HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2019 July 31.

Published in final edited form as:

Oncogene. 2019 May ; 38(21): 3989–4001. doi:10.1038/s41388-019-0713-x.

Control of mTOR Signaling by Ubiquitin

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Abstract

The evolutionarily conserved mTOR signaling pathway plays essential roles in cell growth, proliferation, metabolism and responses to cellular stresses. Hyperactivation of the mTOR signaling is observed in virtually all solid tumors and has been an attractive drug target. In addition to changes at genetic levels, aberrant activation of the mTOR signaling is also a result from dysregulated post-translational modifications on key pathway members, such as phosphorylation that has been extensively studied. Emerging evidence also support a critical role for ubiquitin-mediated modifications in dynamically regulating the mTOR signaling pathway, while a comprehensive review for relevant studies is missing. In this review, we will summarize all characterized ubiquitination events on major mTOR signaling components, their modifying E3 ubiquitin ligases, deubiquitinases and corresponding pathophysiological functions. We will also reveal methodologies that have been used to identify E3 ligases or DUBs to facilitate the search for yet-to-be discovered ubiquitin-mediated regulatory mechanisms in mTOR signaling. We hope that our review and perspectives provide rationales and strategies to target ubiquitination for inhibiting mTOR signaling to treat human diseases.

1. Overview of the mTOR signaling pathway.

The mTOR signaling pathway is an evolutionarily conserved kinase cascade playing essential roles in regulating key cellular functions, including but not limited to cell growth, proliferation, autophagy, metabolism and DNA damage. The TOR genes were firstly identified in yeast by the Hall group in 1991. Afterwards, mTOR was purified by both Schreiber and Snyder groups and its function on cell proliferation and protein synthesis was discovered by multiple groups including Vézina, Sonenberg, Blenis, Thomas, Crabtree, Gelfand and others. The first mTOR-deleted mouse model was established by Peterson and Sabatini groups, which confirms the essential role of mTOR in cell survival and development. Afterwards, the mTOR signaling cascade was characterized by both genetic

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and biochemical approaches (please refer to many great mTOR review articles from Hall, Sabatini and other groups for references) (Figure 1).

2. Overview of the ubiquitin signaling.

Ubiquitin is a small molecule composed of 76 amino acids. Ubiquitination is usually considered as a protein modification by attaching the ubiquitin moiety through one of the seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), or to the amino terminus (M1) in each ubiquitin to form poly-ubiquitin chains. Recently, beyond protein-related function, we found that K63-linked poly-ubiquitin chains also bind DNA to facilitate DNA damage response[1]. Nonetheless, protein ubiquitination is governed by three types of enzymes as a cascade of a three-step reaction, including the E1 ubiquitin activating enzyme, the E2 ubiquitin conjugating enzyme and the E3 ubiquitin ligase. In mammalian genome, there are ~2 E1 enzymes, ~30 E2 enzymes and ~1,000 E3 ligases. There are three major types of E3 ubiquitin ligases in mammals including the Cullin-Ring (really interesting protein) (CRL) family of E3 ligases, HECT-domain containing E3 ligases and RBR (ring-between-ring) E3 ligases. E3 ligases are substrate recognition subunits, which recognize specific protein motif sequences in substrates for binding. These protein motif sequences are defined as “degron”s. CRL is the largest family of E3 ligases and usually CRL E3s are structurally composed of Rbx1 (also named Roc1, binds E2 through its Ring domain), Skp1, Cullin (including one of Cullin 1, 2, 3, 4A, 4B, 5 and 7) and a E3 ligase. F-box proteins is a major type of CRL-E3 ligases bearing a F-box motif with ~50 amino acids (termed as F-box) as a protein binding motif mediating F-box binding to Skp1. There are 69 F-box proteins in mammals[2], thus forming 69 SCF (Skp1/Cullin1/F-box) E3 ligase complexes. Except several well-characterized SCFs including Fbw7, β-TRCP and Skp2, the function of the majority of F-box proteins are just began to be appreciated (please refer to[3–5] for review). In addition to protein degradation, recognition of degrons by E3 ligases can also promote non-degradable ubiquitination via other linkages than degradation oriented K11 and K48 linkages, such as K6 and K63 linkages.

Previously, phosphorylation and its associated physiological functions on key mTOR pathway members have been extensively studied. Recently, ubiquitin-mediated regulation of mTOR signaling emerges as an important approach to diversely regulate functions of mTOR by either degrading the protein targets to terminate the mTOR signaling, modulating protein cellular localization, or promoting protein association with its binding partners to alter its biological functions. Importantly, the ubiquitin system has been considered as a druggable target with various effective agents available. Thus, in this review, we will summarize major ubiquitination events identified to date on major mTOR pathway components, E3 ubiquitin ligases and deubiquitinases regulating these ubiquitination processes, and function of the ubiquitin modifications, hoping to provide the first comprehensive view of ubiquitin-mediated regulation of the mTOR signaling.

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3. Degradation-oriented and ubiquitin-mediated mTOR signaling regulations.

mTOR can be activated by degradation of mTOR inhibitory proteins or inactivated by degradation of mTOR activating partners, both of which are controlled by the protein ubiquitination process. In this section, we will summarize major degradation-oriented ubiquitination modifications on the mTOR signaling components to illustrate roles of these degradation-oriented protein stability control mechanisms in regulating mTOR activity and function in distinct disease and development settings (Figure 1 **and Table 1**).

IRS-1 (insulin receptor substrate 1) is essential for transducing extra-cellular signals such as insulin or insulin-like growth factors to PI3K and mTOR. Loss of or reduced IRS-1 expression results in insulin resistance, which is a major determinant of type 2 diabetes. In early 2000s, IRS-1 protein stability was observed to be controlled in a Ser phosphorylation-dependent manner[6]. Afterwards, inflammation-induced SOCS1 (suppressor of cytokine signaling 1) and SOCS3 expression recruits IRS-1 to the elongin BC ubiquitin ligase for IRS-1 ubiquitination and degradation[7]. Another independent proteomic study found that IRS-1 is also a Fbw8^{CUL7} substrate and its degradation by Fbw8^{CUL7} is controlled by IRS-1 phosphorylation[8]. Furthermore, Fbxo40 was observed to control IRS-1 protein turnover in skeleton muscle[9]. Thus, the IRS-1 protein stability is controlled by a set of E3 ubiquitin ligases under distinct physiological settings.

