

Signaling crosstalk between the mTOR complexes

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Abbreviations: 4E-BP, eIF4E-binding protein; 5'-TOP, 5'-terminal oligopyrimidines; AL, activation loop; AMPK, 5'-AMP-activated protein kinase; aPKC, atypical PKC; CK1 α , casein kinase 1 α ; cPKC, conventional PKC; DEPTOR, dishevelled, eEF, eukaryotic elongation factors; eEF2K, eEF2 kinase; EGF, epidermal growth factor; EGFR, EGF receptor; egl-10, pleckstrin domain-containing mTOR interacting protein; eIF, eukaryotic initiation factor; ELT3, Eker rat uterine leiomyoma cell line 3; ERK, extracellular signal-regulated kinase; Fbw8, F-box 8, also termed as Fbxw8 or Fbx29; FKBP12, FK506-binding protein, 12 kDa; FoxO, forkhead box protein; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; Grb10, growth factor bound-receptor protein 10; GRp58, 58 kDa glucose-regulated protein; GSK3, glycogen synthase kinase 3; HM, hydrophobic motif; IGF, insulin-like growth factor; IGF1R, IGF1 receptor; IKK, inhibitor of NF- κ B (nuclear factor κ -light-chain-enhancer of activated B cells) kinase; IMP, IGF2 mRNA-binding protein; InsR, insulin receptor; IRS, insulin receptor substrate; Maf1, matrix attachment region binding filament-like protein 1 associated factor 1; mNIP7: mammalian nuclear import protein 7; mLST8: mammalian ortholog of lethal with sec thirteen, also known as G β -like protein or G β L; mTOR: mammalian target of rapamycin; mTORC1: mTOR complex 1; mTORC2: mTOR complex 2; NBS1: Nijmegen breakage syndrome protein 1; nPKC: novel PKC; PDCD4: programmed cell death protein 4; PDGF: platelet-derived growth factor; PDK1, phosphoinositide dependent protein kinase 1; PI3K, phosphatidylinositol 3-phosphate kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PKB, protein kinase B; PKC, protein kinase C; PRAS40, Pro-rich Akt substrate of 40 kDa; PROTOR, protein observed with RICTOR; Rac1, Ras-related C3 botulinum toxin substrate 1; RAPTOR, regulatory-associated protein of mTOR; Rbx1, RING-box protein 1; rDNA, ribosomal DNA; Rheb, Ras homolog enriched in brain; Rhes, Ras homolog enriched in striatum; RhoA, Ras homolog gene family, member A; RICTOR, rapamycin-insensitive companion of mTOR; RP, ribosomal protein; rRNA, ribosomal RNA; RSK, p90 RP S6 kinase; RTK, receptor tyrosine kinase; S6K, p70 RP S6 kinase; SCF β TrCP, Skp1/Cul1/F box protein adaptor β -transducin repeat-containing protein; SGK, serum- and glucocorticoid-induced protein kinase; SIN1, stress activated protein kinase interacting protein 1; siRNA, small interfering RNA; TBC1D7, Tre2-Bub2-Cdc16 1 domain family, member 7; Tel2, telomere maintenance 2; TFIIC, transcription factor 3C; TM, turn motif; 5'-TOP, 5'-terminal oligopyrimidine tract; Tti1, Tel2 interacting protein 1; VEGFR, vascular epithelial growth factor receptor; XPLN, exchange factor found in platelets, leukemic, and neuronal tissues

mTOR is a protein kinase which integrates a variety of environmental and intracellular stimuli to positively regulate many anabolic processes of the cell, including protein synthesis. It exists within two highly conserved multi-protein complexes known as mTORC1 and mTORC2. Each of these complexes phosphorylates different downstream targets, and play roles in different cellular functions. They also show differential sensitivity to the mTOR inhibitor rapamycin. Nevertheless, despite their biochemical and functional differences, recent studies have suggested that the regulation of these complexes is tightly linked to each other. For instance, both mTORC1 and 2 share some common upstream signaling molecules, such as PI3K and tuberous sclerosis complex TSC, which control their activation. Stimulation of the mTOR complexes may also trigger both positive and negative feedback mechanisms, which then in turn either further enhance or suppress their activation. Here, we summarize some recently discovered features relating to the crosstalk between mTORC1 and 2. We then discuss how aberrant mTOR complex crosstalk mechanisms may have an impact on the development of human diseases and drug resistance.

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Introduction to the mTOR Complexes

mTOR is a master molecular hub which positively controls anabolic, and conversely negatively regulates some catabolic processes. mTOR is present in two biochemically and functionally distinct multi-component complexes termed mTORC1 and mTORC2. Some components are present in both mTORC1 and 2, for instance, mTOR, mLST8,¹ DEPTOR,² Tti1, Tel2,^{3,4} GRp58⁵ and Rac1.⁶ Yet each of the mTOR complexes possesses a unique subset of components. RAPTOR^{7,8} and PRAS40⁹⁻¹³ can only be found in mTORC1, whereas RICTOR,¹⁴ SIN1,¹⁵⁻¹⁷ PROTOR,¹⁸ XPLN,¹⁹ NBS1,²⁰ IKK α and IKK β ²¹ are specific to mTORC2 (Fig. 1). Each of these components can either support or suppress the activation of the respective mTOR complex (Table 1). Rapamycin (as a complex with the immunophilin FKBP12) rapidly inhibits some activities of mTORC1, whereas mTORC2 is resistant to short-term rapamycin treatment, although prolonged rapamycin treatment does interfere with mTORC2 in some cell types.²²⁻²⁴ This is thought to be due to the binding of rapamycin/FKBP12 to newly synthesized and unbound mTOR molecules, which prevents mTORC2 assembly.²²

