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14. ABSTRACT The long-term goal of this project is geared toward further defining the regulatory mechanisms impinging on the TSC-Rheb circuit and revealing therapeutic strategies to target this signaling network in genetic tumor syndromes and cancer. Specifically, we aim to establish a robust synthetic lethal screening method applied to the study of the TSC network, validate synthetic lethal pairs in an <i>in vivo</i> intestinal stem cell system, and determine whether synthetic lethal combinations found in <i>Drosophila</i> are relevant to mammalian networks. In the first year of funding we have made significant progress at establishing the tools needed for the synthetic screens both in tissue culture and in vivo.					
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INTRODUCTION:

A detailed understanding of how common oncogenic signaling pathways are assembled into larger signaling networks is essential to developing therapeutic strategies to properly target these pathways in cancer and for interpreting clinical outcomes from targeted therapeutics. While the effected oncogenes and tumor suppressors that predominate different classes of human cancer can vary greatly, a small number of highly integrated signaling nodes are affected in the majority of human cancers, regardless of tissue of origin. It is therefore important to understand how these key signaling nodes are regulated. In this project, we focus on one such node, involving the TSC1-TSC2 complex and the Ras related small G protein Rheb, which is aberrantly regulated in nearly all genetic tumor syndromes and the most common forms of sporadic cancer. The long-term goal of this project is geared toward further defining the regulatory mechanisms impinging on the TSC-Rheb circuit and revealing therapeutic strategies to target this signaling network in genetic tumor syndromes and cancer. For this purpose, we will use high-throughput technologies in *Drosophila* to identify synthetic lethal interactions between TSC network tumor suppressors and identified pathway interactors. We will then go on to validate positive hits in an *in vivo Drosophila* model before determining which interactions are conserved using mammalian cell culture.

BODY:

We have made progress towards all the initial goals. Details are provided below according to the original Statement of Work:

Task 1. Establish a robust synthetic lethal screening method applied to the study of the TSC network. (months 1-36).

- Characterize an optimized shRNA targeting each of the core tumor suppressors. (months 1-2) COMPLETED
- Characterize the tumor suppressor genomic rescue constructs. (months 1-2) COMPLETED
- Clone shRNAs targeting each of the candidate genes into each of these constructs to create the desired pairwise combinations. (months 3-8)
- Establish the cell lines with genomic rescue constructs (months 3-8)
- Perform the synthetic screen for viability. (months 8-14)
- Confirm the positives. (months 14-17)
- Perform more quantitative screens using phospho-AKT, dpERK, and phospho-S6K antibodies (months 24-36)

As we were in the process of generating these reagents, breakthrough methods became available based on TALENS and CRISPR approaches to engineer genome. Because of the power of these approaches we decided to modify slightly our original approach. Specifically, rather than using combinatorial RNAi to perform the screens, we now will generate mutant cell lines for the tumor suppressor genes and then perform a single RNAi screen in the mutant cells. The advantage of this approach is that it will decrease the variability associated with combination of RNAi reagents. In addition, we expect that the rate of false positive associated with off target effects of RNAi reagents will be easier to address. We are now at the final stage of generating the cell lines and should be able to initiate the combinatorial screen this fall.

TAL Effector Nucleases (TALENs) and CRISPRs are two newly emerging technologies that are revolutionizing our ability to modify endogenous genomic sequences in a wide variety of organisms (Cermak et al., 2011; Hockemeyer et al., 2011, Liu et al., 2012, Moore et al., 2012; Cong et al., 2013; Mali et al., 2013; Cho et al., 2013; Hwang et al., 2013; Jiang et al., 2013). The use of both of these technologies is becoming well-established in mammalian systems where they have been shown to function with unprecedented efficiency both in cell culture and *in vivo*. Like previous genome editing techniques such as homologous recombination and zinc-finger nucleases, TALENs and CRISPRs can be used to generate double stranded breaks (DSBs) at user-defined locations in the genome. This can be exploited in a number of ways to generate either random mutations or specific alterations to the genome sequence. Unlike previous approaches however, both TALENs and CRISPRs enable greatly improved efficiencies of DSB generation and more specific sequence targeting (**Figure 1**).

The use of both TALENs and CRISPRs to generate DSBs has now been demonstrated in several model organisms and TALENs have been shown to generate both mutations and knock-ins at high frequency in *Drosophila* (Liu et al., 2012). For example, injection of TALENs targeting the *yellow* gene resulted in 17.2% of F0 males demonstrating a heritable yellow mutant phenotype. Furthermore, co-injection of a donor construct was shown to cause heritable insertions of ectopic sequence in 66.7% of F0 males (Liu et al., 2012). In addition, the CRISPR method has very recently be shown to be effective in flies (Gratz et al., 2013)(see also our preliminary data below).

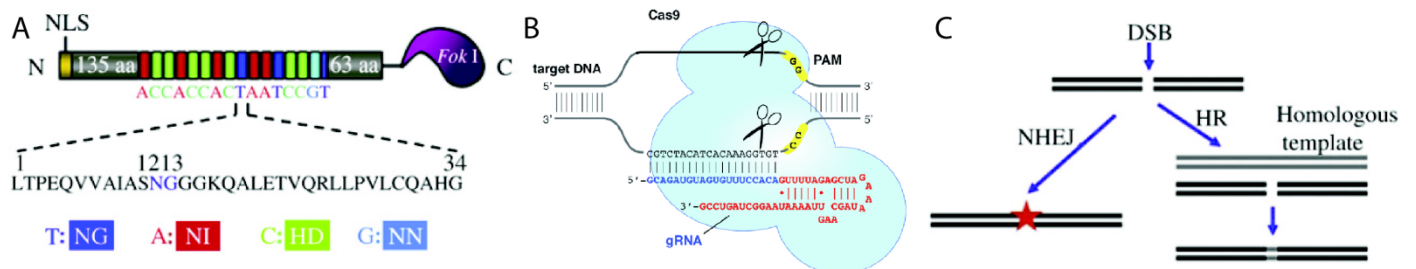


Figure 1: TALE and CRISPR approaches. Although TALENs and CRISPRs are used in similar ways, their structures and mechanisms of function differ considerably. **A.** TALENs consist of a FokI nuclease (an enzyme able to non-specifically cleave DNA) and a DNA binding region, used to target the nuclease to a specific target sequence (modified from Liu et al., 2012). The DNA binding region is comprised of a series of monomer repeats, each of which interacts with a single nucleotide. Therefore, by cloning these monomers together in a specific order, any DNA sequence can be bound and cleaved. This gives the system extremely high versatility with almost no restrictions on the sequences that can be targeted. Furthermore, since FokI acts as a dimer, meaning that the DNA will only be cleaved when two TALENs bind to opposite strands with a limited spacer region in between, extremely high cleavage specificity can be achieved. **B.** Similarly, the CRISPR system requires a DNA binding component and a nuclease enzyme. However, in this case, DNA binding specificity is achieved using a guide-RNA (gRNA) construct with a 20bp region homologous to the target DNA sequence. This interacts with the Cas9 protein, which non-specifically cleaves DNA. One advantage of the CRISPR system is the ease with which gRNA can be produced, therefore increasing the simplicity of experiments. However, greater restrictions on targetable sequences and limited homology length reduce the versatility of this system (review by Charpentier et al., 2013). **C.** Once a DSB has been generated, it can be exploited for several purposes (Liu et al., 2012). The simplest of these is the generation of random mutations at the break site, which can be used to disrupt gene function. This can be achieved by simply generating a DSB within the coding sequence of a gene and allowing the cell to repair the DNA damage. A high proportion of breaks will be repaired by non-homologous end joining (NHEJ). This imperfect mechanism often leads to short insertions or deletions, ideal for generating frame shifts and thereby ablating gene function. Alternatively, a second repair mechanism can be exploited to generate more specific changes including insertions, deletions or substitutions with specificity to the single nucleotide level. A small proportion of DSBs will be repaired by homologous recombination, in which the homologous chromosome is used as a template to repair the damaged locus. By providing a donor construct with homology to the target locus, the repair machinery will often use this as a template instead of the homologous chromosome. Therefore, any alterations made to the donor construct will be incorporated into the endogenous genomic sequence (examples in Liu et al., 2012; Zu et al., 2013).

One challenge in using TALENs is the synthesis of the monomer repeats. Due to the highly repetitive nature and large number of components required conventional cloning techniques are not suitable. Indeed, many of the publications relating to TALENs focus on methods of constructing them (Briggs et al., 2012; Sanjana et al., 2012; Reyon et al., 2012). Due to the large number of TALEN constructs required to perform these experiments, we have modified an existing method of TALEN production to allow high-throughput synthesis. Using a combination of liquid handling robotics and several modifications to the 'iterative capped assembly' TALEN synthesis method (Briggs et al., 2012), we are able to generate 96 TALENs in about one week, a far higher rate than is achievable with other available methods.

To demonstrate functionality of our TALENs in *Drosophila*, we transfected constructs targeting various genes into *Drosophila* S2R+ cells in culture. Using high resolution melt analysis (Dahlem et al., 2012) and qPCR we were able to detect both alterations in melting temperature following TALEN treatment and reductions in mRNA levels, indicating that mutations are produced (see example of the targeted *TSC1* gene in **Figure 2A,B**). Note, that we were also able to validate these genome editing events using the surveyor assay/T7E1 endonuclease assay (data not shown). We also tested the ability of these TALENs to generate knock-ins in cells. Short, single-stranded DNA oligos comprising homology arms of various lengths and a short insert were co-transfected with TALEN constructs targeting *actin5c*. Using PCR to detect successful inserts, we found that the approach was successful and that 15bp homology arms lead to optimal efficiency (**Figure 2C**). Finally, as CRISPR provide an

alternative for genome editing, we tested the ability of CRISPR constructs to generate mutations in S2R+ cells using melt curve assays. Similar to TALENs, a new peak was detected following CRISPR treatment indicating the production of mutations (see example of the targeted *yellow* gene in **Figure 2D**).

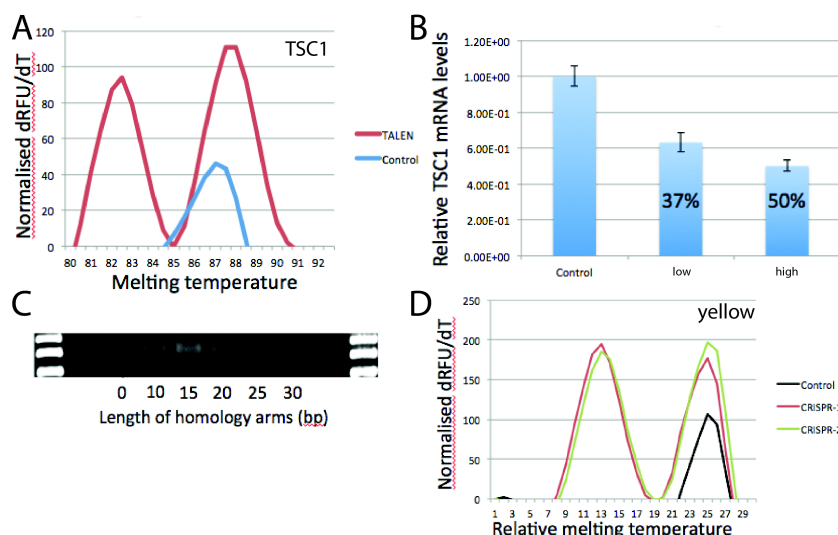


Figure 2: Validation of TALEN and CRISPR reagents. A-B, Melt-curve (A) and qPCR (B) assays to detect alterations in melting temperature following TALEN treatment and reductions in mRNA levels of the targeted *TSC1* gene. For qPCR analysis, TALEN plasmids were co-transfected with a plasmid expressing GFP to mark transfected cells. Cells were then separated by FACS sorting and low GFP (lower 30%) or high GFP (upper 30%) analyzed separately. **C**, To test whether TALENs generate knock-ins in cells, short, single-stranded DNA oligos comprising homology arms of various lengths and a short insert were co-transfected with TALEN constructs

targeting *actin5c*. Using PCR to detect successful inserts, we found that the approach was successful and that 15bp homology arms lead to optimal efficiency. **D**, In similar experiments, plasmids driving expression of a *Drosophila* codon optimized Cas9 and gRNAs under the control of the actin promoter were transfected into S2R+ cells. Genomic DNA was isolated after 3 days and analyzed using melt curve assays. Similar to TALEN experiments, a new peak was detected indicating the production of mutations at the *yellow* gene.

Task 2: Validation of synthetic lethal pairs in an *in vivo* intestinal stem cell system. (months 1-36).

- Characterize the phenotypes and level of knockdown of single shRNAs targeting the five tumor suppressors in ISCs. (months 1-6) COMPLETED
- Characterize shRNAs targeting each gene that show synthetic phenotypes in combinations with the tumor suppressors. (months 6-24) COMPLETED
- Characterize in details the phenotypes of the synthetic interactions using phosphoHistone H3, caspase antibodies, and BrDU. (months 12-36)
- Characterize in further details the phenotypes of the synthetic interactions using pathway specific phospho-antibodies such as phospho-AKT, dpERK, and phospho-S6K (months 14-36)
- Confirm by genomic rescue the specificity of the interactions. (months 15-36)

We have made excellent progress at characterizing the phenotype of tumor suppressor in gut stem cells. In addition, we have developed a quantitative method for monitoring stem cell proliferation. Using these tools we will now be able to analyze quantitatively the effects of combinatorial RNAi experiments.

Drosophila models of human stem cell colon tumors

We have developed 'tumor' fly models for a number of signaling pathways (**Figure 3**) that will be used to test for synthetic interactions with the TSC network. First, reduced function of *wg*, which is specifically expressed in the circular muscles next to ISCs, causes ISC quiescence and differentiation, whereas *wg* overexpression produces excessive ISC-like cells (Lin et al., 2008). Similarly, knockdown of Wnt pathway components, such as *axin* in ISCs, a negative regulator of Wnt signaling, using RNAi

causes excess of these ISC-like cells. Second, we have found that expressing a human gain-of-function RAF transgene, *RAF^{GOF}*, can by itself cause stem cell hyper-proliferation, resulting in both increased stem cells and differentiated cells. Third, we and others have shown that perturbing Notch leads to stem cell hyperproliferation (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006 and 2007). Finally, we have established two additional tumor models involving the reduction of the tumor-suppressors *Hippo* (Karpowicz et al., 2010) and *PTEN*. Like the Raf, Wnt and Notch tumors, these additional models cause the overproliferation of ISCs into tumors of ISC-like cells. We will use these tumor models to characterize the hits identified in Task 1.