PI3K (phosphoinositide 3-kinase) is firstly identified by Lewis C. Cantley and collogues as a lipid kinase for PI(3,4,5)P_3 production. It is a protein kinase complex composed of p110 (α, β and γ) catalytic subunits and p85 (α and β) regulatory subunits. The catalytic subunit p110α is recognized by a plasma membrane attached E3 ligase NEDD4L (neural precursor cell expressed, developmentally down-regulated 4-like) for K29-linked ubiquitination and degradation[10], resulting in compromised mTOR activation. While the complex-free p85β subunit is targeted by Fbxl2 for degradation in a p85β-Tyr655 dephosphorylation-dependent manner[11]. Specifically, Fbxl2 mediates the degradation of PTPL1-dephosphorylated p85β to prevent its competition with active PI3K (composed of p110/p85 heterodimers) for phospho-Tyr docking at plasma membrane[11]. Similarly, Fbxl2 targets IP3R3 for ubiquitination and degradation to limit calcium flux from the ER to mitochondria, mitochondrial calcium overload and apoptosis-events that are counteracted by PTEN[12]. Via these mechanisms, Fbxl2 ensures sufficient activation of the PI3K signaling cascade and promotes cell survival. Intriguingly, viruses (such as hepatitis C) hijack these regulatory mechanisms to inhibit virus-induced apoptosis and establish chronic infection by binding and unmasking IP3R3 degron[13]. In another report, p85 is recruited to HSP70/CHIP-E3 ligase via p42 for ubiquitination and degradation[14]. Ubiquitin-mediated p85 degradation leads to dampened PI3K activation. On the other hand, ubiquitin-mediated activation mechanism for PI3K remains unclear.

As a lipid phosphatase, PTEN antagonizes PI3K function by converting PI(3,4,5)P_3 into PI(4,5)P_2. The E3 ligase WWP2 was identified to negatively govern PTEN protein stability[15]. Interestingly, XIAP1, a pro-apoptotic protein, displays an E3 ligase activity in ubiquitinating and degrading PTEN[16]. Moreover, the E3 ligases DCAF13^{CUL4B}[17] and

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MKRN1[18] were also observed to ubiquitinate and destruct PTEN proteins in osteosarcoma and cervical cancer cells, respectively. Thus, PTEN expression is tightly controlled by multiple E3 ligases to ensure its proper physiological function as a dual lipid and protein phosphatase. In addition to the 26S proteasome pathway, PTEN also undergoes lysosomal degradation, which is mediated by SYNJ2BP[19]. Whether PTEN stability, activity or cellular localization can be regulated by atypical linkage ubiquitination events remain to be determined. Notably, PI(3,4,5)P_3 can also be dephosphorylated into PI(3,4)P_2 by the lipid phosphatase SHIP. The stability of SHIP is governed by the oncogenic-fusion E3 ligase BCR-ABL[20,21] in hematopoietic cancer.

PDK1, the major Akt upstream kinase phosphorylating Akt-T308, has been observed to undergo mono-ubiquitination within its kinase domain[22]. However, the physiological function for mono-ubiquitination of PDK1 remains unclear, but it is not tightly associated with PDK1 kinase activity[22].

mTOR is the essential kinase component for both mTORC2 and mTORC1 complexes. The first mTOR protein stability control mechanism was discovered in 2008 and the tumor suppressive E3 ligase Fbw7 was observed to target mTOR for ubiquitination and degradation in a GSK3-phosphorylation dependent manner[23]. In colorectal cancer, Fbx8, a metastasis suppressive E3 ligase partially exerts its tumor suppressor function through recognizing and degrading mTOR[24].

Deptor is an endogenous mTOR inhibitor suppressing both mTORC1 and mTORC2 activation[25]. Thus, depletion of β-TRCP, a E3 ligase targeting Deptor for ubiquitination and degradation triggered by Deptor phosphorylation via mTOR, CKI, RSK or p38 (see Table 1 for references), led to accumulation of Deptor and subsequently reduced mTORC1 activation. In prostate cancer, the Ring type of E3 ligase SAG (sensitive to apoptosis gene, also named Rbx2) also targets Deptor for destruction, through which SAG activates the mTORC2/Akt signaling to promote prostate tumorigenesis[26].

Rictor is an integral component for the mTORC2 complex and indispensable for mTORC2 kinase activity. Fbw7-mediated Rictor ubiquitination relies on GSK3-mediated Rictor-T1695 phosphorylation[27], resulting in attenuated mTORC2 activity towards phosphorylating Akt, SGK and PKC.

Akt, SGK and PKC are three well-characterized mTORC2 kinase substrates. Akt, also known as PKB (protein kinase B), is the major downstream protein kinase mediating the oncogenic feature of mTORC2. Catalytic-active Akt being phosphorylated on both T308 and S473 is thought to be targeted by multiple E3 ligases for ubiquitination and degradation. For example, TTC3 (tetratricopeptide repeat domain 3) promotes K48-linked poly-ubiquitination and destruction of doubly phosphorylated nuclear Akt[28]. Interestingly, Akt-mediated phosphorylation of TTC3 at Ser378 is necessary to activate TTC3 E3 ligase[28], revealing a feedback mechanism for Akt to restrain its own activity. Another mitochondrial E3 ligase MULAN (mitochondrial ubiquitin ligase activator of NF-κB) targets Akt1 and Akt2, but not Akt3, for ubiquitination and degradation. Specifically, MULAN recognizes phospho-Akt to add K48-linked polyubiquitin chains on Lys284 in Akt kinase domain for Akt destruction[29].

