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mTOR: from growth signal integration to cancer, diabetes and ageing

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Preface

In all eukaryotes, the target of rapamycin (TOR) signaling pathway couples energy and nutrient abundance to the execution of cell growth and division, owing to the ability of TOR protein kinase to simultaneously sense energy, nutrients and stress, and, in metazoan, growth factors. Mammalian TOR complexes 1 and 2 (mTORC1 and mTORC2) exert their actions by regulating other important kinases, such as S6K and Akt. In the last few years, a significant advance in our understanding of the regulation and functions of mTOR has revealed its critical involvement in the onset and progression of diabetes, cancer and ageing.

Introduction

‘Growth’ indicates the set of biochemical processes, intimately linked to the availability of nutrients and energy, by which organisms increase their size and cell number through the synthesis of new cellular components, including proteins, nucleic acids, and lipids. Cells also rely on a complex set of programs to cope with times of nutrient starvation and low energy. To avoid energy imbalance and death, cells quickly suppress biosynthetic programs, increase the recycling of ‘aged’ proteins and organelles to provide an internal source of metabolites, and slow or halt proliferation.

At the interface between growth and starvation stands a signaling pathway centered on a kinase known as the Target of Rapamycin (TOR). The appearance of TOR in early eukaryotes enabled these unicellular organisms to sense the availability of nutrients and to promote growth whenever encountering favorable environmental conditions. With the emergence of multicellularity, TOR acquired additional roles as a central controller of organism growth and homeostasis. As such, mammalian TOR (mTOR) is implicated in disease states where growth is deregulated and homeostasis is compromised, namely cancer, metabolic diseases, and ageing. Dysregulated mTOR signaling fuels the destructive growth of cancers. Over-stimulation of the mTOR pathway by excess food consumption may be a critical factor underlying the diabetes epidemics. Finally, recent findings suggest that mTOR signaling controls the rate at which cells and tissues age, and that inhibiting mTOR may represent a promising avenue to increase longevity.

In this Review we begin by summarizing our current understanding of the regulatory inputs and the cellular actions of mTOR. We then discuss how this integrated knowledge, together with the availability of new chemical agents, could pave the way towards the manipulation

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of human metabolism in order to maximize healthy lifespan and help to stave off ageing, cancer and metabolic diseases.

In the remainder of this review we will use the term mTOR when discussing TOR in mammalian organisms and TOR with species-specific prefixes when discussing non-mammalian organisms (that is, ceTOR in *Caenorhabditis elegans*, dTOR in *Drosophila melanogaster,* dyTOR in *Dictyostelium discoideum* and scTOR in *Saccharomyces cerevisiae*). It should be noted that while mTOR originally stood for 'mammalian TOR', it is now officially an abbreviation for 'mechanistic TOR'.

Organization and actions of mTOR complexes

In yeast and mammalian organisms, genetic and biochemical approaches, respectively, led to the discovery of TOR as the target of the immunosuppressant rapamycin, a macrolide produced by a soil bacterium found on Easter island[1–4]. TOR is a protein kinase belonging to the phosphoinositide 3-kinase related protein kinases (PIKK) family, which comprises large proteins that enable organisms to cope with metabolic, environmental and genetic stress.

Molecular composition of mTORC1 and mTORC2

mTOR is the catalytic subunit of two distinct complexes, called mTOR Complex 1 (mTORC1) and 2 (mTORC2) (reviewed in[5, 6]) (FIG.1). Unique accessory proteins distinguish the two complexes: raptor and rictor define mTORC1 and mTORC2, respectively[7–9]. These companions function as scaffolds for assembling the complexes and for binding substrates as well as regulators[7, 8, 10–15]. Further unique components exist in both complexes: mTORC1 comprises a negative regulator, PRAS40[14, 16], whereas mTORC2 contains Protor and mSin1, which likely help complex assembly and localization, respectively[15, 17–19]. mTORC1 and mTORC2 share mLST8 and the recently identified Deptor, which function as positive and negative regulators, respectively[20] (FIG.1). Biochemical and structural evidence suggests that both mTORC1 and mTORC2 may exist as dimers[12, 21].

Both in yeast and mammals, rapamycin only inhibits the activities of mTORC1, but not mTORC2[9, 22– 25]. Rapamycin binds to the small protein FKBP-12 and, in turn, rapamycin-FKBP-12 binds to and inhibits raptor-bound, but not rictor-bound, mTOR[9, 22, 26, 27]. Rapamycin might inhibit mTORC1 by dissociating raptor from mTOR, thus limiting the access of mTOR to some substrates[8, 21]. Complicating this picture, prolonged treatment with rapamycin can inhibit mTORC2 in a subset of tissues and cell lines[28]. This effect may involve a progressive sequestration of the cellular pool of mTOR in a complex with rapamycin-FKBP12, thus making it unavailable for assembly into mTORC2.

Substrates and actions of mTORC1

The complex in which it participates dictates the substrate specificity of mTOR. The mTORC1 substrates S6 Kinase 1 (S6K1) and eIF-4E binding proten 1 (4E-BP1) associate with mRNAs and regulate both mRNA translation initiation and progression, thus enhancing protein synthesis[29–31] (reviewed in[32]) (FIG.2a). 4E-BP1 suppresses mRNA translation; when phosphorylated by mTORC1, it dissociates from eIF4E, allowing the latter to recruit the translation initiation factor eIF4G to the 5' end of most mRNAs[30, 33]. S6K1 is a positive regulator of translation initiation and elongation when it is phosphorylated by mTORC1, via its ability to phosphorylate multiple substrates including S6[34], eEF2K[35], SKAR[36], CBP80[37] and eIF4B[36, 38, 39].

Ribosome biogenesis is highly energy-intensive and, as such, it is tightly coupled to the energetic status of the cell. Both the synthesis of ribosomal RNAs (rRNAs) and that of ribosomal proteins are positively regulated by mTORC1 (FIG.2a). mTORC1, via S6K1 kinase activity, upregulates the transcriptional activity of the rRNA polymerase, RNA PolI[40, 41]. In yeast, the transcription factors Rrn3 (TIF1 in mammalians), FHL1 and SFP1 mediate the transcription of ribosomal RNAs and proteins downstream of scTORC1[41–44].

Autophagy is the controlled self-degradation of cellular components, ranging from individual proteins (microautophagy) to entire organelles (macroautophagy), which mediates the recycling of damaged, redundant, or even dangerous cellular constituents. Autophagy provides an important source of substrates for energy production during periods of low extracellular nutrients (reviewed in[45]). mTORC1 actively suppresses autophagy, and conversely, inhibition of mTORC1 (by small molecules or by amino acid withdrawal) strongly induces it[46, 47]. In yeast, scTOR-dependent phosphorylation of Atg13 disrupts the Atg1-Atg13 complex that is critical for the formation of the autophagosome[48]. Similarly, mTORC1-mediated phosphorylation negatively regulates the mammalian Atg13 and Atg1 homologues, Atg13 and ULK1 and ULK2[49, 50] (FIG.2b). However, the mammalian-specific autophagic regulators FIP200 and Atg101 are also mTORC1 substrates[51, 52], indicating additional mechanisms for the regulation of autophagy by mTOR.

Substrates and actions of mTORC2

scTORC2 was identified as a mediator of actin cytoskeletal organization and cell polarization[23–25]. scTORC2 controls a number of cytoskeletal regulators, including the chaperonin TCP-1, ROM2 (a GEF for Rho1 and Rho2) and the AGC kinase Ypk2[53, 54]. The role of TORC2 in controlling cytoskeletal polarity has been confirmed in *D. discoideum* and mammalian cells.

Recent findings have revealed novel important roles for mTORC2 in the phosphorylation of AGC kinase family members. mTORC2 phosphorylates and activates Akt, SGK, and PKC, which regulate cell survival, cell cycle progression and anabolism[55–58]. Among AGC kinases, Akt is especially important because of its role in the pathogenesis of cancer and diabetes. Using RNAi in *D. melanogaster* S2 cells, it was found that dTORC2 mediates the phosphorylation of Akt at S505, located in the hydrophobic motif and homologous to S473 in mammals[55, 57, 58]. This is a key finding, because phosphorylation at S473 primes Akt for further phosphorylation at T308, located in the catalytic domain, by PDK1. Together, these two phosphorylation events cause full activation of Akt. Loss of Rictor in worm, fly, mouse and human cells results in complete loss of Akt phosphorylation at S473 but, interestingly, this affects only some substrates of Akt[18, 58–60]. Particularly, phosphorylation of the FOXO1/3 transcription factors was suppressed, but that of Tuberous Sclerosis Complex 2 (TSC2), a substrate of Akt that acts upstream of mTORC1, was not. Because phosphorylation of FOXO1/3 by Akt promotes avoidance of apoptosis, mTORC2 may regulate cell survival[59, 61].

