

Key factors in mTOR regulation

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Received: 19 August 2009 / Revised: 22 September 2009 / Accepted: 23 September 2009 / Published online: 13 October 2009
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Abstract Mammalian target of rapamycin (mTOR) is a protein serine/threonine kinase that controls a wide range of growth-related cellular processes. In the past several years, many factors have been identified that are involved in controlling mTOR activity. Those factors in turn are regulated by diverse signaling cascades responsive to changes in intracellular and environmental conditions. The molecular connections between mTOR and its regulators form a complex signaling network that governs cellular metabolism, growth and proliferation. In this review, we discuss some key factors in mTOR regulation and mechanisms by which these factors control mTOR activity.

Keywords mTOR · TSC2 · Rheb · FKBP38 · PRAS40 · hVps34 · Rag GTPases · Phosphatidic acid

Introduction

TOR, target of rapamycin, is an atypical serine/threonine kinase that was originally identified in the yeast *Saccharomyces cerevisiae* and later in human, mouse and other eukaryotic cells [1, 2]. Rapamycin, in complex with a cytosolic protein FKBP12, specifically binds to TOR and interferes with its function [3, 4]. For most of eukaryotic

cells, inhibition of TOR by rapamycin results in growth arrest. The drug-affected cells withdraw from cell cycle and become unresponsive to growth factor and nutrient stimulation [5]. However, the sensitivity of each type of cells to rapamycin varies [6]. Lymphocytes and certain types of cancer cells are among those highly susceptible to rapamycin inhibition. This differentiated sensitivity allows the use of rapamycin and its derivatives (rapalogs) to selectively block the growth and proliferation of unwanted cells without affecting normal ones in the human body, leading to the development of rapalogs as immunosuppressive drugs and recently as anti-cancer agents [7]. Hence, ever since its identification, TOR has been at the center of extensive studies driven by our desire to understand fundamental mechanisms governing cell growth and by the clinical applications of TOR inhibitors.

In mammals the homologs of the yeast TOR are collectively referred to as mammalian target of rapamycin (mTOR). Like its counterparts in other organisms, mTOR exists in two distinct complexes termed as mTOR complex 1 (mTORC1) and complex 2 (mTORC2), both of which contain several components that are conserved from yeast to human [8, 9]. Despite the presence of mTOR in both complexes, only mTORC1 is sensitive to rapamycin inhibition [8]. However, prolonged rapamycin treatment still affects mTORC2 function, presumably by preventing new mTORC2 generation [10].

Being the target of rapamycin, mTORC1 has been the main focus in most mTOR-related studies. As discussed below, mTORC1 is regulated by an array of diverse intracellular and environmental cues, including those of growth factors, mitogens, phosphatidic acid (PA), energy, nutrient and stresses (Fig. 1). Upon receiving and integrating upstream signals, mTORC1 in turn controls a wide range of growth-related cellular processes, including

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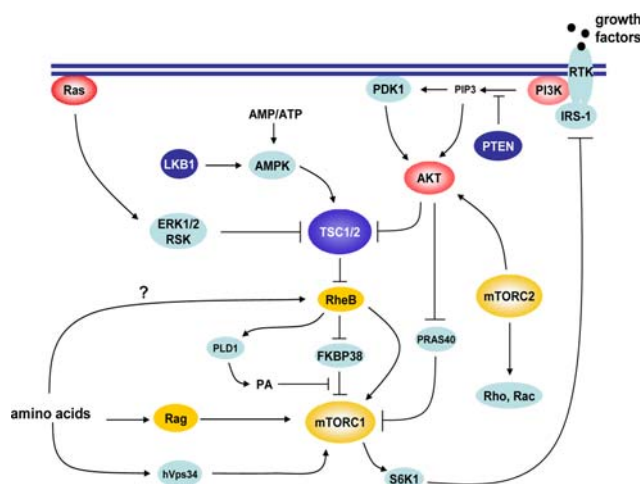


Fig. 1 The signaling network that controls mTOR. mTOR exists in two distinct complexes, mTORC1 and mTORC2. The function of mTORC1 is regulated by signaling pathways responsive to amino acids sufficiency, growth factor stimulation and changes in energy levels. These pathways converge either on mTORC1 itself or on the TSC1/TSC2 complex, making up a complex signaling network. The proximal regulators of mTORC1 include RheB and Rag small GTPases as activators and FKBP38 and PRAS40 as inhibitors. The S6K-directed phosphorylation of IRS-1 constitutes a feedback loop that downregulates the signaling activity from insulin receptors to Akt and mTOR. The mechanisms involved in mTORC2 regulation remain elusive. *Arrows* depict activation and T bars inhibition

translation, transcription, autophagy and hypoxic adaptation [5]. In contrast, mTORC2 is involved in controlling Akt activity and in regulating cell-cycle-dependent organization of actin cytoskeleton [11]. The mechanisms for its regulation have only begun to be revealed. In the following sections, we discuss many recently emerged factors involved in mTOR regulation with emphasis on those directly in contact with mTOR or mTOR complexes.

mTOR

Mammalian target of rapamycin is responsible for the catalytic activity of both mTORC1 and mTORC2. It is a ~289-kDa multi-domain protein that belongs to the family of the phosphatidylinositol 3-kinase (PI3-K)-related kinases (PIKK). Members of this kinase family are characterized by their large sizes (>2,500 amino acids) and a C-terminally located kinase domain that is structurally related to PI3-K [12]. In addition to the kinase domain, mTOR also contains an N-terminal region that is mainly composed of tandem repeats of the HEAT motif, an α -helix-like structure mediating protein–protein interaction [13]. The middle part of mTOR comprises the FAT domain that is found among all the members of the PIKK family and is believed to be involved in interaction with other proteins [14]. The last ~30 residues of mTOR form the

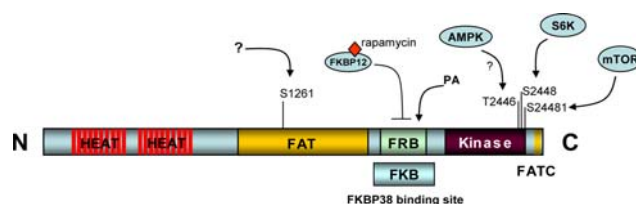


Fig. 2 A schematic presentation of mTOR structures. mTOR is a large protein kinase containing multiple-functional domains. The kinase domain is located at the C-terminus and is responsible for the catalytic activity of mTORC1. The HEAT motif region located at the N-terminus and the FAT domain in the middle are involved in mediating mTOR interaction with other proteins. The FRB domain is where the FKBP12 and rapamycin complex binds, which is within the region that binds to FKBP38. mTOR is also phosphorylated by several kinases, including itself

FATC domain, which consists of an α -helix and a disulfide-bonded loop, which can be regulated by cytosolic redox potential [15]. The FKBP12–rapamycin complex binds to a region lying between the FAT and the kinase domains [16]. Binding of the complex to mTOR is thus not expected to directly affect the kinase activity of mTOR (Fig. 2).