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Interestingly, in head-and-neck cancer, NTP (non-thermal plasma, an ionized gas) induces MULAN expression by elevating cellular ROS levels to facilitate Akt degradation, through which NTP exerts is anti-cancer ability[30]. Moreover, RFP2 (ret finger protein 2), a RBCC (RING finger, B-box, and coiled-coil domain) family of E3 ligases frequently deleted in various tumor types, promotes cell apoptosis in part by degrading both Akt and Mdm2[31]. In addition, BRCA1 (breast cancer susceptibility gene 1) also negatively regulates Akt activation by serving as a E3 ligase that targets phospho-Akt for ubiquitination and degradation[32]. Given that BRCA1 is tightly connected with genome instability and familial breast tumorigenesis, whether DNA damage triggers BRCA1-medaited Akt degradation remains to be explored. Similarly, the U-box E3 ligase CHIP (C-terminal Hsp70 -interacting protein) targets phospho-Akt for degradation[33], through which CHIP exerts a tumor suppressor function. However, a recent report suggests something opposite-that overexpressed CHIP in prostate cancer leads to increased Akt activation[34] to facilitate prostate cancer growth[34]. This discrepancy warrants further investigations to determine whether CHIP differentially regulates Akt ubiquitination and activity in a cellular context-dependent manner. In addition to cancer, Akt stability control also plays a critical role in neurodegeneration diseases. To this end, CHIP degrades Akt in Alzheimer's disease, leading to reduced Tau degradation by CHIP[35]. Thus, CHIP and Akt co-regulate Tau protein stability. A CUL1-based F-box E3 ligase Slimb degrades Akt to facilitate ddaC dendrite pruning by suppressing the Akt signaling[36]. Moreover, ZNRF1 (zinc and ring finger 1) promotes Wallerian degeneration through activating the GSK3/CRMP2 signaling achieved by terminating Akt signaling by promoting Akt ubiquitination and degradation[37]. In addition, the TTC3/Akt/eNOS signaling (TTC3 targets Akt for degradation) also plays a critical role in Down Syndrome[28]. Taken together, most of identified E3 ligases largely target phospho-Akt (pT308 and pS473) for ubiquitination and degradation. In addition to canonical phosphorylation events on S473 and T308, we identified a novel Akt-tail phosphorylation event that may function independent of these doubly phosphorylated Akt to promote Akt activation[38]. Whether and how any E3 ubiquitin ligases recognize tail-phosphorylated Akt or non-modified Akt, which accounts for the majority of Akt species under physiological conditions remains elusive.

In addition to proteasome-mediated protein stability control, Akt has also been demonstrated to be degraded through the autophagy process triggered by AMPK activation upon treatment with curcumin, a yellow bioactive compound from plant turmeric[39]. Moreover, Wnt5a/Wnt11-mediated cell differentiation results in reduced Akt activation, as well as a decrease of total Akt proteins due to caspase-dependent Akt degradation[40]. Interestingly, caspase 3 cleavages Akt at Asp108 and Asp119, which inactivates Akt under detachment-induced cell apoptosis by death receptor signaling in epithelial cells[41]. In Alzheimer's disease, caspase3-meidated Akt cleavage leads to reduced phosphorylation and activation of an Akt substrate GSK3, where GSK3 induces Tau phosphorylation to promote disease development[42]. Taken together, these studies demonstrate that Akt activity/stability is negatively regulated by proteasome-mediated degradation, autophagy and caspase cleavage under distinct pathophysiological conditions.

In addition to Akt degradation, dephosphorylation of Akt also contributes to Akt inactivation, facilitated by phosphatases PP2A (protein phosphatase 2A) and PHLPP1/2 (PH

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Domain And Leucine Rich Repeat Protein Phosphatase 2), on Thr308 and Ser473, respectively. PHLPP1 is degraded by β-TRCP[43] in a GSK3 and CKI-phosphorylation-dependent manner or by SAG[26], leading to increased Akt activity. The PP2A heterotrimeric phosphatase is composed of a structural A, a catalytic C and a regulatory B subunit. $DCAF1^{CUL4}$ targets PP2A-A for destruction to control oocyte meiotic maturation[44]. $KLHL15^{CUL3}$ recognizes and degrades PP2A-B, thus promoting its exchange with other PP2A regulatory subunits[45]. The E3 ligase NOSIP promotes PP2A-C degradation to regulate craniofacial development[46], and the inability of the E3 ligase MID1 to degrade PP2A-C contributes to Opitz Syndrome[47]. Notably, MID1-mediated PP2A-C destruction depends on the Bbox1 domain in MID1 and an MID1 binding protein Alpha4.

There are three isoforms of SGK (serum and glucocorticoid-induced protein kinase) in mammals, namely SGK1, SGK2 and SGK3. SGK1 regulates ion channels, membrane trafficking and cell growth to play critical roles in hypertension, diabetic neuropathy, trauma and neurodegenerative diseases[48]. Basal SGK1 protein levels are low in mammalian cells cultured *in vitro* due to active proteasomal degradation. The E3 ligase NEDD4–2 ubiquitinates and degrades SGK1, while SGK1-mediated NEDD4–2 phosphorylation antagonizes this process[49]. In addition, CHIP co-localizes with SGK1 at ER (endoplasmic reticulum) to promote SGK1 protein turnover under stress conditions[50]. Interestingly, Rictor, an essential mTORC2 component governing SGK phosphorylation and activation, exerts an E3 ligase function by complexing with CUL1 to promote SGK1 degradation[51]. Thus, on one hand, Rictor facilitates SGK1 activation by facilitating SGK1 phosphorylation in a mTORC2 kinase-dependent manner, while on the other hand, Rictor destabilizes SGK1 proteins in a E3 ligase-dependent manner.

PKC is a large family of protein kinases with 15 members. Specifically, isoforms α, β_I, β_{II} and γ belong to classical PKCs; δ, ε, η and θ are defined as novel PKCs and Mζ and ι / λ are atypical PKCs. PKCs usually phosphorylate potent activators for transcription and promote tumorigenesis. Interestingly, PMA and insulin induce ubiquitination and degradation of PKCδ, but not PKCα nor PKCε in a PKCδ-Y311 phosphorylation-dependent manner[52] in muscle. Under nomoxia conditions, atypical PKCζII is recognized and degraded by the tumor suppressive E3 ligase pVHL through its N-terminal PB1 domain[53]. While under hypoxia, PKCζ is rapidly ubiquitinated and degraded by HOIL-1L (heme-oxidized IRP2 ubiquitin ligase 1L) through K48-linked polyubiquitination (see Table 1 for references).