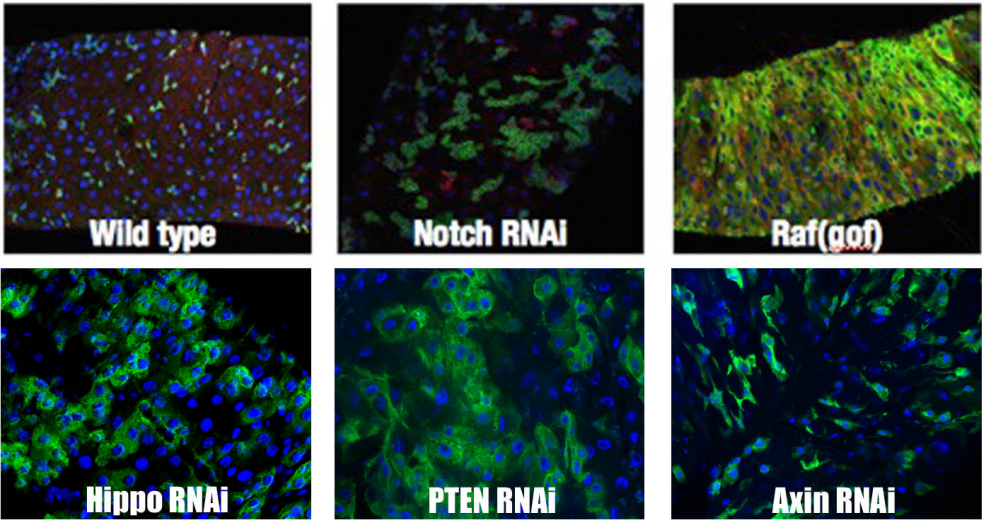


Figure 3: Gut models of tumor stem cells. Notch, RAF, Hippo, PTEN and Axin models of gut stem cell tumors in one-week old adults. The *esg-Gal4* driver was used to induce expression of UAS transgenes encoding *Notch*, *Hippo*, *PTEN* or *Axin* RNAi hairpins, or a *Raf* gain of function (*UAS-^{RAF}GOF*) UAS construct in gut stem cells, in the presence of a *UAS-GFP* transgene. These disruptions induce gut tumors, as evidenced by the strong increase in GFP positive cells.

A quantitative luciferase-based method to measure stem cell number

The *Drosophila* gut is a simplified version of the mammalian gut and can be used to model the early stages of colorectal tumor formation. We adapted a whole-animal luciferase assay that we have previously developed to study position effects (Markstein et al., 2008), to specifically monitor gut stem cell growth in whole-animal homogenates. In short, the *esg-Gal4* driver is used to express a UAS-luciferase transgene in adult ISCs. Adult flies are processed in a 96-well format, and the total luciferase measured can be used as a proxy for the number of stem cells. As shown in **Figure 4**, different levels of luciferase correlate with different levels of tumor formation (e.g. compare the luciferase in one-week-old adults with GFP staining in the RAF and Notch induced tumors). By using luciferase to measure tumor growth, rather than GFP, we will use this luciferase technology to quantify the phenotypes of the combinatorial genotypes.

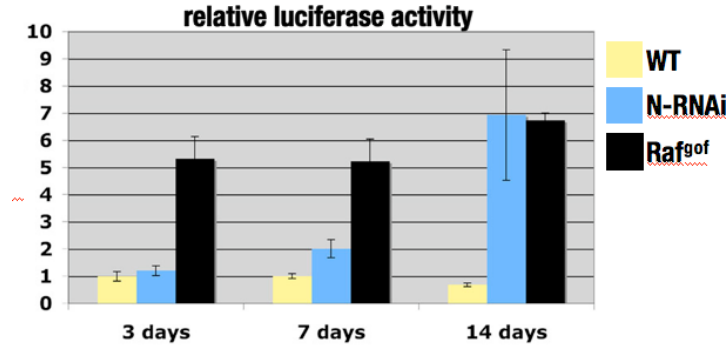


Figure 4. Luciferase provides a quantitative readout of tumor progression. Luciferase measurements from whole flies can be used to detect changes in growth of the adult *Drosophila* stem cells. The *esg-Gal4* driver was used to express luciferase in the stem cells of the adult *Drosophila* gut in wildtype, Notch RNAi (N-RNAi) and *RAF^{GOF}* flies.

Task 3: Determine whether synthetic lethal combinations found in *Drosophila* are relevant to mammalian networks. (months 1-36)

- Characterize the signaling and growth properties of the knockout MEFs for all of the five major tumor suppressor genes of interest (LKB1, NF1, PTEN, TSC1, and TSC2). (months 1-8) COMPLETED
- Identify the mammalian orthologs using DIOTP. (months 8-30) UNDERWAY
- Characterize effective siRNAs against the genes to be targeted using ON-TARGETplus SMARTpool siRNAs from Dharmacon. (months 12-30)
- Test the synthetic lethal interactions with the RTK network core tumor suppressors in MEFs. (months 12-30)
- Confirm the results with neutral base pair substitutions control siRNAs. (months 12-30)
- Test hits that show specificity for killing of one or more tumor suppressor-deficient cell types using available tumor-derived cell lines lacking these tumor suppressors. (months 26-36)
- Test all synthetic lethal interactions, regardless of the MEF results, in a TSC2 null angiomyolipoma-derived cell line. (months 26-36)

We have characterized the signaling induced by loss of the given tumor suppressors in the pairs of wild-type and knockout MEFs, with an emphasis on the major predicted pathways (PI3K-Akt, Ras-ERK, AMPK, and mTORC1). We have also determined their growth and proliferation properties and differential sensitivity to pathway-specific kinase inhibitors. These data provide important baseline measurements moving forward with the validation of targets coming from the *Drosophila* screens. In addition, we have performed IP-MS/MS experiments to identify novel protein-protein interactions that are conserved between fly and mammalian cells, with an emphasis on the core components of the network comprising the TSC complex (TSC1, TSC2, and TBC1D7) and Rheb. This cross-pylum proteomic comparison, made possible with the DIOTP program, will help filter the findings from *Drosophila* and provide a means to prioritize the most promising hits to characterize further in mammalian systems.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrate the efficiency of the TALEN and CRISPR approaches to generate *Drosophila* mutant cell lines
- Develop quantitative luciferase-based approaches to measure stem cell proliferation in gut stem cells
- Establish tumor models for synthetic screens in vivo
- Characterized the mammalian systems for validation of *Drosophila* hits.

REPORTABLE OUTCOMES:

Recent review articles on this topic, citing support from this grant:

1. Ricoult SJH and Manning BD. The multifaceted role of mTOR in the control of lipid metabolism. *EMBO Reports* 2013; 14:242-51.
2. Dibble CC and Manning BD. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat Cell Biol* 2013; 15:555-64.

CONCLUSION:

We are now at the final stage of generating the mutant cell lines using TALEN and CRISPR technologies and should be able to initiate the combinatorial screen this fall. We have made excellent progress at characterizing the phenotype of tumor suppressor in gut stem cells. In addition, we have developed a quantitative method for monitoring stem cell proliferation. Using these tools we will now be able to analyze quantitatively the effects of the combinatorial RNAi experiments and move the confirmed hits into mammalian cells for characterization.

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Signal integration by mTORC1 coordinates nutrient input with biosynthetic output

Christian C. Dibble and Brendan D. Manning

Flux through metabolic pathways is inherently sensitive to the levels of specific substrates and products, but cellular metabolism is also managed by integrated control mechanisms that sense the nutrient and energy status of a cell or organism. The mechanistic target of rapamycin complex 1 (mTORC1), a protein kinase complex ubiquitous to eukaryotic cells, has emerged as a critical signalling node that links nutrient sensing to the coordinated regulation of cellular metabolism. Here, we discuss the role of mTORC1 as a conduit between cellular growth conditions and the anabolic processes that promote cell growth. The emerging network of signalling pathways through which mTORC1 integrates systemic signals (secreted growth factors) with local signals (cellular nutrients — amino acids, glucose and oxygen — and energy, ATP) is detailed. Our expanding understanding of the regulatory network upstream of mTORC1 provides molecular insights into the integrated sensing mechanisms by which diverse cellular signals converge to control cell physiology.

All cells and organisms must coordinate their metabolic activity with changes in their nutrient environment. This is achieved through signalling networks that integrate the sensing of local and systemic nutrient and energy sources, and relay this information to metabolic regulators and enzymes to control cellular anabolic and catabolic processes. One of the master regulators of metabolism and growth is the serine/threonine protein kinase mechanistic target of rapamycin (mTOR; formerly known as mammalian TOR). As part of mTOR complex 1 (mTORC1), it functions at the convergence point of a vast signalling network that senses fluctuations in extracellular and intracellular nutrients. The critical importance of intimately linking nutrient signals to metabolic control in human health is highlighted by the fact that aberrant regulation of mTORC1 signalling has been implicated in the pathophysiology of a diverse set of common human diseases, including cancer, metabolic diseases, neurological disorders, and inflammatory and autoimmune diseases^{1–4}.

mTORC1, comprised of three essential and evolutionarily conserved core subunits (mTOR, Raptor and mLST8)^{3,5}, is responsive to both organismal and cellular nutritional status, and controls downstream metabolic processes accordingly. Systemic changes in the metabolism of the organism are sensed by mTORC1 through pathways activated by secreted growth factors, cytokines and hormones. Activation of mTORC1 is also dependent on sufficient levels of essential intracellular nutrients, including amino acids, glucose and oxygen. Nutrients seem to be the more ancient input for mTORC1, as its activation in yeast depends strictly on nutrient availability⁵. In higher eukaryotes, cell culture experiments suggest that intracellular nutrients only basally activate mTORC1 but are essential for its robust stimulation by extracellular growth factors^{6,7}.

Here, we focus on mTORC1 as a key link between nutritional status and metabolic control, with an emphasis on recent advances in understanding the mechanisms of nutrient sensing and signal integration by this protein kinase complex.

Promotion of anabolic metabolism downstream of mTORC1

To understand the physiological importance of the network of signalling inputs upstream of mTORC1, we must first consider the downstream processes regulated by mTORC1. Under nutrient and energy-replete conditions, mTORC1 is activated to stimulate anabolic processes that convert nutrients and energy into macromolecules, including protein, lipid and nucleic acids. The control of cellular and systemic metabolism by mTORC1 signalling has been the subject of several recent review articles^{2,3,8–10}, and we briefly summarize some of the major mechanisms of metabolic regulation here (Fig. 1).

Best known for its role in promoting protein synthesis, mTORC1 activation leads to both an acute increase in the translation of specific mRNAs and a broader increase in the protein synthetic capacity of the cell. mTORC1 regulates 5'-cap-dependent mRNA translation through two sets of direct downstream targets: the eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BP1 and 2) and the ribosomal S6 kinases (S6K1 and 2)¹¹. 4E-BP appears to have the most profound effect on mRNA translation downstream of mTORC1, binding to eIF4E at the 5'-cap of mRNAs and blocking assembly of the translation initiation complex. The phosphorylation of 4E-BP1 and 2 by mTORC1 stimulates its release from eIF4E, allowing translation initiation to proceed. This mechanism is particularly important for initiating the translation

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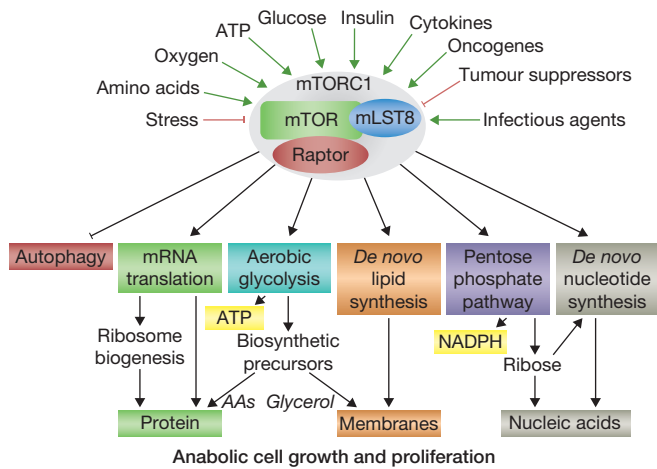


Figure 1 mTORC1 signalling links cellular growth conditions with metabolic processes underlying anabolic cell growth and proliferation. Many physiological and pathological signals affect the activation status of mTORC1, including cellular nutrients and energy, growth factors, oncogenes and tumour suppressors, and a variety of intracellular pathogens (that is, infectious agents). When activated, mTORC1 regulates a number of cellular processes. The processes affecting the metabolic state of the cell are shown here. Through various downstream mechanisms, mTORC1 signalling inhibits autophagy and stimulates mRNA translation, glycolysis, lipid synthesis, the pentose phosphate pathway and *de novo* pyrimidine synthesis, thereby promoting the production of energy (ATP), reducing equivalents (NADPH) and the major macromolecules required for cell growth.

of mRNAs with 5'-terminal oligopyrimidine (5'-TOP) or 5'-TOP-like motifs, rendering this class of mRNAs sensitive to mTORC1 activation and inhibition^{12–14}. Importantly, 5'-TOP mRNAs are enriched for those encoding ribosomal proteins and translation factors. Therefore, the acute translational control over this class of mRNAs allows mTORC1 signalling to globally enhance cellular protein synthesis. Additional mTORC1-dependent mechanisms are also believed to contribute to its role in increasing ribosome biogenesis to promote protein synthesis¹⁵.

mTORC1 signalling can also promote lipid and nucleic acid synthesis, as well as stimulate glucose uptake, glycolysis and NADPH production to support these anabolic processes. This is currently understood to occur largely through the regulation of transcription factors by mTORC1¹⁶, but post-translational mechanisms have also recently been uncovered^{17,18}. For example, mTORC1 signalling increases the translation of hypoxia-inducible factor 1 α (HIF1 α), which induces expression of glucose transporters and glycolytic enzymes and promotes a switch from mitochondrial oxidative metabolism to glycolysis^{19–24}. This switch to aerobic glycolysis, referred to as the Warburg effect in cancer cells, is observed in most proliferating cells, and is believed to promote metabolic flux from intermediates of glycolysis into biosynthetic branches²⁵. The sterol regulatory element-binding proteins (SREBP1 and 2) globally induce the expression of enzymes involved in *de novo* fatty acid and sterol biosynthesis²⁶, and mTORC1 signalling promotes lipid synthesis through the activation of these transcription factors^{19,27,28}. mTORC1 signalling also promotes the expression of pentose phosphate pathway (PPP) genes and metabolic flux specific to the oxidative, NADPH-producing branch of the PPP (ref. 19). The mTORC1-induced expression of the rate-limiting enzyme in the oxidative PPP, glucose-6-phosphate dehydrogenase (G6PD), was found to be dependent on SREBP1. Given that lipid synthesis is one of

the most NADPH-demanding metabolic pathways, its co-regulation with the oxidative PPP by mTORC1 and SREBP is likely to help satisfy this requirement. Of equal importance, the mTORC1-mediated control of the PPP also results in increased production of ribose required for nucleotide synthesis¹⁹. In parallel to this transcriptional mechanism, mTORC1 also acutely stimulates metabolic flux through *de novo* pyrimidine synthesis through the S6K1-mediated phosphorylation of the enzyme CAD (carbamoyl phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase), thereby increasing the pool of nucleotides available for RNA and DNA synthesis^{17,18}.

