, as described above. Lysates were cleared by centrifugation at 40,000 x g for 20 min. and incubated with pre-washed anti-FLAG M2 affinity gel (300 µL slurry per 500 mL culture) for 2 hours at 4°C. Beads were washed once in Triton lysis buffer, twice in Triton lysis buffer supplemented with 300 mM NaCl, and once in CHAPS buffer (0.1% CHAPS, 50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$). Proteins were eluted from the beads with 0.5 mg/mL FLAG peptide in CHAPS buffer for 2 hours and concentrated with 10 kDa (for Rap2A) or 30 kDa (for Unmet) MWCO centrifugal filters (Millipore Sigma). Further purification was performed by size-exclusion chromatography on a Superose6 10/300 column (Cytiva) pre-equilibrated in CHAPS buffer supplemented with 2 mM DTT. Elution fractions were resolved by SDS-PAGE and stained with InstantBlue Coomassie Protein Stain (Abcam). Pure protein fractions were pooled and concentrated, supplemented with 10% glycerol, and snap frozen in liquid nitrogen before storage at -80°C.

Radioactive SAM-binding assay

Radioactive SAM-binding assays were performed as previously reported (Gu et al., 2017). Briefly, pre-blocked anti-FLAG M2 agarose beads (Millipore Sigma) were incubated with purified proteins (30 µL bead slurry and 10 µg protein per condition) to allow for rebinding of the proteins. The beads were then washed and incubated for 1 hour on ice in cytosolic buffer with 5 µM [^{3}H]-labeled SAM and the indicated concentrations of unlabeled SAM, SAH, SFG, or carnosine. After this incubation, beads were aspirated dry, rapidly washed four times with binding wash buffer (cytosolic buffer supplemented with 300 mM NaCl), and resuspended in 80 µL cytosolic buffer. 15 µL aliquots from each sample were quantified using a TriCarb scintillation counter (Perkin Elmer). The SAM-binding capacity of Rap2A, wild-type Unmet, Unmet G195D, and Unmet E30A were assayed in the same experiment.

K_d calculations

The affinity of Unmet for SAM was determined by normalizing the bound [^{3}H]-labeled SAM concentrations across three separate binding assays performed with varying amounts of unlabeled SAM. These values were plotted and fit to a hyperbolic equation (the Cheng-Prusoff equation) to estimate the IC_{50} value. K_d values were derived from the IC_{50} value using the equation: $K_d = IC_{50} / (1+([^3H]SAM/K_d))$.

Generation of fly cells stably expressing Unmet mutant cDNAs

For stable expression of the E30A and G195D mutants of Unmet, an N-terminal 3x-FLAG tag sequence and cDNAs encoding the indicated Unmet mutants were cloned into the pAc5-STABLE2 vector by Gibson assembly. pAc5-STABLE2 contains an mCherry cassette followed by a T2A site, followed by an eGFP cassette, a second T2A site, and a neomycin (G418) resistance cassette (González et al., 2011). Tagged Unmet mutant cDNA replaced the mCherry cassette.

3 million S2R+ cells expressing copper-inducible FLAG-Unmet from the endogenous locus were plated in 6-well culture dishes and transfected with 1 µg of the stable expression vector using Effectene, as described above. 24 hours after transfection, cells were transferred into Schneider's media containing 1 mg/mL G418 (Thermo Fisher Scientific) and passaged for 3-4 weeks until control untransfected cells died. Because G418 selection is often incomplete in

S2R+ cells, the selected population was sorted by GFP intensity via FACS to generate a stable pool of cells expressing the mutant Unmet proteins at roughly comparable levels. To prevent silencing or changes in expression, stable pools expressing Unmet mutant cDNAs were used in dTORC1 signaling experiments within 2 weeks of isolation by FACS.

Fly stocks, diets, and husbandry

All flies were reared at 25°C and 60% humidity with a 12 hours on/off light cycle on standard lab food (12.7 g/L deactivated yeast, 7.3 g/L soy flour, 53.5 g/L cornmeal, 0.4 % agar, 4.2 g/L malt, 5.6 % corn syrup, 0.3 % propionic acid, 1% tegosept/ethanol).

Synthetic food was formulated and prepared as previously described (Piper et al., 2014). For food containing 10 μM rapamycin, a 20 mM stock solution of rapamycin in ethanol was diluted 2000-fold in freshly prepared food before the agar hardened.