Akt-mediated TSC2 phosphorylation is the critical signaling node for connecting mTORC2 to mTORC1. The TSC complex is composed of TSC2, TSC1 and TBC1D7 and considered tumor suppressive given to its function in suppressing the Rheb/mTORC1 signaling. Loss of *TSC2* or *TSC1* is observed as the cause of TSC (tuberous sclerosis complex) disease with formation of benign tumors in kidney, lung, heart and skin. Thus, understanding regulatory mechanisms governing TSC complex protein stability control may provide new insights into TSC treatments. TSC2 protein stability is controlled by multiple E3 ligases, including $Fbw5^{CUL4/DDB1}$, Pam[54], E6AP (independent of HPV E6) and HERC1 (see Table 1 for references). Interestingly, TSC1 binding to TSC2 protects TSC2 from being recognized and degraded by Fbw5, Pam and HERC1. RB1CC1 (RB1-inducible coiled-coil 1) binds TSC1

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and induces TSC1 ubiquitination and degradation with help from unknown E3 ligase(s)[55]. In hepatocellular carcinoma, TRIM31 exerts an oncogenic function by targeting both TSC1 and TSC2 for degradation[56]. The identities of E3 ubiquitin ligases governing TBC1D7 protein turnovers remain completely unknown.

mTORC1 is activated by GTPases including Rheb and Rags upon growth factor and amino acid stimulation, respectively. The first evidence indicating that Rheb protein stability is regulated by proteasomes comes from the observation that hydrogen peroxide reduces Rheb protein abundance in RAW264.7 cells[57]. To date, only one report suggests that nitrosylated GAPDH complexes with the E3 ligase Siah1 to target Rheb for K48-linked ubiquitination and degradation[58]. More detailed investigations are necessary to provide more insights into whether growth signaling plays a role in this Rheb degradation process and how Rag protein stability is controlled.

The Ragulator complex (composed of LAMTOR3 (MAPKSP1 or MP1), LAMTOR2 (ROBLD3 or p14), LAMTOR1 (p18), LAMTOR4 (c11orf59) and LAMTOR5 (HBXIP)) recruits Rag GTPases to lysosome for mTORC1 activation in response to amino acids. Thus, proteasomal degradation of Ragulator negatively regulates mTORC1 activity, retards cell growth but promotes autophagy. Notably, similar to the TSC2/TSC1 complex, loss of either of the Ragulator components destabilizes other subunits in a proteasome-dependent manner[59]. The exact identities for E3 ligases governing Ragulator protein stability are elusive.

FLCN, together with its binding partner FNIP1/2, serve as a GAP for RagC and RagD[60] to suppresses mTORC1 activation. The E3 ligase β-TRCP targets FNIP1/2 for ubiquitination and degradation in a CKI phosphorylation-dependent manner, leading to elevated mTORC1 activity and increased renal cancer growth[61]. GATOR1 (composed of DEPDC5, NPRL2 and NPRL3) exerts a GAP activity towards RagA and RagB to suppress mTORC1 activation; while GATOR2 (consists Mios, WDR24, WDR59, Seh1L and sec13) promotes mTORC1 activation through negatively regulating DEPDC5 in GATOR1[62]. Upon amino acid addition, release of GATOR1-mediated suppression is necessary to facilitate Rag/mTORC1 activation, which is achieved by KLHL22-mediated DEPDC5 degradation[CUL363]. KICSTORs (GATSL3/GATSL1/GATSL2) recruit GATOR1 to lysosome to suppress mTORC1 activation. mTORC1 senses distinct amino acids through various amino acid sensors, such that CASTORs are arginine sensors for mTORC1 activation and Sestrin-2 is a leucine sensor. In addition to amino acids, SAMTOR is the S-adenosylmethionine sensor for mTORC1 activation, while lysosomal cholesterol is sensed by the SLC38A9/NPC1 complex that promotes mTORC1 activation. Given that these mTORC1 regulators were identified recently, the protein stability control mechanisms for these proteins remain to be further determined.

mTORC1 promotes protein synthesis by phosphorylating S6K and 4EBP1, inhibits autophagy through phosphorylating ULK1, facilitates nucleotide metabolism by phosphorylating CAD and regulates SREBP1 expression and lipid metabolism through phosphorylating Lipin1. mTORC1 and CKI-mediated phosphorylation of Lipin1 primes Lipin1 for recognition, ubiquitination and degradation by β-TRCP[64]. SREBP1, the target of

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Lipin 1, is recognized and degraded by Fbw7 upon phosphorylation by GSK3, or by RNF20 upon phosphorylation by PKA (see Table 1 for references). How the protein stability of other major mTORC1 substrates in addition to Lipin1 is controlled and whether deregulation of these ubiquitin signaling contributes to human diseases warrants further in-depth investigations.

4. Non-degradation-oriented ubiquitin-mediated mTOR signaling regulations

In addition to K48-linked poly-ubiquitination on mTOR signaling components that promotes 26S-proteasome-mediated protein degradation, K63-linked poly-ubiquitination has also been observed on mTOR components to dynamically modulate their functions in a non-degradation oriented manner. In this section, we will summarize these identified protein ubiquitination events, their modifying E3 ligases and biological consequences (Figure 1 **and Table 1**).

The E3 ligase Cbl-b negatively regulates p85 in a degradation-independent manner, where Cbl-b binding to p85 inhibits the recruitment of p85 to CD28 and TCRζ in T cells (see Table 1 for references). Moreover, TRAF6 promotes K63-linked poly-ubiquitination of p85α to enhance p85α binding with TβRI (TGF-β type I receptor), which subsequently activates PI3K signaling[65]. Interestingly, atypical-linkage ubiquitination of PTEN by RFP (Ret finger protein) inhibits PTEN phosphatase activity[19] but the underlying molecular mechanism(s) remains to be determined.