Collectively, these findings place mTORC2 upstream of key cellular processes such as cell-cycle progression, anabolism and cell survival.

Upstream regulators of mTOR complexes

mTORC1 acts as a signal integrator for four major regulatory inputs: nutrients, growth factors, energy, and stress (FIG.2). These inputs can cooperate and antagonize each other, enabling the cell to fine-tune the action of mTORC1. In contrast, the regulation of mTORC2 is only beginning to be discovered, but the available evidence seems to suggest that only growth factors directly regulate this complex.

Nutrients regulate mTORC1

Amino acids are the building blocks of proteins, and are also used in the synthesis of DNA, glucose and ATP. Early studies showed that amino acids are absolutely required for mTORC1 signaling in cultured cells, and cannot be compensated for by other activating stimuli[13, 62, 63]. However, the molecular components of amino acid signaling to mTORC1 have long remained mysterious. We now know that amino acids regulate mTORC1 via the Rag family of small GTPases[13, 64] (FIG.2c). Rag GTPases are heterodimers of either Rag A or B with either Rag C or D; crucially, the two members of the heterodimer have opposite nucleotide loading states. In the absence of amino acids, RagA or RagB is GDP loaded and RagC or RagD contains GTP, and this is the inactive Rag conformation. Amino acids cause Rag GTPases to switch to the active conformation, where RagA or RagB is GTP loaded and RagC or RagD is GDP loaded. The active Rag heterodimer physically interacts with raptor, causing the clustering of mTORC1 onto the surface of late endosomes and lysosomes, where the Rags reside[13, 64, 65]. This relocalization may enable mTORC1 to interact with the small GTPase Rheb, an essential activator of mTORC1 that is controlled by growth factor inputs[13, 66, 67]. This model has been largely confirmed in *S. cerevisiae*, where the Rag GTPase homologs, GTR1 and GTR2, interact physically and genetically with scTORC1[68–71]. However, the rheb homologue in *S. Cerevisiae* is not essential for life and does not appear to function upstream of scTORC1[72]. Thus, some aspects of scTORC1 regulation may differ from metazoans and await clarification.

Rag proteins do not show any obvious membrane targeting signals. However, three small molecules p14, MP1 and p18, collectively known as the *Ragulator*, form a molecular scaffold that binds the Rag GTPases to the lysosomal surface. When the ragulator is genetically inactivated, the Rag GTPases become cytoplasmic, recruitment of mTORC1 to the lysosome fails, and amino acid signaling to mTORC1 is blocked[65].

Collectively, these findings add to growing evidence for a key role of endomembranes in controlling the activity of mTOR (BOX 2); moreover, they suggest that subcellular localization of mTORC1 underlies its ability to integrate nutrient and growth factor signals (BOX 1).

Growth factors regulate mTORC1

Multicellular organisms rely on long-range communication to coordinate the distribution of nutrients and the growth of cell populations throughout the body. With the emergence of metazoans, the mTOR pathway became wired to signaling pathways initiated by growth factors, such as insulin, which carry information on the nutritional state of the organism as a whole. One key end point of growth factor signaling to mTORC1 is the small GTPase Rheb that, when GTP-loaded, stimulates the kinase activity of mTORC1[14, 66, 67]. In metazoans Rheb plays an essential role, because its loss abolishes the activation of mTORC1 by both growth factors and nutrients. Conversely, overexpression of Rheb can maintain mTORC1 activity even when nutrients and growth factors have been withdrawn (BOX 1).

How do growth factors regulate the GTP-loading of Rheb? Binding of insulin to its receptor activates the phosphatidylinositol 3-kinase (PI3K) pathway, which leads to phosphorylation and activation of Akt. In turn, Akt phosphorylates tuberin/TSC2, a large protein that, together with hamartin/TSC1, forms the Tuberous Sclerosis Complex (TSC)[73–75] (FIG.2d). TSC acts as a GAP for Rheb[76–79]; because GDP-loaded Rheb is unable to activate mTORC1, TSC effectively shuts off mTORC1 signaling[80, 81]. Akt-mediated phoshorylation of TSC2 inhibits its GAP activity for Rheb[82, 83] and thus promotes the activation of mTORC1. Akt also phosphorylates PRAS40, causing its binding to 14-3-3 proteins and

preventing it from inhibiting mTORC1[14, 16, 84]. Moreover, in a positive feedback loop, mTOR phosphorylates and inhibits PRAS40[85, 86].

Growth factors can signal to mTORC1 via alternative pathways to the PI3K-Akt axis. For example, extracellular signal-regulated kinase (Erk) activates TSC1/2 downstream of the Ras-Raf-Erk axis[87]. Moreover, the Wnt pathway has been implicated in mTORC1 signaling[88, 89]. Glycogen synthase kinase 3 (GSK3) acts as a negative regulator of mTORC1 by phosphorylating TSC1/2; by inhibiting GSK, Wnt activates mTORC1.

The convergence of multiple growth factor-initiated pathways onto TSC2 likely allows mTORC1 to participate in many developmental and physiological processes. This is supported by the absolute requirement for mTORC1 in early embryonic development[59, 90, 91].

Energy and stress regulate mTORC1

Chemical inhibitors of glycolysis and mitochondrial function suppress mTORC1 activity, indicating that mTORC1 senses cellular energy[8, 92]. Energy sensing by mTORC1 is critical, because mTORC1-driven growth processes consume a large fraction of cellular energy, and thus could be deleterious to starving cells.

Glycolysis and mitochondrial respiration convert nutrients into energy, stored in the form of ATP, and cellular ATP quickly drops upon nutrient deprivation. The mTORC1 pathway indirectly senses low ATP, via a mechanism centered on the AMP-activated protein kinase (AMPK) (reviewed in[93]) (FIG.2e). Both AMP and ATP are allosteric regulators of AMPK: when the AMP:ATP ratio increases, AMPK phosphorylates TSC1/2, possibly stimulating its GAP activity toward Rheb[89, 94, 95]. Moreover, AMPK phosphorylates raptor, causing its binding to 14-3-3, which leads to the inhibition of mTORC1 through allosteric mechanisms[96].

Numerous stressors affect ATP levels, and thus may regulate mTOR via the AMP-AMPK axis. During hypoxia, mitochondrial respiration is impaired, leading to low ATP levels and activation of AMPK (reviewed in[97]). Hypoxia also affects mTORC1 in AMPK-independent ways, by inducing the expression of the REDD1/2 genes, which then suppresses mTORC1 through a mechanism that requires TSC1/2[98, 99] (FIG.2f). Conversely, other stressors that do not primarily impinge on cellular energy signal through AMPK. DNA damage results in inhibition of mTORC1 activity via p53-dependent upregulation of AMPK[95, 100, 101] (FIG. 2g). Sestrin 1 and 2 are two transcriptional targets of p53 implicated in the DNA damage response. Recently, it was shown that sestrins potently activate AMPK, thus mediating the p53-dependent suppression of mTOR activity upon DNA damage[102].

Upstream regulation of mTORC2

Surprisingly little is known about the upstream activators of mTORC2. Given its role in regulating Akt, SGK and PKC, it is generally thought that growth factors control mTORC2, directly or indirectly. In fact, insulin stimulation of cultured cells promotes S473 phosphorylation of Akt by mTORC2[58].

Because Akt, SGK and PKC respond to different growth factors, the range of upstream regulators of mTORC2 may be quite wide. Thus, how can signaling specificity be achieved? One potential solution may come from the existence of multiple isoforms of mSin1. Three out of the five known splice variants of mSin1 participate in mTORC2, effectively defining three distinct complexes; of these, only two are regulated by insulin[17]. Thus, mSin1 may function as an adaptor between mTORC2 and specific growth factor receptors. The mSin1

orthologue in *D. discoideum*, RIP3, mediates the enhanced migratory behavior driven by activated RAS; thus, RAS may provide a link between growth factors and mTORC2[103, 104].

mTOR regulates metabolism in mammals

Due to the intermittency of food intake and the necessity to keep nutrient levels in the bloodstream within a narrow physiological range, multicellular organisms have acquired mechanisms to store energy after feeding and to later mobilize this energy. Long-range signals, primarily insulin, provide whole-body coordination of energy consumption and storage. mTOR regulates all these responses by its ability to integrate insulin and nutrients, mediate energy storage and consumption and prevent the triggering of conflicting metabolic signals by suppressing catabolism (FIG.3).

mTOR in fasting and starvation

Having evolved in conditions of limited nutrient availability, mammals (including humans) have developed a striking ability to maximize the available resources of energy anticipating periods of shortage.