mTORC1 and protein synthesis

mTORC1 is acutely activated by a variety of anabolic stimuli including growth factors, hormones and nutrients via different signaling pathways, many of which, with the notable exception of amino acid signaling,²⁵ impinge on TSC2. TSC2 serves as a GAP for the small G protein Rheb.²⁶⁻²⁸ The inhibition of TSC2 upon its phosphorylation, induced by upstream stimuli, favors the accumulation of GTP-bound Rheb, which in turn activates mTORC1 through a yet-to-be-determined mechanism (Fig. 1).²⁶⁻²⁸ Insulin, for example, inhibits TSC2 function via the PI3K-PKB pathway.²⁹⁻³² PI3K catalyzes the conversion of PIP2 to PIP3.³³ The latter recruits PKB to the plasma membrane, allowing PDK1 to phosphorylate PKB on Thr308 in its activation loop, leading to its activation,³⁴ PKB in turn phosphorylates and inhibits TSC2 (Fig. 1).²⁹⁻³² Notably, the full activation of PKB also requires its phosphorylation on a hydrophobic site, Ser473, which is catalyzed by mTORC2.³⁵ Interestingly, in mouse striatal tissue, another small G protein Rhes has also been shown to stimulate mTORC1 in its GTP-bound state.³⁶ Nevertheless, unlike Rheb, Rhes also activates mTORC2.³⁶

mTORC1 is tightly linked to several steps of protein synthesis including ribosome biogenesis, translation initiation and elongation. In order to meet the demand for global protein synthesis in response to rapid changes in extracellular nutrient supply, mTOR plays an important role in the control of total ribosome levels within the cell, and this is mediated through the regulation of RNA polymerases and other transcription factors which positively controls the expression of RP or rRNA genes (Fig. 1).³⁷⁻⁴² mTORC1 upregulates the transcriptional activity of RNA polymerases Pol I (makes 5S rRNA) and Pol III (makes the other 3 major rRNAs: 28S, 18S and 5.8S), which are involved in ribosome biogenesis.³⁸ mTORC1 directly phosphorylates Maf1, a repressor of Pol III, and thereby abolishes the inhibitory effect of Maf1 on Pol III transcription (Fig. 1).^{39,40} mTORC1 also activates TFIIC, a transcription factor for Pol I and Pol III.³⁹ In

yeast, TOR translocates into the nucleus in response to nutrient supply, and is associated with rDNA promoter to regulate the transcription of RP genes and 35S rRNA synthesis.³⁷

The translation of 5'-TOP mRNAs, which encode ribosomal proteins (and other proteins involved in anabolic processes including some translation factors) is positively regulated by mTORC1.⁴³⁻⁴⁵ However, it is still not fully understood how mTORC1 controls 5'-TOP mRNA translation and this remains a key question in the field. In addition, Sonenberg's group has discovered that the phosphorylation and inhibition of 4E-BPs is responsible for the proliferative effect of mTORC1 signaling,⁴⁶ and the same group has also recently reported that the translation of many cell cycle-related mRNAs is indeed controlled by mTORC1/4E-BP pathway.⁴⁷ Of interest, another report from Ruggero's group has indicated that RICTOR is highly expressed during S phase, and mTORC2 may be a key regulator of the translation of mRNAs encoding proteins that are important in S-phase.⁴⁸

Translation initiation is generally considered the rate-limiting phase of protein synthesis and especially as the step at which mRNA-specific control is exerted. It relies on a subset of highly organized protein-protein interactions between the eIFs, which serve to recruit the mRNA to the 43S preinitiation complex (comprised of the 40S ribosome subunit, eIF2-GTP-methionyl(Met)-tRNA_i ternary complex, eIF3 and the eIF2 specific GAP eIF5). mRNA recruitment is mediated by the eIF4F (comprising the mRNA helicase eIF4A, eIF4E and the scaffold protein eIF4G) group of factors (reviewed in^{49,50}). The formation of eIF4F is prevented by the binding of 4E-BPs to eIF4E, and this inhibitory interaction is abolished upon 4E-BP phosphorylation by mTORC1 (Fig. 1).⁵¹⁻⁵⁶ Inhibition of mTORC1 strongly impairs the translation of mRNAs containing a 5'-TOP sequence, and recent studies have indicated that this is largely dependent on the effect of mTORC1 on 4E-BPs.⁴³ Another well-known mTORC1 downstream target is S6K (two main isoforms, S6K1/2), which are phosphorylated and activated by mTORC1.⁵⁷ Active S6Ks can phosphorylate and activate eIF4B,⁵⁸ an RNA-binding protein that enhances eIF4A's helicase activity; or phosphorylate and inactivate PDCD4, by promoting its degradation via binding to the ubiquitin ligase SCF^{FBTRCP}, and thereby releasing eIF4A from the inhibitory association with PDCD4.⁵⁹ On the other hand, inactive S6K1 binds to eIF3, a scaffold protein which provides the platform for the association of mRNA and the 43S preinitiation complex, and the inhibitory binding of S6K1 to eIF3 can be prevented upon S6K1 activation by mTORC1 (Fig. 1).^{60,61}

eEF2 mediates the translocation of the ribosome along the mRNA by one codon at a time. The activity of eEF2 is inhibited upon phosphorylation at Thr56 by the highly specific Ca²⁺/calmodulin-dependent kinase, eEF2K.⁶² eEF2K also undergoes rapamycin-sensitive inhibitory phosphorylation at Ser78, Ser359 and Ser366.⁶³⁻⁶⁵ The phosphorylation of Ser359 and Ser366 (the latter catalyzed by S6Ks) impairs eEF2K kinase activity while the phosphorylation of Ser78 prevents the recruitment of calmodulin to eEF2K and therefore also inhibits eEF2K (Fig. 1).⁶³⁻⁶⁵