The E3 ligase TRAF2 (TNF Receptor Associated Factor 2) governs K63-linked GβL poly-ubiquitination at Lys305 and Lys313 under serum-starvation conditions, which promotes Raptor binding to favor mTORC1 formation, while repelling Sin1 binding to dissociate mTORC2[66]. Therefore, K63-linked polyubiquitination of GβL serves as a major mechanism to maintain mTOR complex homeostasis. However, the upstream signaling governing TRAF2 activity warrants further investigations. On the other hand, TRAF6, with help from p62, poly-ubiquitinates mTOR through a K63-linkage to facilitate mTORC1 activation by promoting mTOR recruitment to lysosome in response to amino acid stimulation[67]. In addition, K63-linked ubiquitination of Raptor by DDB1/CUL4 is also necessary for mTORC1 activation at lysosome[68], and this process can be antagonized by the ubiquitin hydrolase UCHL-1-mediated Raptor deubiquitination[69]. Under mitochondrial stress, the E3 ligase PARKIN interacts with and ubiquitinates mTOR at Lys2066 and Lys2306 through unknown ubiquitin linkage(s) to maintain mTORC1 activity[70]. Notably, the detailed molecular mechanisms underlying poly-ubiquitination-mediated mTOR activation remain to be determined.

In addition to mTOR, TRAF6 also promotes K63-linked poly-ubiquitination of Akt at Lys8 and Lys14 in the Akt-PH domain to facilitate its plasma membrane attachment and activation by IGF-1[71]. In addition, NEDD4 induces K63-linked poly-ubiquitination of phosphor-Akt, which facilitates nuclear retention of active Akt[72]. Interestingly, Skp2, but not TRAF6, mediates Akt poly-ubiquitination and membrane recruitment in response to activation of HER2 receptors by EGF in breast cancer to promote glycolysis and resistance

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to HER2 inhibition[73]. While in lung cancer, TRAF4, but not Skp2, promotes Akt poly-ubiquitination and activation in response to EGF[74]. In glioma, the Fbxl18 E3 ligase is significantly amplified to promote glioma growth through catalyzing K63-linked Akt ubiquitination to facilitate Akt activation[75]. It remains to be determined how distinct upstream stimuli, such as IGF-1 and EGF, triggers differential E3 ligases binding to Akt to induce Akt poly-ubiquitination, as well as why Akt poly-ubiquitination is governed by different E3 ligases responding to the same physiological cue in different cancer settings.

Moreover, the lysosome-anchored E3 ligase RNF152 promotes RagA poly-ubiquitination through a K63-linkage upon amino acid stimulation to promote mTORC1 activation[76] by engaging RagA to lysosome. Subsequently, the E3 ligase Skp2-mediated K63-linked ubiquitination of RagA at Lys15 recruits GATOR1 to turn off mTORC1[77]. In addition, TRAF6-mediated K63-linked poly-ubiquitination of Rac1 at Lys16 promotes Rac1 activation and subsequent activation of both mTORC2 and mTORC1[78], while the detailed molecular mechanism(s) governing Rac1-mediated mTOR activity control remains unknown.

5. Deubiquitination in mTOR signaling.

Given that protein ubiquitination is a reversible reaction that the ubiquitin moiety can be removed by deubiquitinases (DUBs, a type of proteases), many deubiquitinases have been identified to antagonize the identified ubiquitin signaling mentioned above (**Table 2**). For example, USP7 stabilizes IRS-1 by removing K48-linked ubiquitin chains, and IGF-1 stimulation dissociates USP7 from binding to ubiquitinated IRS-1, thus leading to enhanced IRS-1 degradation[79]. PTEN can be stabilized by DUBs including USP10 in lung cancer and hepatocellular carcinoma, USP13 and OTUD3 in breast cancer, and Ataxin-3 in lung cancer through removing K48-linked ubiquitin moieties (see Table 2 for references). In addition to stability control, USP7 (also called HAUSP, herpesvirus-associated ubiquitin-specific protease) deubiquitinates PTEN to promote PTEN nuclear export, and this process can be antagonized by binding and sequesting USP7 from PTEN by PTEN phosphorylation at Ser380 by S6K[80]. Deptor is stabilized by OTUB1[81] by removing K48-linked ubiquitin chains. Although no E3 ligase(s) has been identified to promote Raptor destruction, Raptor is stabilized by USP9X in mouse embryonic brains[82] to facilitate mTORC1 activation. PHLPP1, an Akt phosphatase, is stabilized by various DUBs including USP46 and WRD48/USP12 in colon cancer, USP1 in lung cancer, and Uaf1/WDR20/USP12 in prostate cancer (see Table 2 for references). Similarly, the kinase ULK1 is stabilized by USP20, which is necessary to initiate autophagy[83].

Mono-ubiquitination of PDK1 is removed by USP4[22], which occurs at the plasma membrane where PDK1 is activated. CYLD (cylindromatosis), originally identified as a DUB inactivating the NF-κB signaling, removes K63-linked poly-ubiquitin chains from Akt to retard Akt membrane recruitment in prostate cancer[84]. In addition, OTUD7B is the deubiquitinase antagonizing TRAF2-goverend K63-linked GβL ubiquitination to promote GβL incorporation into mTORC2 complexes[66]. UCHL-1, a protease-like deubiquitinase, promotes the removal of K63-linked poly-ubiquitin chains on Raptor and GβL to modulate mTOR complex formation[69]. Moreover, K63-linked mTOR poly-ubiquitination is removed

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by USP9X[85]. Notably, only a handful of DUBs have been well-characterized and assigned with certain physiological functions. Thus, the physiological function and regulations on many orphan DUBs functioning in mTOR signaling need to be further determined by both mouse genetic studies and biochemical analyses. More importantly, unlike E3 ubiquitin ligases (which are not REAL enzymes but rather help E2 to transfer ubiquitin), DUBs exert enzymatic activities. Thus, DUB inhibitors might be a new line of therapeutic choices for human diseases.