Generation and validation of *unmet*$^{-/-}$ and *dSAMTOR*$^{-/-}$ fly lines

unmet$^{-/-}$ and *dSAMTOR*$^{-/-}$ flies were generated with CRISPR-Cas9-mediated deletion of the gene loci. Two sgRNAs with cutting sites bracketing each gene locus were cloned into the pCFD3 expression vector using the following oligonucleotide sequences (Housden et al., 2014):

unmet guide 1:
sense: GTCGCCGAACCTTCGTCATCAACG
antisense: AAACCGTTGATGACGAAGGTTCGG

unmet guide 2:
sense: GTCGTTGGACTTGATTGTGGTGTT
antisense: AAACAACACCACAATCAAGTCCAA

dSAMTOR guide 1:
sense: GTCGAAGCCTGCGCCAGTTGACTA
antisense: AAACTAGTCAACTGGCGCAGGCTT

dSAMTOR guide 2:
sense: GTCGCTTATCTAGCTATCGTCCTG
antisense: AAACCAGGACGATAGCTAGATAAG

For each gene, both pCFD3-sgRNAs were microinjected into *y,sc,v; nos-Cas9* embryos, and emerging adults were crossed to *Lethal/FM7* (for *unmet*$^{-/-}$) or *Lethal/CyO* (for *dSAMTOR*$^{-/-}$). Progeny were screened by PCR for deletion of the whole locus using the following primers:

unmet:
F: CAGTGTAACCAGATCTAAAGTGGCGACT
R: GAGCGAGAAATTGTCCTAAAATTTGCATCC

dSAMTOR:
F: TGAATATTGGTTCTGAACGGTAAACTCGC
R: GCAATAGCATTTGTCCATTTACGACATCC

Individual *y,sc,v; unmet*$^{-/-}$ stocks were established along with *y,sc,v;* + control lines that followed the same cross scheme. Mutant stocks were sequence-verified using the primers above. To verify that *unmet*$^{-/-}$ flies no longer expressed *unmet* mRNA, total RNA was extracted from homogenized flies with TRIzol (Thermo Fisher Scientific). qPCR was performed on synthesized

cDNA using a QuantStudio6 RT-PCR system (Applied Biosystems). Relative expression levels were quantified by the ΔΔCt method using the qPCR primers described above. α-tubulin served as an internal standard.

Ovarian staining and immunofluorescence assays

To assess cell death in ovaries, 5-day old age-synchronized, mated flies (20 females, 3 males) were flipped into vials of chemically-defined diets and maintained on those diets for 1 or 5 days. Flies were transferred to fresh vials every 2 days. Ovaries were dissected in ice-cold PBS, fixed for 20 minutes with 4% paraformaldehyde at room temperature, and washed three times in PBS supplemented with 0.3% Triton X-100 (0.3% PBST) for 10 minutes each. Samples were then blocked for 30 min. (PBST, 5% BSA, 2% FBS, 0.02% NaN_3) and incubated in blocking buffer with primary antibodies overnight at 4°C. Primary antibodies were used at the following concentrations for immunostaining: mouse anti-hts (1B1, DHSB) at 1:50, rabbit anti-cleaved Dcp-1(Cell Signaling Technology) at 1:100. Ovaries were washed four times with PBST for 15 min. and treated with Alexa 488 and 555-conjugated secondary antibodies diluted 1:400 in blocking buffer for 1 hour at room temperature. After secondary antibody treatment, tissues were washed four times with PBST for 15 min. before mounting in Vectashield containing DAPI (Vector Laboratories).

Ovarian images were acquired on a Zeiss LSM 710 laser-scanning confocal microscope using a 20x objective. The Zeiss ZEN software package was used to control the hardware and image acquisition. Images were captured with the 405 nm, 488 nm, and 561 nm excitation lasers.

Statistical analyses

Two-tailed t-tests were used for comparison between two groups. All comparisons were two-sided, and p-values of less than 0.05 were considered to indicate statistical significance. For comparisons with two categorical factors (e.g. ovarian degeneration in flies of different genotypes on different diets), two-way ANOVAs were used to evaluate whether the interaction term between the factors was significant, followed by post-hoc analysis with Tukey-Kramer multiple comparison tests. For Tukey-Kramer multiple comparison tests, adjusted p-values of less than 0.05 were considered to indicate statistical significance.

Construction of phylogenetic trees

Homologs of mTOR, Unmet (CARNMT1), WDR24, Mios, and Seh1L were drawn from the OMA Orthology Database, supplemented with sequences manually curated from BLASTp searches seeded by the *Drosophila melanogaster* protein sequences. Protein sequences from *Drosophila melanogaster*, *Drosophila simulans*, *Drosophila elegans*, *Drosophila busckii*, *Lucilia cuprina*, *Aedes aegypti*, *Apis mellifera*, *Daphnia pulex*, *Branchiostoma floridae*, *Callorhinchus milii*, *Homo sapiens*, and *Schizosaccharomyces pombe* were aligned using ClustalO. Maximum likelihood trees were constructed from protein alignments using RAxML-NG (Kozlov et al., 2019) with a bootstrapping cutoff of 0.03. Trees were visualized in Dendroscope 3.8.4.

Data availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

References

Alves, A.N., Sgrò, C.M., Piper, M.D.W., and Mirth, C.K. (2022). Target of Rapamycin Drives Unequal Responses to Essential Amino Acid Depletion for Egg Laying in Drosophila Melanogaster. In Front Cell Dev Biol, pp. 822685.