The regulation and functions of mTORC2

Table 1. List of mTOR complex components and their contribution to substrate recognition or complex activity

Complex Component	Presence in mTORC1 or 2	Contribution to Substrate Recognition or Complex Activity
mTOR	Both	Positive to both
RAPTOR	1	Positive
mLST8	Both	No effect on 1, positive to 2
GRp58	Both	Positive to 1, no effect on 2
DEPTOR	Both	Negative to both
Rac1	Both	Positive to both
PRAS40	1	Negative and/or a substrate?
Tti1	Both	Positive to both
Tel2	Both	Positive to both
RICTOR	2	Positive
SIN1	2	Positive
PROTOR	2	Positive on SGK1, no effect on others
XPLN	2	Negative
NBS1	2	Positive
IKK α	2	Positive
IKK β	2	Positive

In comparison to mTORC1, the mechanism by which mTORC2 is regulated is far less well understood. Nonetheless, mTORC2 activity and signaling to PKB can also be acutely stimulated by insulin via a PI3K-dependent manner (Fig. 1),⁶⁶⁻⁶⁸ so further study is essential to elucidate the mechanism by which PI3K activates mTORC2. mTORC2 is responsible for the phosphorylation and full activation of several AGC kinases, including PKB,³⁵ SGK1,⁶⁹ cPKCs (including α , β I, β II and γ) and ϵ , an nPKC (Fig. 1).^{14,70,71} During synthesis, these AGC kinases are folded and stabilized upon phosphorylation of their TM by mTORC2, and for full activation, mTORC2 phosphorylates a further site within the HM.^{70,71} For example, mTORC2 associates with polysomes and phosphorylates the nascent PKB polypeptide at Thr450 (TM), which allows PKB to escape from being ubiquitinated.⁷² Upon stimulation of the PI3K pathway, PKB translocates to the plasma membrane and is phosphorylated by PDK1 at Thr308 (AL)³⁴ and on Ser473 (HM) by mTORC2 (Fig. 1).³⁵

mTORC2 was originally identified as a positive regulator of actin cytoskeletal organization and polarization,^{14,73-75} it also regulates cell growth,^{76,77} proliferation,^{76,78} cell survival^{19,24,79} and differentiation^{19,80-82} in certain cell types. In contrast to mTORC1, the role of mTORC2 in mRNA translation is far from clear. It has been shown that the effect of mTOR on 5'-TOP mRNA translation is mediated through mTORC1 but not mTORC2.⁴³ However, mTORC2 may also control mRNA translation in

other cell types which have not been tested before. Interestingly, in many cells,^{24,83} but not all,⁸⁴ the stimulation of the mTORC2-PKB pathway leads to phosphorylation and inhibition of GSK3 β . Active GSK3 β phosphorylates eIF2B ϵ (the catalytic subunit of eIF2B) and inhibits eIF2B's activity.⁸⁵ eIF2B is the GEF that generates active GTP-bound eIF2. eIF2-GTP, in turn, binds to the initiator Met-tRNA_i and thereby promotes 43S preinitiation complex formation and hence translation initiation. Therefore, it is plausible that the suppression of GSK3 β by mTORC2-PKB activation releases eIF2B ϵ from the inhibitory phosphorylation by GSK3 β , which may result in increased rates of translation initiation. However, remains to be tested.

“On/Off” Switches for the mTOR Complexes

Although mTORC1 and 2 share a number of components in common, the effects of them on each of the complexes may differ (Table 1). For instance, mLST8 is essential for the integrity and activation of mTORC2, yet its deletion has no effect on mTORC1.⁸⁴ In contrast, GRp58 is required for the assembly and stimulation of mTORC1, but it is dispensable for mTORC2.⁵ Nevertheless, some mTORC components do exert similar effects on both mTOR complexes, such as Rac1, deletion of which in MEFs and lymphocytes leads to a reduction in cell size.⁶ Rac1 is a positive regulator of both mTORC1 and mTORC2.⁶ Tel2, an essential protein for mammalian embryonic