Mammalian target of rapamycin is phosphorylated at multiple sites, including Ser2448, Ser2481, Thr2446 and Ser1261. Phosphorylation at Ser2448 is mediated by p70 ribosomal S6 kinase (S6K) and occurs predominantly to mTOR in mTORC1 [17–19]. Ser2481 is an autophosphorylation site. Phosphorylation at this site happens mainly to mTOR in mTORC2 and is insensitive to acute rapamycin treatment [19, 20]. Thr2446 is phosphorylated presumably by cAMP activated protein kinase (AMPK), and the phosphorylation levels increase upon nutrient withdrawal and decrease upon insulin stimulation [21]. Ser1261 was identified recently as a site whose phosphorylation promotes mTORC1 activity and is required for mTOR autophosphorylation at Ser2481 [22]. In all the cases examined, phosphorylation of mTOR appears to alter its kinase activity rather than its association with other components in the mTOR complexes [19, 23].

mTORC1

mTOR complex 1 is sensitive to rapamycin and thus is responsible for all the rapamycin-sensitive processes in mammalian cells. It consists of mTOR, regulatory associated protein of mTOR (raptor) and mLST8 (mammalian lethal with sec13 protein 8, also known as G β L), all of which are essential for the function of the complex (Fig. 3) [8, 24, 25].

mLST8 is the only mTOR partner found in both mTORC1 and mTORC2. It is a 36-kDa protein composed entirely of seven WD40 repeats, which are short sequence motifs of ~40 amino acids, often terminating in a

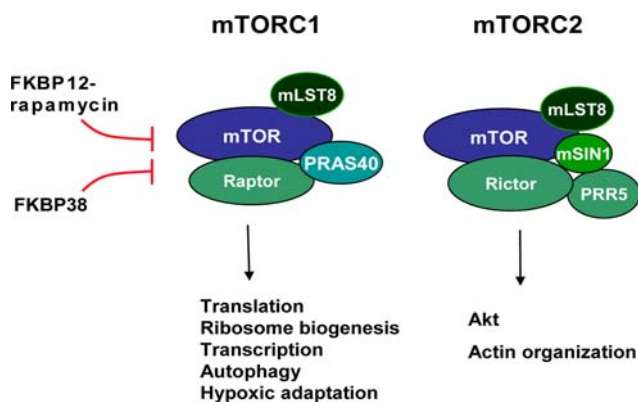


Fig. 3 Two mTOR complexes and their functions. mTORC1 is composed of mTOR, mLST8, raptor and PRAS40. Its function is involved many growth-related processes and oxygen adaptation and is sensitive to rapamycin. mTORC2 shares mTOR and mLST8 with mTORC1, but contains a few other unique components, including rictor, mSIN1 and PRR5

tryptophan-aspartic acid (W-D) dipeptide [26]. mLST8 has been found to associate with the kinase domain of mTOR and stimulate mTOR kinase activity [25]. However, it appears to be dispensable for association of mTOR with raptor, and hence may not play a critical role in mTORC1 function [27].

Raptor, also known as p150 TOR-scaffold protein, is the unique component of mTORC1. It acts as an adapter protein in mTORC1 by binding and presenting substrates to mTOR [24, 28]. Raptor is a large protein with a M_w of ~150 kDa that contains an N-terminal RNC (raptor N-terminal conserved) domain followed by three HEAT repeats and seven WD-40 repeats in the C-terminus of the protein [24, 25]. Analysis of the interaction between the yeast TOR and Kog1, the yeast counterpart of raptor, reveals that the C-terminal WD40 repeats of Kog1 bind to the N-terminal HEAT repeats of TOR, and the RNC domain is in proximity to the TOR kinase domain. The RNC domain is thus likely to function in bringing in substrates into the vicinity of the catalytic region [29]. Raptor binds to a sequence motif, commonly referred to as the TOR signaling motif (TOS), which is found in all known substrates of mTORC1, including S6K, 4E-BP1 and PRAS40 [30, 31]. Mutations in TOS motif of S6K and 4E-BP1 that abolish the binding with raptor also eliminate the ability of these polypeptides to be phosphorylated by mTORC1 in cells. In addition to TOS motif, 4E-BP1 contains another raptor-interacting motif (RAIP) that is required for its binding with raptor and efficient phosphorylation by mTORC1 in cells [32, 33]. Recently, a new raptor interacting motif, SAIN (Shc and IRS-1 NPXY-binding) domain, has been found in insulin receptor substrate-1 (IRS-1), which mediates the mTORC1-dependent phosphorylation of IRS-1 at Ser636 and Ser639 [34].

Besides its function to bring protein substrates to mTOR, raptor itself is also phosphorylated at multiple sites by several kinases, including AMPK, p90 ribosomal S6 kinase (RSK) and mTORC1. AMPK phosphorylates raptor on two well-conserved serine residues, Ser722 and Ser792. The phosphorylation recruits binding of 14-3-3 to raptor, resulting in inhibition of mTORC1 activity [35]. RSK phosphorylates raptor at three sites, including Ser719, Ser721 and Ser722. Phosphorylation at these sites has been shown to promote mTORC1 kinase activity [36]. In addition, a few mTORC1-directed phosphorylation sites have been found in raptor. Mutations in one of the sites, Ser863, attenuate the effect of small GTP-binding protein *Ras-homology enriched in brain* (Rheb) on mTORC1 activation [37]. Collectively, these findings suggest that raptor is able to integrate various signals for mTOR regulation.