Bar-Peled, L., Chantranupong, L., Cherniack, A.D., Chen, W.W., Ottina, K.A., Grabiner, B.C., Spear, E.D., Carter, S.L., Meyerson, M., and Sabatini, D.M. (2013). A tumor suppressor complex with GAP activity for the Rag GTPases that signal amino acid sufficiency to mTORC1. Science *340*, 1100-1106.

Bridgham, J.T., Carroll, S.M., and Thornton, J.W. (2006). Evolution of Hormone-Receptor Complexity by Molecular Exploitation. Science *312*, 97-101.

Brunkard, J.O. (2020). Exaptive evolution of Target of Rapamycin signaling in multicellular eukaryotes. Developmental Cell *54*, 142-155.

Cao, R., Zhang, X., Liu, X., Li, Y., and Li, H. (2018). Molecular basis for histidine N1 position-specific methylation by CARNMT1. Cell Research *28*, 494-496.

Chantranupong, L., Scaria, S.M., Saxton, R.A., Gygi, M.P., Shen, K., Wyant, G.A., Wang, T., Harper, J.W., Gygi, S.P., and Sabatini, D.M. (2016). The CASTOR proteins are arginine sensors for the mTORC1 pathway. Cell *165*, 153-164.

Condon, K.J., and Sabatini, D.M. (2019). Nutrient regulation of mTORC1 at a glance. Journal of Cell Science *132*.

Coyle, S.M., Flores, J., and Lim, W.A. (2013). Exploitation of latent allostery enables the evolution of new modes of MAP kinase regulation. Cell *154*, 875-887.

Cristea, I.M., and Chait, B.T. (2011). Conjugation of magnetic beads for immunopurification of protein complexes. Cold Spring Harb Protoc *2011*, pdb.prot5610.

Drozak, J., Piecuch, M., Poleszak, O., Kozlowski, P., Chrobok, L., Baelde, H.J., and de Heer, E. (2015). UPF0586 protein C9orf41 homolog is anserine-producing methyltransferase. Journal of Biological Chemistry *290*, 17190-17205.

Drummond-Barbosa, D., and Spradling, A.C. (2001). Stem cells and their progeny respond to nutritional changes during Drosophila oogenesis. Developmental Biology *231*, 265-278.

Fink, P., Pflitsch, C., and Marin, K. (2011). Dietary Essential Amino Acids Affect the Reproduction of the Keystone Herbivore Daphnia pulex. PLOS ONE *6*, e28498.

Fowler, D.M., Araya, C.L., Fleishman, S.J., Kellogg, E.H., Stephany, J.J., Baker, D., and Fields, S. (2010). High-resolution mapping of protein sequence-function relationships. Nature Methods *7*, 741-746.

González, A., and Hall, M.N. (2017). Nutrient sensing and TOR signaling in yeast and mammals. The EMBO Journal *36*, 397-408.

González, M., Martín-Ruíz, I., Jiménez, S., Pirone, L., Barrio, R., and Sutherland, J.D. (2011). Generation of stable Drosophila cell lines using multicistronic vectors. Sci Rep *1*, 75-75.

Gracheva, E.O., Ingolia, N.T., Kelly, Y.M., Cordero-Morales, J.F., Hollopeter, G., Chesler, A.T., Sánchez, E.E., Perez, J.C., Weissman, J.S., and Julius, D. (2010). Molecular basis of infrared detection by snakes. Nature *464*, 1006-1011.

Grandison, R.C., Piper, M.D.W., and Partridge, L. (2009). Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. Nature *462*, 1061-1064.

Gu, X., Orozco, J.M., Saxton, R.A., Condon, K.J., Liu, G.Y., Krawczyk, P.A., Scaria, S.M., Harper, J.W., Gygi, S.P., and Sabatini, D.M. (2017). SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. Science *358*, 813-818.

Housden, B.E., Lin, S., and Perrimon, N. (2014). Cas9-based genome editing in Drosophila. Methods Enzymol *546*, 415-439.

Julius, D., and Nathans, J. (2012). Signaling by sensory receptors. Cold Spring Harb Perspect Biol *4*, a005991.