6. Evolution of E3 ubiquitin ligases, DUBs and mTOR signaling components.

Most of the mTOR signaling components are highly conserved from yeast to mammals, with only Rheb and TSC2/TSC1 missing in certain metazoan and fungi (refer to[86] for review). Phosphorylation events that play important roles in regulating mTOR signaling, is also quite conserved through evolution, given that a co-evolution pattern of both mTOR components and their modifying kinases is observed[86]. On the other hand, compared with conserved phosphorylation events, most E3 ligases and deubiquitinases identified are only conserved in mouse and human, but not in lower eukaryotes such as yeast and drosophila (Figure 2). These observations suggest that ubiquitin-mediated modifications on mTOR signaling components may be a late evolutionary event and ubiquitin modifications may provide new layers of regulations in higher eukaryotes in order to adapt to more sophisticated cellular environments, and may also be specifically associated with human diseases. However, more thorough studies are warranted towards these directions.

7. Approaches used to identify E3 ligase/substrate and DUB/substrate pairs for mTOR signaling components.

In the following section, we will summarize the major experimental approaches used in previous studies to identify ubiquitin-mediated mTOR regulations. We hope these methods will inspire the characterization of unknown ubiquitin modifying enzymes for mTOR signaling components (**Table 1**), as well as more intensive studies for ubiquitin modifications in other major signal transduction pathways.

(1) Proteomics-mediated protein interactome approach.

In this method, either a substrate or an E3 ligase is used as the bait to pulldown its interacting proteins for further mass spectrometry analyses. This method is based on the principle that E3 ligases or DUBs form relatively stable interactions with substrates. For example, to understand the function of the orphan F-box protein Fbxl2, Fbxl2 is expressed in HeLa or HEK293T cells and immunoprecipitated for MudPIT analyses[11]. By this approach, p110-free p85 is identified as a *bona fide* Fbxl2 substrate, through which Fbxl2 negatively controls the PI3K signaling. On the other hand, TAP tagged-mTOR (as a substrate) is used as a bait in HEK293T cells and mTOR interactome was established by TAP-tag pulldown coupled mass spectrometry analyses, where USP9X was identified as a novel mTOR binding partner and a DUB to deubiquitinate mTOR for suppressing mTOR[85]. Similarly, triply-tagged PTEN (by S-protein, Flag and streptavidin-binding peptide, SBP) is used as the bait

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in a tandem purification using lysates from HEK293T cells followed by mass spectrometry analyses, and WWP2 is identified as a PTEN binding protein that targets PTEN for ubiquitination and degradation[15]. To further stabilize the interactions between E3/DUB and substrate, MG132, a 26S-proteasome inhibitor, is commonly used before cell collection to preserve more interactions to increase the detection sensitivity.

(2) Loss-of-function genetic screens.

Another large-scale and non-biased screening approach is to utilize the loss-of-function screens facilitated by siRNAs, shRNAs or CRISPR. To this end, a siRNA screen against a DUB library identifies DUBs to remove PDK1 mono-ub[22]; a loss of function screen using siRNAs targeting 99 DUBs finds candidates regulating ULK1 stability[83], and a siRNA library against 92 DUBs is employed to identify PTEN DUBs[87]. Notably, this approach is more appropriate for substrates with relatively higher levels of endogenous ubiquitination. Due to off-target effects from knockout/knockdown constructs, as well as influences from phenotypes derived from loss of essential genes, necessary controls and cautions need to be taken into consideration for this approach.

(3) Yeast-two-hybrid (Y2H) assays.

This approach is to use yeasts to perform interaction studies. For example, using a Y2H assay with a full-length Akt2 as a bait, TTC3 is found as a direct Akt binding partner that promotes Akt ubiquitination and degradation[28]. In addition, a direct interaction between PTEN and its E3 ligase DCAF13 is also confirmed by Y2H[17]. The same approach has also been used to identify Fbw5 as an E3 ligase controlling TSC2 protein stability[88]. Given to the presence of homologous genes in yeast, Y2H studies may generate false positive results. In addition, due to the possibility that certain post-translational modifying enzymes are not conserved in yeast, if the targeted protein-protein interactions depend on certain post-translational modifications, the Y2H assay may not faithful recapitulate the physiological conditions in mammals.

(4) Bioinformatic approaches to search for E3 ligase degrons.

With the identification of more substrates for a given E3 ligase and availability of crystal structures for E3/substrate complexes, a handful of well-defined protein motifs in substrates for specific E3 ligases have been deciphered. For example, Fbw7 prefers a consensus CDC phospho-degron (CPD) sequence I/L-I/L/P-T-P-XXXX for recognition, where a priming phosphorylation at the TP site is critical to trigger CPD binding. The E3 ligase Keap1 recognizes "ETGE" and "DLG" degrons, β-TRCP prefers a "DSGxxS" degron, and Cop1 targets a "[D/E][D/E]xxVP[D/E]" degron for binding (see[89] for review). This approach has led to the successful identification of quite some substrates for E3 ligases. For example, through a genome-wide search for Fbw7 degron-containing proteins, mTOR is identified as a *bona fide* Fbw7 substrate through which Fbw7 negatively regulates mTOR signaling[23]. In addition to searching for known degrons, a bioinformatic search using Biocarta and KEGG databases from an RNA microarray analyses links Fbx8 to mTOR, and biochemical assays demonstrates that Fbx8 is an E3 ligase facilitating mTOR degradation[24]. Notably, the presence of a degron sequence is not necessarily indicate the candidate as a substrate, as the

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structure constrains around the degron, and whether degron recognition requires priming modifications also need to be taken into consideration.

(5) In-cell ubiquitination screen.

Ubiquitination assays are the most direct evidence to demonstrate whether a candidate protein exerts any E3 ligase or DUB function. For example, to identify E3 ligase(s) responsible for K63-linked Akt ubiquitination, a panel of selected E3 ubiquitin ligases are expressed in HEK293T cells in the presence of Akt1 and His-tagged ubiquitin. Using in-cell ubiquitination assays, the authors narrowed down a handful of candidates as E3(s), among which TRAF6 is further characterized as the major E3 ligase ubiquitinating and activating Akt[71]. Similarly, in-cell deubiquititination assays are used to identify DUBs removing K63-linked ubiquitin chains on Akt, and CYLD is found to be a major hit[90]. The caveats for in-cell ubiquitination assays at least include that this assay may not be able to distinguish direct or indirect effects of a given E3/DUB on ubiquitination status of a given substrate.