Early during fasting, glucose and amino acid blood levels decrease, causing a drop in circulating insulin. In addition, the imbalance between energy expenditure and food intake leads to an increased AMP:ATP ratio. These factors converge as inhibitory inputs on mTORC1, placing a brake on energyintensive biosynthetic processes and upregulating macroautophagy[105]. Mitophagy (the autophagic degradation of mitochondria) provides an immediate source of energy at the expense of nutrient-intensive, long-term ATP production (reviewed in[106]). The prosurvival role of autophagy was elegantly demonstrated *in vivo* in mice with ATG5 genetically deleted. Immediately after birth, interruption of the placental nutrient supply causes an energetic shortage that is compensated for by upregulation of autophagy in several organs. ATG5-deficient mice are unable to overcome this energetic challenge and die within one day after birth[107].

In the liver, induction of autophagy causes simultaneous recycling of mitochondria, cytoplasmic proteins and stored glycogen. This effect is so dramatic that murine liver reduces to around 30% of its normal size in a 24h fasting period. Furthermore, during starvation, white adipose tissue (WAT) and liver mobilize lipid stores, converting them into free fatty acids that are utilized by the liver and muscle via β-oxidation. Recent evidence indicates that, during fasting, autophagosomes sequester lipid droplets and break them down into free fatty acids[108]. Autophagy also mediates massive protein breakdown in the muscle[105], releasing amino acids to the bloodstream. The liver absorbs these amino acids, especially alanine, and converts them into glucose, which is then exported to the blood. The flow of nutrients released by autophagy feeds back onto mTORC1, causing its partial reactivation[109].

Changes in mitochondrial function stemming from inhibition of mTORC1 may also contribute to fasting responses. Activation of 4E-BP1 under limiting nutrients in *D. melanogaster* led to the selective translation of mRNAs encoding the mitochondrial respiratory chain[110], consistent with an attempt to increase the efficiency of ATP production. Moreover, mTORC1 promotes mitochondria biogenesis and enhances respiration through a ternary complex with the transcription factors PGC-1α and YY1[111]. Thus, inhibition of mTORC1 during starvation acts on mitochondrial function at 3 different levels: by placing a brake on the synthesis of new mitochondria, eliminating a subset of the existing mitochondria via mitophagy and by increasing the efficiency of existing mitochondria through the 4E-BP1 translational program.

mTOR, over-feeding, and insulin sensitivity

Growth control programs have evolved under conditions of scarce nutrients that were prevalent during mammalian evolution, and not in the western-life conditions of the last few decades. Overfeeding may be pathological because selection has favored organismal responses, partly mediated by mTOR, that accumulate and store energy in anticipation of periods of shortage. This translates into aberrant cellular responses when food and energy are plentiful and constantly available.

One of the most efficient forms of energy storage are triglycerides, because they provide a high energetic yield per unit of mass. mTORC1 mediates lipid accumulation in fat cells and favors formation of WAT (FIG.3). Adipocyte-specific deletion of Raptor in mice leads to reduced WAT tissue and enhanced fatty-acid oxidation[112]. Although the molecular mechanisms underlying these effects are not fully-understood, mTORC1 facilitates both differentiation of preadipocytes[113] and lipid accumulation[114] by indirectly upregulating Peroxysome proliferator-activated receptor (PPAR)-γ, a factor necessary and sufficient for adipocyte differentiation[115, 116]. Consistently, TSC2-deficient MEFs, which have increased mTORC1 activity as a result, show enhanced adipogenesis and PPAR-γlevels[117].

mTORC1 may impact on PPAR-γ activity by increasing its translation[118] and by activating the transcription factor SREBP-1c (FIG.3a). Active SREBP-1c enhances PPAR-γ activity[119, 120], and transactivates a set of genes directly involved in lipid synthesis[121]. At present, the molecular links between mTORC1, SREBP-1c and PPAR-γ activity remain to be clarified.

Recent evidence also implicates mTORC2 in lipid biogenesis. Akt activation by mTORC2 leads to induction of PPAR-γ through parallel mechanisms that include mTORC1 activation as well as direct inhibition of FoxO1[122, 123]. In worms, ceTORC2 participates in lipid accumulation through both Akt and SGK[60].

From a clinical perspective, chronic mTORC1 hyperactivation mediates the aberrant lipogenesis in WAT, liver and muscle that occurs in obesity, and which contributes to insulin resistance. Furthermore, mTORC1 hyperactivation during overfeeding may trigger an S6K-dependent negative feedback loop; activated S6K dampens the function of IRS1, an adaptor protein that recruits key downstream effectors to the insulin receptor, thus leading to insulin desensitization. The resulting dampened Akt activation leads to reduced glucose uptake and glycogen synthesis, and promotes gluconeogenesis in the liver. Collectively, these effects lead to a worsening of the hyperglycemic and hyperinsulinemic condition (FIG. 3b). Supporting the role of the S6K-IRS1 feedback loop in the pathogenesis of type 2 diabetes, S6K1-deficient mice displayed enhanced insulin sensitivity even when chronically maintained on a high fat diet[124].

Of note, mTORC1 hyperactivation in the context of insulin insensitivity poses a paradox: how can mTORC1 be constitutively active in an insulin insensitivity state if insulin is responsible for the activation of mTORC1? This apparent paradox can be explained by mTORC1 being hyperactive as a consequence of the nutrient branch. Chronic high blood levels of amino acids, as occurs in obesity[125], will keep mTORC1 at work together with an active S6K-IRS loop and, consequently, within a context of insulin insensitivity (FIG.3b).

Strikingly, the same molecular circuitry that controls metabolism in peripheral tissues also influences food intake in the central nervous system. Leucine locally applied to the hypothalamus induced satiety through activation of mTORC1; conversely, inhibition of mTORC1 in the hypothalamus by rapamycin injection increased food intake[126].

Thus, mTORC1 coordinates food intake with energy storage at multiple levels, from central control of food seeking to energy storage and expenditure in peripheral tissues. This multi-level regulation explains the profound consequences that dysregulated mTOR signaling exerts on human metabolism.

mTOR in cancer etiology and therapy

The most direct evidence that mTOR can drive tumorigenesis comes from familial cancer syndromes arising from mutations of negative mTOR regulators such as TSC1/2, LKB1 and PTEN (see Supplementary FIG.1). mTOR-dependent mechanisms also contribute to the growth of many sporadic cancers. For example, upon mTORC1-mediated inhibition of 4E-BP1, activated eIF4E preferentially drives the translation of mRNAs for pro-tumorigenic genes, including cell cycle regulators (FIG.4a). Indeed, eIF4E promotes cell survival in *in vivo* mouse models of lymphoma by upregulating the translation of the anti-apoptotic protein Mcl-1[127–129]. mTOR also upregulates Fatty Acid Synthase (FAS), an enzyme involved in lipid synthesis that favors rapid proliferation of cancer cells (FIG.4a) (reviewed in[130]).

Increasing evidence suggests that autophagy plays a role in tumor suppression. The most direct data supporting the tumor suppressive roles of autophagy come from mice heterozygous for the autophagic protein Beclin[131, 132] and from Atg4C-deficient mice[133], both of which are tumor-prone. Thus, constitutive mTORC1 activation may indirectly favor tumorigenesis by suppressing autophagy (FIG.4a).

By activating Akt[58] and SGK[56] mTORC2 may directly drive tumorigenesis. Akt promotes proliferation, survival and nutrient uptake in cancer cells (reviewed in[134]). Tumors driven by mutations in the tumor suppressor PTEN (which usually inhibits Akt signalling) or by oncogenic mutations in PI3K (which promotes Akt signalling) may be especially dependent on the pro-survival activities of Akt, and this is where targeting mTORC2 may prove especially useful. In fact, Rictor is required for the growth of prostate tumors caused by PTEN deletion in mice[61].

Rapamycin as a mTOR-centered cancer therapy

The existence of a potent, naturally occurring inhibitor of mTOR, rapamycin, appeared to be a lucky strike for cancer therapies. However, to date the limited success of rapamycin as an anti-cancer drug in clinical trials has generated some disappointment. Here we discuss the limitations of rapamycin and the current efforts to move beyond this drug towards an effective mTOR-centered therapy of cancer.