Kim, J., and Guan, K.-L. (2019). mTOR as a central hub of nutrient signalling and cell growth. Nature Cell Biology *21*, 63-71.
Kozlov, A.M., Darriba, D., Flouri, T., Morel, B., and Stamatakis, A. (2019). RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics *35*, 4453-4455.
Kunzelmann, S., Böttcher, R., Schmidts, I., and Förstemann, K. (2016). A comprehensive toolbox for genome editing in cultured Drosophila melanogaster cells. G3: Genes|Genomes|Genetics *6*, 1777-1785.
LaFever, L., and Drummond-Barbosa, D. (2005). Direct control of germline stem cell division and cyst growth by neural insulin in Drosophila. Science *309*, 1071-1073.
Lebo, D.P.V., and McCall, K. (2021). Murder on the ovarian express: a tale of non-autonomous cell death in the Drosophila ovary. Cells *10*, 1454.
Lee, B.C., Kaya, A., Ma, S., Kim, G., Gerashchenko, M.V., Yim, S.H., Hu, Z., Harshman, L.G., and Gladyshev, V.N. (2014). Methionine restriction extends lifespan of Drosophila melanogaster under conditions of low amino-acid status. Nat Commun *5*, 3592-3592.
Leulier, F., and Lemaitre, B. (2008). Toll-like receptors — taking an evolutionary approach. Nature Reviews Genetics *9*, 165-178.
Li, H., Janssens, J., De Waegeneer, M., Kolluru, S.S., Davie, K., Gardeux, V., Saelens, W., David, F., Brbić, M., Leskovec, J.*, et al.* (2021). Fly Cell Atlas: a single-cell transcriptomic atlas of the adult fruit fly. bioRxiv, 2021.2007.2004.451050.
Liu, G.Y., and Sabatini, D.M. (2020). mTOR at the nexus of nutrition, growth, ageing and disease. Nature Reviews Molecular Cell Biology *21*, 183-203.
Longo, P.A., Kavran, J.M., Kim, M.-S., and Leahy, D.J. (2013). Transient mammalian cell transfection with polyethylenimine (PEI). In Methods in Enzymology, J. Lorsch, ed. (Academic Press), pp. 227-240.
Melick, C.H., and Jewell, J.L. (2020). Regulation of mTORC1 by upstream stimuli. Genes *11*, 989.
Nei, M., and Rooney, A.P. (2005). Concerted and Birth-and-Death Evolution of Multigene Families. Annual Review of Genetics *39*, 121-152.
Oteiza, P., and Baldwin, M.W. (2021). Evolution of sensory systems. Current Opinion in Neurobiology *71*, 52-59.
Peng, M., Yin, N., and Li, M.O. (2017). SZT2 dictates GATOR control of mTORC1 signalling. Nature *543*, 433-437.
Piper, M.D.W., Blanc, E., Leitão-Gonçalves, R., Yang, M., He, X., Linford, N.J., Hoddinott, M.P., Hopfen, C., Soultoukis, G.A., Niemeyer, C.*, et al.* (2014). A holidic medium for Drosophila melanogaster. Nature Methods *11*, 100-105.
Sancak, Y., Peterson, T.R., Shaul, Y.D., Lindquist, R.A., Thoreen, C.C., Bar-Peled, L., and Sabatini, D.M. (2008). The Rag GTPases bind Raptor and mediate amino acid signaling to mTORC1. Science *320*, 1496-1501.
Saxton, R.A., Chantranupong, L., Knockenhauer, K.E., Schwartz, T.U., and Sabatini, D.M. (2016a). Mechanism of arginine sensing by CASTOR1 upstream of mTORC1. Nature *536*, 229-233.
Saxton, R.A., Knockenhauer, K.E., Wolfson, R.L., Chantranupong, L., Pacold, M.E., Wang, T., Schwartz, T.U., and Sabatini, D.M. (2016b). Structural basis for leucine sensing by the Sestrin2-mTORC1 pathway. Science *351*, 53-58.
Shimada, Y., Burn, K.M., Niwa, R., and Cooley, L. (2011). Reversible response of protein localization and microtubule organization to nutrient stress during Drosophila early oogenesis. Developmental Biology *355*, 250-262.