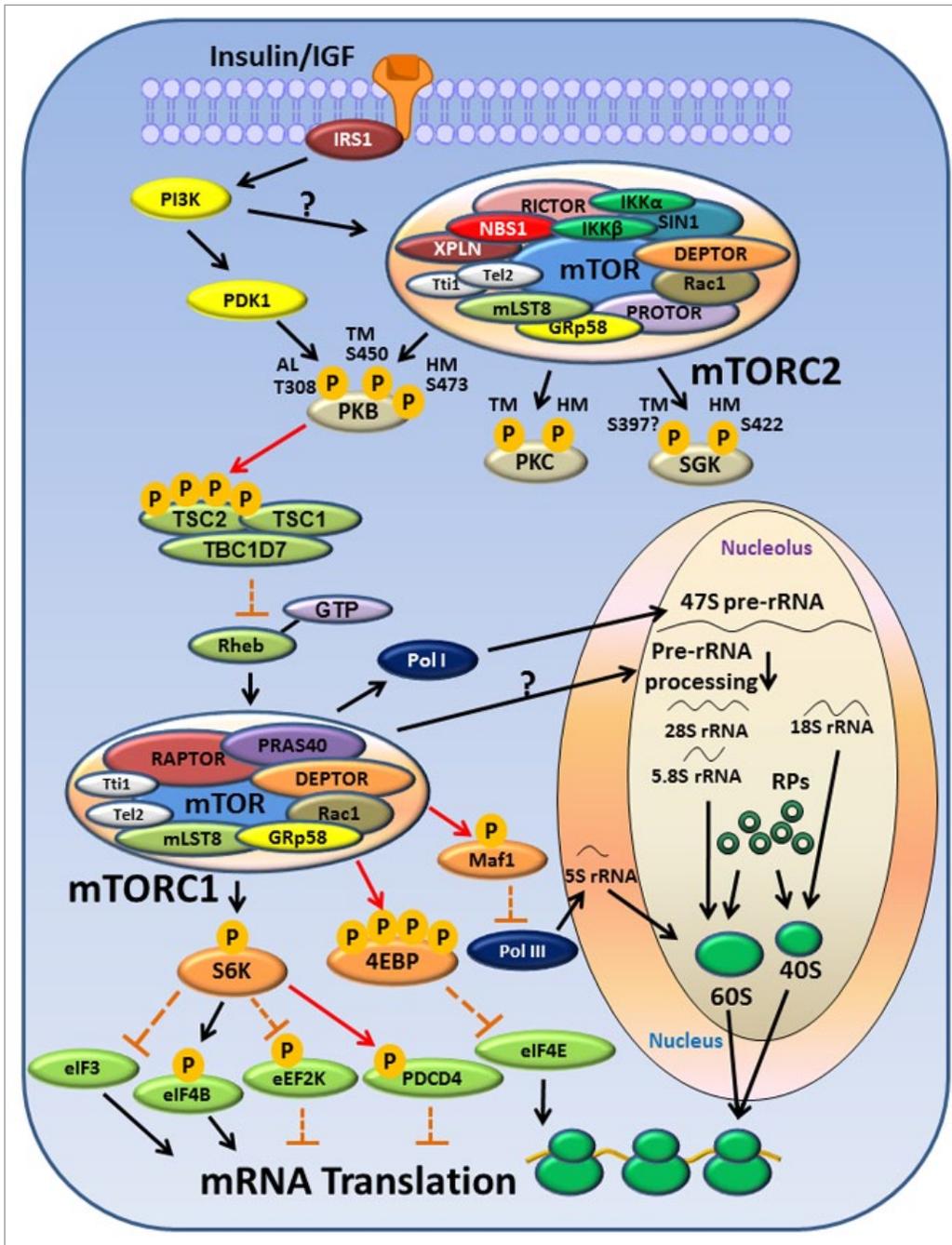


Figure 1. Pathway upstream and downstream of mTOR complexes in response to insulin/IGF stimulation and the regulation of protein synthesis by mTORC1. Insulin/IGF activates mTORC1 through IRS-PI3K-PKB pathway which in turn inhibits the GAP activity of TSC complex toward Rheb, allowing Rheb to activate mTORC1. How PI3K leads to mTORC2 activation remains to be investigated. mTORC2's downstream targets include PKB, PKC and SGK. mTORC1 may regulate several steps in mRNA translation through its substrates S6K (and its own substrates) and 4E-BP1/2. For instance, eIF3, eIF4B, PDCD4 and eIF4E are implicated in the initiation step of translation, whereas eEF2K is involved in translation elongation. mTORC1 also positively regulates ribosome biogenesis, and this can be mediated through the activation of RNA polymerases and the processing of 47S pre-rRNA. Black arrows: positive effect or activation; red lines: phosphorylation events causing a negative effect or inactivation; discontinuous orange lines: an inhibitory mechanism inactivated in response to upstream stimulation. We indicate all the proteins reported to associate with mTORC1/2, but do not intend to imply all are necessarily associated simultaneously.

development and cell growth, positively regulates the maturation and stability of mTOR,³ and it has been demonstrated that Tel2 and its associated protein Tti1 are crucial for the assembly of both mTOR complexes.⁴

DEPTOR

DEPTOR is a negative regulator of both mTORCs,² and is expressed at low levels in most cancer cells, coincident with enhanced activities of mTORC1 and 2 and improved

resistance to nutrient deprivation-induced apoptosis, yet striking exceptions occur in many multiple myelomas where DEPTOR is often overexpressed.² Although the activation of mTORC1 is suppressed in those cells, mTORC2 is “re-activated” as a result of the alleviation of the negative feedback mechanism exerted by S6K, downstream of mTORC1, toward PI3K. Therefore survival rates of these tumor cells are also improved.² As a positive feedback mechanism, the phosphorylation of DEPTOR (by S6K1, RSK or CK1 α) in response to extracellular stimuli results in its ubiquitination and degradation, which releases mTOR from inhibition by DEPTOR.⁸⁶

PKB and the TSC complex

As mentioned before, phosphorylation of PKB by mTORC2 at Ser473 (HM) is required for the full activation of PKB against only some substrates,^{35,84} and although PKB lies upstream of mTORC1, this phosphorylation event is not required for the activation of mTORC1, because knocking-down RICTOR⁸⁴ or SIN1¹⁵ (essential mTORC2 component) in MEFs had no effect on the phosphorylation of mTORC1 upstream regulator TSC2 or mTORC1 downstream targets S6K1 and 4E-BPs, and thus, in this regard, mTORC2 cannot be considered as an upstream regulator of mTORC1. In contrast, the phosphorylation of PKB at Thr308 (AL) by PDK-1 is crucial for insulin signaling to mTORC1.³⁴ Nevertheless, PKB Ser473 phosphorylation is essential for the regulation of FoxO, transcription factors which are excluded from the nucleus and thus inactivated upon mTORC2-PKB stimulation.⁸⁴ Therefore, there is a differential requirement for the phosphorylation of Ser473 for different PKB substrates. However, in primary, non-transformed, non-immortalized, diploid human fibroblasts, mTORC2 does lie upstream of mTORC1, because knocking-down RICTOR in those cells abolishes the phosphorylation of S6K1.⁷⁶