The function of mTORC1 has been implicated in many cellular processes by the nature of their sensitivity to rapamycin inhibition. However, only a few direct substrates of mTORC1 have so far been identified. Among them, 4E-BP1 and S6K are two well-characterized targets. As such, the mTORC1-directed phosphorylation of both proteins is commonly used as an indicator for mTORC1 activity in cells. 4E-BP1 is a translation repressor, which binds and inhibits the translation initiation factor 4E (eIF-4E), a key factor for translation of 5' capped mRNAs, among which are transcripts encoding growth promoting factors, such as Myc, cyclin D1, vascular endothelial growth factor (VEGF) and signal transducer and activator of transcription 3 (STAT3) [38]. mTORC1 phosphorylates 4E-BP1 at several sites, including Thr37, Thr46, Ser65, Thr70 and Ser83 [39–41]. Phosphorylation at Thr37 and Thr 46 appears to prime 4E-BP1 for additional phosphorylation at the other sites, which leads to its dissociation from eIF-4E [42]. S6K is phosphorylated by mTORC1 at Thr389 within a hydrophobic motif that links its catalytic domain with the C-terminal autoinhibition domain [40, 43, 44]. This phosphorylation permits further phosphorylation by phosphoinoside-dependent kinase 1 (PDK1) at Thr229 in the activation loop of the kinase domain of S6K, resulting in its activation [45, 46]. Once activated, S6K phosphorylates ribosomal protein S6, a protein required for translation of 5' terminal oligopyrimidine (TOP) mRNAs encoding ribosomal proteins and elongation factors. Although mTORC1 is likely to have many other substrates, 4E-BP1 and S6K are the key factors contributing to the ability of mTORC1 to promote increased cell size [47].

In addition to S6 protein, S6K also phosphorylates several other proteins, including IRS-1, glycogen synthase kinase 3 (GSK3), translation elongation factor 2 (eEF2) kinase and pro-apoptotic protein Bad [48–52]. Of these targets, phosphorylation of IRS-1 is of great importance in mTOR regulation. IRS-1 binds to insulin receptor or

insulin-like growth factor-1 (IGF-1) receptor and is required for activation of PI3-K in response to insulin or IGF-1 stimulation [53]. S6K phosphorylates IRS-1 at Ser302, which disrupts its interaction with the receptors, leading to a blockage in insulin signaling to Akt and mTOR [50, 54]. The S6K directed phosphorylation of IRS-1 thus constitutes a negative feedback that downregulates the signaling activity from insulin receptor to mTOR (Fig. 1).

mTORC2

mTOR complex 2 shares mTOR and mLST8 with mTORC1. However, it contains several unique components, including rapamycin-insensitive companion of mTOR (riCTOR), mammalian stress-activated protein kinase-interacting protein 1 (mSIN1) and proline-rich repeat protein-5 (PRR5) or PRR5-like (also known as protor1 and protor 2) (Fig. 3) [55–60]. The association of rictor to mTOR is critical for mTORC2 kinase activity and requires the presence of mLST8 and mSIN1, which makes the two proteins indispensable for mTORC2 function [27, 55, 60, 61]. PRR5 and its close homolog PRR5L are two newly discovered mTORC2 components. These proteins bind to rictor or mSIN1, but are not essential for mTORC2 stability or kinase activity [56, 58]. In addition to the components of mTORC2, rictor has also been shown to interact with other proteins, such as integrin-linked kinase (ILK), Myo1c and heat shock protein 70 (Hsp70) [62–64]. The significance of these interactions is unclear. It is possible that rictor, through interaction with these proteins, acts as an adaptor to bring mTORC2 to its targets. Alternatively, these proteins may function as regulators to control mTORC2 through rictor.

The FKBP12–rapamycin complex does not bind to mTORC2. Hence, mTORC2 is insensitive to acute rapamycin treatment [8, 57]. However, prolonged treatment with rapamycin has been found to disrupt mTORC2 assembly in some cell lines, resulting in decreased levels of functional mTORC2 [10, 65]. The effect of rapamycin may be mediated by the drug-induced dephosphorylation of rictor and mSIN1 [66, 67]. It has been found that short-term treatment with rapamycin triggers dephosphorylation of rictor and mSIN1 exclusively in the cytoplasm, but does not affect mTORC2 assembly. Prolonged rapamycin treatment leads to complete dephosphorylation and cytoplasmic translocation of nuclear rictor and mSIN1, which correlates with reduction in mTORC2 levels [66, 67].

Two major functions have been ascribed to mTORC2, including regulation of Akt and cell cycle-dependent organization of actin cytoskeleton. mTORC2 phosphorylates Akt at Ser473 in its C-terminal hydrophobic motif,

which, in conjunction with PDK1-mediated phosphorylation at Thr308, drives full activation of Akt [61]. mTORC2 regulates actin cytoskeleton through a mechanism that involves the small GTPases Rho and Rac, although the molecular details of the regulation remain elusive [9, 57]. In addition, mTORC2 has been found to phosphorylate PKC and serum- and glucocorticoid-induced protein kinase 1 (SGK1), and may be involved in controlling cell size [68–71]. Thus, mTORC2, like its partner mTORC1, has pleiotropic roles in cell growth control.

TSC1 and TSC2

TSC1 and *TSC2* were originally identified as two tumor suppressor genes. Inactivating mutations of the genes cause tuberous sclerosis complex syndrome, an inherited genetic disorder that is manifested by occurrence of tumor in multiple organs [72, 73]. Early studies suggested that the gene products, TSC1 and TSC2, also known as hamartin and tuberin, form a heterodimeric complex that displays a GTPase-activating protein (GAP) activity [74–76]. However, the function of the TSC1/TSC2 complex remained unclear until genetic studies in *Drosophila* ascribed a role for the complex in cell size control and placed it in a pathway that converges the insulin signaling from Akt to mTOR and S6K [77–79]. Subsequent studies in *Drosophila* identified small GTPase Rheb as a downstream target of TSC1 and TSC2 and upstream of TOR [80, 81]. Biochemical analyses confirmed Rheb as a bona fide effector of the TSC1/TSC2 complex [82–84]. In mammalian cells, coexpression of TSC1 and TSC2 reduces the levels of GTP-bound Rheb, while insulin stimulation increases the levels in a PI-3K-dependent manner [85]. These seminal studies in *Drosophila* and mammalian cells established a signaling scheme underlying insulin-stimulated mTOR activation that involves, in a sequential order, PI3-K, Akt, the TSC1/TSC2 complex, Rheb and mTOR (Fig. 1).