As well as promoting the synthesis of macromolecules, mTORC1 potentially inhibits autophagy (or macroautophagy), a cellular recycling and quality control mechanism. Autophagy is a multi-stage process in which membranous structures called autophagosomes engulf cytosolic organelles and macromolecules and, through fusion with lysosomes, target their constituents for degradation into nutrient building blocks²⁹. Under nutrient-rich, growth-promoting conditions, inhibition of this catabolic process favours cell growth. It is likely that mTORC1 inhibits autophagy at multiple steps, but the most well-characterized mechanism is through the direct control of ULK1 (also known as ATG1), a protein kinase that regulates the initiation of autophagosome formation^{30–33}. mTORC1 also directly phosphorylates the transcription factor EB (TFEB), a master regulator of lysosomal and autophagy genes, thereby exerting an inhibitory input that is likely to attenuate autophagy^{34,35}. The major signals that stimulate the induction of autophagy include cellular nutrient and energy depletion, which lead to a decrease in mTORC1 signalling and relief of its inhibition of autophagy.

The general effect of mTORC1 activation is to promote an increase in biomass for cell growth and proliferation. However, mTORC1 signalling plays specialized roles in terminally differentiated tissues, such as promoting localized mRNA translation in neurons, which is critical for the control of synaptic plasticity³⁶, and suppression of ketogenesis in the liver following feeding³⁷. Regardless of the setting, nutrient sensing by mTORC1 serves as a critical decision point between anabolic and catabolic metabolism. The broad control that mTORC1 exerts over metabolism provides a rationale for why it must be particularly responsive to the local and systemic availability of metabolic raw materials.

Systemic nutrient sensing through secreted growth factors

Systemic integration of signals reflecting the physiological state of the organism, including nutritional status, is critical for maintaining homeostasis. These signals are communicated between tissues and cell types through secreted ligands classified as growth factors, hormones and cytokines (collectively referred to as growth factors here). The archetypal systemic nutrient signal is insulin, which is produced by pancreatic β cells in response to increased blood glucose levels and stimulates adaptive signalling events in liver, fat and muscle. mTORC1 is activated by insulin and most other growth factors through either receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs) at the cell surface. Downstream of these receptors, two major signalling pathways are involved in mTORC1 activation: the phosphatidylinositol-3-OH kinase (PI(3)K)–Akt and Ras–Erk pathways (Fig. 2). These pathways are differentially activated downstream of specific receptors, with the PI(3)K–Akt pathway dominating downstream of the insulin and insulin-like growth factor (IGF) receptors. Importantly, many components of these signalling pathways are oncogenes or tumour suppressors, resulting

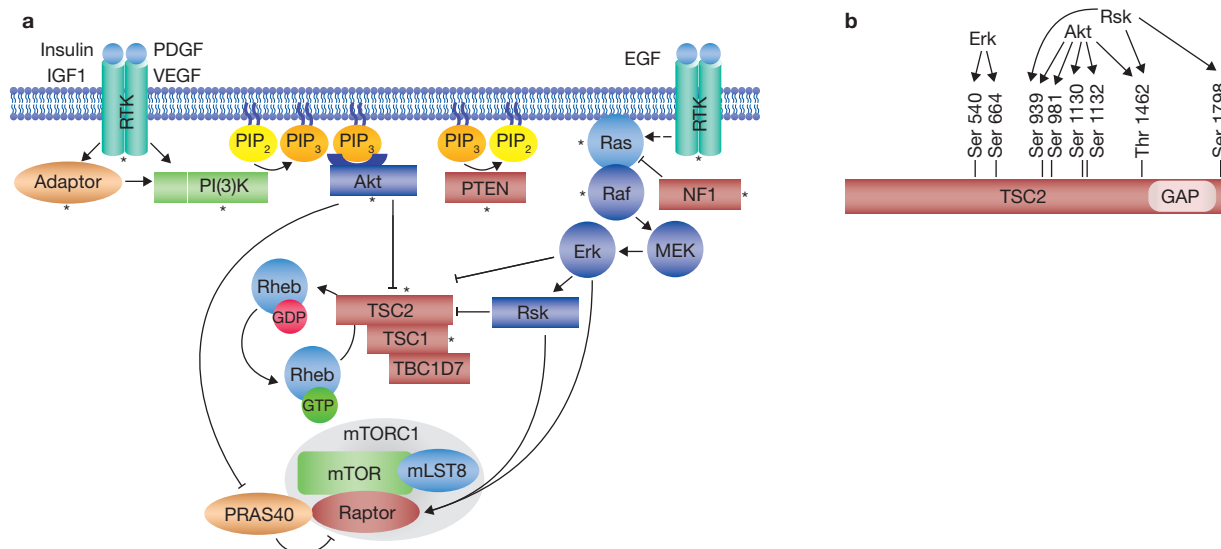


Figure 2 Secreted growth factors stimulate mTORC1 activity through the PI(3)K–Akt and Ras–Erk pathways. **(a,b)** Through binding to receptor tyrosine kinases (RTKs) and scaffolding adaptor proteins, a variety of secreted growth factors (insulin, IGF1, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) shown here) stimulate the recruitment and activation of PI(3)K. PI(3)K activity generates phosphatidylinositol-3,4,5-trisphosphate (PIP₃), which recruits Akt to the plasma membrane, where it is activated by upstream kinases (not shown). Ras is also activated downstream of

RTKs and stimulates a kinase cascade leading to the activation of Erk and Rsk. In response to growth factors, the Akt, Erk and Rsk protein kinases phosphorylate specific residues on TSC2 **(b)** within the TSC complex, thereby negatively regulating the ability of this complex to act as a GAP for Rheb. Consequently, GTP-bound Rheb accumulates and activates mTORC1. Parallel inputs into mTORC1 from these kinases also exist, with Akt phosphorylating PRAS40 and Erk and Rsk both phosphorylating residues on Raptor. Oncogenes and tumour suppressors mutated in human cancers are indicated with asterisks.

in growth-factor-independent activation of mTORC1 in up to 80% of human cancers, across nearly all lineages¹ (Fig. 2).

Growth-factor-dependent pathways stimulate mTORC1 signalling by regulating a small G protein switch directly upstream of mTORC1. Rheb (Ras homolog enriched in brain) is a ubiquitous small GTPase of the Ras superfamily, which, in its GTP-bound form (Rheb^{GTP}), is a direct, potent and essential activator of mTORC1^{38–44}. The Akt and Erk protein kinases promote the accumulation of Rheb^{GTP} by phosphorylating and inhibiting the tuberous sclerosis complex 2 (TSC2) protein, a GTPase-activating protein (GAP) specific for Rheb. TSC2 functions in a complex (referred to here as the TSC complex) containing two other proteins, tuberous sclerosis complex (TSC1) and Tre2–Bub2–Cdc16 (TBC) 1 domain family member 7 (TBC1D7), which are required for the stability and full GAP activity of TSC2 (refs 40,45). In the absence of growth factor signalling, the TSC complex maintains Rheb in its GDP-bound state (Rheb^{GDP}), thereby blocking the activation of mTORC1. Although the molecular mechanism is not well understood, phosphorylation of specific residues on TSC2 by Akt and Erk, as well as Rsk downstream of Erk (Fig. 2, inset), in response to growth factors, inhibits the ability of the TSC complex to regulate Rheb, allowing Rheb^{GTP} to accumulate and activate mTORC1 (refs 46–51). Other parallel mechanisms can also contribute to mTORC1 regulation by growth factors, including the Akt-mediated phosphorylation of PRAS40, a non-essential component of mTORC1 (refs 44,52), and the Erk and Rsk-dependent phosphorylation of Raptor (Fig. 2)^{53–55}. However, the TSC complex is absolutely essential for inhibition of mTORC1 signalling in the absence of growth factors. It is now recognized that many, but not all, upstream inputs into mTORC1 signalling impinge on control of the TSC complex and Rheb⁵⁶. Thus, growth factor signalling pathways stimulate an acute and robust increase in mTORC1 activity. However, they can also contribute

to mTORC1 activation more indirectly through the stimulated uptake of nutrients, which are essential for the basal activation of mTORC1, as discussed below.

Amino acid sensing through the Rag GTPases

Amino acids are essential for mTORC1 activation⁷. Cell culture experiments suggest that mTORC1 is particularly sensitive to decreases in leucine, arginine or glutamine^{7,57,58}. However, it is unclear whether mTORC1 truly senses individual amino acids or the total intracellular pool of amino acids, which can be differentially affected by removal of specific amino acids. It is believed that an intracellular sensor exists that probably interacts directly with amino acids, or their derivatives, and initiates a signalling mechanism to basally activate mTORC1 and allow its further stimulation by growth factors. Whereas the molecular nature of the upstream amino acid sensor is currently unknown, progress has been made on the mTORC1 proximal signalling mechanism by which the amino acid sensor ultimately communicates to mTORC1.

Rheb is essential for amino acids to activate mTORC1, but the primary amino acid sensing pathway appears to function in parallel to Rheb^{59,60} and involves a second class of small G proteins, the Rag GTPases^{61–63}. The Rag proteins belong to a highly conserved family of GTPases that consist of two subtypes, which associate to form heterodimers essential for their stability and function. In mammals, RagA or RagB (orthologues of the budding yeast protein Gtr1) form a heterodimer with RagC or RagD (orthologues of yeast Gtr2)⁶⁴. RagA/B–RagC/D heterodimers bind directly to Raptor in mTORC1 (ref. 63). This association is highly dependent on the nucleotide-binding state of the heterodimer, with mTORC1 binding predominantly to heterodimers consisting of a GTP-bound RagA/B (RagA/B^{GTP}) and a GDP-bound RagC/D (RagC/D^{GDP}). Importantly, the nucleotide-binding states of Rag proteins are influenced

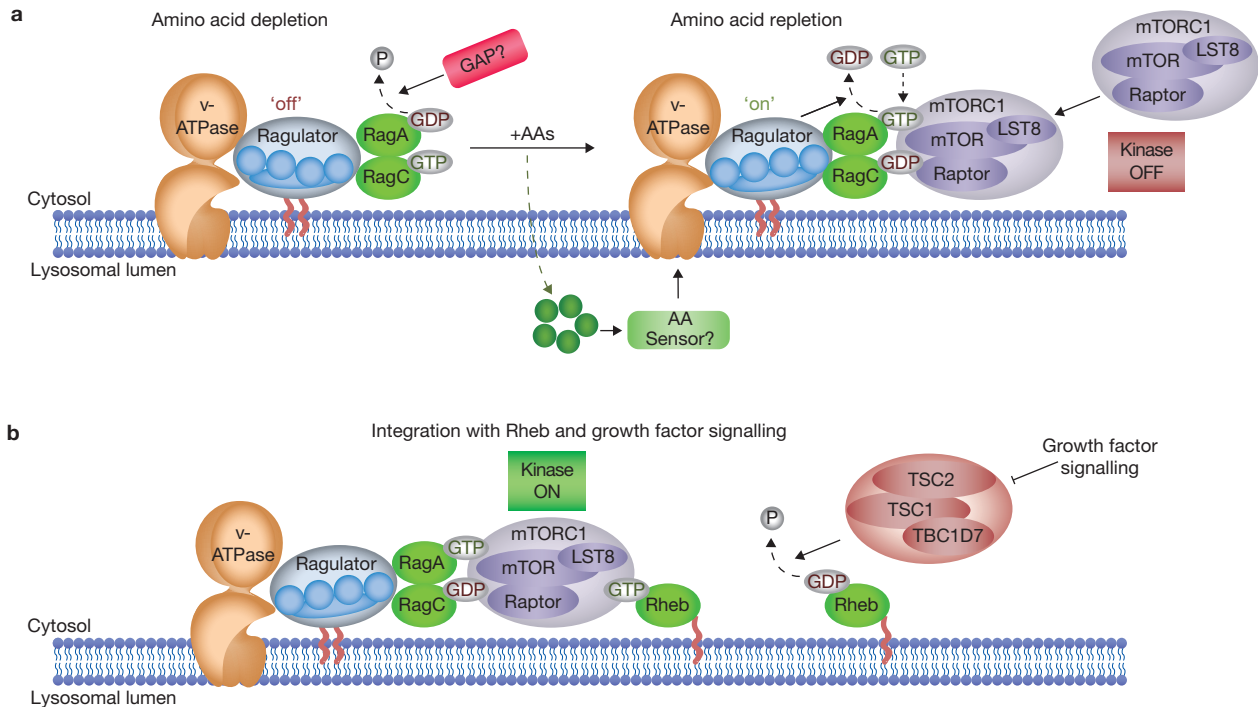


Figure 3 Model of the Rag-dependent recruitment of mTORC1 to the lysosome in response to amino acids and the integration with Rheb-dependent growth factor signalling. **(a)** Amino-acid-stimulated recruitment of mTORC1 to the lysosome. Under amino-acid-deplete conditions, the Ragulator is not active as a RagA/B GEF, and RagA/B^{GDP}–RagC/D^{GTP} heterodimers that accumulate are unable to recruit mTORC1 to the lysosome. In the presence of amino acids, which enter the lysosome, an unknown signal or sensor within the lysosomal lumen triggers a conformational change within the v-ATPase that, through direct interactions,

promotes Ragulator GEF activity. In this manner, amino acids stimulate the formation of RagA/B^{GTP}–RagC/D^{GDP} heterodimers, which bind directly to mTORC1 and recruit the inactive kinase complex to the lysosomal surface. **(b)** The amino acid signal allows mTORC1 to come in contact with its essential upstream activator Rheb. In the absence of growth factors, Rheb is maintained in its GDP-bound state through the GAP activity of the TSC complex, and mTORC1 remains inactive. Growth factor signalling inhibits the TSC complex, allowing formation of Rheb^{GTP}, which binds to and activates mTORC1.

by amino acids, with starvation promoting the accumulation of RagA/B^{GDP}–RagC/D^{GTP} heterodimers, which cannot associate with mTORC1, and re-feeding stimulating a change to the mTORC1-binding RagA/B^{GTP}–RagC/D^{GDP} state⁶³. In both mammalian and *Drosophila melanogaster* cells, expression of constitutively GTP-bound mutants of RagA or RagB renders mTORC1 signalling resistant to amino acid starvation, but not to growth factor withdrawal^{62,63}. The critical nature of the nucleotide-loading state of the RagA/B subunit in amino acid sensing by mTORC1 was further confirmed with a mouse knock-in allele of RagA that is constitutively bound to GTP, which in the homozygous state renders mTORC1 signalling in cells and tissues resistant to nutrient withdrawal⁶⁵. However, unlike Rheb^{GTP}, the RagA/B^{GTP}–RagC/D^{GDP} heterodimer does not seem to directly activate mTORC1, but rather spatially regulates mTORC1 in a manner that permits its ultimate activation by Rheb (Fig. 3).