(6) Targeted binding screen.

Compared with proteomics approach, a selected list of E3 or DUB candidates are tested for their ability to bind a substrate. For example, using a bioinformatic motif search approach, GβL is demonstrated as a TRAF2 substrate[66]. In a search for DUBs antagonizing this process, all OUT family of DUBs are co-expressed with GβL and OTUD7B is identified as a major GβL binding partner and the DUB deubiquitinating GβL[66]. Similarly, the same panel of DUBs are ectopically expressed in HCT116 cells to examine their effects on endogenous PTEN abundance and OTUD3 is found as a major DUB negatively regulating PTEN protein stability[91]. In addition, through co-expression of 30 DUB ORFs and PTEN in HEK293T cells, USP13 is identified as a DUB stabilizing PTEN through deubiquitinating PTEN[92]. Again, this is a correlative approach that does not address a causal relationship between E3s/DUBs and substrates, thus further biochemical analyses are needed for in-depth characterization.

(7) Biochemical Arrays.

The E3/substrate pair can also be established by biochemical arrays. For example, to understand the oncogenic property of MKNR1, lysates from MKNR1-depleted HeLa cells were analyzed by a phospho-kinase array, where MKNR1 is found to target phospho-Akt for ubiquitination and degradation[18]. This is a more targeted approach and usually requires some hints for potential hits.

(8) Biochemical enzyme purification and LC-MS/MS identification.

This is a mixed approach to partially purify the experimental materials to facilitate mass spectrometry detection. For examine, to identify E3 ligases for p110, MEFs were fractionated by a S100 ion exchange column and each fraction was used in *in vitro* p110 ubiquitination assays to determine the major fractions containing the E3 ligase activity. After further purification steps the resulting proteins were analyzed by mass spectrometry for protein identifications. As a result, NEDD4L was identified as the major E3 ligase in the active fractions promoting p110 ubiquitination and degradation[10].

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(9) Hypothesis-driven research.

In some cases, previous literature may suggest some connections between a E3/substrate pair. For example, in a study to elucidate how mitochondrial status affects mTORC1 activity, the authors found that mTORC1 protects cells from death under mitochondria stress. Given that Parkin has a similar function with mTORC1 in this regard, the authors hypothesized and investigated whether Parkin is involved in mTORC1 regulation. As a result, Parkin indeed interacts with and ubiquitinates mTOR to maintain mTORC1 activity under mitochondria stress conditions[70].

In addition to the above mentioned methods, the identification of a di-glycine signature for ubiquitin linkages enables the development of a GPS (global protein stability) profiling system based on a fluorescence-mediated genetic screen in combination with a QUINT (quantitative ubiquitination interrogation) proteomic approach created by the Elledge lab[93]. This non-biased system has been widely used for genome-wide measurement of protein stability that can identify either substrates for a given E3 ligase/DUB, or possible E3(s)/DUB(s) for a given substrate. In addition, multiple commercially available assay kits and services are provided to facilitate research towards these directions.

8. Concluding remarks

In addition to well-characterized phosphorylation-mediated mTOR regulatory mechanisms, mTOR signaling components also undergo modifications by mono-ubiquitination (such as PDK1[22]) or poly-ubiquitination in a variety of linkages (such as K29-linked ubiquitination and degradation of p110 by NEDD4L, K48-linked ubiquitination and degradation of Akt by TTC3, K63-linked ubiquitination and activation of Akt, and multiple-linkage ubiquitination and degradation of ULK1 by NEDD4L, see Table 1 for references) that determine distinct protein fates with different effects on protein functions. In addition to the ubiquitination modification on lysine residues, recently mTOR-Lys1218 malonylation has also been reported to negatively regulate mTORC1 activity[94] with unknown mechanisms, which may be due to reduced K63-linked mTOR ubiquitination.

Moreover, it is common that one E3 ligase regulates multiple substrates in the mTOR signaling. For example, CHIP promotes degradation of multiple mTOR signaling components including p85[14], Akt[33] and SGK1[50] through K48-linked poly-ubiquitination, while CHIP also facilitates K63-linked poly-ubiquitination of PKCζ for its kinase activation[95]. TRAF6 usually promotes K63-linked poly-ubiquitination to facilitate activation of p85, mTOR, Akt, Rac1 and ULK1 (see Table 1 for references). Other TRAF family of E3 ligases such as TRAF2 and TRAF4 have been shown to aid K63-linked ubiquitination of GβL[66] and Akt[74], respectively. The E3 ligase $SCF^{\beta\text{-TRCP}}$ targets multiple proteins regulating mTOR signaling for ubiquitination and degradation including Deptor, PHLPP1, PDCD4, FNIP2 and Lipin1 (see Table 1 for references). Thus, β-TRCP functions as a hub that is utilized for the regulation of multiple aspects of the mTOR signaling following pro-growth stimuli, coordinating increased protein synthesis with pro-survival signaling. Specifically, (1) upon mitogenic stimulation, the tumor suppressor PDCD4, which inhibits the translation initiation factor eIF4A, is degraded in a β-TRCP- and S6K1 phosphorylation-dependent manner, therefore allowing efficient protein translation and cell growth[96]; (2) mTOR

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cooperates with CK1α and β-TRCP to induce the degradation of the mTOR inhibitor, DEPTOR, generating an auto-amplification loop that promotes the further activation of mTOR[97]; (3) alternatively, β-TRCP promotes mTOR activation through degrading FNIP2 (component of the FNIP1/FNIP2/FLCN tumor suppressive complex upstream of mTORC1) in a CKI and nutrient sensitive manner[61]; (4) β-TRCP regulates cell survival in cooperation with the ERK-RSK pathway by targeting BimEL for degradation[98]; and (5) β-TRCP indirectly promotes Akt activation by targeting PHLPP1, a phosphatase dephosphorylating and inactivating Akt, for ubiquitination and degradation in a CKI and GSK3-dependent manner[43]. On the other hand, NEDD4L promotes a unique K29-linked polyubiquitination of p110 to mediate p110 protein degradation, while NEDD4L produces K48-linked ubiquitin chains on ULK1 for ULK1 destruction (see Table 1 for references). Similarly, NEDD4 facilitates both K48-linked ubiquitination of SGK1 for SGK1 destruction[49] and K63-linked ubiquitination of phospho-Akt to facilitate its nuclear transportation and activation[72].