TSC2 is a 180-kDa protein that contains a GAP homology domain at its C-terminus. When assayed in vitro, TSC2 stimulates GTP hydrolysis of Rheb, but not other related small GTPases [82, 86]. TSC1 does not possess any GAP activity and is not required for that of TSC2 in vitro. However, in cells TSC1 appears to be essential for TSC2 function, as mutations in TSC1 have been found to reduce the function of the TSC1/TSC2 complex [86, 87]. TSC1 contains a transmembrane (TM) domain and is involved in targeting the TSC1/TSC2 complex to membrane structures [74, 88]. The membrane association allows the complex to directly interact with Rheb and modulate its GTP hydrolysis activity [84].

TSC2 is phosphorylated by multiple kinases involved in various signaling pathways, including Akt, AMPK,

RSK and extracellular signal-regulated kinase (ERK). In response to growth factor, such as insulin stimulation, TSC2 is phosphorylated by Akt at multiple sites, including Ser939, Ser981 and Thr1462 [79, 89–91]. A mutant TSC2 lacking these phosphorylation sites acts dominantly in cells to block Akt signaling to mTOR, suggesting that the Akt-directed phosphorylation is to downregulate the function of TSC2. However, the phosphorylation does not appear to affect the GAP activity of TSC2 directly. Instead, it creates binding sites for a cytosolic protein, 14-3-3, which binds to phosphorylated TSC2 and inhibits its function [88]. The mechanism by which 14-3-3 downregulates TSC2 function is not fully understood. Some studies have suggested that binding with 14-3-3 prevents TSC2 from associating with TSC1 and thus reduces the function of the TSC1/TSC2 complex toward Rheb [79, 90], while others show that 14-3-3 may directly inhibit TSC2 function without disrupting the TSC1/TSC2 complex [89, 91].

In addition to being a target of Akt, TSC2 is also phosphorylated at Ser1345 by AMPK. Unlike the Akt-directed phosphorylation, which leads to inhibition of TSC2 function, the AMPK-directed phosphorylation enhances the GAP activity of TSC2 and consequently downregulates of mTORC1 [92]. As part of intracellular energy-sensing pathway, AMPK is activated when intracellular ATP is depleting and AMP levels arise [93]. The AMPK-dependent regulation of TSC2 thus couples intracellular energy levels to mTORC1 activity. The AMPK-dependent phosphorylation at Ser1345 also promotes subsequent phosphorylation at Ser 1337 and Ser1341 by glycogen synthase kinase 3 β (GSK3 β). These coordinated phosphorylation events lead to full activation of the GAP activity of TSC2 [92, 94]. Since GSK3 β is a component in the Wnt signaling pathway, the GSK3 β -directed phosphorylation links the pathway to mTORC1 regulation, whereby an activated Wnt signaling pathway, which inhibits GSK3 β activity [95], is able to enhance mTORC1 activity by repressing GSK3 β -dependent activation of TSC2 [92, 94].

TSC2 is also phosphorylated by ERK and RSK in response to mitogen stimulation and Ras activation. Activated ERK phosphorylates TSC2 at Ser664, which causes dissociation of TSC2 from TSC1 and impairs its function [96, 97]. RSK phosphorylates TSC2 at Ser1798, resulting in inhibition of TSC2 function. The mechanism for the inhibition is not clear. However, it has been found to be additive to the inhibitory effect of Akt-directed phosphorylation [98, 99].

The ability to receive phosphorylation by multiple kinases and alter its GAP activity accordingly provides a mechanism for TSC2 to integrate signals from various signaling pathways for mTOR regulation.

Rheb

Ras-homology enriched in brain was identified as a gene whose expression was induced in rat brain by synaptic activity and growth factor stimulation [100, 101]. Rheb is a small GTPase that is structurally more close to Ras than to other small GTPases. The regions in Rheb involved in GTP binding are highly similar to those in Ras. Particularly, the core effector domain of Rheb, the region that involves in targeting and binding to downstream effectors, shares six out of nine identical and two similar amino acid residues with that of Ras. It is thus believed that the action mechanism of Rheb is similar to that of Ras, which involves binding to its effector through the effector domain in a GTP-dependent manner [102, 103]. One striking difference, however, is that Rheb contains an arginine residue at position 15 rather than a glycine residue that is present at the corresponding site in Ras. This difference results in a low intrinsic GTPase activity in Rheb, which correlates with a relatively high level of GTP-bound Rheb in cells [102, 104, 105].

The activity of the small GTPases of Ras family depends on their nucleotide-binding states, which are regulated reciprocally by their cognate GAPs and guanine nucleotide exchange factors (GEFs) [102]. The GAP for Rheb has been shown to be the TSC1/TSC2 complex, which binds GTP-bound Rheb and stimulates GTP hydrolysis [82–84, 86]. However, the identity of the Rheb GEF remains elusive, although recent studies have suggested that the translationally controlled tumor protein (TCTP) may fill this role [106, 107].

Because the GAP activity of the TSC1/TSC2 complex is regulated by Akt- and AMPK-dependent phosphorylation of TSC2, the nucleotide-binding state of Rheb is expected to be controlled by these kinases, which couples, respectively, growth factor and energy signals to the TSC1/TSC2 complex. In support of this notion, insulin has been shown to regulate the nucleotide-binding states of Rheb through TSC2 [85, 108, 109]. However, whether Rheb is regulated by amino acids is controversial. Studies have shown that starving cells for amino acids reduces the levels of GTP-bound Rheb, while re-addition of amino acids increases the levels in the cells, suggesting that Rheb is regulated by amino acid conditions [109]. In contrast, two other studies have failed to detect any significant changes in the nucleotide-binding states of Rheb upon re-addition of amino acids to amino acid-starved cells [83, 110]. It is worth noting that in the latter cases, the GTP/GDP loading assays were done against overexpressed recombinant Rheb, while in the first cases, the assays were against endogenous Rheb or recombinant Rheb expressed at levels similar to those of the endogenous protein [108, 109]. When overexpressed in cells, Rheb stays largely in GTP-bound state, and its activity for mTOR activation becomes insensitive to amino

acids [104, 108, 111], which raises a possibility that the overproduced Rheb rendered it unresponsive to changes in amino acid conditions in these studies. Since there is no evidence suggesting that the function of the TSC1/TSC2 complex is affected by amino acid conditions. If amino acids regulate Rheb, they may do so through TSC-independent mechanisms. One possibility is that amino acids control Rheb through a putative GEF of Rheb. Importantly, while Rheb is largely GTP-bound in *tsc2* null cells, starving the cells for amino acids still results in mTORC1 inactivation, which suggests that amino acids are able to control mTORC1 activity independent of Rheb [108]. Although this finding does not answer whether Rheb is regulated by amino acids, it makes the issue less significant in mTOR regulation.