A major breakthrough in understanding the spatial regulation of mTORC1 came with the discovery of a protein complex dubbed the Ragulator, which is responsible for both the subcellular localization of the Rags and regulation of their nucleotide-binding state^{66,67}. In yeast, Gtr1–Gtr2 heterodimers interact with Ego1 and Ego3 to form the EGO complex, which associates with the outer surface of the vacuole, the yeast equivalent of the lysosome^{5,61}. In mammalian cells, RagA/B–RagC/D heterodimers localize to the lysosome through an interaction with the Ragulator, which is similar in architecture to the EGO complex but whose components are not orthologues of the Ego proteins⁶⁶. Among the five

Ragulator subunits (LAMTOR1–5), LAMTOR1 (also known as p18) anchors the pentameric complex, and hence the Rag heterodimers, to the lysosomal surface via dual N-terminal lipid modifications (myristoyl and palmitoyl moieties). LAMTOR2 (also known as p14), LAMTOR3 (also known as MP1), LAMTOR4 (also known as C7orf59), and LAMTOR5 (also known as HBXIP) share common structural elements called Roadblock domains, which are also present in the Rag proteins but serve an unknown molecular function^{67,68}. Like the Rag proteins, all components of the Ragulator are required for amino acid sensing to mTORC1 (refs 66,67). mTORC1 acutely translocates to the Rag–Ragulator complex at the lysosome in response to amino acid re-feeding, and this requires a switch to the activated RagA/B^{GTP}–RagC/D^{GDP} state^{66,67}. This translocation to the lysosome is both necessary and sufficient for amino acid sensing by mTORC1. Importantly, the Ragulator not only serves as a lysosomal scaffold, but also stimulates the nucleotide switch in the Rag proteins in response to amino acids by acting as a guanine nucleotide exchange factor (GEF) towards RagA and RagB (ref. 67). The molecular origin of this activity within the pentameric complex is unknown, but it induces the RagA/B subunit within Rag heterodimers to release GDP, allowing subsequent loading with GTP. Through an unknown mechanism, this change coincides with a switch of the RagC/D subunit from a GTP- to GDP-bound form. Therefore, by promoting the RagA/B^{GTP}–RagC/D^{GDP} state, the Ragulator GEF activity stimulates the recruitment of mTORC1 to the lysosome in response to amino acids.

How amino acids are sensed by the Ragulator at the lysosome is unknown, but seems to involve intra-lysosomal amino acids and v-ATPase, a large protein complex spanning the lysosomal membrane that acts as proton pump to acidify the lysosome⁶⁹. The v-ATPase complex was found to be essential for amino acid sensing by mTORC1 in both *Drosophila* and mammalian cells. Ragulator subunits co-purify with those of v-ATPase, and the two complexes make multiple contacts that vary in the presence or absence of amino acids. Importantly, v-ATPase catalytic activity is required for an amino-acid-induced conformational shift between the two complexes and the stimulation of the switch to the RagA/B^{GTP}–RagC/D^{GDP} state that recruits mTORC1 to the lysosome. However, this mechanism does not seem to involve the lysosomal proton gradient. Therefore, it seems that v-ATPase stimulates the GEF activity of the Ragulator in response to amino acids⁶⁷. Somewhat unexpectedly, it was found that signals affecting the v-ATPase–Ragulator interactions and mTORC1 activation originate from intralysosomal, rather than cytosolic, amino acids⁶⁹. These data provide compelling evidence that the unknown amino acid sensor lies at, and probably within, the lysosome.

Here, we briefly discuss a few of the many other factors that have been found to influence amino acid sensing by mTORC1. For any putative amino-acid-sensing pathway upstream of mTORC1, it will be critical to determine how it interfaces with the Rag proteins, as their regulation seems to represent the most proximal event to mTORC1 activation in response to amino acids. Leucyl-tRNA synthetase (LRS) has been proposed through independent studies in yeast⁷⁰ and mammalian cells⁷¹ to function as an amino acid sensor that regulates the Rag proteins, but major mechanistic differences exist between these studies. Most notably, the yeast study suggested that LRS regulates Gtr1, the RagA/B orthologue, whereas the mammalian study suggested specific regulation of RagD, a Gtr2 orthologue. Another study has indicated that p62 (also known as sequestrome 1, or SQSTM1), which targets proteins for degradation via autophagy and is itself a substrate of autophagy, is involved in amino acid sensing by mTORC1 through a direct interaction with RagC/D (ref. 72). However, unlike components of the Rag–Ragulator circuit, p62 is dispensable for insulin to stimulate mTORC1. MAP4K3 (mitogen-activated protein kinase kinase kinase 3) is activated in response to amino acids and functions upstream of the Rag GTPases to promote mTORC1 signalling in both *Drosophila* and mammalian systems^{73–75}. Understanding the nature of the molecular connection of MAP4K3 to both amino acids upstream and the Rags downstream requires further study. Interestingly, the two deamination steps of glutaminolysis, which convert glutamine to α -ketoglutarate, have been shown to influence the ability of leucine and glutamine to stimulate mTORC1 signalling⁷⁶. Although the molecular basis of this link is unknown, it seems that glutaminolysis promotes GTP-loading of RagB, and the product α -ketoglutarate can stimulate mTORC1 translocation to the lysosome even in the absence of amino acids. The vertebrate-specific protein SH3BP4 has been found to directly bind to the Rags and attenuate the stimulation of mTORC1 signalling by amino acids⁷⁷. The relationship between other emerging amino-acid-sensing pathways and the Rag GTPases is poorly understood. These include the class III PI(3)K Vps34 and phospholipase D (discussed further in Box 1), the G-protein-coupled taste receptors T1R1 and T1R3 (ref. 78), and the inositol polyphosphate multikinase (IMPK)⁷⁹. It is clear from these and other studies that there will be many cellular pathways and

BOX 1 Signals from endogenous and dietary lipids

Relatively little is known about how intracellular and dietary lipids influence mTORC1 signalling. Two lipid signalling molecules found on endomembranes, phosphatidylinositol-3-phosphate (PtdIns(3)P) and phosphatidic acid — and the enzymes that produce them, Vps34 and phospholipase D (PLD), respectively — have been found to promote mTORC1 signalling in some settings^{127–129}. These enzymes and phospholipids have been partially localized to late endosomes and lysosomes, and their regulation of mTORC1 signalling might be tied together. The activities of Vps34 and PLD seem to be responsive to both amino acids and glucose, and their knockdown can impair acute mTORC1 activation by these nutrients^{130–134}, although it has been pointed out that these effects can often be compensated for under steady-state conditions¹²⁸. Through the production of PtdIns(3)P, Vps34 activity has been proposed to recruit PLD to the lysosome, where its product phosphatidic acid is believed to stimulate mTORC1 (refs 128,133). However, genetic ablation of Vps34 in *Drosophila* or in specific mouse tissues has no apparent effect on mTORC1 signalling^{135,136}, and PLD-deficient flies as well as *PLD1*^{−/−} and *PLD2*^{−/−} mice are viable, suggesting a non-essential role for these enzymes in activating mTORC1 (refs 137,138). It is worth noting that other cellular sources of these lipids do exist, but the enzymes responsible for their production have not been specifically implicated in mTOR signalling. Further studies are required to clarify how these lipid signalling pathways fit into the emerging model of spatial integration of signals by mTORC1 at the lysosome.

In mammals, high-fat diets and obesity are associated with elevated mTORC1 signalling in metabolic tissues, which is believed to contribute to the development of insulin resistance under such conditions^{139–142}. The molecular nature of this chronic mTORC1 activation and whether there are cell-autonomous effects of dietary lipids on mTORC1 signalling are not well understood. Free fatty acids have been described in a few studies to acutely stimulate mTORC1 signalling^{142–146}. The upstream events that mediate this effect are unknown but could depend on either energy production through the β -oxidation of fatty acids or signalling from extracellular fatty acids engaging specific GPCRs¹⁴⁷. However, it is somewhat paradoxical that mTORC1 would be activated by free fatty acids, given that it can promote the *de novo* synthesis of fatty acids through its induction of SREBP (refs 19,27). The relationship between lipid sensing and synthesis by mTORC1 remains an important area of investigation, as aberrant activation of mTORC1 under conditions of obesity is believed to be a molecular factor underlying many of the pathological manifestations of obesity.

processes that impinge, either directly or indirectly, on the emerging amino-acid-sensing system at the lysosome to influence the basal state of mTORC1 activation.

Integrating nutrient sensing with growth factor signalling

It has been proposed that spatial regulation of mTORC1 through the Ragulator–Rag circuit, in conjunction with its requirement for the GTP-loading of Rheb, serves as a coincidence detector or a molecular ‘and gate’, allowing a hierarchy of signals to be integrated by mTORC1

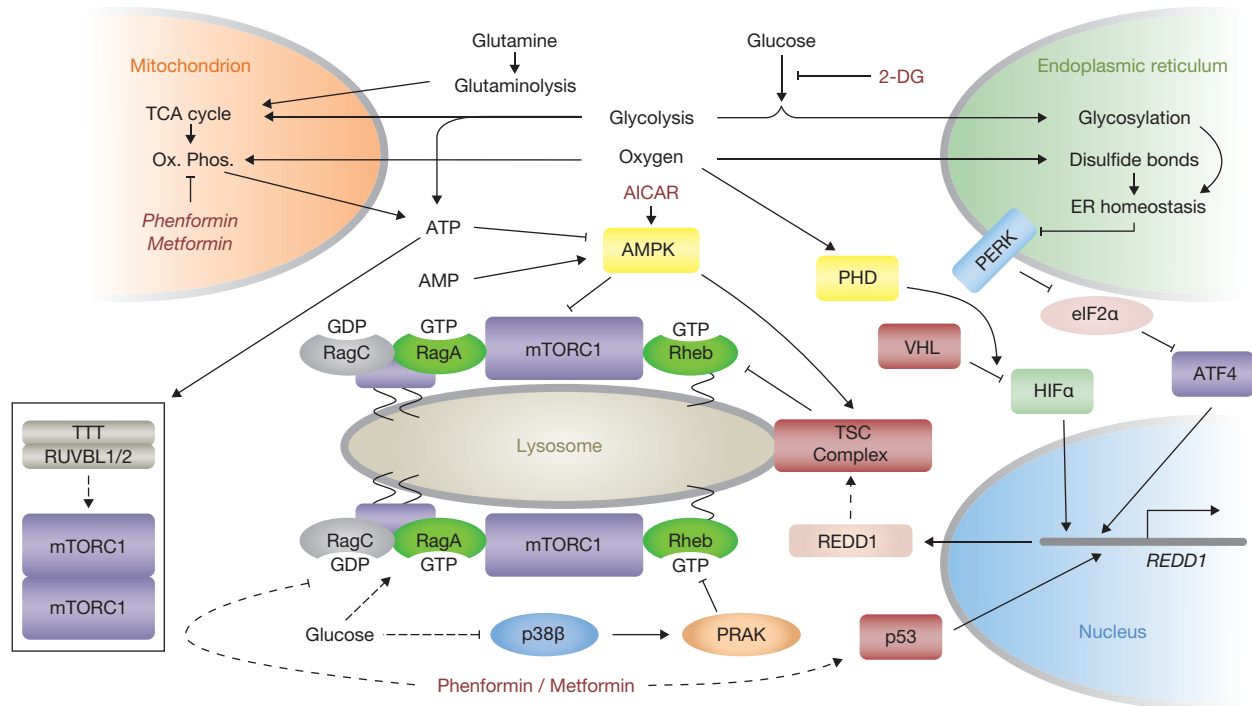


Figure 4 Transcriptional and post-translational regulation of mTORC1 by glucose, oxygen and cellular energy. Sufficient glucose and oxygen levels are required for mTORC1 activation, and many sensing mechanisms have been identified. Glucose, glutamine and oxygen are utilized for ATP production through glycolysis, the citric acid (TCA) cycle and oxidative phosphorylation (ox. phos.). Decreased availability of these nutrients can lower ATP levels, with a subsequent rise in AMP levels — conditions that stimulate the activation of AMPK. AMPK inhibits mTORC1 through activation of the TSC complex (which inhibits Rheb) and phosphorylation of Raptor within mTORC1. Glucose or oxygen deprivation, as well as other forms of energy stress, also stimulates the transcription of *REDD1* through the action of either the HIF1, ATF4 or p53 transcription factors. REDD1 somehow cooperates with the TSC complex to inhibit Rheb and mTORC1. Through their sensing of AMP and oxygen, respectively, AMPK and the prolyl hydroxylase proteins (PHD) represent the only known direct sensors

of cellular metabolic status within this network. As well as energy stress, glucose and oxygen can also be sensed through endoplasmic reticulum (ER) homeostasis, as they are required for proper protein glycosylation and disulfide bond formation, respectively. Disrupting these processes results in activation of PERK and inhibition of eIF2 α , resulting in the selective translation of ATF4. Glucose starvation and energy stress also seem to signal to mTORC1 through the Rag GTPases, albeit through unknown mechanisms, and through a pathway involving the p38 β and PRAK kinases (leading to direct phosphorylation of Rheb). Inset box: severe states of ATP depletion inhibit the ability of the TTT–RUVBL1/2 complex to promote formation of functional mTORC1 dimers. Note that mTORC1 is depicted as a single unit at the lysosome for simplicity. Compounds such as 2-deoxyglucose (2-DG), AICAR and the biguanides metformin and phenformin also have inputs into these different mechanisms of mTORC1 inhibition. Dashed lines denote unknown molecular mechanisms.

(refs 3,66). Whereas growth factor signalling pathways that increase Rheb^{GTP} levels by impinging on the TSC complex are required for maximal mTORC1 activation, they fail to activate mTORC1 in the absence of amino acids. Rheb localizes on the surface of multiple endomembrane compartments, at least in part through a C-terminal sequence that is farnesylated^{80–82}, a modification required for Rheb to activate mTORC1 signaling^{40,83}. Importantly, a subpopulation of Rheb resides at the lysosome⁶⁶. Therefore, the recruitment of mTORC1 to this compartment through the Ragulator–Rag system in response to amino acids brings mTORC1 in contact with its essential upstream activator Rheb (Fig. 3b). This mechanism helps explain the dominance of amino acid signalling over growth factor signalling to mTORC1. Through the lysosomal shuttling of mTORC1, the amino-acid-sensing pathway facilitates the association of Rheb and mTORC1, but the ultimate activation of mTORC1 seems to be determined by the GTP/GDP-loading state of Rheb, which is controlled by the TSC complex. Interestingly, growth factor signalling pathways might also impinge on Rheb regulation directly at the lysosome, as the TSC complex also localizes to the lysosomal surface⁴⁵. This current spatial model of signal integration is enticing, but it will be

important to more completely define the molecular mechanisms and temporal nature of these and other signals influencing both the activation and inhibition of mTORC1 signalling.

Multiple inputs from glucose, oxygen and cellular energy levels

As well as amino acids, the presence of two other essential cellular nutrients, glucose and oxygen, are sensed by mTORC1; they are also required for both its basal and growth-factor-stimulated activation. A decrease in the availability of glucose or oxygen to cells results in profound changes in cellular metabolism and can cause an acute, but often transient, drop in cellular energy levels, in the form of ATP. Cells respond to such changes by tipping the metabolic balance from anabolic processes that consume energy and carbon (for macromolecular biosynthesis) to catabolic pathways that produce energy. As a major promoter of anabolic processes, mTORC1 is a key target in this metabolic adaptation and is negatively regulated by decreases in glucose, oxygen and/or energy levels^{84–86}. This regulation is now known to occur through multiple interconnected adaptive response mechanisms lying upstream of mTORC1 (Fig. 4).