Shiotani, S., Yanai, N., Suzuki, T., Tujioka, S., Sakano, Y., Yamakawa-Kobayashi, K., and Kayashima, Y. (2013). Effect of a dipeptide-enriched diet in an adult Drosophila melanogaster laboratory strain. Bioscience, Biotechnology, and Biochemistry *77*, 836-838.
Slavik, K.M., Morehouse, B.R., Ragucci, A.E., Zhou, W., Ai, X., Chen, Y., Li, L., Wei, Z., Bähre, H., König, M.*, et al.* (2021). cGAS-like receptors sense RNA and control 3′2′-cGAMP signalling in Drosophila. Nature *597*, 109-113.
Steck, K., Walker, S.J., Itskov, P.M., Baltazar, C., Moreira, J.M., and Ribeiro, C. (2018). Internal amino acid state modulates yeast taste neurons to support protein homeostasis in Drosophila. Elife *7*.
Tafur, L., Hinterndorfer, K., Gabus, C., Lamanna, C., Bergmann, A., Sadian, Y., Hamdi, F., Kyrilis, F.L., Kastritis, P.L., and Loewith, R. (2022). Cryo-EM structure of the SEA complex. Nature.
Tang, X., Zhang, Y., Wang, G., Zhang, C., Wang, F., Shi, J., Zhang, T., and Ding, J. (2022). Molecular mechanism of *S*-adenosylmethionine sensing by SAMTOR in mTORC1 signaling. Science Advances *8*, eabn3868.
Tenthorey, J.L., Young, C., Sodeinde, A., Emerman, M., and Malik, H.S. (2020). Mutational resilience of antiviral restriction favors primate TRIM5α in host-virus evolutionary arms races. eLife *9*, e59988.
Valenstein, M.L., Rogala, K.B., Lalgudi, P.V., Brignole, E.J., Gu, X., Saxton, R.A., Chantranupong, L., Kolibius, J., Quast, J.-P., and Sabatini, D.M. (2022). Structure of the nutrient-sensing hub GATOR2. Nature *607*, 610-616.
Valvezan, A.J., and Manning, B.D. (2019). Molecular logic of mTORC1 signalling as a metabolic rheostat. Nature Metabolism *1*, 321-333.
Wang, S., Tsun, Z.-Y., Wolfson, R.L., Shen, K., Wyant, G.A., Plovanich, M.E., Yuan, E.D., Jones, T.D., Chantranupong, L., Comb, W.*, et al.* (2015). Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1. Science *347*, 188-194.
Wei, Y., and Lilly, M.A. (2014). The TORC1 inhibitors Nprl2 and Nprl3 mediate an adaptive response to amino-acid starvation in Drosophila. Cell Death & Differentiation *21*, 1460-1468.
Wolfson, R.L., Chantranupong, L., Saxton, R.A., Shen, K., Scaria, S.M., Cantor, J.R., and Sabatini, D.M. (2016). Sestrin2 is a leucine sensor for the mTORC1 pathway. Science *351*, 43-48.
Wolfson, R.L., Chantranupong, L., Wyant, G.A., Gu, X., Orozco, J.M., Shen, K., Condon, K.J., Petri, S., Kedir, J., Scaria, S.M.*, et al.* (2017). KICSTOR recruits GATOR1 to the lysosome and is necessary for nutrients to regulate mTORC1. Nature *543*, 438-442.
Wolfson, R.L., and Sabatini, D.M. (2017). The dawn of the age of amino acid sensors for the mTORC1 pathway. Cell Metabolism *26*, 301-309.
Zheng, B., Wu, Z., and Xu, B. (2014). The effects of dietary protein levels on the population growth, performance, and physiology of honey bee workers during early spring. J Insect Sci *14*, 191.

Acknowledgments:

We thank R.A. Weinberg and H.S. Malik for helpful discussions and critical reading of the manuscript. We gratefully acknowledge all members of the Sabatini and Perrimon Laboratories for their insights. In particular, we thank K.J. Condon and J.M. Roberts for experimental discussions and advice; M.L. Valenstein for assistance with protein purification and insights into GATOR2; B. Ewen-Campbell for advice about phylogenetic tree construction, and E. Spooner for mass spectrometric analysis of proteomics samples. Figures 8D and 8H were created using Biorender.com. This work was supported by grants from the NIH (R01 CA103866, R01 CA129105, and R01 AI47389 to D.M.S.; 5P01 CA120964-04 and R01 AR057352 to N.P.; T32 GM007287 and F31 CA232340 to G.Y.L.), the Lustgarten Foundation to D.M.S., and the Cystinosis Research Foundation to P.J. and N.P. N.P. is an Investigator of the Howard Hughes Medical Institute.

Author contributions: G.Y.L. and D.M.S. conceived the study and formulated the research plan. G.Y.L. and D.M.S. interpreted experimental results with input from P.J. and N.P. G.Y.L. designed and performed biochemical experiments with assistance from R.E.B. P.J. generated the *unmet*$^{-/-}$ and *dSAMTOR*$^{-/-}$ fly strains with assistance from G.Y.L. P.J. performed larval fat body signaling experiments, while G.Y.L. performed phenotypic characterization of *unmet*$^{-/-}$ flies. G.Y.L. and D.M.S. wrote the manuscript. G.Y.L., P.J., N.P., and D.M.S. edited the manuscript.

Competing interests: Authors declare that they have no competing interests.

Materials & correspondence: The data and reagents that support the findings of this study are available from the authors and the Whitehead Institute (sabadmin@wi.mit.edu) upon request. Plasmids generated in this study are available on Addgene.

Chapter 3: Summary and Future Directions

A. Summary

As a master regulator of growth and metabolism, the mTORC1 pathway toggles the balance between anabolism and catabolism in response to environmental signals. These signals include amino acid levels, which license mTORC1 activity when nutrients are plentiful. Over the past fifteen years, we and others have identified more than 20 proteins that relay nutrient signals within the pathway, including three mammalian nutrient sensors that bind directly to leucine, arginine, and *S*-adenosylmethionine[1-4]. Despite our progress in cataloguing these factors, it is not known how the mTORC1 pathway evolved its elaborate nutrient sensing machinery, nor is it clear whether the same proteins regulate mTORC1 signaling in different species.