Overexpression of Rheb has been shown to activate mTORC1, and the activation is dominant over the effect of serum or amino acid withdrawal, suggesting that Rheb is a proximal activator of mTORC1 [80, 82]. Indeed, recombinant Rheb has been found to associate with mTOR, and this association is stimulated by amino acids [112]. However, the association does not appear to be GTP-dependent. It has been shown that Rheb mutants devoid of nucleotide-binding associate more strongly with mTOR than wild-type or active Rheb. Despite this, the kinase activity of mTOR has been found to be higher when it is copurified with recombinant wild-type Rheb than with inactive mutants, suggesting that the ability of Rheb to stimulate mTOR relies on its activity. Consistent with the notion, it has been found that GTP-loaded Rheb, when added to partially purified mTORC1 *in vitro*, increases its kinase activity [113, 114]. These findings strongly support that Rheb is able to bind to mTOR and stimulate its activity. However, evidence is lacking showing that Rheb directly binds with mTOR [112, 114, 115].

FKBP38

FKBP38, also known as FKBP8, belongs to the peptidyl prolyl *cis/trans* isomerase (PPIase) family of FK506-binding protein (FKBP) [116, 117]. Members of this protein family share a PPIase or PPIase-like domain, commonly referred to as the FKBP-C domain, and function as chaperones to facilitate protein folding and functions [118, 119]. The prominent member is FKBP12, which consists of entirely the FKBP-C domain and serves as the receptor for rapamycin [120, 121]. FKBP38 contains a N-terminally located FKBP-C domain, a tetratricopeptide repeat (TPR) motif sitting in the middle of the protein, a putative Ca^{2+} /calmodulin (CaM)-binding site lying immediately adjacent to a TM domain at the very C-terminus. The TPR motif is found in other members of the

FKBP family proteins and is believed to be involved in mediating protein-protein interaction. The Ca^{2+} /CaM-binding site is responsible for binding with CaM in a Ca^{2+} -dependent manner. The TM domain in FKBP38 is unique among all the FKBP proteins, which is required for anchoring FKBP38 to mitochondria, where it functions [122–125].

FKBP38 has been suggested to be the endogenous inhibitor of mTOR [126]. In cells, the expression levels of FKBP38 have been found to correlate inversely with the activity of mTORC1 [114, 127, 128]. *In vitro*, recombinant FKBP38 has been shown to inhibit mTORC1 kinase activity [126, 128]. FKBP38 inhibits mTORC1 by directly binding to mTOR. The FKBP-C domain of FKBP38, the region that is highly similar to FKBP12, is responsible for the binding. On the other hand, the minimal region in mTOR that is capable of binding with FKBP38 comprises amino acids 1967–2191, which embraces the FKBP12–rapamycin-binding (FRB) domain (amino acids 2015–2114). These attributes suggest that FKBP38 acts in the same way as the FKBP12–rapamycin complex to interfere with mTOR function. In accordance with this notion, it has been found that FKBP38, like the FKBP12–rapamycin complex, does not associate with mTORC2 [126].

The interaction of FKBP38 with mTOR is regulated by Rheb, which binds directly to FKBP38 and prevents it from interaction with mTOR [126]. Unlike its association with mTOR, the binding of Rheb with FKBP38 is GTP-dependent. It has been shown that Rheb binds to FKBP38 strongly when loaded with GTP, but weakly when loaded with GDP *in vitro* [126]. Rheb interacts with FKBP38 through its effector domain within the switch I region. Mutations in the effector domain impair the ability of Rheb to interact with FKBP38 and eliminate the ability of Rheb to stimulate mTORC1 [129]. This effector domain-mediated and GTP-dependent binding is a common mechanism for other members of the Ras family of small GTPases to interact with their targets [102], indicating that Rheb elicits its signaling activity as do by other members of the Ras family and that FKBP38 is a bona fide effector of Rheb [129].

Rheb binds to the FKBP-C domain in FKBP38, the same region that interacts with mTOR. However, the interaction of FKBP38 with Rheb and that with mTOR are not mutually exclusive. A dominant inactive mutant of Rheb, D60K, has been shown to bind to FKBP38 without displacing mTOR [126]. It is likely that Rheb controls FKBP38 allosterically in a GTP-dependent manner, resulting in the release of mTOR from its inhibition.

The binding of Rheb with FKBP38 in cells is regulated by growth factor and amino acid conditions. In cells starved for serum or amino acids, Rheb exhibits a weak binding with FKBP38, which is strongly stimulated upon

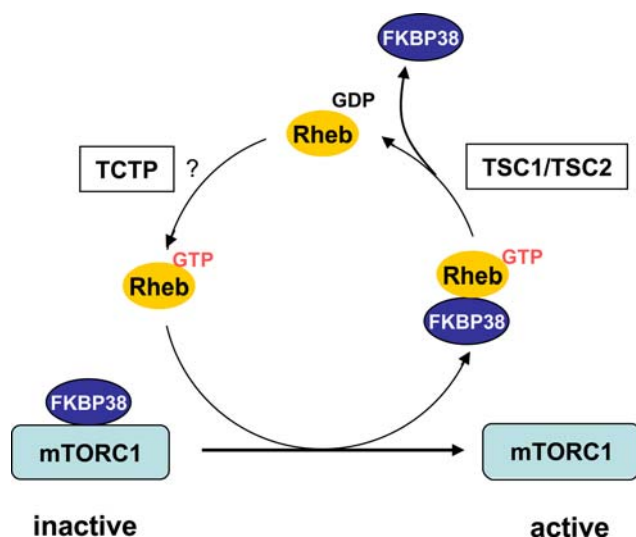


Fig. 4 A model for the role of FKBP38 in mTORC1 regulation. FKBP38 inhibits mTOR activity through direct binding to mTOR. In GTP-bound form, Rheb interacts with FKBP38 and liberates mTORC1 for activation. The action of Rheb is terminated by the TSC1/TSC2 complex, which promotes the hydrolysis of bounded GTP to GDP. Reloading of Rheb with GTP by a putative guanine exchange factor repeats the cycle

re-addition of the omitted ingredients. Accompanied with the increased binding of Rheb with FKBP38 is a reduction in the association of FKBP38 with mTOR. These observations suggest that growth factors and amino acids control the association of FKBP38 with mTOR through Rheb, presumably by modulating its nucleotide-binding states [126]. The same observations also support a signaling model in which Rheb activates mTORC1 by antagonizing the inhibitory effect of FKBP38 on mTOR (Fig. 4). Consistent with this scheme, it has been shown that addition of recombinant GTP-loaded Rheb blocks the inhibitory effect of FKBP38 on mTORC1 activity in vitro [128].