Rapamycin-based therapeutic approaches may have encountered a stumbling block in the S6Kmediated feedback loop, which leads to a severe upregulation of PI3K signaling, and provides important pro-survival and proliferative signals through Akt and other AGC kinases, when mTORC1 is inhibited and unable to activate S6K. Additionally, S6K inhibition activates the MEK-ERK signaling cascade[135], as well as transcription of platelet-derived growth factor receptor (PDGFR)[136]. These loops counteract the effects of rapamycin, dampening its effectiveness in cancer models and in patients (FIG.4b)[135, 137] (reviewed in[138]), and may explain why rapamycin is cytostatic but not cytotoxic in many tumors.

While high doses of rapamycin or its prolonged delivery can block mTORC2 in some cell lines[28, 139], rapamycin is largely selective for mTORC1. Given the role of mTORC2 and especially Akt as drivers of tumorigenesis[61], this poses a major concern. Furthermore, rapamycin does not inhibit all the functions of mTORC1; of notice, rapamycin only affects

4E-BP1 phosphorylation transiently and partially[47, 139–142]. Thus rapamycin, by suppressing the S6K1-IRS1 feedback loop and hyperactivating the PI3K-AKT pathway, may ultimately stimulate 4E-BP1 phosphorylation and perhaps other tumor-promoting functions of mTORC1. These two major drawbacks have motivated the search for second-generation inhibitors of mTOR function.

mTOR and mTOR-PI3K catalytic inhibitors

Recently, independent groups generated a series of synthetic small molecules[47, 142–145], which function as ATP-competitive inhibitors and block all known mTORC1 and mTORC2 activities. For example, Torin 1 inhibits 4E-BP1 phosphorylation and triggers autophagy to a far greater extent than rapamycin[47]. Moreover, unlike rapamycin, Torin 1 and the other catalytic inhibitors also completely block mTORC2-mediated phosphorylation of Akt[47, 142–145]. Supporting the applicability of this drug in a clinical setting, pre-clinical data in genetically engineered mice argue that even full inhibition of mTORC1 and 2 could be well tolerated in adult tissues[61, 146]. Moreover, the mTOR-catalytic inhibitor PP-242 showed a better response than rapamycin in a mouse model of experimental leukemia, together with a surprising milder effect on normal lymphocytes[147].

However, mTOR catalytic inhibitors are not immune to potential drawbacks. Loss of the S6Kmediated feedback loop resulting from mTORC1 inhibition enhances Akt phosphorylation at T308 by PDK1 (FIG.4b). Consequently, when suboptimal doses of mTOR catalytic inhibitors were used, the residual mTORC2 activity towards S473 potently activated Akt[20]. Furthermore, while acute inhibition of mTORC2 by one such inhibitor, Ku-0063794, effectively suppressed T308 phosphorylation in wild-type cells, it failed to do so in cells where mTORC2 is genetically inactivated[144]. This result suggests that under chronic inhibition of mTORC2, which may occur in a clinical setting, alternative pathways may ensure Akt phosphorylation at T308 even in the absence of the priming S473 phosphorylation.

The similarity between the catalytic domains of mTOR and class I PI3-Kinase has also enabled the design of ATP-competitive drugs that simultaneously block the activity of both kinases (FIG.4b). When two inhibitors of this kind, PI-103 and NVP-BEZ235, were delivered to tumor cells driven by PI3K, they strongly suppressed both S6K1 and Akt activation[148, 149]. More importantly, this type of compound suppressed the proliferation of cancer cells more efficiently than rapamycin or the PI3K inhibitor LY294002, and to a similar degree as a combination of the two.

Dual PI3K-mTOR inhibitors may be conceptually superior to catalytic mTOR inhibitors, because they disable both inputs to Akt, namely PI3K-PDK1 and mTORC2. However, this broad inhibition of signaling pathways may be toxic to normal cells. In fact, while both the ATP-competitive mTOR inhibitor PP-242 and the dual PI3K-mTOR inhibitor PI-103 showed an antileukemic effect *in vivo,* the latter also harmed normal lymphocytes, suggesting that the therapeutic window of dual inhibitors might be narrow[147].

It still remains unclear to what extent mTOR inhibitors and mTOR/PI3K dual inhibitors are effective in inducing the death of cancer cells[143, 148–150], although apoptotic responses were observed in cells from gliomas, breast and hematological tumors[147, 151–153]. Nevertheless, these compounds show a consistently good effect against tumors driven by PI3K/AKT, while they were ineffective against tumors driven by mutations of Ras, which has the ability to signal through multiple pathways, such as the MEKERK pathway[148]. In this case, a combination therapy of dual mTOR-PI3K inhibitors together with a MEK inhibitor was required to achieve antitumoral effects.

mTOR and ageing

Ageing can be defined as a time-dependent decline of the physiological functions of cells, tissues and organs. Ageing can favor the insurgence of sporadic diseases such as cancer, or can itself lead to death through organ failure. Because of this, ageing is increasingly viewed as a disease in its own right, for which molecular therapies can be designed.

In recent years, the manipulation of nutrient sensing and stress response pathways has resulted in an extended lifespan of organisms from yeast to mammals. The rationale behind this observation is that growth-promoting programs may accelerate ageing by generating metabolic by-products and by directly inhibiting homeostasis and repair. Conversely, suppression of growth programs through chemical and genetic manipulations, or by reducing food intake, results in the activation of salvage programs that preserve the functionality of cells and tissues for extended periods of time (reviewed in[154]).

Due to its role at the interface of growth and starvation, mTOR is a prime target in the genetic control of ageing, and evidence from genetic studies supports the view that mTOR may be a master determinant of lifespan and ageing in yeast[155, 156], *C. elegans*[157, 158], flies[159, 160] and mice[161].

The only 'natural' method available to counter ageing is dietary restriction (DR), where the caloric intake is decreased anywhere from 10% to 50%. DR appears to act mainly through the inhibition of mTORC1, and genetic inactivation of mTORC1 pathway components provides no additional benefit over DR[156, 159, 160]. In mice, DR causes lifespan extension and changes in gene expression profile similar to those resulting from loss of S6K1[162], further supporting the view that DR acts through inhibition of mTORC1.

mTOR and cellular ageing

Cellular ageing may stem from the cumulative action of metabolic by-products, exogenous chemicals and ionizing radiation, and order-degrading stochastic processes. To cope with these sources of chaos and damage, the cell relies on rescue processes that either attempt to repair the damaged molecules (such as DNA repair), or mediate their clearance (such as autophagy and proteasomal degradation).

Inhibition of mTORC1 may both counter the sources of damage and enhance repair mechanisms (FIG.5a). Inhibiting mTORC1, and translation as a consequence, extended the lifespan of yeast, worms and flies[163–166]. Reduced translation may place smaller demands on the protein folding systems, thus decreasing the by-production of misfolded protein aggregates. Accordingly, prolonged rapamycin treatment in a mouse model of Huntington Disease decreased the formation of toxic huntingtin aggregates[167].

Lifespan extension can also be explained by an increase in autophagy following mTORC1 inhibition. Autophagy was required for lifespan extension by virtually all protocols in worms and flies[159, 168, 169]. Interestingly, an age-dependent drop in the expression of LAMP2A, which mediates chaperonmediated autophagy, contributed to the ageing of hepatocytes[170]. DR and rapamycin deliver a boost to the autophagic pathway that may compensate for its age-dependent decline.

Interestingly, inhibiting mTORC1 may lead to the increased translation of genes that exert a protective function. In a recent report, the activation of 4E-BP1 by DR in *D. melanogaster* resulted in the increased translation of several components of the mitochondrial electron transport chain[110]. This selective upregulation led to improved mitochondrial respiration.

One may speculate that the resulting decrease in ROS production should result in less cellular damage.

Finally, inhibition of mTORC1 may result in the activation of specific gene expression programs related with the regulation of lifespan; stress-response programs controlled by the transcription factor Gis-1 function downstream of scTOR in regulating *S cerevisiae* lifespan[170].

mTOR and tissue ageing

Tissue-specific stem cells are key in maintaining organ function, by replacing differentiated cells undergoing turnover as well as those that have succumbed to damage (for comprehensive reviews, see[171]). There is evidence that the number of stem cells, as well as their propensity to undergo novel divisions for tissue turnover and repair purposes, declines over time, leading to an irreversible degradation of organ function and thus ageing. For instance, the cell cycle regulators $p16^{Ink4a}$ and $p19^{Arf}$ act as a brake to limit the proliferation of the stem cell pool, but their age-dependent accumulation ultimately causes stem cells to undergo senescence[172, 173].