One of the first lines of defence against energy stress — defined as the depletion of cellular ATP — is acute activation of the AMP-dependent protein kinase (AMPK), which is activated by even subtle decreases in cellular ATP levels⁸⁷. Through numerous downstream targets, AMPK initiates an adaptive program that promotes catabolic metabolism and inhibits anabolic processes. For instance, AMPK stimulates autophagy while inhibiting lipid and protein synthesis^{33,87–89}. Critical to this adaptive response is the inhibition of mTORC1 signalling, which occurs through the AMPK-mediated phosphorylation of at least two pathway components, TSC2 and Raptor. Through phosphorylation of TSC2 on Ser 1387, which acts as a priming event for subsequent phosphorylation of more sites by GSK3, AMPK promotes the inhibition of Rheb and mTORC1 by the TSC complex^{90–92}. Hence, loss of any TSC complex component renders the mTORC1 pathway at least partially resistant to energy-stress-inducing conditions, including glucose starvation, inhibition of glycolysis or oxidative phosphorylation, or hypoxia^{45,90,92–94}. AMPK has also been found to have a direct inhibitory effect on mTORC1 by phosphorylating Raptor on Ser 792 (ref. 95). The relative contributions of the AMPK-mediated activation of the TSC complex, resulting in decreased levels of Rheb^{GTP} and this more direct inhibition of mTORC1, are unknown. However, one possibility is that Raptor phosphorylation blocks basal activation of mTORC1, whereas AMPK-mediated phosphorylation of TSC2 overrides growth factor signalling through the TSC complex that would otherwise activate mTORC1. Importantly, the regulation of mTORC1 by AMPK renders mTORC1 signalling sensitive to a rapidly expanding list of chemicals, xenobiotics and natural products that activate AMPK, including commonly prescribed drugs such as metformin and aspirin⁹⁶.

Another major regulator of mTORC1 signalling that is involved in the adaptation to hypoxia, glucose starvation and perhaps other cellular stresses is the protein REDD1 (regulated in development and DNA damage responses 1; also known as DDIT4, Dig2 and RTP801). The *Drosophila* orthologues of REDD1 were identified in a genetic screen for regulators of cell growth, with their overexpression or loss resulting in a respective decrease or increase in cell and organ growth, similarly to that observed with TSC1 and TSC2 (ref. 97). Importantly, hypoxia stimulates the expression of these genes and mammalian *REDD1* through the stabilization and activation of HIF1^{97,98} — which, in the presence of oxygen, is degraded through the action of oxygen-dependent prolyl-hydroxylases and the von-Hippel Lindau (VHL) E3 ubiquitin ligase⁹⁹. The HIF1-mediated induction of *REDD1* seems to be required for the sustained inhibition of mTORC1 signalling under hypoxia⁹³ (that is, 1% oxygen). Genetic and cell biological evidence indicates that REDD1 inhibits mTORC1 signalling through the TSC complex, although the mode of action of REDD1 has yet to be fully elucidated^{93,97,100,101}. The activation of HIF1, its induction of REDD1, and the subsequent inhibition of mTORC1 signalling in response to hypoxia, seems to also require upstream input from the ATM tumour suppressor, a protein kinase best known for its role in the DNA-damage response¹⁰². Interestingly, REDD1 is also required for the inhibitory effects of energy stress on mTORC1 signalling, including the effects induced by glucose starvation, 2-deoxyglucose, and metformin^{103,104}. Under these and other stress conditions that inhibit mTORC1, it is likely that *REDD1* expression is driven by the transcription factors p53 or ATF4 rather than HIF1 (refs 104–106). The ATF4-mediated expression of *REDD1* seems to be independent of energy stress. Rather, it is stimulated downstream of endoplasmic

reticulum (ER) stress, resulting from the deleterious effects of glucose starvation, 2-deoxyglucose or hypoxia on protein maturation in the ER (refs 105,107,108). In general, the sensitivity of protein glycosylation and disulfide bond formation to the availability of glucose and oxygen, respectively, renders the ER (and the adaptive stress response originating therein, known as the unfolded protein response) a key component of cellular nutrient sensing. It seems likely that the relative contributions of acute activation of AMPK and the transcriptional induction of REDD1 to inhibitory signals affecting mTORC1 in response to hypoxia will depend on many factors, including: the duration of exposure to hypoxia; the dependence of the cell on oxidative metabolism; the secretory properties of the cell; and the tissue microenvironment^{94,109,110}. Similar principles are likely to apply to glucose deprivation and various other forms of nutrient and energy stress.

In addition to the mechanisms discussed above, pathways by which glucose and energy stress impinge on mTORC1 signalling independently of the TSC complex have also emerged. Depletion of cellular energy levels can activate the stress-responsive mitogen-activated protein kinase p38 and its direct target p38-regulated/activated kinase (PRAK), albeit through an unknown mechanism^{111,112}. In cells deficient for either the β isoform of p38 (p38 β) or PRAK, mTORC1 signalling is largely unresponsive to 2-deoxyglucose, which blocks glycolysis and other glucose-utilizing processes, suggesting that the p38 β –PRAK pathway is required to suppress mTORC1 signalling under such conditions¹¹². It seems that this pathway inhibits mTORC1 signalling through the PRAK-mediated phosphorylation of Ser 130 on Rheb, which is proposed to disrupt its ability to bind GTP. Other studies, however, have found that p38-dependent signalling stimulates, rather than inhibits, mTORC1 (refs 113–115). Severe conditions of energy stress, such as that caused by combined glucose and glutamine starvation in cell culture, can even impair the assembly of mTORC1, thereby overriding all other upstream regulatory events¹¹⁶. This occurs by disrupting the association between mTOR and the Tel2–Tti1–Tti2 (TTT)–RUVBL1/2 complex, which is required for the proper folding and stability of mTOR and related kinases^{117,118}. Within the TTT–RUVBL complex, the ATPase activity of RUVBL is susceptible to cellular ATP depletion, and loss of this activity results in a defect in higher-order assembly of mTORC1 into a homodimer¹¹⁶, which is the functional signalling complex¹¹⁹.

There is genetic evidence that the Rag GTPases are also involved in glucose sensing by mTORC1. It has been found that mouse embryonic fibroblasts that are homozygous for a constitutively GTP-bound mutant of RagA (*RagA^{GTP}*) are resistant to the inhibitory effects of either amino acid or glucose withdrawal on mTORC1 signalling⁶⁵. Furthermore, due to an inability to downregulate mTORC1 in response to a natural drop in blood glucose levels immediately after birth, *RagA^{GTP/GTP}* neonates perish before suckling. An independent study also provided evidence that the Rag GTPases are involved in the sensing of some forms of energy stress by mTORC1 (ref. 120). It is predicted from these studies that the GTP/GDP-loading state of the RagA/B–RagC/D heterodimer is affected by glucose withdrawal or energy-stress-inducing conditions, but as is the case for amino acids, the sensing mechanism is currently unknown. It is interesting to note that in both yeast and mammalian cells, glucose starvation has been found to induce an acute disassembly of v-ATPase, and this is rapidly reversed by reintroduction of glucose^{121,122}. Such a mechanism could influence the ability of the v-ATPase complex to stimulate Regulator GEF activity, thereby blocking RagA/B–GTP loading and

the recruitment of mTORC1 to the lysosome during glucose starvation.

The myriad of mechanisms (that have been uncovered so far) by which decreases in intracellular glucose, oxygen and/or ATP lead to inhibition of mTORC1, even in the presence of growth factors, underscore the importance of attenuating mTORC1 signalling and its downstream anabolic processes for adapting to nutrient and energy depletion.

Conclusions and outstanding questions

The mTORC1 signalling network integrates information about the complex nutrient environment of individual cells, tissues and organisms to mount an appropriate physiological response. Although impressive progress has been made over the past decade in understanding mTORC1 signalling, critical questions remain. For instance, apart from AMPK and the prolyl hydroxylases upstream of HIF1, direct sensors of nutrients and metabolites within the upstream signalling network have not yet been identified. Importantly, the molecular details by which signals impinge on mTORC1 regulation have been revealed, almost exclusively, through cell culture studies under largely non-physiological conditions. The experimental comparison of two extreme conditions, such as complete removal of a specific nutrient followed by acute re-feeding, has been essential to provide robust biochemical and cell biological readouts to characterize the signalling mechanisms underlying a given response. Although the molecular pathways characterized in such studies are likely to be similar *in vivo*, we must define the signals that dominantly control mTORC1 in different tissues — where, unlike in cell culture models, mTORC1 is generally in the ‘off’ state and only transiently activated in response to specific stimuli. Cell culture experiments suggest that nutrients only basally activate mTORC1, but the relative contribution of nutrient and growth factor signals to mTORC1 activation *in vivo* is poorly understood. Conditions referred to as ‘energy stress’ in cell culture models, which are maintained under super-physiological levels of growth factors and nutrients, are likely to be closer to the homeostatic state *in vivo*. This is illustrated by the fact that loss of the LKB1 tumour suppressor, which is required for AMPK activation by energy stress, results in the formation of gastrointestinal polyps that exhibit high levels of mTORC1 signalling relative to the normal epithelium⁹². Therefore, removal of the inhibitory signal from AMPK in this setting is sufficient to activate mTORC1.

Consistent with cell culture studies, mTORC1 signalling in the liver is inhibited under fasting conditions through a pathway dependent on the TSC complex¹²³. However, feeding induces a robust and transient activation of mTORC1 in this tissue through a mechanism that seems to be independent of insulin signalling¹²⁴, suggesting that an unknown nutrient input might be dominating this response. Under conditions of dietary (or calorie) restriction, which has been shown to prolong the lifespan of many organisms through mechanisms believed to involve mTORC1 inhibition, the attenuation of mTORC1 signalling in different tissues is likely to reflect decreases in specific local nutrients, as well as circulating insulin and IGF1 (refs 125,126). The importance of defining the molecular mechanisms and hierarchy of signals that regulate mTORC1 signalling *in vivo* is highlighted by the diverse disease settings in which mTORC1 is aberrantly activated, including ageing-related diseases such as cancer and diabetes.

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The multifaceted role of mTORC1 in the control of lipid metabolism

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The mechanistic target of rapamycin is a protein kinase that, as part of the mechanistic target of rapamycin complex 1 (mTORC1), senses both local nutrients and, through insulin signalling, systemic nutrients to control a myriad of cellular processes. Although roles for mTORC1 in promoting protein synthesis and inhibiting autophagy in response to nutrients have been well established, it is emerging as a central regulator of lipid homeostasis. Here, we discuss the growing genetic and pharmacological evidence demonstrating the functional importance of its signalling in controlling mammalian lipid metabolism, including lipid synthesis, oxidation, transport, storage and lipolysis, as well as adipocyte differentiation and function. Defining the role of mTORC1 signalling in these metabolic processes is crucial to understanding the pathophysiology of obesity and its relationship to complex diseases, including diabetes and cancer.

Keywords: adipocytes; Akt; insulin; liver; mTOR

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See the Glossary for abbreviations used in this article.

Introduction

Of the four main classes of biological macromolecule, our understanding of the molecular mechanisms by which cellular signalling pathways regulate lipid metabolism has lagged behind that of carbohydrates, proteins and nucleic acids. However, lipids are crucially important both structurally and functionally in all living organisms. An obvious reason for this dependence is the lipid makeup of the plasma membrane and many subcellular organelles. Moreover, lipids act as signalling molecules on both a cellular, for example phosphoinositides, and organismal, for example steroid hormones, scale. Lipids are also used for energy storage, primarily as triacylglycerides in adipocytes, and as an alternative to glucose for catabolic metabolism. Despite the dependence of living organisms on lipids, we know little about how lipid homeostasis is controlled by the intricate network of cellular signalling pathways that sense cellular growth conditions. As detailed in this review, the mechanistic target of rapamycin (mTOR) protein kinase has emerged as a crucial link between cellular and systemic growth signals and the regulation of lipid metabolism.

mTOR is an evolutionarily conserved serine/threonine kinase that exists within two functionally distinct protein complexes, the mechanistic target of rapamycin complexes 1 (mTORC1) and 2 (mTORC2). mTORC1 senses and integrates a diverse array of cellular signals, with mTOR kinase activity within the complex being influenced by a variety of nutrients—for example, amino acids, glucose and oxygen, cellular energy levels, such as ATP, and many secreted growth factors, cytokines and hormones, including insulin. All of these signals require the Ras-related small G protein Rheb, which on GTP-loading is an essential upstream activator of mTORC1 [1]. Many of the signals that regulate mTORC1 do so by altering the GTP-binding status of Rheb through activation or inhibition of a GTPase-activating protein complex, comprised of TSC1, TSC2 and TBC1D7—the TSC–TBC complex [2]. For instance, insulin, IGF1 and other growth factors inhibit the complex to activate Rheb and mTORC1 through Akt-mediated phosphorylation of TSC2 [3,4]. By contrast, a decrease in cellular ATP, such as the decrease that occurs during glucose depletion, activates the complex to inhibit Rheb and mTORC1, at least in part, through the action of AMPK (Fig 1; [5–7]). On activation, mTORC1 directly phosphorylates S6K1 and S6K2, 4E-BP1 and 4E-BP2, and a growing number of other downstream targets [8]. Whilst the overall effects of mTORC1 signalling differ in cells and tissues, it has an evolutionarily conserved role in promoting anabolic cell growth and inhibiting the catabolic process of autophagy. On the other hand, mTORC2 seems to be regulated primarily by growth factor signalling and phosphorylates a conserved hydrophobic motif in the protein kinases Akt, SGK and some isoforms of PKC, thereby increasing their kinase activity [9]. Through these targets, and probably through others, mTORC2 signalling is believed to promote cell survival, proliferation, metabolism and changes in the actin cytoskeleton. The two mTOR complexes can be distinguished from one another by their differential sensitivity to rapamycin, an allosteric and partial inhibitor of mTOR (Sidebar A).

Many studies in cell and mouse models, combined with pre-clinical and clinical data on mTOR inhibitors, have revealed a pivotal role for mTOR—particularly within mTORC1—in controlling lipid homeostasis in many settings, both physiological and pathological. We review this evidence below, with a focus on the key aspects of lipid synthesis, storage and mobilization. The emerging picture is that, through a variety of molecular mechanisms, mTORC1 signalling promotes processes to synthesize and store lipids, whilst inhibiting those leading to lipid consumption (Fig 1).