Although the model provides a logical explanation for the role of Rheb and FKBP38 in mTOR regulation, controversies exist. One major concern is that the inhibitory effect of FKBP38 overexpression on mTORC1 activity has been found to be modest [114, 127, 130], which has led to the suggestion that FKBP38 is not the sole mediator of Rheb in mTORC1 regulation and that Rheb may control mTORC1 through both FKBP38-dependent and independent mechanisms [131].

TCTP

Activity of the Ras family of small GTPases is normally regulated by their cognate GAPs and nucleotide exchange factors (GEFs) [102]. With the identification of the TSC1/TSC2 complex as the GAP for Rheb, the search for its GEF

has drawn extensive attention. Recently, TCTP, a highly conserved protein in eukaryotes, has been suggested to be the long-sought GEF for Rheb [106]. TCTP, also called histamine-releasing factor (HRF), is a key protein in the process of tumor reversion and well conserved through phylogeny as a pro-survival, growth-stimulating and anti-apoptotic factor [132]. Hsu et al. [106] showed that in *Drosophila*, dTCTP acts upstream of dS6K, and down-regulation of dTCTP reduced cell size, cell number and organ size. Importantly, they found that dTCTP displayed a guanine nucleotide exchange activity toward Rheb in cells and in vitro. Consistent with these findings, Dong et al. [107] demonstrated that human TCTP accelerated its GDP release in vitro and activated the mTORC1 pathway in cells. However, the identity of TCTP as Rheb GEF has been challenged by several other studies in mammalian cells showing that TCTP is unable to interact with Rheb in vitro and is largely ineffective in regulating S6K phosphorylation in cells [130, 133, 134]. The reason for these discrepancies is not clear. Further independent studies are needed to clarify the role of TCTP in controlling mTORC1 signaling.

PRAS40

PRAS40 was originally identified as a proline-rich Akt substrate that is capable of binding to 14-3-3 upon phosphorylation by Akt [135]. It was later recognized as a component of the mTORC1 complex by mass spectrometry analysis of proteins associated with mTOR [136]. PRAS40 has been shown to associate with raptor and inhibit mTORC1 kinase activity both in vitro and in cells [58, 113, 137, 138]. Furthermore, the association of PRAS40 with mTORC1 increases under conditions that inhibit mTOR signaling, such as nutrient or serum deprivation or mitochondrial metabolic inhibition [136]. These findings suggest that PRAS40 is an inhibitor of mTORC1. Intriguingly, while overexpression of PRAS40 inhibits mTORC1 activity, knocking down the expression of PRAS40 also reduces the insulin-stimulated S6K phosphorylation. These findings suggest that the role of PRAS40 in mTORC1 function is not simply inhibitory [136, 139].

PRAS40 contains a TOS motif that is involved in binding with raptor. This motif is commonly found in mTORC1 substrates, such as S6K and 4E-BP1, indicating that PRAS40 is a substrate of mTORC1. Hence, it has been suggested that PRAS40 inhibits mTORC1-directed phosphorylation of S6K and 4E-BP1 by competing with these proteins for raptor binding [137, 138]. PRAS40 is phosphorylated by mTORC1 at several sites, including Ser183, Ser212 and Ser221 [138–140]. Phosphorylation at Ser183

and Ser221 appears to reduce the association of PRAS40 with raptor and relieve its inhibition on mTORC1 [138, 139], which may constitute a positive feed-forward mechanism for mTORC1 activation, such that a suboptimal mTORC1 signaling activity potentiates its further activation by reducing PRAS40 association.

PRAS40 is also phosphorylated at Thr246 by Akt in response to insulin stimulation. The Akt-directed phosphorylation creates a docking site for 14-3-3, which binds to PRAS40 and interferes with its association with mTORC1 [113, 137]. This Akt-PRAS40 connection counts for the second mechanism, in addition to Akt-TSC2-Rheb signaling axis, whereby Akt activates mTORC1 and explains why in *Drosophila* Akt is able to maintain a normal development in the absence of TSC2 [141]. In addition, Thr246 has also been found to be phosphorylated by PIM1, a serine/threonine kinase that has diverse biological roles in cell survival, proliferation and differentiation [142]. Like the Akt-directed phosphorylation, the phosphorylation by PIM1 also reduces the association of PRAS40 with mTORC1 and increases the mTORC1-directed phosphorylation of 4E-BP1 and S6K [143].

IKK

Inhibitor of nuclear factor κ B (NF κ B) kinase (IKK) complex is a key component in the tumor necrosis factor α (TNF α)/NF- κ B signaling pathway that is involved in systemic inflammation [144]. It consists of three tightly associated subunits, IKK α , IKK β and IKK γ , of which IKK α and IKK β serve as the catalytic subunits and IKK γ is the regulatory subunit [145]. IKK is activated by proinflammatory cytokines, such as TNF α and lipopolysaccharide (LPS) [146]. In addition to activation of IKK, TNF α has been known to stimulate mTORC1 activity [147]. However, the underlying mechanism remained unclear until a recent finding that IKK β associates with TSC1 [148]. Lee et al. [148] have found that in response to TNF α treatment, IKK β interacts with TSC1 and phosphorylates it at Ser487 and Ser511. The phosphorylation represses the function of the TSC1/TSC2 complex, resulting in mTORC1 activation. In addition, the IKK-directed phosphorylation has been found to correlate with TNF α -induced VEGF production in multiple tumor types, indicating that TNF α promotes VEGF expression and angiogenesis through IKK-dependent mTORC1 activation.