Moreover, aberrant growth signals or stress signals can accelerate stem cell senescence and tissue ageing (FIG.5B). In a recent report, persistent expression of Wnt proteins in mouse epidermis caused hyperproliferation of epithelial stem cells, ultimately causing them to undergo senescence and exhausting the stem cell niche. Importantly, these actions seemed to occur through Wnt-mediated activation of the mTOR pathway: rapamycin treatment prevented both the hyperproliferation and premature senescence of epidermal stem cell exposed to excess Wnt[88]. In mouse hematopoietic stem cells (HSCs), constitutive mTORC1 activation via deletion of TSC1 led to increased expression of $p16^{Ink4a}$, $p19^{Arf}$ and $p21^{CIP1}$, resulting in HSCs depletion. An age-dependent increase in the activity of mTORC1 was detected and, moreover, prolonged rapamycin treatment preserved the HSCs pool to levels similar to young animals[174].

Finally, over-activation of the PI3K pathway via deletion of PTEN also led to HSCs hyperproliferation followed by their depletion, likely via mTORC1. In fact, treatment with rapamycin restored the capacity of PTEN-null HSCs to reconstitute the blood lineage of irradiated mice[175].

Altogether, these findings point to mTORC1 as a key mediator of growth signals that drive exit of tissue stem cells from quiescence, and to mTORC1 inhibition as a viable approach to preserve the stem cell pool, and thus the functionality of tissues and organs over time.

Concluding remarks

The recent identification of novel regulators and their mode of action further strengthen the idea that the basic layout of the mTOR pathway is that of a signal integrator. The TSC node computes signals from growth factors, stressors and energy to regulate Rheb. A second node is mTORC1 itself, where raptor and PRAS40 directly relay energy and growth factors. Finally, the lysosomal membrane acts as a platform for integration of nutrient inputs with the Rheb axis. It remains to be determined whether signal integration also occurs at the level of mTORC2, and whether mTORC1 and mTORC2 are coordinated to a greater extent than is currently known. The identification of upstream regulators of mTORC2 will likely shed light on these questions.

Given its many cellular actions, it is puzzling that only few substrates of mTOR have been identified so far. This is partly due to the weak and transient nature of the mTOR-substrate

interaction; the continuous improvement of mass spectrometry techniques, combined with the use of the novel mTOR catalytic inhibitors will likely bring important advances in this area. Moreover, the emerging concept that whole classes of genes may be co-regulated by mTORC1-mediated translational control further extends the range of its downstream effectors.

A more integrated understanding of the mTOR pathway will pave the way for novel approaches to old diseases; mTOR has evolved to accelerate growth, but it also speeds up cancer, metabolic derangement and ageing in adulthood. For these reasons, a chronic but well tolerated inhibition of mTOR starting in mid-life could bring significant improvements to human health.

This lifestyle improvement may come at a cost. For instance, it has been observed that lifespan extension by various manipulations comes at the expense of fertility and reproductive success, but recent findings indicate that there may be a way around it: in *D. melanogaster*, supply of a single amino acid, methionine, allows maintenance of reproductive potency in the context of CR-induced lifespan extension[176]. Another potential risk is that over-stimulation of autophagy by chronic mTOR inhibition may actually speed up ageing. Furthermore, the many feedback loops in which mTOR participates may actually result in harmful outcomes. Thus, the particular regimen of mTOR inhibition may have to be carefully chosen while considering the advantages of rapamycin versus catalytic inhibitors and chronic versus intermittent administration.

Finally, it remains to be seen whether limiting mTOR activity in adult humans would really enable a longer lifespan, or it would only bring an increase in the quality of life and the way we age, without necessarily affecting how long we live.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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BOX1: mTORC1 as a signal integrator

How are mTORC1 inputs integrated to generate a coherent signaling response? Signal integration by mTORC1 is likely based on convergent regulation of physical interactions and localization; growth factors induce GTP loading of Rheb, enabling it to physically interact with mTORC1, while amino acids cause the Rag GTPase-mediated shuttling of mTORC1 to the endomembrane system, where Rheb resides. This scenario provides an explanation for the requirement of both inputs for the activation of mTORC1. In the absence of growth factors and amino acids, the Rag and Rheb GTPases are inactive and mTORC1 is physically removed from Rheb (figure, part a). In the presence of abnormally high growth factor inputs, (caused, for instance, by activating mutations in the PI3K pathway), Rheb is hyperactive (green). However, because mTORC1 is not recruited to Rheb, it remains inactive (figure, part b). Amino acids activate the Rag GTPases, which recruit mTORC1 and allow it to bind to Rheb. However, in the absence of growth factors Rheb is inactive, and so is mTORC1 (figure, part c). When both amino acids and growth factors are present, mTORC1 is recruited to active Rheb and activated (figure, part d).

Importantly, nutrients and energy can hardly be regarded as an on/off switch: their concentration varies smoothly in time as a function of the feeding cycle. Thus, mTORC1 likely senses fine variations in these parameters, continuously adjusting the rate of biosynthetic processes accordingly. Once activation of mTORC1 has been achieved, increasing concentrations of amino acids and growth factors can drive it further (figure, part e). Experimental overexpression of Rheb bypasses the requirement for Rag-mediated recruitment of mTORC1 and allows its activation in the absence of amino acids (figure, part f), supporting the signal integration model. Other regulatory inputs, such as energy-sensing AMPK, as well as feedback mechanisms can modulate or entirely suppress this signal integration mechanism, ensuring that nutrients, growth factors and energy do not generate conflicting signals. Thus, the regulation of mTORC1 is a multi-step decision process that takes into account multiple indicators of the energy status of the cell before making a commitment to grow and proliferate.

BOX2: mTOR and membranes

Increasing evidence points to a role for membrane traffic in the regulation of mTOR signaling. Amino acid signaling through the Rag GTPases causes mTORC1 to shuttle to lysosomes, which enables mTORC1 to respond to amino acids and to insulin signal-induced Rheb activation (Fig. 2). The lysosomal surface hosts the molecular machinery for mTORC1 activation that includes the Rag GTPases, the trimeric ragulator complex, and possibly GAPs and GEFs for the Rag GTPases[13, 65]. Vps39, a GEF for Rab7, has been implicated in regulating the nucleotide loading of GTR1, the RagA/B orthologue in yeast[68]. Moreover, work from the Thomas lab has implicated Vps34, the type III pPI3 Kinase that controls endosomal maturation and autophagy, in mediating amino acid signaling to mTORC1[177].

The presence of mTORC1 at the lysosome also suggests a physical basis for the regulation of autophagy: because autophagic membranes fuse with lysosomes to promote degradation of their contents, mTORC1 may be optimally placed to phosphorylate and inhibit key autophagy-promoting proteins. Of notice, yeast GTR1 and GTR2 are part of the EGO complex, which are involved in the regulation of microautophagy[69]. It would be interesting to assess whether mammalian Rag GTPases play similar roles; in fact, recent evidence points to a role for mTORC1 in the reformation of primary lysosomes following autophagy[178] In addition, nutrient storage and release by the lysosome may allow the rapid activation of nutrient signals upstream of mTORC1.

In contrast to mTORC1 the subcellular localization of mTORC2 is unclear, although recent studies suggest it may also associate with membranes. Experimental approaches based on genetic fluorescent tags revealed that yeast TORC2 localizes to discrete, spot-like domains on the plasma membrane. Notably, membrane localization of scTORC2 depends on Avo1, the mammalian orthologue of mSin1, which has a phospholipid-binding PH domain that may mediate the association of mTORC2 with membranes[179]. The localization of mTORC2 on peripheral membranes would be consistent with its activation by PI3K and the insulin receptor, and with its role in phosphorylating Akt. However, indirect evidence suggests that mTORC2 might localize, and be activated at, signaling endosomes near the cell surface.