Intriguingly, although genetic ablation of the mTORC1 upstream negative regulator TSC2 (TSC2^{-/-}) in MEFs greatly enhances mTORC1 activity, the stimulation of mTORC2 and hence PKB Ser473 phosphorylation are abolished in these cells.⁸⁷ This effect is independent of the GAP activity of TSC2 toward Rheb, or negative feedback mechanisms (see below) that inactivate PI3K.⁸⁷ The TSC1/2 complex can also associate with mTORC2.⁸⁷ Recently, a third subunit of the TSC1/2 complex called TBC1D7 was identified.⁸⁸ TBC1D7 is required for the GAP activity of TSC1/2 and is therefore inhibitory to mTORC1 activation. shRNA knockdown of TBC1D7 in HeLa cells also causes a reduction in insulin-induced PKB Ser473 phosphorylation, implying that as well as TSC2, TBC1D7 is also a positive regulator of mTORC2.⁸⁸ In contrast, mTORC2 activity is reportedly unaffected in TSC2^{-/-} ELT3 cells, implying that the positive effect of TSC2 on mTORC2 activity may be cell type-dependent.⁸⁹ Notably, knocking-down of RICTOR by siRNA in TSC2^{-/-} ELT3 cells impair cell proliferation and survival, and this was apparently caused by a reduction in RhoA activity, because constitutively active RhoA could restore the decrease in DNA synthesis caused by RICTOR knock-down in these cells.⁸⁹

FoxO

On the other hand, FoxO, which is regulated by the mTORC2-PKB pathway, also plays an important role in maintaining the

homeostatic balance between mTORC1 and 2. Under energy stress conditions, activated FoxO1 can promote the expression of Sestrin3,⁹⁰ a member of the sestrin family of PA26-related proteins which regulate cell growth and survival.⁹¹ In turn, Sestrin3 suppresses the activation of mTORC1 in a TSC2-dependent manner.⁹⁰ In contrast, active FoxO1 also induces the expression of RICTOR at both mRNA and protein levels, which promotes mTORC2 complex formation and PKB stimulation.⁹⁰

Negative Feedback Interplay between mTORCs

IRS

Knocking-down RAPTOR, an mTORC1 essential component, enhances the activation of mTORC2 by insulin, because mTORC1 inhibition alleviates mTORC2 from the suppression by several negative feedback loops (Fig. 2A). In the first, prolonged mTORC1 activation leads to a decrease in the transcription of IRS1, which is required for insulin or IGF signaling to activate PI3K (reviewed in⁹²). Chronic mTORC1 activation also causes the inhibitory phosphorylation of IRS1 which disrupts its association with InsR or IGF1R. This was first reported to be mediated by S6K1 (Fig. 2A).⁹³ Since then, many additional IRS1 phosphorylation sites have been discovered which negatively affect IRS1 function. Besides mTORC1 itself and S6K1, several other protein kinases are also able to phosphorylate these sites, some of which are mTORC2 downstream targets like PKB and GSK3; others also include mTORC1 upstream regulators such as ERK and AMPK (reviewed in⁹²). In a negative feedback mechanism, the inhibition of IRS1 upon prolonged mTORC1 stimulation diminishes the activation of PI3K, which normally promotes the activation of both mTORC1 and mTORC2 (Fig. 2A).⁶⁶⁻⁶⁸

Interestingly, it has recently been found that mTORC2 can also negatively regulate IRS1 by inducing the phosphorylation (at Ser86) and stabilization of Fbw8, which in turn promotes IRS1 degradation (Fig. 2A).⁹⁴ Fbw8 is a substrate recognition subunit of the Cullin7 E3 ubiquitin ligase complex which promotes the ubiquitination and degradation of IRS1 (reviewed in⁹⁵). Activation of mTORC2 downregulates the levels of IRS1 via Fbw8.⁹⁴ There is an increase in IRS1 levels in MEF cells lacking the mTORC2 essential component SIN1 (SIN1^{-/-}). This enhances serum-mediated S6K1 phosphorylation on Thr389, indicative of increased mTORC1 activity,⁹⁴ and provides an indirect IRS1-mediated feedback mechanism whereby mTORC2 stimulation negatively regulates the activity of mTORC1.

Grb10

Another negative feedback loop involves a recently identified mTORC1 substrate Grb10.^{96,97} Grb10 is a negative regulator of the insulin/IGF signaling pathway because it inhibits the autophosphorylation of InsR (Tyr1150/Tyr1151)/IGF1R (Tyr1135/Tyr1136), tyrosine phosphorylation of IRS1/2, and the recruitment of PI3K to IRS1 (Fig. 3).^{96,97} Several phosphorylation sites in Grb10 have been found to be phosphorylated by mTORC1 in vitro or sensitive to treatment of cells with mTOR inhibitors. The phosphorylation of Grb10 stabilizes it, which in turn destabilizes IRS1 (Fig. 2A).^{96,97} The