Contributions of this thesis

In this thesis, we identify the first unique non-mammalian nutrient sensor and leverage our discovery to understand how the mTORC1 pathway integrates new inputs. Unmet, a fly-specific *S*-adenosylmethionine (SAM) sensor, is an evolutionary "missing link": a preexisting enzyme co-opted by the mTORC1 pathway. In vivo, loss of this sensor leads to profound, methionine-dependent defects in the female germline, offering a tantalizing hint that tissue-specific enrichment of the sensor may reflect local demand for a nutrient and a tissue-specific role for mTORC1.

By tracing the incorporation of Unmet into the mTORC1 pathway, we identify GATOR2—an enigmatic signaling hub upstream of mTORC1—as a platform for the evolution of new nutrient sensors. We show that variable loops extending from the beta-propellers of GATOR2 underwrite the evolvability of the mTORC1 nutrient sensing pathway and repurpose ancestral enzymes as allosteric regulators of the pathway. This mechanism for sensor acquisition offers us a blueprint for building artificial sensors into the mTORC1 pathway. Exploiting differences

between methionine sensing in flies and vertebrates, we rewire the mTORC1 pathway by enforcing a ligand-responsive interaction between the core machinery of the mTORC1 pathway and a metabolite-binding protein. Our insight into the modular architecture of the pathway suggests a design strategy that can be used to engineer artificial switches to regulate mTORC1 activity with much greater precision.

Our work suggests that exaptation—repurposing existing proteins to enhance fitness in a new context—is a recurrent theme in the evolution of sensory complexity[5]. For the mTORC1 pathway, which must evolve new sensors quickly enough to keep up with dietary adaptations, co-option of metabolite-binding enzymes by conserved pathway components serves as an evolutionary shortcut, exchanging the difficult task of evolving a ligand-binding site for the simpler one of evolving a new protein-protein interaction[6,7]. Interestingly, GATOR2 may itself be a product of exaptive evolution. Recent structural studies show that the cage-like scaffold of the GATOR2 complex uses coatomer motifs similar to those seen in COPII coats and the nuclear pore[8,9], suggesting that core nodes of the mTORC1 pathway—in addition to the accessory nutrient sensors—also derive from reuse and co-option of preexisting proteins.

B. Future Directions and Discussion

While the work presented here sheds new light on the structure and logic of the nutrient sensing pathway, several aspects of mTORC1 sensor evolution remain mysterious. At a molecular level, what residues define the compound Unmet-binding site on GATOR2? Building on the in vivo characterization of Unmet in the fly ovary, does differential sensor expression in cells or tissues reflect the biological importance of the corresponding nutrient in that tissue or niche? Which selection pressures led to acquisition and retention of nutrient sensors in particular organisms? Moreover, what is the role of Unmet in humans or other organisms where it does not appear to function as a nutrient sensor? We discuss some of these questions in greater detail below.

Molecular basis of Unmet sensor acquisition

Drawing upon biochemical and phylogenetic evidence, we identify residues on the WDR beta-propellers of GATOR2—especially those on Mio and WDR24—as critical sites for Unmet binding. However, these residues do not cohere into a single interaction surface; in fact, a recent structure of the human GATOR2 complex shows the WDR domains of Mios and WDR24 splayed nearly 90 angstroms apart[8]. Computational structure predictions using AlphaFold fail to generate a plausible binding surface between Unmet and the *Drosophila* Mio/WDR24/Nup44A propellers[10]. AlphaFold may struggle with this task because the stoichiometry is unclear and because Unmet likely binds to a compound interface, with contact sites that span multiple proteins. AlphaFold's reliance on multiple sequence alignments also makes it more challenging to identify interaction surfaces when the interaction emerges so late in insect evolution.

Obtaining a co-structure of fly GATOR2 bound to Unmet would allow us to understand how GATOR2 assimilated a new sensor in mechanistic detail. We speculate that structural transformations in the GATOR2 complex during signaling would place the Mio and WDR24 propellers in close apposition, perhaps by flipping the WDR24 propeller inward toward the central Mio-Mio brace on the GATOR2 scaffold. Indeed, GATOR1-Rag signaling mechanisms suggest that large-scale rearrangements occur during mTORC1 signal transduction. Recent cryo-EM structures of GATOR1 show that the Rag-GTPases flip from one side of the complex to another upon nutrient starvation, perhaps because GATOR2 orients GATOR1 differently with respect to the lysosomal surface[11,12]. Although we do not yet know the function of GATOR2, Unmet binding may regulate mTORC1 signaling by shifting the balance between active and inactive conformations of GATOR2.

Discovery of additional niche-specific nutrient sensors

Our identification of Unmet reveals that nutrient sensors derive from ancestral enzymes. Both Unmet and SAMTOR were methyltransferases before their co-option by the mTORC1

pathway; meanwhile, distant homology to bacterial enzymes suggests that Sestrin and CASTOR descend, respectively, from an alkyl hydroperoxide reductase and an aspartate kinase[13,14]. Because the architecture of the mTORC1 pathway allows it to rapidly swap in new sensors under environmental pressure, we predict that other niche-specific nutrient sensors exist across evolution.