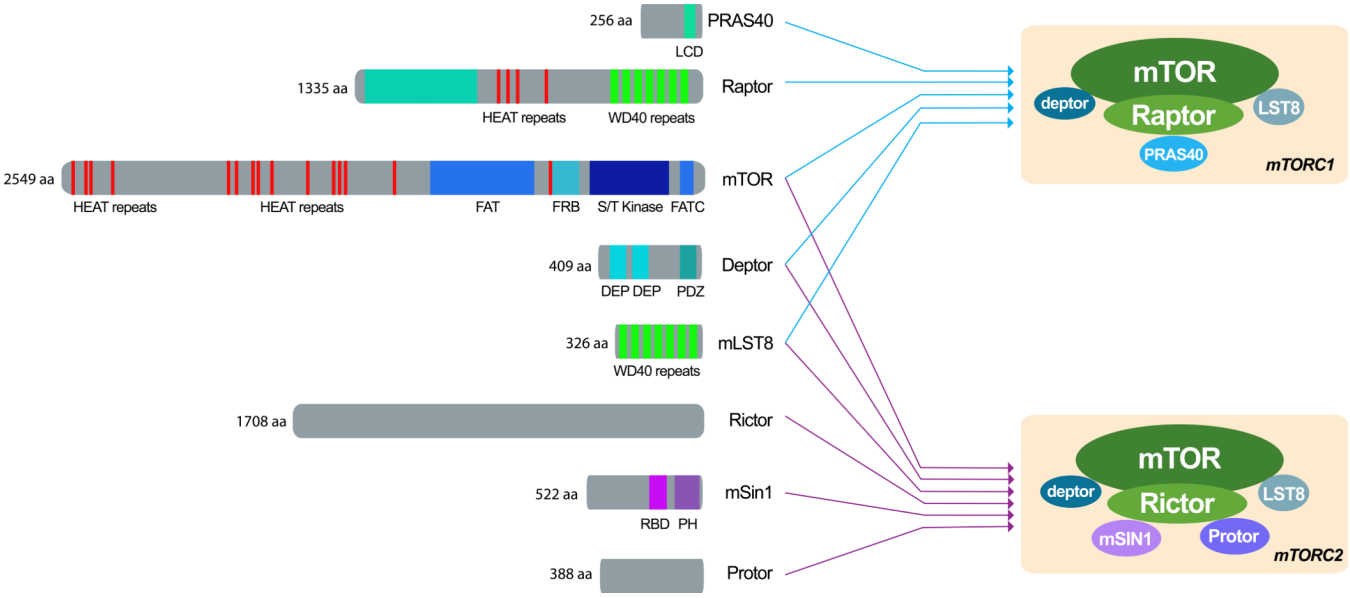


Figure 1. Domain organization of mTOR and mTOR-interacting proteins

A. mTORC1 and mTORC2 have shared (mTOR, mLST8 and deptor) and unique components. Raptor and PRAS40 are unique to mTORC1 and rictor, mSin1 and Protor are specific to mTORC2. The domain organization of mTOR resembles that of other PIKK family members. At the N-terminus there is a cluster of Huntingtin, Elongation factor 3, a subunit of protein phosphatase 2A and TOR1 (HEAT) repeats, which mediate protein-protein interactions. These are followed by, a FRAP, ATM and TRRAP (FAT) domain, the FKBP12-Rapamycin Binding (FRB) domain (which mediates the inhibitory action of rapamycin), the serine/threonine kinase catalytic domain and the C-terminal FATC domain. mLST8 is highly conserved; its seven WD40 domains form a beta propeller that mediates protein-protein interactions. Deptor consists of a tandem dishevelled, egl-10, pleckstrin (DEP) domains, followed by a single postsynaptic density 95, discs large, zonula occludens-1 (PDZ) domain. The scaffolding function of raptor is reflected by its composition of protein-binding domains; it consists of several HEAT repeats, followed by seven WD40 motifs, probably arranged in a beta propeller.Rictor has no clearly identifiable domains or motifs despite its key role in mTORC2 function; likewise, Protor lacks identifiable domains or motifs, and its function awaits clarification.

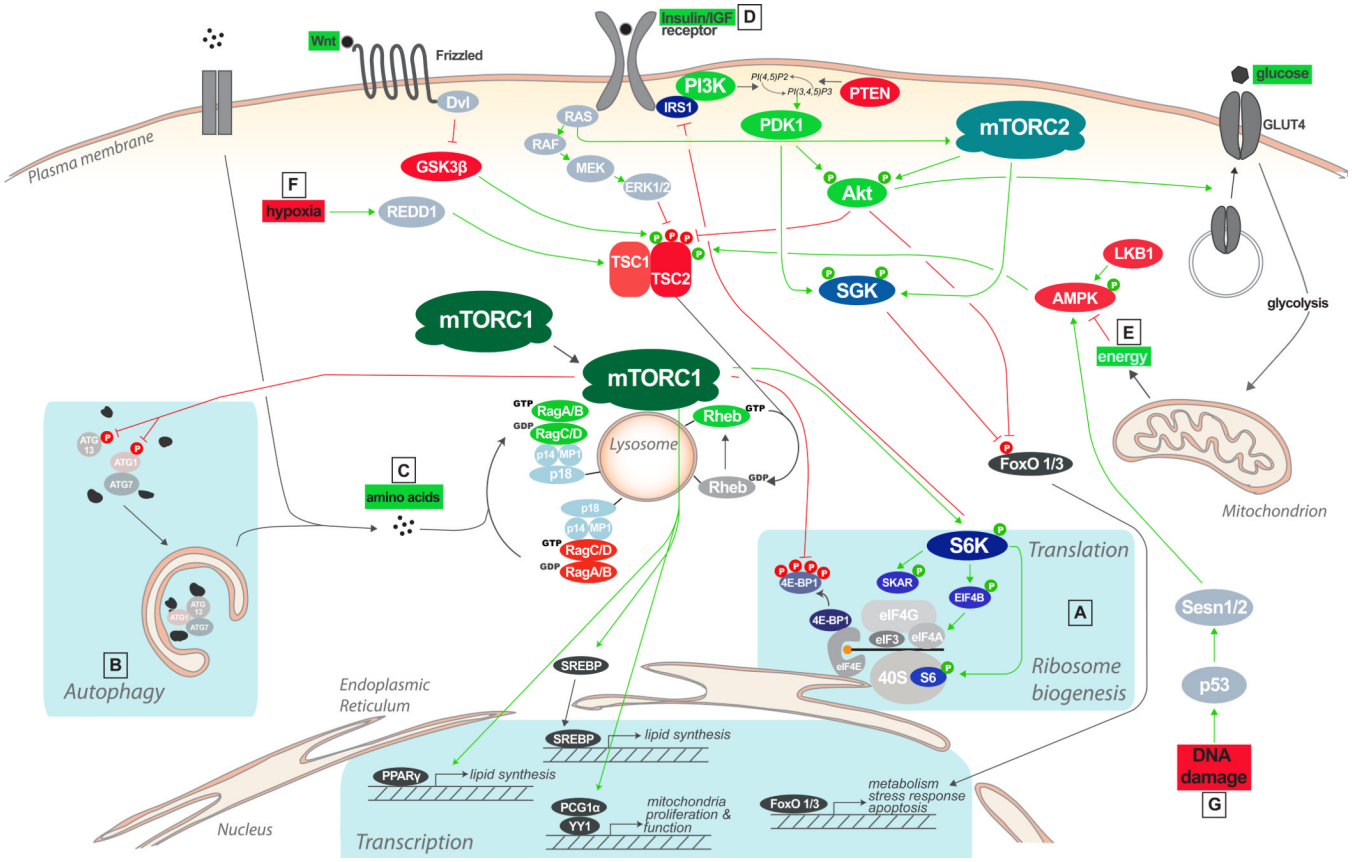


Figure 2. The mTOR signaling pathway

mTORC1 promotes translation (A) and inhibits autophagy (B), by integrating nutrient signals generated by amino acids (C), growth factor signals relayed by the insulin and insulin-like growth factors (D), energy signals acting through AMP-activated kinase (AMPK) (E) and various stressors including hypoxia (F) and DNA damage (G). A first level of integration occurs at the level of the Tuberous Sclerosis Complex (TSC). Akt and extracellular regulated kinase (ERK) 1/2 phosphorylate and inhibit TSC, while AMPK, glycogen synthase kinase (GSK) 3-beta and hypoxic factor REDD1 activate it. A second level of integration occurs at the lysosome: the Rag GTPases (held in place by the p18/p14/MP1 ragulator) recruit mTORC1 to the lysosomal surface in response to amino acids; in turn, lysosomal recruitment enables mTORC1 to interact with GTP-bound Rheb, the end point of growth factor, energy and stress inputs. Growth factor receptors activate mTORC2, probably via Ras, near the plasma membrane, where mTORC2 may be recruited through binding of mSin1 to phospholipids. Because of its role in phosphorylating and activating Akt, mTORC2 forms a core component of the PI3K pathway.