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Sidebar A | mTORC1 versus mTORC2 and the differential effects of mTOR inhibitors

In studying the mechanistic target of rapamycin (mTOR) signalling network, or interpreting the mTOR literature, it is crucial to understand some of the basic complexities of mTOR signalling and inhibition. The mechanistic target of rapamycin complex 1 (mTORC1) is composed of the core essential components mTOR, mTOR-associated protein, LST8 homologue (mLST8) and the regulatory-associated protein of mTOR (Raptor), whereas mTORC2 is composed of mTOR, mLST8, SAPK-interacting protein 1 (SIN1) and the Raptor-independent companion of mTOR (Rictor). Although these complexes are functionally distinct, they can have an influence on each other's activity. For instance, as mTORC2 stimulates an increase in Akt activity [84], it might influence its downstream signalling from mTORC1. On the other hand, several negative feedback mechanisms are triggered by mTORC1 activation, which influences mTORC2 activity, including one leading to direct phosphorylation of Rictor within mTORC2 by ribosomal S6 kinase 1 (S6K1) downstream from mTORC1 [85,86]. Regarding mTOR inhibitors, the widely used rapamycin and its many analogues, which on interaction with the ubiquitous protein FK506 binding protein of 12 kDa (FKBP12) binds to an allosteric site amino terminal to the mTOR kinase domain—the FKBP12-rapamycin binding domain—only has access to mTOR within mTORC1. However, it is evident that in both cell culture and mice, prolonged exposure to rapamycin can block the assembly of mTORC2 by sequestering uncomplexed mTOR [82,87]. Therefore, although rapamycin is specific to mTORC1 for acute inhibition and generally leads to an increase in upstream signalling from mTORC2 and Akt by blocking negative feedback mechanisms, one must consider that the observed effects of long-term rapamycin treatment might be due to loss of mTORC2 in some experimental systems, which affects the many processes downstream from Akt. Also, the development of mTOR kinase domain inhibitors, which completely block mTOR within both complexes, has revealed that rapamycin only partly inhibits mTORC1 activity. Whilst the nature of this differential sensitivity is unknown, rapamycin strongly affects the phosphorylation of some mTORC1 targets (for example, S6K1) but only modestly inhibits other targets (for example, eIF4E-binding protein 1; [88]).

Lipogenesis

The regulation of *de novo* sterol and fatty acid synthesis by signalling pathways, especially insulin signalling, has garnered intense interest. Unlike most terminally differentiated cells, hepatocytes and adipocytes synthesize significant amounts of lipid *de novo* through pathways in which cytosolic acetyl-CoA, derived from glucose or amino acid catabolism, is used to form the hydrophobic carbon backbone of lipids. Acetyl-CoA is either committed to sterol and isoprenoid biosynthesis through the action of HMG-CoA synthase or to fatty acid biosynthesis through acetyl-CoA carboxylase. Both the sterol and fatty acid synthesis branches comprise many steps requiring many specific enzymes. Importantly, the SREBPs are transcription factors that stimulate the expression of genes encoding nearly all of these lipogenic enzymes [10]. The three SREBP isoforms, encoded by two genes, are produced as inactive transmembrane proteins at the endoplasmic reticulum (Fig 2). Under conditions of abundant sterols, full-length SREBP, through its sterol-sensing binding partner SCAP, is retained in the endoplasmic reticulum by the INSIG proteins [11]. Depletion of intracellular sterols results in release of the SREBP-SCAP complex from Insig and their transport to the Golgi apparatus, in which two proteolytic cleavage events by the site-specific proteases S1P and S2P liberate the active amino-terminus of SREBP. This fragment then enters the nucleus and

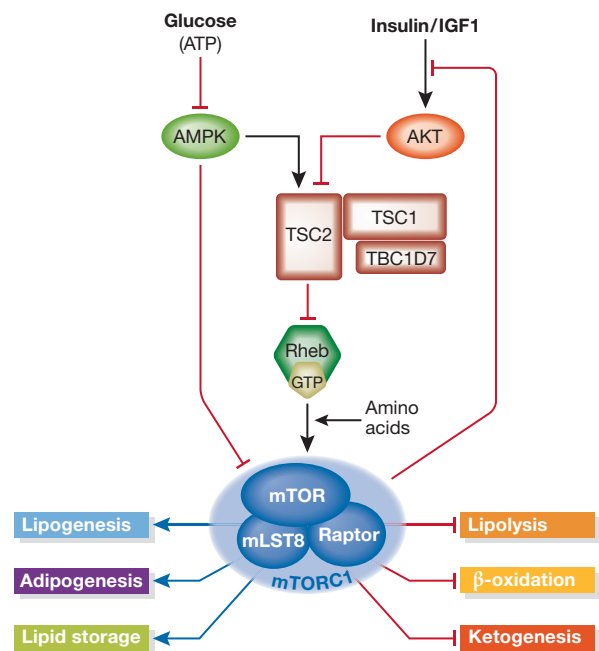


Fig 1 | Upstream regulation from the mTORC1 and its downstream functions related to lipid metabolism. The presence of amino acids is required for the activation of mTORC1 by GTP-bound Rheb. Upstream from Rheb, the TSC–TBC complex receives signals about systemic and local nutrient and energy availability, in part through AMPK and Akt. These signals either activate or inhibit the ability of the TSC–TBC complex to act as a GAP for Rheb, thereby inhibiting or activating mTORC1, respectively. Activated mTORC1 leads to enhanced phosphorylation of IRS1, which serves as negative feedback to dampen the insulin response. mTORC1 has many roles in regulating lipid metabolism, including the promotion of lipid synthesis and storage and inhibition of lipid release and consumption, which are detailed in the text. AMPK, adenosine monophosphate-activated protein kinase; GAP, GTPase-activating protein; IRS1, insulin receptor substrate 1; IGF1, insulin-like growth factor 1; mTORC1, mechanistic target of rapamycin complex 1; Raptor, regulatory-associated protein of mTOR; TSC, tuberous sclerosis complex; TBC, Tre-2/Bub2/Cdc16 domain-containing protein.

induces transcription from SREs within target genes. SREBP1a and 1c are products of alternative splicing of the *SREBF1* gene and have been primarily implicated in the control of genes involved in fatty acid synthesis, although SREBP1a is thought to activate most SRE-containing genes [12]. SREBP2 is encoded by *SREBF2* and is believed to have a more important role in the transcription of steroidogenic genes, including those involved in cholesterol synthesis in the liver [13,14]. Although the SREBPs preferentially activate transcription of different sets of genes, there is substantial overlap between the targets of the SREBP isoforms and the tissue specificity of these preferences, which has not been fully established. Importantly, independent studies have identified the SREBPs as major transcriptional effectors of mTORC1 signalling and have demonstrated that mTORC1 activation promotes lipogenesis through this family of transcription factors [15,16].

mTORC1 signalling promotes SREBP activation and lipogenesis in response to both physiological and genetic stimuli. In primary rodent hepatocytes and the intact liver, insulin or feeding has been shown to increase the expression of the major liver isoform

Glossary

4E-BP1/2	eIF4E-binding protein 1/2
AMPK	adenosine monophosphate-activated protein kinase
ATG5/7	autophagy-related 5/7
ATGL	adipose triglyceride lipase
C/EBP	CCAAT/enhancer-binding protein
CPT1	carnitine palmitoyltransferase 1
DAG	diacylglycerol
eIF4E	eukaryotic translation initiation factor 4E
GSK3	glycogen synthase kinase 3
HSL	hormone-sensitive lipase
IDL	intermediate density lipoprotein
IGF1	insulin-like growth factor 1
Insig	insulin-induced gene
LDL	low density lipoprotein
LDLR	LDL receptor
lipin 1	phosphatidate phosphatase LPIN1
LPL	lipoprotein lipase
LST8	lethal with SEC13 protein 8
MAG	monoacylglycerol
MEF	mouse embryonic fibroblast
N-CoR1	nuclear receptor co-repressor 1
PCSK9	proprotein convertase subtilisin/kexin type 9
PKA/C	protein kinase A/C
PPAR α / γ	peroxisome proliferator-activated receptor α / γ
Raptor	regulatory-associated protein of mTOR
Rictor	Raptor-independent companion of mTOR
S6K1/2	ribosomal S6 kinase 1/2
SCAP	SREBP cleavage-activating protein
SCD	stearoyl-CoA desaturase
SGK	serum and glucocorticoid regulated kinase
shRNA	short hairpin RNA
siRNA	small interfering RNA
SRE	sterol response element
SREBP	sterol regulatory element-binding protein
TAG	triacylglycerol
TBC1D7	TBC1 domain family, member 7
TCA	tricarboxylic/citric acid
TSC1/2	tuberous sclerosis complex 1/2
VLDL	very low density lipoprotein

of SREBP (SREBP1c) and its targets, and to promote *de novo* lipid synthesis in a manner that is sensitive to rapamycin [17–19]. Insulin activates mTORC1 through a pathway involving the Akt-mediated phosphorylation and inhibition of TSC2, within a complex with TSC1 and TBC1D7 [2–4]. Expression of constitutively active Akt or loss of either TSC1 or TSC2, both of which result in insulin-independent activation of mTORC1 signalling, stimulates the global expression of SREBP1 and SREBP2 targets and drives lipogenesis through mTORC1 [15,16]. These latter studies found that mTORC1 signalling promotes accumulation of the processed, mature form of SREBP1, which resides in the nucleus to induce its own expression and that of genes involved in both steroid and fatty acid biosynthesis. In exploring the molecular mechanism of this regulation, it was found that S6K1 is required downstream from mTORC1 to stimulate the increase in levels of active SREBP1, expression of SREBP1 and SREBP2 targets, and *de novo* lipogenesis in TSC2-deficient cells [15]. SREBP1 regulation in this setting is independent of the effects on the proteasomal degradation of its active form, suggesting that S6K1 promotes the processing of SREBP1. Consistent with these findings, S6K1 has been found

to promote the activation of hepatic SREBP1c by having an effect on its processing [20,21], and to affect the processing of SREBP2 in a hepatocellular carcinoma cell line [22]. mTORC1 signalling has also been suggested to increase SREBP1 activation in an S6K1-dependent manner in cultured myotubes [23].

Genetic mouse models have demonstrated that mTORC1 activation is essential, but not sufficient, to stimulate hepatic SREBP1c and its lipogenic targets in response to feeding [18,24]. Mice lacking mTORC1 in their liver, through liver-specific *Raptor* knockout, fail to induce SREBP1c and lipogenesis [24], and have reduced levels of both liver triglycerides and circulating cholesterol on a 'Western' diet [25]. However, characterization of mice with a liver-specific knockout of *Tsc1* (LTsc1KO) and constitutive activation of mTORC1, which is independent of insulin and feeding, revealed that mTORC1 signalling, although essential, is not capable of activating SREBP1c and hepatic lipid synthesis on its own [18]. In fact, these mice were found on two independent strain backgrounds to be resistant to the development of both age- and diet-induced hepatic steatosis due to decreased SREBP1c activation [18,26]. These seemingly paradoxical findings are the result of a strong feedback attenuation of Akt signalling that accompanies loss of function of the TSC1–TSC2 complex in all settings [27]. A crucial role for Akt signalling in the induction of SREBP1c and lipogenesis in the liver has been established through rodent models [28–30], and this has been extended by using mice with liver-specific *Rictor* knockout, which results in the loss of mTORC2 activity and its activating phosphorylation of Akt [31]. Consistent with the essential nature of Akt signalling to hepatic SREBP1c, a restoration of Akt activity in LTsc1KO hepatocytes restores SREBP1c activation and lipogenesis [18]. Whilst many mTORC1-independent pathways might function in parallel downstream from Akt to help to promote the activation of hepatic SREBP1c, including GSK3 inhibition [32], data from the LTsc1KO mice suggest that one pathway involves the repression of an isoform of the SREBP inhibitor Insig, Insig2a, which is only expressed in the liver [18]. A liver-specific mechanism is also consistent with the fact that mTORC1 activation alone is sufficient to promote SREBP activation and lipogenesis in other settings, even in the absence of Akt signalling [15].

The molecular mechanism by which S6K1 promotes SREBP processing is unknown, and it is clear from additional studies that S6K1 is not the only direct target downstream from mTORC1 involved in SREBP isoform regulation, which might vary by cellular context. For instance, siRNA knockdown of the mRNA cap-binding protein eIF4E, which is normally activated by mTORC1 signalling through the phosphorylation and release of its inhibitory binding partner 4E-BP1, decreases overall levels of SREBP1 and its canonical target SCD in breast cancer cell lines [33]. The potential involvement of 4E-BP1 regulation by mTORC1 in some cells might explain the resistance of SREBP1 or SREBP2 activation to rapamycin in specific settings [22,34]. The resistance of some mTORC1 targets to rapamycin (Sidebar A) is an important consideration when examining the role of mTORC1 signalling in any aspect of lipid metabolism. Another direct target of mTORC1 that, as with 4E-BP1, is partly resistant to rapamycin for its regulation is the phosphatidic acid phosphatase lipin 1, which has also been implicated in SREBP regulation [25,35]. Lipin 1 seems to have a role in the remodelling of the nuclear lamina, which is inhibited by mTORC1-mediated phosphorylation of many residues on this enzyme. Lipin 1 phosphorylation also coincides