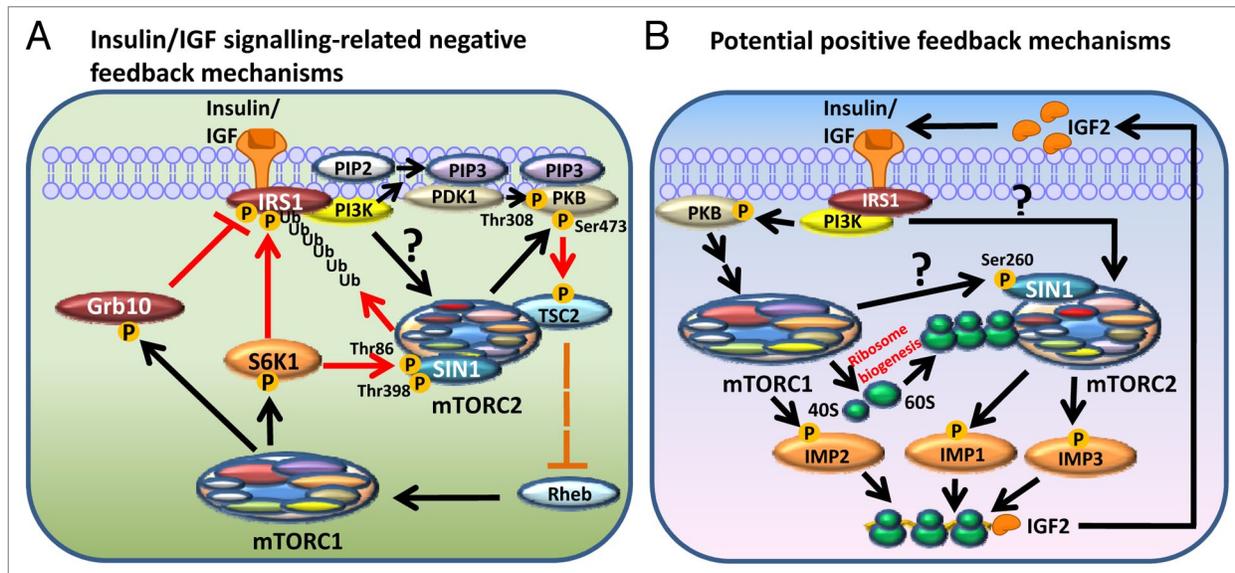


Figure 2. Negative and possible positive feedback mechanisms of the mTOR complexes. **(A)** Negative feedback loops in insulin/IGF signaling. This includes (i) the stabilization of Grb10 by mTORC1; (ii) the inhibition of IRS1 by S6K1, or ubiquitination and degradation of IRS1 induced by mTORC2; and (iii) SIN1 phosphorylation by S6K1. **(B)** Potential positive feedback mechanisms of the mTOR complexes. This includes upregulation of *IGF2* mRNA translation via the phosphorylation and stimulation of IMPs; mTORC1 increases the synthesis of ribosomes which promote mTORC2 activity; and the phosphorylation of SIN1 at Ser260 by mTOR, which is required for mTORC2 complex assembly and activation. Black arrows: upregulation or phosphorylation event leads to activation; red arrows: downregulation or phosphorylation event leading to inactivation; red "T" lines: inhibition of the phosphorylation event which leads to inactivation; discontinuous orange lines: an inhibitory mechanism released in response to upstream stimulation.

discovery of Grb10 as an mTORC1 substrate and IRS1 as a S6K1 substrate are of great importance to understanding type 2 diabetes mellitus, as abnormal insulin signaling is a major feature of insulin resistance. It is believed that nutrient overload, as in the case, e.g., of obese individuals, leads to over-stimulation of mTORC1 and subsequent downregulation of insulin signaling pathways, although it is not fully clear what mechanisms underlie this effect. On the other hand, long-term inhibition of mTORCs by rapamycin also leads to the development of insulin resistance and impaired glucose homeostasis, and this is primarily due to the inhibition of mTORC2.⁹⁸

Phosphorylation of RICTOR on Thr1135

Several groups have identified Thr1135 as a phosphorylation site in RICTOR, an essential component of mTORC2.⁹⁹⁻¹⁰² Thr1135 phosphorylation is modulated by growth factor and nutrient availability. Interestingly, S6K1 is the kinase responsible for phosphorylating this site, which promotes the sequestration of RICTOR by 14-3-3 proteins.^{99,101,102} This may provide a negative feedback loop between the two mTOR complexes. Indeed, PKB phosphorylation at Ser473 is increased upon insulin stimulation in RICTOR-null (RICTOR^{-/-}) MEFs expressing RICTOR T1135A (non-phosphorylatable threonine to alanine mutant), in comparison to when wild-type RICTOR is expressed in those cells, whereas the phosphorylation of other mTORC2 downstream targets, such as PKCs and SGK1, remains unaffected.^{99,101} However, RICTOR Thr1135 phosphorylation does not affect the integrity of mTORC2 or its kinase activity.⁹⁹⁻¹⁰² One explanation for this is that RICTOR Thr1135 phosphorylation reduces the affinity of mTORC2 for PKB, but not PKCs or SGK1, independently of mTOR kinase activity. Moreover, this

phosphorylation event is unaffected in SIN1^{-/-} MEFs, implying that the phosphorylation of RICTOR at Thr1135 does not require mTORC2.¹⁰⁰ Therefore, the significance of RICTOR Thr1135 phosphorylation is far from understood and may play roles in mTORC2-independent RICTOR functions.¹⁰⁰ Furthermore, it was also shown by Gao and colleagues¹⁰³ that RICTOR Thr1135 phosphorylation has a negative effect on its association with cullin-1 and Rbx1 to form a functional E3 ubiquitin ligase, which promotes the ubiquitination of SGK1. Therefore, RICTOR Thr1135 phosphorylation stabilizes SGK1 by impairing SGK1 degradation, which provides a rationale for the accumulation of SGK1 in several human cancers. The same group has also found that RICTOR Thr1135 is not only phosphorylated by S6K1, but also by ectopically expressed PKB and SGK1 in 293T cells.¹⁰³