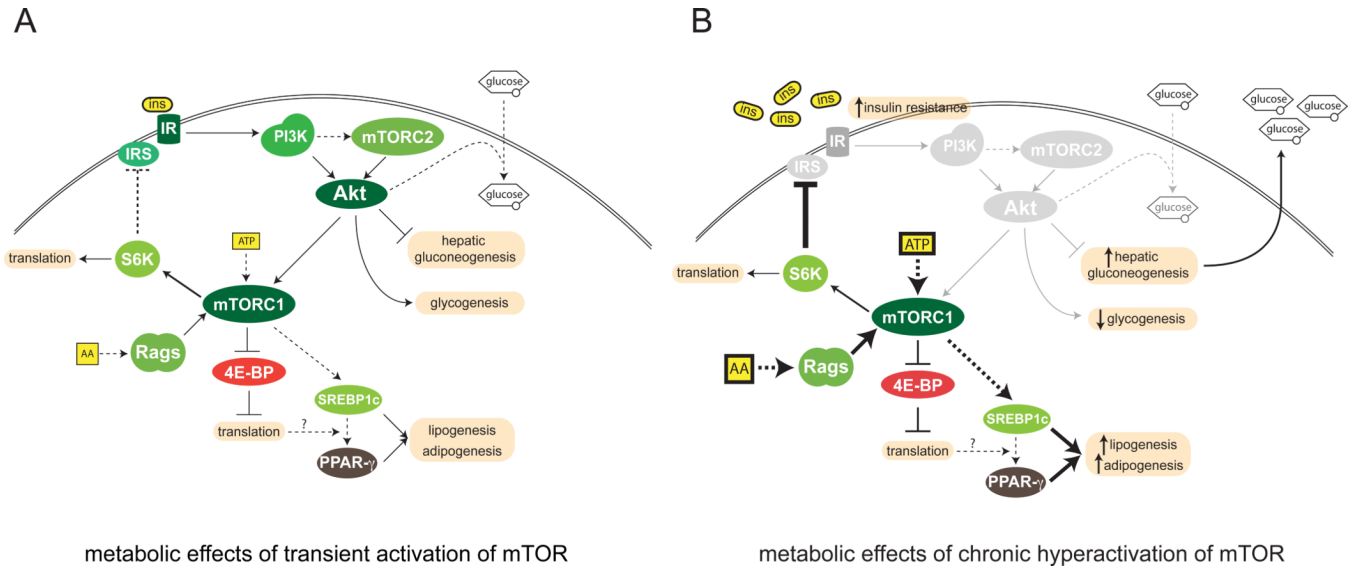


Figure 3. mTOR in metabolism and insulin resistance

A. Effects of mTOR in metabolism. mTOR links nutrient abundance with growth and the accumulation of energy stores in anticipation of future nutrient shortage. Feeding triggers a raise in insulin and nutrient levels in the bloodstream; these converge as activating inputs to mTOR. In turn, mTORC1 activates protein synthesis, cell mass increase, and lipid accumulation, whereas mTORC2 promotes glucose usage by the cell by positively regulating glucose import, glycogen synthesis and by inhibiting gluconeogenesis. B. In the western diet, overabundance of nutrients leads to chronic mTOR activation, which disrupts energetic homeostasis. During chronic insulin stimulation, as occurs in over-feeding states, mTORC1 activity towards S6K inhibits insulin receptor signaling at the cellular membrane, contributing to the onset of the diabetic state. In an insulin-resistance state, phosphatidylinositol 3 kinase (PI3K) and Akt are not activated, leading to decreased glucose uptake and to hepatic gluconeogenesis, which in turn worsen hyperglycaemia. Despite decreased insulin signaling, mTORC1 remains active, maintaining the negative feedback loop to IR. The nutrient inputs to mTORC1 may explain sustained mTORC1 activity in the context of insulin insensitivity.

(AA: amino acids; ins: insulin; IR: insulin receptor; IRS; insulin receptor substrate)

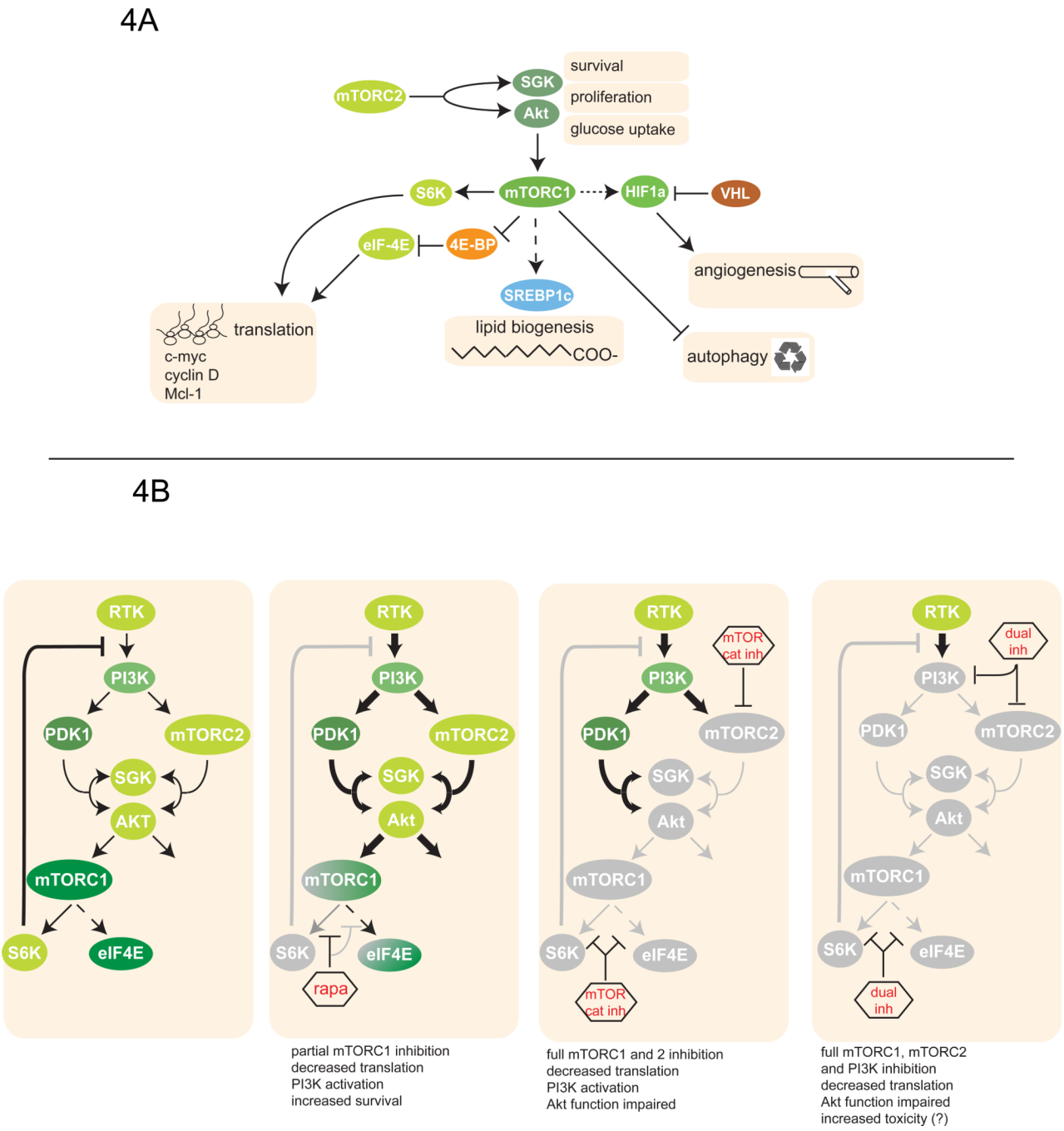


Figure 4. mTOR in cancer

A. mTOR-regulated cellular processes that play a role in cancer. mTORC1 favors tumorigenesis by driving translation of oncogenes, by inhibiting autophagy, by upregulating angiogenesis, and by enhancing the accumulation of lipids. mTORC2 plays a role in tumorigenesis by activating Akt and other AGC family proteins which promote proliferation and survival. Moreover, by promoting Akt-mediated glucose uptake, mTORC2 fuels the metabolism of cancer cells.

B. Therapeutic inhibition of mTOR activity by rapamycin, mTOR catalytic inhibitors (mTOR cat inh) and dual PI3K-mTOR inhibitors (dual inh). Rapamycin only partially suppresses mTORC1 function, efficiently inhibiting S6K but not eIF4E; thus, it only

partially inhibits translation. Moreover, due to the inhibition of the S6K-dependent feedback loops, rapamycin indirectly upregulates phosphatidylinositol 3 kinase (PI3K) activity and promotes cell survival. In contrast, mTOR ATP-competitive inhibitors target all known functions of mTORC1 as well as mTORC2; thus, they inhibit translation more potently. Although PI3K overactivation still occurs, Akt phosphorylation by mTORC2 is impaired. Dual PI3K/mTOR inhibitors block all functions of PI3K, including PDK1- and mTORC2-mediated activation of Akt. However, they might cause increased toxicity.