Moreover, studies from Baldwin's [149, 150] group have revealed an association between IKK α and mTORC1 in PTEN-deficient cancer cells. The association is regulated by Akt and is required for Akt-stimulated mTORC1 activation. Overexpression of IKK α has been found to enhance

mTORC1 kinase activity in vitro, suggesting that IKK α is a positive regulator of mTORC1. It has thus been proposed that IKK α may activate mTORC1 by phosphorylating mTOR or components in mTORC1.

Phosphatidic acid and phospholipase D

Phosphatidic acid is a phospholipid metabolite that serves as a second messenger in mitogenic signaling pathways [151–153]. An elevated PA level in cells, either by serum stimulation or treatment with exogenous PA, results in an increase in mTORC1 activity. In contrast, inhibition of PA accumulation by treatment with 1-butanol represses mTORC1 activity [154]. The action of PA on mTORC1 is mediated through a direct binding of PA to mTOR to a region within the FKBP12-rapamycin binding (FRB) domain. Residue Arg2109 in this domain has been found to be critical for the binding. Mutations at this site reduce the interaction of PA with mTOR and repress mTORC1 signaling [154]. How binding of PA leads to mTORC1 activation is not fully understood. Direct activation of mTORC1 kinase activity has not been observed. However, two mechanisms have been suggested that may mediate the effect of PA on mTORC1. Sun et al. recently reported that PA was able to reduce the association of FKBP38 with mTOR both in vitro and in cells, which suggests that PA may activate mTORC1 by alleviating the inhibitory of FKBP38 [131, 155]. On the other hand, Toschi et al. [156] showed that suppression of PA production blocked the association of mTOR with raptor, suggesting that PA binding is required for the integrity of mTORC1. These two mechanisms are not necessarily mutually exclusive. Given the role of FKBP38 in mTORC1 regulation, it is possible that an enhanced binding of FKBP38 with mTOR upon PA depletion alters the association of mTOR with raptor.

Mitogen and growth factor stimulate PA production by activating phospholipase D (PLD), which catalyzes the hydrolysis of membrane phosphatidylcholine to produce PA and choline [152]. In mammalian cells, two isoforms of PLD, PLD1 and PLD2, have been identified. Consistent with their role in PA production, overexpression of either protein has been shown to activate mTORC1 activity and knockdown of their expression to reduce mitogen-stimulated mTORC1 activity [32, 157–160]. The activity of PLD1 in cells is regulated by protein kinase C and several small GTPases, including Arf, Cdc42, Rac1 and RhoA [161]. Cdc42 and RhoA have been shown to interact directly with PLD1 and stimulate its activity. Interestingly, while overexpression of Cdc42 increases mTORC1 activity, overexpression of RhoA does not [162]. The reason for this dilemma is not clear. However, we have recently

observed that RhoA is able to associate with mTORC1 and downregulates its kinase activity (Y. Lai and Y. Jiang, unpublished observation), suggesting that RhoA, in addition to stimulating PA production through PLD1 activation, may have a negative effect that directly acts on mTORC1. In addition to the Rho family of small GTPases, PLD1 has been found to be activated by Rheb. Sun et al. [160] have shown that Rheb binds to PLD1 in a GTP-dependent manner and stimulates its activity. Downregulation of Rheb, either by siRNA knockdown or TSC2 overexpression, reduces mitogen-stimulated PLD1 activation. Importantly, downregulation of PLD1 or inhibition of PA production blocks Rheb-stimulated mTORC1 activation, suggesting that the activation effect of Rheb on mTORC1 requires PLD1-dependent PA production [160]. In addition to PA production, PLD2 may also regulate mTORC1 through physical interaction. PLD2 has been found to interact with raptor, and the interaction appears to be important for PLD2-stimulated mTORC1 activation [158].

The Rag small GTPases

The Rag small GTPases are a group of unique small GTPases remotely related to Ras [163, 164]. Four members of this type of GTPase have been identified in mammalian cells, including RagA, RagB, RagC and RagD [164, 165]. RagA and RagB are closely related. RagC and RagD differ significantly from RagA and RagB, but share high similarity with each other. The Rag small GTPases function as heterodimers containing one subunit from either RagA or RagB and the other from either RagC or RagD [165–167]. Like other small GTPases, the activity of the Rag proteins is regulated by their nucleotide-binding states. However, a functional configuration of the dimer requires a GTP-bound RagA or RagB in complex with a GDP-bound RagC or RagD [168]. The activity of the complex is believed to be determined mainly by the GTP-bound subunit, but its stability requires the other subunit in GDP-bound state [169].

In mammalian cells, the Rag small GTPases have recently been found to associate with mTORC1 as heterodimers [168]. It has been found that GTP-bound RagA or RagB (RagA/B^{GTP}), when complex with GDP-bound RagC or RagD (RagC/D^{GDP}), associates with mTORC1, likely through a direct binding to raptor. The association is regulated by amino acid conditions; re-addition of amino acids to amino acid-starved cells stimulates the association. Since amino acids also stimulate the GTP-loading of RagA/B, it is likely that amino acids promote the association by controlling the GTP-loading of RagA/B. Importantly, in cells expressing the RagA/B^{GTP}–RagC/D^{GDP} heterodimer, the mTORC1 activity becomes insensitive to deprivation of

amino acids, but remains responsive to other conditions known to regulate mTORC1, including oxidative stress, mitochondrial inhibition, serum and energy deprivation [168]. This observation suggests that the Rag GTPases mediates mTORC1 activity in response exclusively to amino acid availability and that expression of RagA/B^{GTP} mimics amino acids sufficiency. However, despite the ability to activate mTORC1 in cells, the Rag heterodimers are ineffective in stimulating mTORC1 kinase activity *in vitro*, suggesting that the GTPases controls mTORC1 activity by means other than increasing its kinase activity [168].

In amino acid-starved cells, mTOR is distributed throughout cytoplasm on punctate membrane structures resembling endosomal compartments [168]. Upon restimulation with amino acids, mTOR, as well as raptor, accumulate to late endosomal or lysosomal compartments that are enriched with Rab7. This relocation appears to be dependent on the Rag small GTPases, since it does not occur in cells when expression of the Rag proteins is downregulated by siRNA [168]. Interestingly, it has been previously shown that the Rab7-enriched compartments are also the place where Rheb resides [170, 171]. This colocalization thus promotes a model which explains the role of amino acids and Rag proteins in mTORC1 regulation. In this model, amino acids stimulate the activity of the Rag GTPases, which in turn binds to mTORC1 and recruits it to late endosomal and lysosomal compartments, where it is activated by Rheb (Fig. 5). Accordingly, it can be envisaged that the role of growth factors in mTORC1 activation is to activate Rheb and that of amino acids is to present mTORC1 to an activated Rheb. This explains why mTORC1 cannot be activated in the absence of either amino acids or growth factors.