A regulatory input via SIN1

Because RICTOR Thr1135 phosphorylation does not affect mTORC2 kinase activity,⁹⁹⁻¹⁰² and RICTOR and SIN1 are essential components of mTORC2,^{14-17,73} it is possible that mTORC1 activation triggers a negative feedback loop via SIN1. Indeed, Liu et al. have recently discovered that upon stimulation, S6K1 can phosphorylate SIN1 at Thr86 and Thr398, which will lead to the dissociation of mTORC2 complex and inactivation (Fig. 2A).¹⁰⁴ In comparison to the negative feedback loops mentioned above, this mechanism does not apply only to insulin/IGF signaling, but also to other growth factors like EGF and PDGF.¹⁰⁴ Upon EGF stimulation, HeLa cells expressing the non-phosphorylatable SIN1 T86A or T398A mutant display sustained PKB phosphorylation on Ser473 and Thr308, whereas EGF treatment only leads to transient PKB phosphorylation in SIN1^{-/-} MEFs expressing wild-type SIN1.¹⁰⁴ Paradoxically, an

earlier study performed by Humphrey et al. reported that PKB, rather than S6K1, is the SIN1 Thr86 kinase.¹⁰⁵ In their study, the phosphorylation of SIN1 on Thr86 had no effect on mTORC2 complex assembly and, instead of suppressing mTORC2 activation as reported by Liu et al., it actually enhanced the activity of mTORC2.¹⁰⁵ However, the study of Liu et al. was performed mainly in HeLa cells,¹⁰⁴ an epithelial cancer cell line, whereas Humphrey et al. primarily used adipocytes.¹⁰⁵ Indeed, in agreement with Humphrey et al., Liu et al. found that both PKB and S6K1 can phosphorylate SIN1 at Thr86 in adipocytes.¹⁰⁴ Nevertheless, it is difficult to reconcile the functional differences regarding SIN1 Thr86 phosphorylation between these two studies – stimulatory to mTORC2 according to Humphrey et al.,¹⁰⁵ but inhibitory to mTORC2 (and perhaps also mTORC1) in the work of Liu et al.¹⁰⁴ Interestingly, some ovarian cancer patients carry SIN1 mutations [R81T (arginine to threonine) and S84L (serine to leucine)] close to Thr86.¹⁰⁶ These mutations impair the phosphorylation of SIN1 at Thr86, which disrupts the S6K1-SIN1 negative feedback loop, resulting in sustained PKB phosphorylation at Ser473 and elevated oncogenic/pro-survival signaling.¹⁰⁴

Implications of negative feedback loops for cancer therapies

The discovery of these negative feedback mechanisms indeed greatly contributes to a better understanding of cancer development and cancer drug resistance. For instance, a well-known secondary effect related to rapamycin analogs is the alleviation of several negative feedback loops, including those mentioned above, leading to sustained and enhanced stimulation of PI3K, which may then result in the over-activation of mTORC2 and PKB, hence promoting cancer cell survival. mTOR inhibition also triggers feedback activation of RTKs.¹⁰⁷ For instance, the tyrosine phosphorylation and trans-activation of the EGFR in response to rapamycin treatment may activate other prosurvival kinases like ERK and RSK.¹⁰⁸ Similarly, second generation mTOR inhibitors which simultaneously suppress the activity of mTORC2 as well as mTORC1 may also trigger growth factor receptor (VEGFR, IGF1R and EGFR) tyrosine phosphorylation and activation. This is mediated by an increase in the activity of FoxO upon mTORC2-PKB inhibition, because transient knock-down of FoxO by siRNA can block the phosphorylation events induced by the mTOR inhibitors.¹⁰⁹ However, it remains to be elucidated how activation of FoxO, a transcriptional regulator, leads to phosphorylation of growth factor receptors.

Possible Positive Feedback Crosstalk of the mTOR Complexes

IMPs

In the last section, we listed examples whereby, upon mTORC1 activation, negative feedback loops start to operate to shut down mTORC2 (and also mTORC1) upstream signals. However, the stimulation of mTOR complexes may also trigger some positive regulatory mechanisms which consequently further enhance their activation. One such possible mechanism involves IMPs and the translational regulation of IGF2 (Fig. 2B).^{110,111} It has been demonstrated that active mTORC1 phosphorylates IMP2 at

Ser162 and Ser164,¹¹⁰ while IMP1 and IMP3 are phosphorylated by mTORC2 at Ser181 or Ser183, respectively.¹¹¹ All these phosphorylation events are stimulatory to IMPs, promoting the binding of IMPs to the *IGF2* mRNA and enhancing its translation by cap-independent internal ribosomal entry.^{110,111} Presumably, increased levels of IGF2 will further enhance the activation of mTORC1 and 2, although this is yet to be confirmed.

Ribosome biogenesis

It has been shown that active mTORC2 associates with ribosomes and that this binding can be promoted by insulin signaling and PI3K stimulation.⁶⁸ Importantly, transient knock-down of mNIP7, which is required for the maturation of rRNA and therefore ribosome production,¹¹² or genes encoding RPs such as L7, L26 and S16, by siRNA in HeLa cells, severely impairs mTORC2 activation in response to insulin whereas the stimulation of mTORC1 remains unaffected.⁶⁸ Therefore, it is plausible that the upregulation of ribosome biogenesis by mTORC1 may actually promote mTORC2 activation (Fig. 2B). However, this also requires experimental confirmation.

SIN1

We have mentioned that in HeLa cells, upon growth factor stimulation, the essential mTORC2 component SIN1 appears to undergo inhibitory phosphorylation on Thr86 and Thr398, leading to the dissociation of mTORC2 and hence inactivation,¹⁰⁴ although according to Humphrey et al. SIN1 Thr86 positively regulates mTORC2 activity in adipocytes.^{104,105} Another SIN1 phosphorylation site found by Sarbassov's group, Ser260, which is sensitive to glucose starvation and ATP depletion, positively regulates mTORC2 assembly (Fig. 2B).¹¹³ Phosphorylation of SIN1 at Ser260 is catalyzed by mTOR,¹¹³ although it remains unclear which mTOR complex (or perhaps both) is (are) responsible for its phosphorylation. Notably, tumors induced by subcutaneous injection of H-Ras transformed MEFs constitutively expressing S260A (non-phosphorylatable serine to alanine mutant) SIN1 in mice, have only approximately 15–20% of the volume of the corresponding cells that are expressing wild-type or S260D (phosphomimetic serine to aspartic acid mutant) SIN1, further illustrating an important role of SIN1 in cancer development.¹¹³