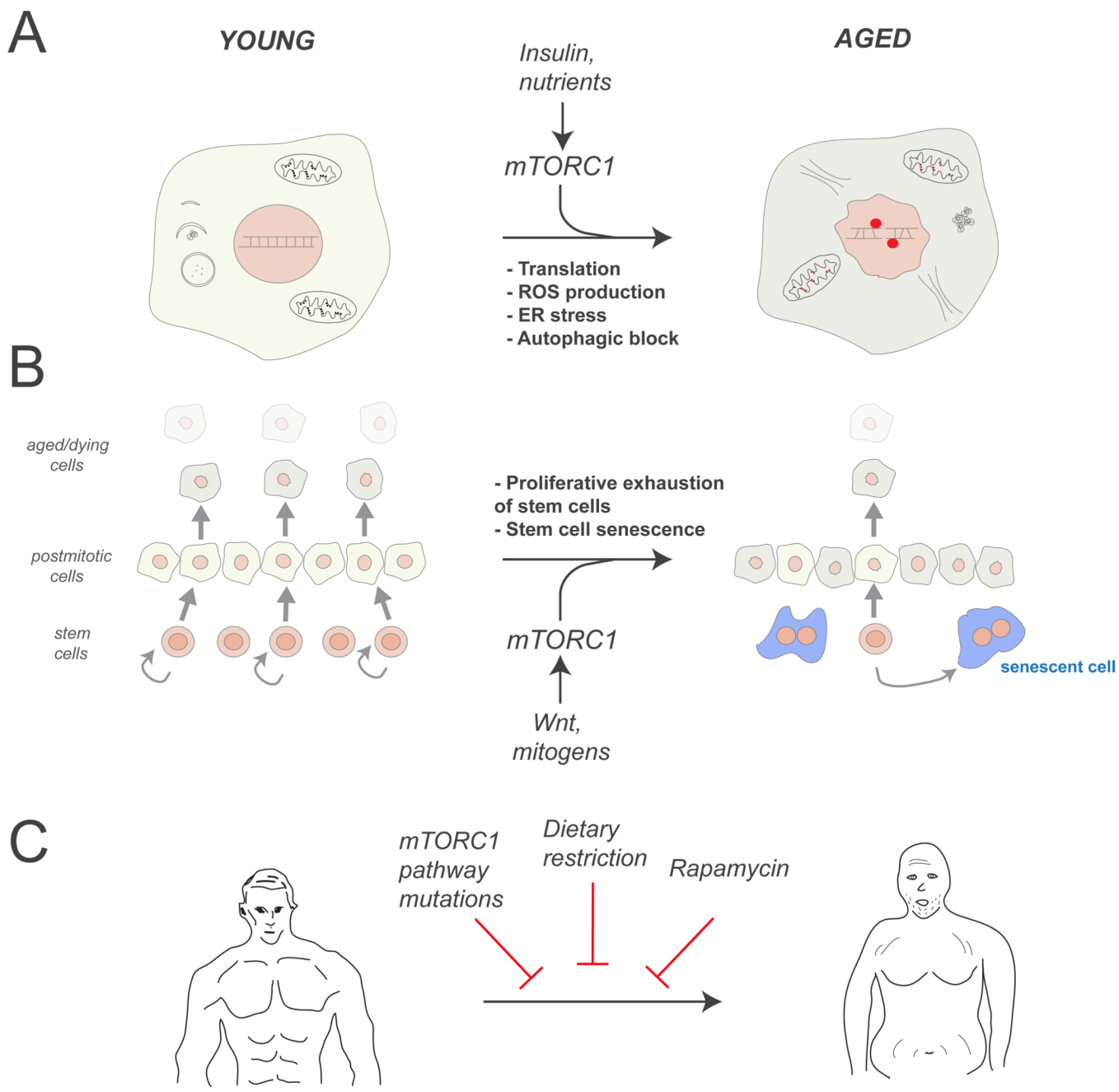


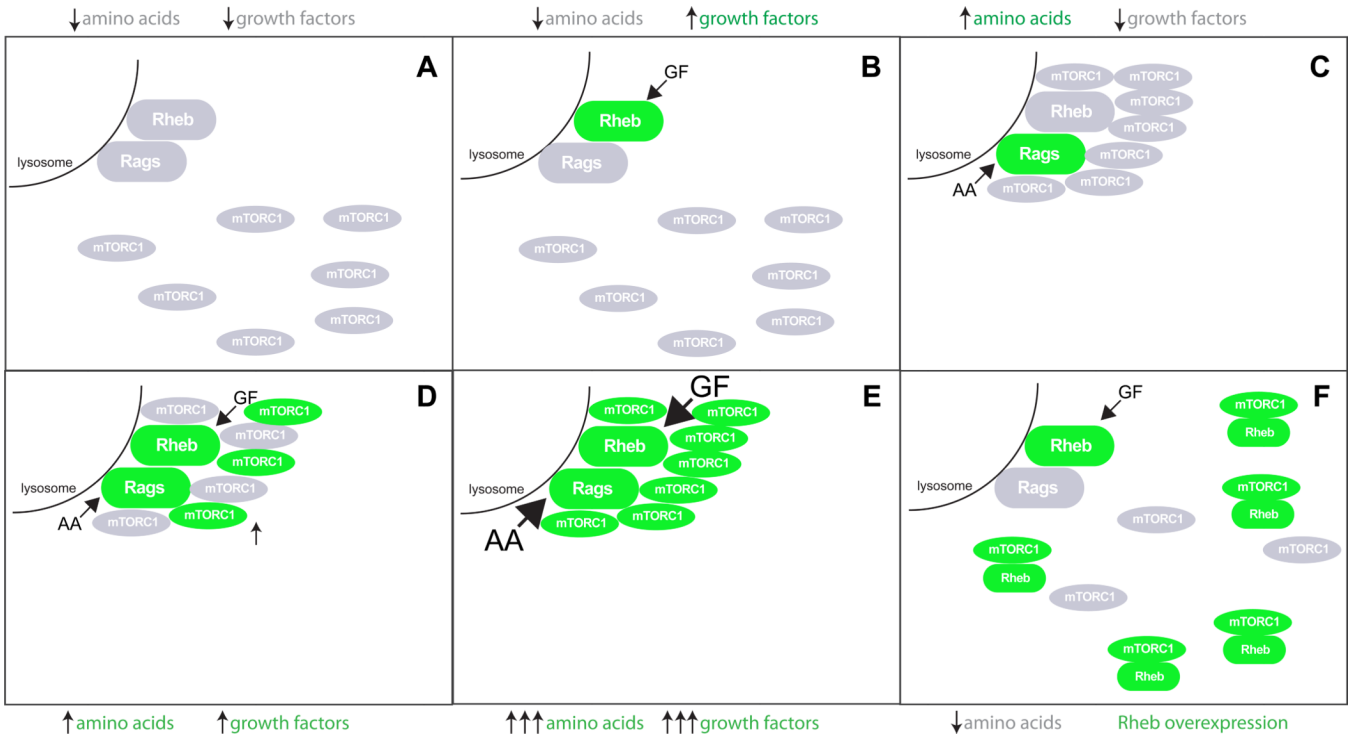
Figure 5. mTOR in ageing

A. mTORC1-regulated processes that promote cellular ageing. mTORC1-dependent translation may overcome the protein folding capacity of the cell, resulting in accumulation of unfolded proteins and ER stress. Stimulation of mitochondrial function may increase ROS production, resulting in oxidative damage to DNA, proteins and membranes. Inhibition of autophagy by mTORC1 reduces the turnover of cellular components and promotes the accumulation of their damaged forms (red).

B. mTORC1 promotes stem cell exhaustion and tissue ageing. In young tissues, stem cells divide asymmetrically to generate new stem cells as well as postmitotic cell that replace those that have undergone turnover (left). Continued exposure to mitogens that signal through mTORC1 causes stem cell exhaustion through hyperproliferation or senescence

(right); thus, in aged tissues post-mitotic cells are no longer replaced and the overall performance of the tissue is degraded.

C. Inhibiting mTORC1 activity by various means allows lifespan extension in multiple organisms, and may have beneficial effects on human ageing.



Box1 Figure.