For example, we show that the *D. melanogaster* TORC1 pathway acutely senses not just leucine and *S*-adenosylmethionine but also—directly or indirectly—the amino acids threonine, glutamine, phenylalanine, and tryptophan. These amino acids do not regulate the human mTORC1 pathway, and their direct sensors are unknown. Given that genetic CRISPR screens have comprehensively identified nutrient-dependent regulators of mTORC1 signaling in human cells, similar efforts in fly cells could reveal the identity of multiple new sensors[15-17]. We therefore propose a screen in fly S2R+ cells that stably express the human S6 protein. Because S6 phosphorylation is regulated by mTORC1 kinase activity, we can use a FACS-compatible phospho-S6 antibody to identify genetic lesions that render the mTORC1 pathway insensitive to threonine, glutamine, phenylalanine, or tryptophan starvation. One advantage of this approach over an analogous screen in human cells is that the more compact fly genome contains fewer paralogs that might mask the effect of individual gene knockouts.

Further afield, in prototrophic species that can synthesize all twenty amino acids, are there nutrient sensors for other metabolites? Is there a nitrogen sensor in yeast? Full understanding of the mTORC1 pathway likely awaits the discovery of additional nutrient sensors in diverse organisms. Sensors initially characterized in other species may even be conserved in humans, expressed in so-far poorly-characterized rare cell types that have specialized metabolic environments or needs.

Tissue-specific roles of mTORC1 nutrient sensing

Unmet expression is highly enriched in young germ cells of the fly ovary. These cells are uniquely sensitive to damage upon nutrient starvation, and aberrant mTORC1 activity in early egg chambers can drive a mismatch between protein availability and protein demand, leading to a lasting collapse in egg production[18]. It is possible that the sensor is differentially expressed in a cell type where its cognate metabolite is under heavy demand. For example, methionine supplementation alone is sufficient to restore fecundity in flies during dietary restriction, indicating that methionine may be a limiting nutrient for ovarian function[19]. Moreover, germline development in flies is tightly regulated by epigenetic modifications, and the SAM pool may be depleted during egg production due to the need to methylate nucleic acids and protein.

Interestingly, the emergence of Unmet as a novel sensor in insects appears to coincide with a change in diet toward less proteinaceous food sources. At the evolutionary branch point between honeybees and Dipterans, insects may transition from diets of microorganisms or pollen, which have consistently high levels of protein, to blood or rotting fruit, where protein content is lower or variable[20-22]. Acquisition of Unmet may therefore have conferred selective advantages by allowing Dipterans to use a newly limiting nutrient to gate reproduction.

These observations buttress an emerging theme in mTORC1 nutrient sensing, in which sensors may be spatially segregated within tissues to match local nutrient gradients or to mediate tissue-specific functions of mTORC1. A recent study shows that Sestrin expression is compartmentalized in the liver zonules of mice to enable zone-specific induction of FGF21 hormone production downstream of mTORC1[23]. Sestrin is also expressed in esophagus-adjacent glia in flies to activate mTORC1 in the brain upon dietary consumption of leucine[24]. Within the fly ovary, Sestrin is poorly expressed in the young germ cells that express Unmet, suggesting that specific sensors may be preferentially enriched in different cell types[25]. This compartmentalization of the nutrient sensors may also explain, in part, why animals have

evolved so many different sensors for amino acids when any individual amino acid would probably suffice as a proxy for protein availability.

If the nutrient sensors play tissue-specific roles, they may allow us to pharmacologically modulate mTORC1 signaling in a tissue-specific manner. To date, efforts to restrict the effects of mTORC1 inhibitors to specific organs have largely relied upon differential drug permeability[26]. The nutrient sensors may offer an alternative route to tissue-specific mTORC1 inhibition, especially because they already possess druggable small-molecule binding clefts. Rational design of novel, tissue- and complex-specific mTORC1 therapeutics may allow us to avoid some of the metabolic side effects of existing mTOR inhibitors and transform our treatment of metabolic disorders, cancers, neurodegeneration, and aging.

References

1 Liu, G. Y. & Sabatini, D. M. mTOR at the nexus of nutrition, growth, ageing and disease. *Nature Reviews Molecular Cell Biology* **21**, 183-203, doi:10.1038/s41580-019-0199-y (2020).

2 Chantranupong, L. *et al.* The CASTOR proteins are arginine sensors for the mTORC1 pathway. *Cell* **165**, 153-164, doi:10.1016/j.cell.2016.02.035 (2016).

3 Gu, X. *et al.* SAMTOR is an S-adenosylmethionine sensor for the mTORC1 pathway. *Science* **358**, 813-818, doi:10.1126/science.aao3265 (2017).

4 Wolfson, R. L. *et al.* Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science* **351**, 43-48, doi:10.1126/science.aab2674 (2016).