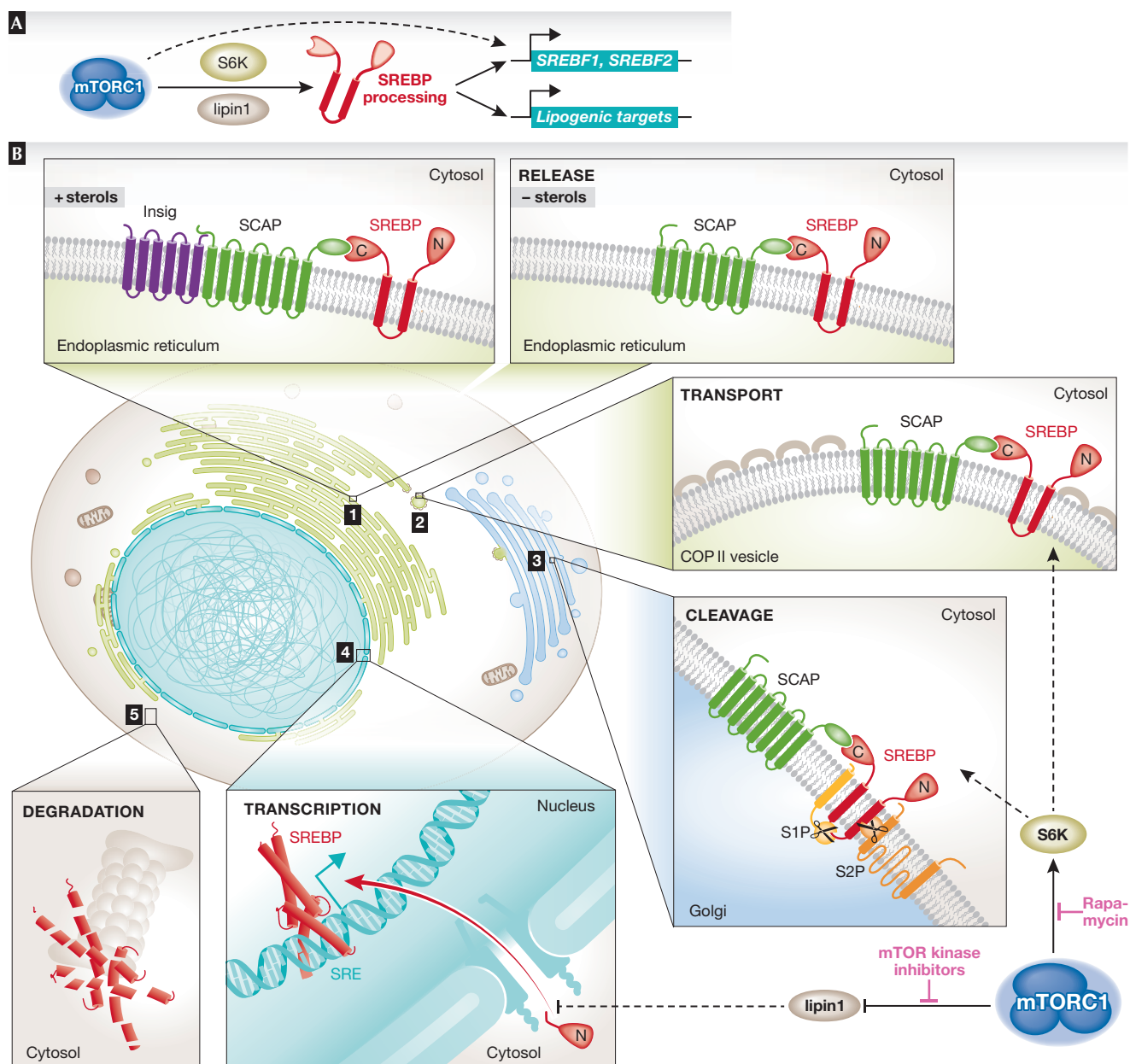


Fig 2 | The complex steps leading to SREBP activation and input from mTORC1 signalling. **(A)** SREBP processing and activation is regulated by mTORC1 through S6K and lipin 1 leading to the transcriptional induction of the *SREBF1* and *SREBF2* genes, encoding SREBP1 and SREBP2, respectively, and genes encoding many lipogenic enzymes involved in both fatty acid and sterol synthesis. The mTORC1-mediated transcriptional activation of *SREBF1* could result from either autoregulation by SREBP1 or from an unknown parallel pathway downstream from mTORC1. **(B)** In the presence of sterols, SREBP resides in the endoplasmic reticulum bound to SCAP and the Insig proteins. When sterols become scarce SCAP undergoes a conformational change, which releases the SCAP–SREBP complex from the Insig, allowing its transport from the endoplasmic reticulum to the Golgi apparatus through COPII vesicles. Once in the Golgi, SREBP comes into contact with two site-specific proteases. S1P cleaves the luminal loop of SREBP and S2P cleaves the amino-terminal transmembrane region of SREBP, which releases the N-terminal region of SREBP containing the DNA-binding and -transactivating domains. The NLS-containing processed form of SREBP enters the nucleus to activate transcription of genes containing SREs in their promoters. Finally, the processed form of SREBP is unstable and subject to proteasome-mediated degradation. In some settings, SREBP processing has been found to require S6K1 downstream from mTORC1 and is therefore sensitive to rapamycin. However, the nuclear shuttling of SREBP has been found to require lipin 1 downstream from mTORC1, the phosphorylation of which is largely resistant to rapamycin but sensitive to mTOR kinase domain inhibitors (Sidebar A). The precise molecular mechanisms by which either of these two mTORC1 targets regulates SREBP activation are unknown. COPII, coatamer protein II; Insig, insulin-induced gene; lipin 1, phosphatidate phosphatase LPIN1; mTORC1, mechanistic target of rapamycin complex 1; NLS, nuclear localization signal; S1/2P, site 1/2 protease; S6K1, ribosomal S6 kinase 1; SCAP, SREBP cleavage-activating protein; SRE, sterol response element; SREBP1/2, sterol regulatory element-binding protein 1/2.

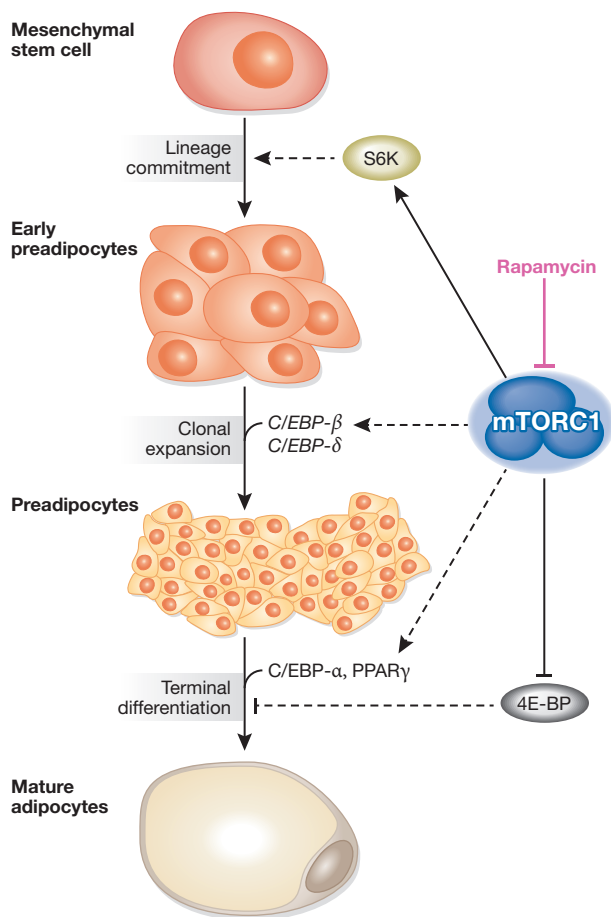


Fig 3 | mTORC1 signalling has been implicated in promoting the three main steps of adipogenesis. Adipogenesis consists of the differentiation of a mesenchymal stem cell to a mature adipocyte, which makes up a significant part of adipose tissue in which energy is stored as lipids. The commitment of the mesenchymal stem cells to the adipocyte lineage is the first step of adipogenesis and is facilitated by S6K1 activity. C/EBP-β and -δ are the primary drivers of clonal expansion, which is crucial for preadipocyte maturation, and the former has been suggested to be activated by mTORC1 signalling. The terminal differentiation of preadipocytes to mature adipocytes is mediated by PPARγ and C/EBP-α. mTORC1 promotes this final step through both its inhibition of 4E-BP and its activation of PPARγ through a poorly understood mechanism. Although the precise molecular mechanisms have yet to be defined, rapamycin blocks adipogenesis. 4E-BP, eIF4E-binding protein; C/EBP-α/β/δ, CCAAT/enhancer-binding protein-α/β/δ; mTORC1, mechanistic target of rapamycin complex 1; PPARγ, peroxisome proliferator-activated receptor γ; S6K1, ribosomal S6 kinase 1.

with an increase in the levels of processed, nuclear SREBP1 and SREBP2, and the expression of SREBP targets. Although the phosphatidic acid phosphatase activity of lipin 1 was shown to be important for its inhibitory effect on nuclear SREBP levels [35], the molecular mechanism and tissue specificity of this regulation, as with S6K1 and 4E-BP1, remains unknown. Finally, it is clear that mTORC1 signalling also increases the transcript levels of SREBP1 and SREBP2 in cell culture models [15], and SREBP1c in both rodent hepatocytes and the intact liver in response to insulin or feeding [18–21]. This

mTORC1-dependent transcriptional response leads to an increase in full-length SREBP isoforms that accompany the increased processing and activation of SREBP. However, it remains unclear whether this transcriptional effect is simply a result of autoregulation by processed SREBPs at the *SREBF1* or *SREBF2* promoter or a parallel pathway independent from the effects of mTORC1 on SREBP processing (Fig 2). Both *SREBF1* and *SREBF2* contain a characterized SRE in their promoters [36,37]. In cell culture models, exogenous expression of processed SREBP1a stimulates the expression of endogenous SREBP1 and SREBP2 transcripts in a manner that is no longer sensitive to rapamycin, suggesting that the transcriptional effects of mTORC1 signalling on SREBP expression are upstream from processed SREBP [15]. However, elegant studies with a transgenic version of SREBP1c in rats suggest that the role of mTORC1 in SREBP1c processing and gene expression is separable [21]. More studies are needed to understand the many inputs of mTORC1 signalling, especially *in vivo*, into the regulation of SREBP isoforms.

Adipogenesis

Adipocytes are specialized mesenchymal cells that either store lipids as energy reserves (white adipose tissue) or burn lipids through oxidation to generate heat (brown adipose tissue). Pharmacological and genetic studies have demonstrated that the differentiation of mesenchymal stem cells into mature adipocytes—adipogenesis—requires mTOR signalling (Fig 3). Rapamycin treatment has been reported to reduce adipogenesis in a variety of cell culture models. Rapamycin seems to block the early determination step in brown adipocyte differentiation, in which a mesenchymal stem cell commits to becoming a preadipocyte [38]. Similarly, rapamycin treatment or shRNA-mediated knockdown of *S6K1* in embryoid bodies hinders their commitment to preadipocytes [39]. However, much of our knowledge of adipogenesis comes from cell culture models of preadipocytes after lineage commitment and also from MEFs, and has therefore been focused on the later steps of white adipose differentiation. Treatment of preadipocytes with rapamycin leads to a marked decrease in adipocyte differentiation [40–44]. mTOR has been implicated in hormonal induction of clonal expansion, which is an initial step of differentiation that occurs through the action of two C/EBP family transcription factors, C/EBP-β and -δ. Overall levels of C/EBP-β have been found to decrease on rapamycin treatment, which corresponds with a repression of clonal expansion of preadipocytes [41]. However, rapamycin has also been shown to inhibit preadipocyte differentiation after clonal expansion, thereby ruling out the anti-proliferative effects of rapamycin as its primary mode of inhibiting adipogenesis [42–44].

Several genetic models have further supported a crucial role for mTORC1 activation in terminal adipocyte differentiation, in which it seems to be both necessary and sufficient. For instance, MEFs lacking TSC1 or TSC2, which have sustained, insulin-independent activation of mTORC1 signalling, have an mTORC1-dependent enhanced capacity to differentiate into adipocytes despite these cells being severely resistant to insulin, a major adipogenic factor [45]. Reciprocally, TSC2-deficient MEFs that express a phosphorylation site mutant of TSC2, which blocks the ability of mTORC1 to be activated by insulin and Akt signalling, show reduced adipogenesis [45]. The enhanced adipogenesis in mesenchymal cells lacking the TSC tumour suppressors probably explains the common development of adipocyte-rich renal angiomyolipomas in patients with TSC [46]. Consistent with an essential role

for mTORC1, RNA interference knockdown of *Raptor* also blocks adipogenesis in preadipocytes [47]. Downstream from mTORC1, genetic evidence suggests a role for both S6K and 4E-BP in the control of adipogenesis. The involvement of S6K in the commitment of stem cells to preadipocytes was reinforced by the reduced size of this progenitor cell population in *S6K1* knockout mice and a defect in the capacity of embryonic stem cells from these mice to commit to the adipocyte lineage [39]. Reciprocally, *4E-BP1/2* double-knockout MEFs show enhanced differentiation towards adipocytes [48], suggesting that the ability of mTORC1 to both activate S6K and inhibit 4E-BP contributes to its role in promoting adipogenesis. Interestingly, the *S6K1* knockout mice have a lean phenotype on both normal and high-fat diets [39,49], whereas the *4E-BP1/2* double-knockout mice are more sensitive to diet-induced obesity than their wild-type counterparts [48]. However, the differences in adiposity in these systemic mouse models probably reflect many effects of mTORC1 signalling on lipid synthesis and mobilization, discussed elsewhere in this review, in addition to its role in promoting the development of adipose deposits.

The molecular mechanisms by which mTORC1 and its downstream targets stimulate adipocyte differentiation have yet to be fully defined. The temporal activation of two transcription factors, C/EBP- α and PPAR γ —the master regulator of terminal adipocyte differentiation—is responsible for inducing the final stages of differentiation [50]. mTORC1 signalling has been shown to increase PPAR γ transcript and protein levels, as well as its transactivating activity [45,47,51,52], albeit through unknown mechanisms. Cell culture experiments have suggested that regulation of the final differentiation steps is primarily independent of S6K and is probably dependent on 4E-BP inhibition downstream from mTORC1 [40,48]. However, a study has indicated that PPAR γ activation can also be suppressed by hyperactive mTORC1 signalling through its negative feedback effects on insulin signalling [53]. These findings indicate that there are probably mTORC1-dependent and -independent inputs into PPAR γ activation and adipocyte differentiation downstream from insulin signalling, with more *in vivo* experiments needed.

Lipolysis

In addition to its role in stimulating lipogenesis through SREBP, mTORC1 signalling is believed to promote the storage of fatty acids in lipid stores by inhibiting lipolysis. Neutral lipids, in the form of MAG, DAG and TAG inside the cell are subject to lipolysis to mobilize free fatty acids for energy production or remodelling into new lipid species, including specific membrane and signalling lipids. Patients treated with rapamycin frequently have dyslipidaemia, one facet of which is elevated levels of plasma free fatty acids, which could reflect an increase in lipolysis in adipose tissue [54,55]. Mice treated with rapamycin show a reduction in adipocyte size and overall adiposity, and rapamycin stimulates lipolysis in cultured adipocytes [56–58]. Genetic manipulations of mTORC1 signalling in several mouse models have reinforced the link between mTORC1 activation and an inhibition of lipolysis. The adipose tissue of *4E-BP1/2* double-knockout mice shows decreased lipolysis [48], and *S6K1* knockout mice are leaner with elevated rates of lipolysis [49]. However, mice with adipose-specific *Raptor* knockout, whilst also lean with reduced adiposity, do not show an obvious increase in lipolysis [47]. This suggests that the lipolysis phenotypes observed in the whole-body *4E-BP* and *S6K1* knockout models could be due to systemic effects rather than those intrinsic to the

adipocyte. Interestingly, adipose-specific *Atg7* knockout mice that have a defect in autophagy, show decreased adipocyte lipolysis [59], suggesting that the inhibitory effects of mTORC1 on lipolysis could be, at least in part, through its attenuation of autophagy.

Although the molecular mechanisms of lipolytic regulation by mTOR are not fully understood, mTORC1 signalling has been found to influence three distinct lipases: ATGL, HSL and LPL [60]. In adipocytes, ATGL catalyses the lipolysis of TAGs to DAGs within lipid droplets. HSL then converts the DAGs to MAGs. In 3T3-L1 adipocytes, mTORC1 suppression increases the transcription of ATGL, which parallels the enhanced lipolysis induced by rapamycin or siRNA knockdown of *Raptor* [57]. The phosphorylation of HSL at Ser563, an established PKA site, is associated with an increase in its lipase activity. A decrease in HSL phosphorylation correlates with mTORC1 activation and the diminished release of free fatty acids [58]. However, as with ATGL transcriptional suppression, how mTORC1 signalling negatively affects HSL phosphorylation on this PKA site is unknown. Similarly to mTORC1 inhibition, adipocyte-specific *Rictor* knockout also leads to the phosphorylation of HSL at Ser563 [61]. In addition to adipocyte lipolysis, mTORC1 has been implicated in the control of the extracellular lipase LPL. LPL is a water-soluble lipase present in plasma, as well as on the surface of endothelial cells, primarily in muscle and adipose tissue. It hydrolyses TAG in circulating VLDL to promote conversion to IDL and LDL, which facilitates the uptake of lipoprotein into tissues [62]. Systemic rapamycin treatment has been found to decrease LPL activity in mouse adipose tissue, and mouse and human plasma, albeit through an unknown mechanism [63,64]. The collective studies in patients treated with rapamycin and a variety of cell and mouse models suggest that mTORC1 activation, which occurs in metabolic tissues after feeding, promotes the synthesis and storage of lipids. By contrast, mTORC1 inhibition, such as during fasting, stimulates lipolysis and the release of free fatty acids into the circulation.