Concluding Remarks

mTOR is a key modulator of anabolic cellular processes and importantly, it positively controls many aspects of mRNA translation as well as other anabolic processes. mTOR exists in two major multi-protein complexes termed as mTORC1 and mTORC2. Although, since the discovery of mTORC2 in 2004,^{14,73} a number of crosstalk mechanisms between the mTOR complexes have been described, there are still many unanswered questions within the mTORC interplay puzzle which await further investigation. First of all, it is crucial to determine whether the phosphorylation of SIN1 on Thr86 and Thr398 exerts positive or negative effects on mTORC2 in response to growth factor stimulation, and whether it plays the same role in response to other stimuli or under stress conditions. It is likely that these phosphorylation sites also affect mTORC1 activity

because there is also a sustained phosphorylation of PKB at Thr308 in EGF-stimulated HeLa cells transfected with either SIN1 T86A or T398A, which should then upregulate mTORC1 activity via the phosphorylation and inhibition of TSC2. It implies that the phosphorylation of SIN1 can potentially serve as a negative feedback loop for both mTOR complexes,¹⁰⁴ although further study is still required to determine the effect of SIN1 phosphorylation on the activity of mTORC1. Moreover, it is still unknown which of the mTOR complexes is responsible for SIN1 Ser260 phosphorylation.

Previously, insulin/IGF-mediated mTORC1 and 2 feedback mechanisms were the best characterized, whereas feedback loops in response to other stimuli were much less understood. The discovery of SIN1 phosphorylation sites (Thr86,^{104,105} Ser260¹¹³ and Thr398¹⁰⁴), which can be regulated by several growth factors (Thr86 and Thr398)^{104,105} and energy stress (Ser260),¹¹³ opened doors in the search of mTORC feedback mechanisms in non-insulin/IGF related signaling pathways. For instance, would be an interesting field to explore mTORC crosstalk in amino acid signaling. The mechanisms by which amino acids activate mTORC1 have been extensively studied in the last decade and are now relatively well-documented (reviewed in¹¹⁴). One study, by Tato et al.,⁶⁷ indicated that mTORC2 activity can also be acutely stimulated by amino acids in a PI3K-dependent manner, yet 1) the mechanism by which PI3K activates mTORC2; 2) also; how does mTORC1 actually sense amino acids; and 3) whether there is any similarity between the signaling pathways by which amino acids activate the two mTOR complexes, is far from understood.

Another intriguing question is how TSC1/2 and TBC1D7 positively regulate mTORC2 activity. TSC1/2 binds to mTORC2.⁸⁷ Although deletion of TSC2 does not interrupt the association of RICTOR and mTOR, it severely affects mTORC2 activation in MEFs.⁸⁷ There are several possible mechanisms by which the TSC complexes might contribute to mTORC2 activation, either dependent on mTORC1 (discussed in this article) or independent of it. Indeed, TSC2^{-/-} MEFs display high levels of S6K1 stimulation which disrupts SIN1-RICTOR binding, presumably due to an increase in SIN1 phosphorylation at Thr86 and Thr398.¹⁰⁴ Nevertheless, transient knock-down of RAPTOR, the defining mTORC1 component, in TSC2^{-/-} MEFs, does not restore mTORC2 activity, implying that the interaction between TSC complex and mTORC2 can also be mTORC1-independent.⁸⁷ A recent computational model based on experimental data obtained by Dalle Pezze et al. suggests that TSC1/2 makes no input into mTORC2, and a PI3K variant insensitive to negative feedback loops involving mTORC1 is responsible for mTORC2 activation.¹¹⁵ It is important to note that the model proposed

by these authors is mainly based on research data using mTOR Ser2481 phosphorylation as a specific readout of mTORC2 activity,¹¹⁵ yet mTOR Ser2481 phosphorylation also occurs in mTORC1 and can therefore cannot be used as a specific marker of mTORC1 or mTORC2.¹¹⁶ Consequently, the conclusions of this study must be treated with caution,¹¹⁷ and questions remain regarding the existence of such a PI3K variant.

Deregulation of the translational control resulting from disruptions in the interplay between the mTORC1 and mTORC2 circuitry may have a profound impact on disease pathogenesis. For instance, chronic excess nutrients, which give rise to obesity, cause chronic activation of mTORC1 and subsequently S6K1, which results in the downregulation of IRS and therefore the inhibition of insulin signaling, leading to the development of insulin resistance.⁹³ On the other hand, mTORC2 activation is cytoprotective to cardiomyocytes.^{118,119} mTORC1 stimulation, which causes cardiac hypertrophy as well as downregulation of mTORC2 signaling, leads to increased cardiomyocyte apoptosis and tissue damage after myocardial infarction.^{118,119} Conversely, blocking mTORC1 activation by overexpressing PRAS40 can promote mTORC2 signaling and hence myocardial protection.^{118,119} In addition, as mentioned above, many components within the complex cross-talk network between mTORC1 and mTORC2, such as SIN1^{104,113} and TSC2 (reviewed in¹²⁰), play important roles in the development of cancer.

In summary, we have described the crosstalk mechanisms between the two mTOR complexes. Each mTOR complex regulates a distinct subset of downstream targets involving in many essential cellular functions, especially cell growth, proliferation and survival. Abnormal regulation of the mTOR complexes is implicated in the development of a variety of human illnesses such as diabetes, cardiovascular diseases (cardiac hypertrophy) and cancer. Interestingly, the regulation of mTOR complexes is tightly linked, and therefore it is of great interest and potential importance to understand the implications of mTORC crosstalk mechanisms in the pathogenesis and treatment of these diseases. We expect that future studies will shed more light on the yet-to-be completed picture of mTORC cross-regulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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