5 Brunkard, J. O. Exaptive evolution of Target of Rapamycin signaling in multicellular eukaryotes. *Developmental Cell* **54**, 142-155, doi:10.1016/j.devcel.2020.06.022 (2020).

6 Tenthorey, J. L., Young, C., Sodeinde, A., Emerman, M. & Malik, H. S. Mutational resilience of antiviral restriction favors primate TRIM5α in host-virus evolutionary arms races. *eLife* **9**, e59988, doi:10.7554/eLife.59988 (2020).

7 Fowler, D. M. *et al.* High-resolution mapping of protein sequence-function relationships. *Nature Methods* **7**, 741-746, doi:10.1038/nmeth.1492 (2010).

8 Valenstein, M. L. *et al.* Structure of the nutrient-sensing hub GATOR2. *Nature* **607**, 610-616, doi:10.1038/s41586-022-04939-z (2022).

9 Tafur, L. *et al.* Cryo-EM structure of the SEA complex. *Nature*, doi:10.1038/s41586-022-05370-0 (2022).

10 Jumper, J. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583-589, doi:10.1038/s41586-021-03819-2 (2021).

11 Shen, K. *et al.* Architecture of the human GATOR1 and GATOR1–Rag GTPases complexes. *Nature* **556**, 64-69, doi:10.1038/nature26158 (2018).

12 Egri, S. B. *et al.* Cryo-EM structures of the human GATOR1-Rag-Ragulator complex reveal a spatial-constraint regulated GAP mechanism. *Molecular Cell* **82**, 1836-1849.e1835, doi:10.1016/j.molcel.2022.03.002 (2022).

13 Saxton, R. A., Chantranupong, L., Knockenhauer, K. E., Schwartz, T. U. & Sabatini, D. M. Mechanism of arginine sensing by CASTOR1 upstream of mTORC1. *Nature* **536**, 229-233, doi:10.1038/nature19079 (2016).

14 Saxton, R. A. *et al.* Structural basis for leucine sensing by the Sestrin2-mTORC1 pathway. *Science* **351**, 53-58, doi:10.1126/science.aad2087 (2016).

15 Condon, K. J. *et al.* Genome-wide CRISPR screens reveal multitiered mechanisms through which mTORC1 senses mitochondrial dysfunction. *Proceedings of the National Academy of Sciences* **118**, e2022120118, doi:doi:10.1073/pnas.2022120118 (2021).

16 Huang, H. *et al.* In vivo CRISPR screening reveals nutrient signaling processes underpinning CD8(+) T cell fate decisions. *Cell* **184**, 1245-1261.e1221, doi:10.1016/j.cell.2021.02.021 (2021).

17 Viswanatha, R., Li, Z., Hu, Y. & Perrimon, N. Pooled genome-wide CRISPR screening for basal and context-specific fitness gene essentiality in Drosophila cells. *eLife* **7**, e36333, doi:10.7554/eLife.36333 (2018).

18 Wei, Y. & Lilly, M. A. The TORC1 inhibitors Nprl2 and Nprl3 mediate an adaptive response to amino-acid starvation in Drosophila. *Cell Death & Differentiation* **21**, 1460-1468, doi:10.1038/cdd.2014.63 (2014).

19 Grandison, R. C., Piper, M. D. W. & Partridge, L. Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. *Nature* **462**, 1061-1064, doi:10.1038/nature08619 (2009).

20 Zheng, B., Wu, Z. & Xu, B. The effects of dietary protein levels on the population growth, performance, and physiology of honey bee workers during early spring. *J Insect Sci* **14**, 191, doi:10.1093/jisesa/ieu053 (2014).
21 Fink, P., Pflitsch, C. & Marin, K. Dietary Essential Amino Acids Affect the Reproduction of the Keystone Herbivore Daphnia pulex. *PLOS ONE* **6**, e28498, doi:10.1371/journal.pone.0028498 (2011).
22 Steck, K. *et al.* Internal amino acid state modulates yeast taste neurons to support protein homeostasis in Drosophila. *Elife* **7**, doi:10.7554/eLife.31625 (2018).
23 Cangelosi, A. L. *et al.* Zonated leucine sensing by Sestrin-mTORC1 in the liver controls the response to dietary leucine. *Science* **377**, 47-56, doi:10.1126/science.abi9547 (2022).
24 Gu, X. *et al.* Sestrin mediates detection of and adaptation to low-leucine diets in Drosophila. *Nature* **608**, 209-216, doi:10.1038/s41586-022-04960-2 (2022).
25 Li, H. *et al.* Fly Cell Atlas: a single-cell transcriptomic atlas of the adult fruit fly. *bioRxiv*, 2021.2007.2004.451050, doi:10.1101/2021.07.04.451050 (2021).
26 Zhang, Z. *et al.* Brain-restricted mTOR inhibition with binary pharmacology. *Nature* **609**, 822-828, doi:10.1038/s41586-022-05213-y (2022).