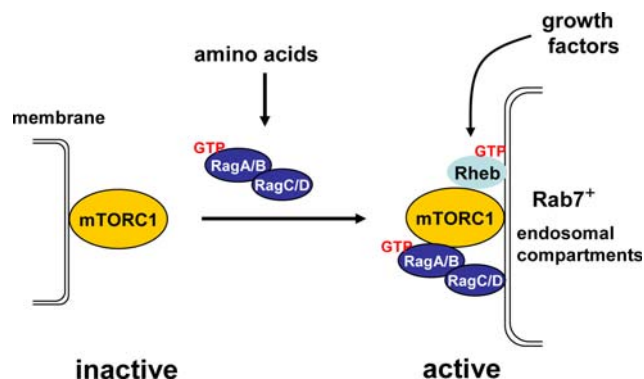


Fig. 5 A model for the role of the Rag GTPases in mTORC1 regulation. In cells starved for amino acids, mTORC1 resides on membrane structures distributed throughout the cytoplasm. Restimulation with amino acids activates the heterodimers of the Rag small GTPases, which causes mTORC1 redistribution to Rab7-enriched endosomal compartments, where it is activated by Rheb

hVps34 and calcium

Vps34 was identified in yeast as an unconventional (Class III) PI3-K that converts phosphatidylinositol to phosphatidylinositol 3-phosphate (PI3P), a bioactive messenger [172]. It is structurally highly similar to the catalytic subunit of classic (Class I) PI3-K [the 110-kilodalton (p110) subunit of PI 3-kinase] [173]. In yeast, Vps34 is involved in protein sorting to the yeast lysosome-like vacuole [174].

Nobukuni et al. [111] observed that wortmanin, a potent inhibitor of PI3-K, prevented amino acid-stimulated mTORC1 activation. However, downregulation of class I PI3-K by siRNA, while blocks insulin-induced mTORC1 activation, had no effect on amino acid-stimulated mTORC1 activation. This observation suggests that the effect of wortmanin on amino acid-induced mTORC1 activation is mediated by another PI3-K. In support of this notion, it was found that addition of amino acids to amino acid-starved cells induced a sharp increase in the intracellular levels of PI3P, which coincided with an increase in the kinase activity of human Vps34 (hVps34), supporting a role for hVps34 in amino acid-mediated mTORC1 activation [111]. Consistent with this notion, overexpression of hVps34 has been shown to activate mTORC1, while downregulation of hVps34, either by siRNA or antibodies specifically against hVps34, to reduce amino acid-induced mTORC1 activation. In addition, siRNA knockdown of hVps15, a protein kinase that activates hVps34, also inhibits amino acid-induced mTORC1 activation [111, 175]. These studies demonstrate that hVpd34 mediates the effect of amino acids for mTORC1 activation.

How does hVps34 activate mTORC1 in response to amino acid stimulation? Gulati et al. [176] showed that amino acid-stimulated mTORC1 activation was accompanied by a rapid rise in intracellular $[Ca^{2+}]$. Treating cells with the endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin (Tg), which increased $[Ca^{2+}]$ in cytosol, was able to activate mTORC1 in the absence of amino acids. Conversely, depletion of Ca^{2+} suppressed amino acid-induced mTORC1 activation. These results suggest that a rise in $[Ca^{2+}]$ is required and sufficient for mTORC1 activation. Interestingly, the Ca^{2+} -induced mTORC1 activation is attenuated when hVp34 expression levels are reduced by siRNA knockdown, suggesting that Ca^{2+} mediated mTORC1 activation requires hVps34 [176]. Since hVps34 contains a putative Ca^{2+} /CaM binding domain, it is possible that Ca^{2+} and CaM may bind to hVps34 and regulate its activity. Indeed, Gulati et al. [176] showed that CaM bound directly to hVps34 in the presence of Ca^{2+} , and the binding of CaM was required for the lipid kinase activity of hVps34. Collectively, these results support a model whereby amino acids stimulate mTORC1 activity through Ca^{2+} -dependent activation of hVps34,

which results in an increase in PI3P in cells. However, it remains to be determined how PI3P activates mTORC1. In this regard, it is interesting to note that hVps34 is colocalized with Rab7 on late endosomal compartments, where the Rag small GTPases bring mTORC1 for activation by Rheb [168, 177]. A potential connection among PI3P, Rheb and Rag GTPases is thus worth exploring.

Conclusion

Recent advances in mTOR study have uncovered a complex regulatory network that governs mTOR activity. As discussed above, mTOR is regulated at multiple levels, including direct phosphorylation, oligomerization and interactions with its regulators, which in turn are controlled by an array of signaling cascades that are responsive to changes in intracellular and environmental conditions. Such a complex regulation network ensures a fine tuning of mTOR activity in response to numerous conditions and fits the pivotal role of mTOR in cell growth control.

Despite these advances, one important level of regulation remains largely unexplored, that is, the spatial control. mTOR has been found to associate with various intracellular compartments, including endosomal membranes, the Golgi complex, mitochondria and shuttling between cytoplasm and nucleus [67, 178–182]. This wide distribution pattern of mTOR suggests that localization is likely to play a critical role in mTOR function. This notion is highlighted by the recent finding that the Rag small GTPases control mTORC1 by targeting it to late endosomal compartments for activation by Rheb [168]. Other issues pertaining to spatial control of mTOR include how mTOR function is affected by its localization and whether mTOR localized at various places is regulated differently. In this regard, the use of S6K and 4E-BP1 phosphorylation as a surrogate for mTORC1 activity carries a risk of overlooking regulatory mechanisms that control mTORC1 for functions other than translation. In future studies, incorporation of spatial control in mTOR regulation may be the key for answering the fundamental question in mTOR study, that is, how does mTOR integrate signals of different origins to control cell growth?

Acknowledgments The authors thank their colleagues for stimulating discussion and critical reading of this manuscript. This work was supported by NIH grants CA129821 and GM068832 to YJ.

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