β -oxidation and ketogenesis

Consistent with the inhibition of mTORC1 signalling promoting fatty acid release and consumption, there is growing evidence that mTORC1 suppresses the β -oxidation of fatty acids for energy or ketogenesis. Rapamycin has been found to increase β -oxidation in rat hepatocytes and this has been attributed to increased expression of β -oxidation enzymes, including long-chain acyl-CoA dehydrogenase and carnitine acyltransferase [17,65]. This effect of rapamycin could be due to the induction of autophagy, which seems to promote the β -oxidation of fatty acids from TAGs in hepatocytes [66]. However, genetic evidence suggests that autophagy has inhibitory effects on β -oxidation in adipose tissue [59,67]. Mice with whole-body knockout of *S6K1* seem to have enhanced β -oxidation, as evidenced by increased levels of CPT1 transcript in isolated adipocytes [49]. Consistent with mTORC1 signalling attenuating β -oxidation, myoblasts isolated from *S6K1/S6K2* double-knockout mice also show enhanced β -oxidation of fatty acids [68]. However, this phenotype was attributed to indirect effects from energy stress and AMPK activation in this setting. As with the *S6K1* knockout and the *S6K1/S6K2* double-knockout mice, mice with adipose-specific *Raptor* knockout are lean with adipocytes that show increased mitochondrial uncoupling, which could allow them to burn lipids rapidly without generating ATP [47,49,68]. Paradoxically, mTORC1 activation has also

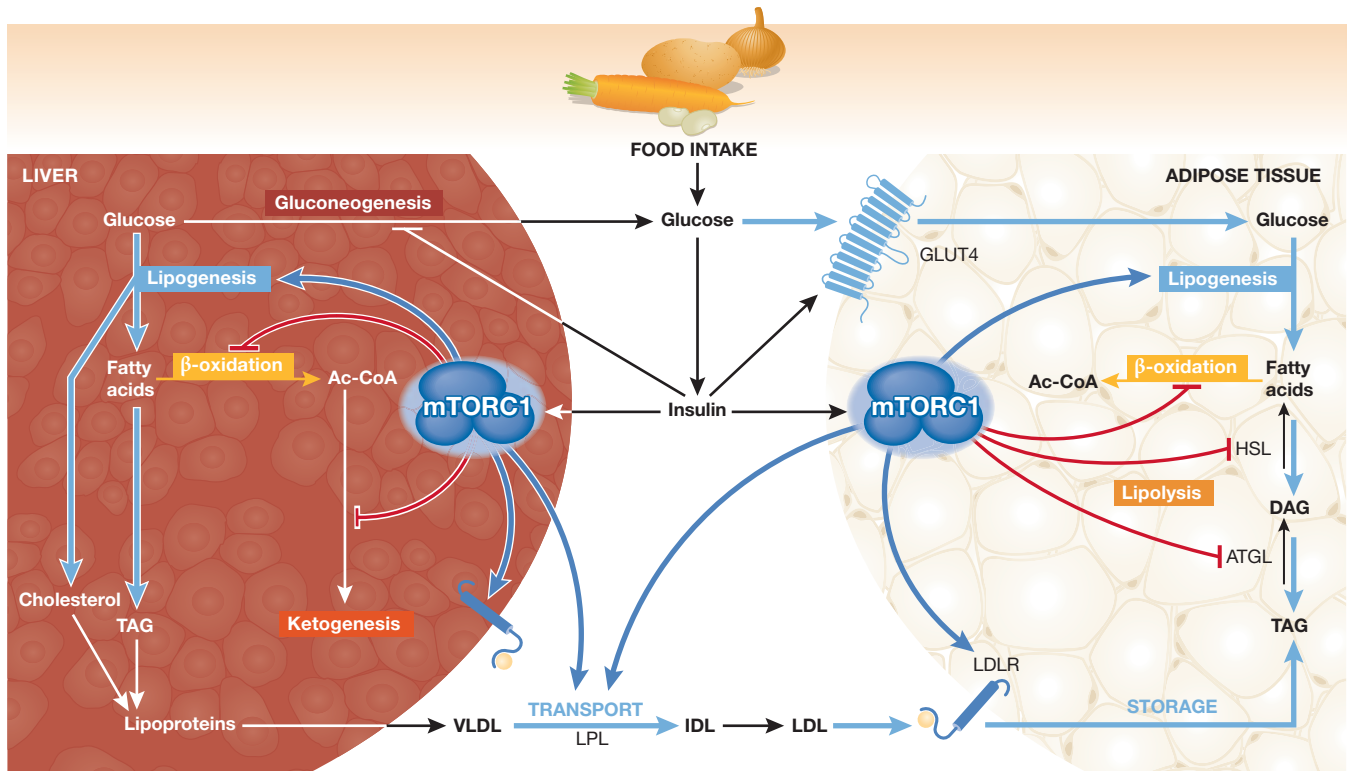


Fig 4 | The increase in insulin levels after a meal alters hepatic and adipose lipid metabolism, at least in part, through mTORC1 signalling (a working model). In the liver, mTORC1 promotes lipid synthesis through SREBP1c activation. In addition, mTORC1 signalling blocks lipid catabolism by blocking β -oxidation and ketogenesis in the liver. Consequently, mTORC1 activation in the liver promotes the synthesis of TAGs and perhaps cholesterol, which are incorporated into VLDL for transport to peripheral tissues. Evidence suggests that mTORC1 signalling positively influences LPL activity, which promotes lipid delivery to peripheral tissues by hydrolysing VLDL to IDL, which is then converted to LDL. Lipoprotein-bound TAGs are taken up by tissues, including adipocytes, through the LDLR. Both the expression and stability of LDLR, at least in the liver, are probably promoted by mTORC1 activation. In response to insulin, mTORC1 has been suggested to inhibit lipolysis in adipocytes by downregulating ATGL and HSL. Therefore, the systemic effects of postprandial mTORC1 activation are to promote the flux of carbon from glucose towards TAG storage in adipose tissue. See text for details regarding the evidence underlying this model. Ac-CoA, acetyl-CoA; ATGL, adipose triglyceride lipase; DAG, diacylglycerol; GLUT4, glucose transporter type 4; HSL, hormone-sensitive lipase; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LDLR, LDL receptor; LPL, lipoprotein lipase; mTORC1, mechanistic target of rapamycin complex 1; SREBP1c, sterol regulatory element-binding protein 1c; TAG, triacylglycerol; VLDL, very low density lipoprotein.

been linked to increased mitochondrial biogenesis in some settings [69]. This could explain the decrease in oxidative capacity of muscle [69–71] and Jurkat T cells [72] after the inhibition or complete loss of mTORC1 signalling. However, further studies are needed to determine how the observed changes in mitochondrial gene expression and oxygen consumption in these settings influence the β -oxidation of fatty acids. The collective data suggest that mTORC1 signalling inhibits fatty acid oxidation, whilst also promoting mitochondrial biogenesis in some settings.

The acetyl-CoA released from β -oxidation can either enter the TCA cycle or, under fasting conditions in the liver, be converted to ketone bodies. Genetic evidence suggests that mTORC1 signalling in the liver, which is respectively inhibited and activated by fasting and feeding, suppresses ketogenesis [73]. Mice with *LTsc1KO* that show sustained mTORC1 signalling under fasting have a defect in ketogenesis, whereas mice with liver-specific *Raptor* knockout show an increase in fasting-induced ketogenesis. mTORC1 seems to suppress the expression of ketogenic enzymes through its regulation of N-CoR1 and PPAR α [73], by a mechanism probably dependent on S6K2 [74]. These inhibitory effects on PPAR α and its

transcriptional targets could also explain the negative regulation of fatty acid oxidation by mTORC1. The repression of β -oxidation and ketogenesis by mTORC1 probably acts together with its stimulation of lipogenesis, further promoting the flux of acetyl-CoA towards lipid synthesis and storage.

Lipid transport

Several lines of evidence suggest a role for mTORC1 signalling in the control of lipid mobilization and transport. As stated above, patients treated with mTORC1 inhibitors suffer frequently from a dyslipidaemia consisting of hypertriglyceridaemia and hypercholesterolaemia, as well as increased levels of plasma free fatty acids [55]. The source of the elevated circulating lipids in these patients is unknown. However, TAG and cholesterol transport out of the liver involves their packaging into apolipoprotein complexes, and plasma levels of both apolipoprotein B-100 and apolipoprotein C-III have been found to be increased in patients treated with rapamycin [54]. A study in guinea pigs revealed that the increase in circulating TAGs observed in the response to rapamycin correlates with an increase in VLDL, the primary mode of

TAG export from the liver [75]. In cultured hepatocytes, the ability of insulin to repress the expression of both apolipoprotein B and apolipoprotein A-5 is sensitive to rapamycin, suggesting that the increase in apolipoproteins observed on rapamycin treatment *in vivo* might be due to direct effects on hepatocytes [76,77]. How mTORC1 negatively regulates the expression or protein levels of specific apolipoproteins is unknown and could be secondary to changes in apolipoprotein uptake or degradation. Conversely, mTORC1 signalling seems to upregulate LDLR, which facilitates the uptake of cholesterol-rich LDL from the plasma into the liver and peripheral tissues. *LDLR* gene expression is controlled by SREBP [78] and would, therefore, be predicted to be stimulated by insulin in an mTORC1-dependent manner. In addition, mTORC1 signalling downstream from the insulin receptor in the liver has been found to repress the expression of PCSK9, a known negative regulator of LDLR protein levels [79]. Consequently, rapamycin treatment decreases LDLR levels in a PCSK9-dependent manner, thereby reducing LDL uptake and increasing its circulating levels. Combined with the rapamycin-stimulated increase in lipolysis and apolipoprotein levels, these effects on the LDLR suggest a mechanistic basis for the dyslipidaemia observed in patients treated with mTORC1 inhibitors.

mTORC1 in physiology, obesity and diabetes

The global effects of the mTORC1-mediated regulation of lipid metabolism detailed above are predicted to promote the systemic flux of carbon into lipids and their storage as TAGs within adipose tissue (Fig 4). The postprandial increase in both glucose and insulin stimulates the acute activation of mTORC1 within metabolic tissues, in which mTORC1 has contextual roles in controlling lipid metabolism. In the liver, and probably in adipose tissue, mTORC1 activation induces lipogenesis. At the same time, mTORC1 probably blocks the β -oxidation of fatty acids in the liver, adipose, and perhaps muscle, instead promoting the use and storage of glucose in these tissues. TAGs and cholesterol produced in the liver facilitate the packaging and release of VLDL into circulation. mTORC1 signalling might enhance uptake of lipids by peripheral tissues through the activation of LPL, which hydrolyses VLDL to IDL, and an increase in the levels of LDLR. In adipose tissue, the insulin-stimulated activation of mTORC1 is predicted to contribute to the inhibition of lipolysis, further promoting the storage of TAGs, either mobilized from the liver or produced *de novo* within the adipocytes.

Whilst mTORC1 is activated transiently within metabolic tissues by normal feeding, conditions of nutrient overload and obesity can lead to chronically elevated mTORC1 signalling in these tissues [49,80]. The mechanism by which obesity leads to hyperactivation of mTORC1 is unknown but happens probably through a combination of hyperglycaemia and hyperinsulinaemia under these conditions. Furthermore, evidence suggests that increased circulating levels of branch-chain amino acids, which are known to activate mTORC1, correlates with the development of obesity and insulin resistance [81]. In addition to potentially exacerbating obesity by further promoting lipid storage in adipose depots, chronic mTORC1 activation under such conditions is believed to contribute to the development of insulin resistance, which frequently accompanies obesity. Increased mTORC1 signalling can trigger several distinct feedback mechanisms, which in a cell-autonomous manner, dampens the cellular response to insulin. The *in vivo* contribution of

Sidebar B | In need of answers

- (i) What are the molecular mechanisms by which mTORC1 regulates SREBP1 and SREBP2?
- (ii) Which lipid species are most influenced by the activation state of mTORC1 signalling?
- (iii) Does mTORC1 stimulate the synthesis of membrane lipids in addition to storage lipids?
- (iv) How do lipids influence mTORC1 signalling?
- (v) How does mTORC1 become dysregulated under conditions of obesity?
- (vi) Does mTORC1 inhibition contribute to the effects of AMPK-activating compounds on cellular and systemic metabolism?
- (vii) What is the role of mTORC1 activation in the common lipogenic phenotype of cancer cells?
- (viii) How is lipid metabolism differentially regulated by mTORC1 in different tissues?

these feedback mechanisms to insulin resistance is well illustrated by loss- and gain-of-function mouse models of mTORC1 signalling. For instance, *S6K1* knockout mice have enhanced peripheral insulin sensitivity [49], whereas mice with *LTsc1* KO show hepatic insulin resistance with greatly reduced Akt signalling [18]. Therefore, under conditions of obesity, mTORC1 activation in metabolic tissues probably both perpetuates obesity and promotes insulin resistance, thereby expediting the progression to type II diabetes.

The fundamental role of mTORC1 in regulating whole-body lipid homeostasis, paired with its frequent upregulation in obesity and type 2 diabetes, suggests that mTOR inhibitors might offer some therapeutic benefit in metabolic diseases. In theory, mTORC1-specific inhibitors should suppress lipid synthesis and promote lipolysis and lipid catabolism, in addition to blocking mTORC1-dependent feedback mechanisms to resensitize tissues to insulin. However, important caveats arise from the use of mTORC1 inhibitors to combat obesity and diabetes. First, prolonged treatment with rapamycin disrupts mTORC2 and therefore Akt activation downstream from the insulin receptor, further exacerbating the insulin-resistant phenotype (Sidebar A; [82]). Second, patients treated with rapamycin frequently have increased levels of circulating TAGs, cholesterol and free fatty acids [55]. Therefore, whilst rapamycin treatment might help mobilize lipids and deplete fat stores, lipid clearance offers an additional pathological challenge. Targeting mTORC1 signalling indirectly might offer a more promising avenue. AMPK is a potent negative regulator of mTORC1, blocking its function through phosphorylation of both the TSC–TBC complex [2,5] and Raptor [6]. Therefore, mTORC1 signalling is blocked on activation of AMPK, which is stimulated by a large variety of natural and synthetic compounds, including metformin, resveratrol and aspirin [83]. Importantly, metformin is the most widely prescribed anti-diabetes drug in the world. Whether any of the beneficial metabolic effects of metformin are attributed to its inhibition of mTORC1 signalling is one of several important outstanding questions (Sidebar B).

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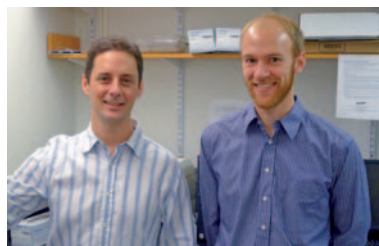
CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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