Furthermore, one mTOR signaling component can be targeted by different E3 ligases or DUBs for regulation in a cancer type- or cellular context-dependent manner. For example, PTEN deubiquitination is regulated by USP10 in lung cancer[99] and hepatocellular carcinoma[100], while the same process is regulated by USP13[92] or OTUD3[91] in breast cancer. It remains to be determined whether cellular context-dependent upstream signaling events may determine the function of differentially identified DUBs in modulating the function of the same protein.

In summary, ubiquitin modifications play important roles in regulating the mTOR signaling by either degrading the protein targets to terminate the mTOR signaling, modulating protein cellular localization for enzyme activation/inactivation, triggering protein binding to other partners and through other unknown mechanisms. Post-translational modifications of the “degron” add an additional layer of regulation to the ubiquitination process and this might in part explain why in some cases, no inverse correlation/mutually exclusivity is observed for E3 ligases and substrates in cancer. Interestingly, it seems that ubiquitin-mediated mTOR regulation is a late evolutionary event as a gained modification and function for higher eukaryotes, which may facilitate the adaption of mammals to a more complexed and sophisticated biological system. Compared with well-studied phosphorylation-mediated regulations of the mTOR signaling, there are much to be investigated for roles of ubiquitin on many mTOR signaling components. Identification of the E3 ligases, DUBs and function of the unknown ubiquitination events in mTOR signaling will greatly advance mTOR biology and shed new lights into clinic therapeutics targeting E3 ligases or DUBs for human disease treatments by modulating mTOR activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors sincerely apologize to all those colleagues whose important work was not cited in this paper owing to space limitations, especially many prominent and pioneer work in the mTOR filed. We thank other Liu members for critical reading of the manuscript and helpful discussions. This work was supported by the NIH grants (P.L.

R00CA181342), a V Foundation Research Scholar Grant (P.L. V2018–009), a UNC IBM Junior Faculty Development Award (P.L) and the UNC University Cancer Research Fund (P.L.).

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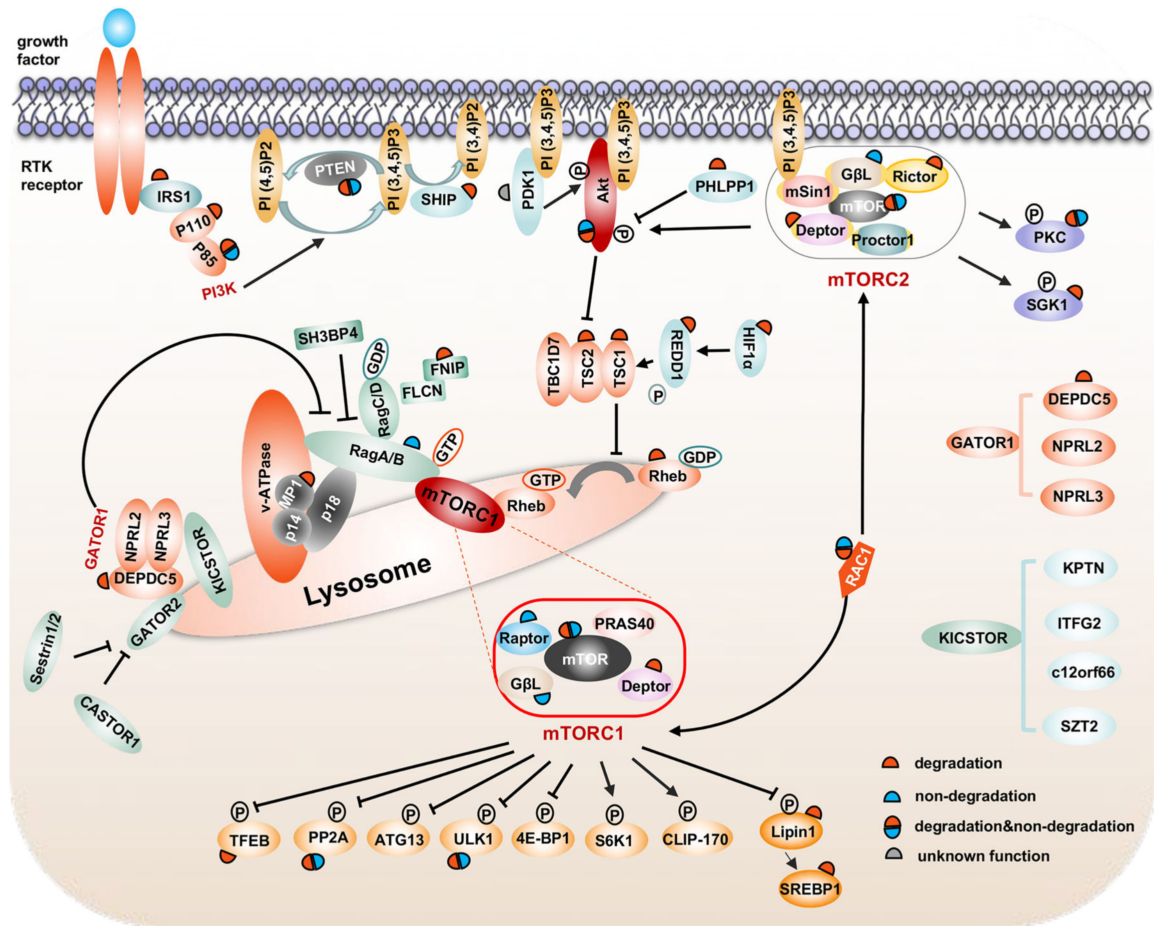

Figure 1. Overview of the ubiquitin regulations on mTOR signaling components.

This is a cartoon illustration of key mTOR signaling pathway members, their cellular localization and reported ubiquitin-mediated regulations. The red half circle indicates degradation-oriented ubiquitination, the blue half circle indicates non-degradation-oriented ubiquitination, and the gray half circle indicates ubiquitination with unknown function.

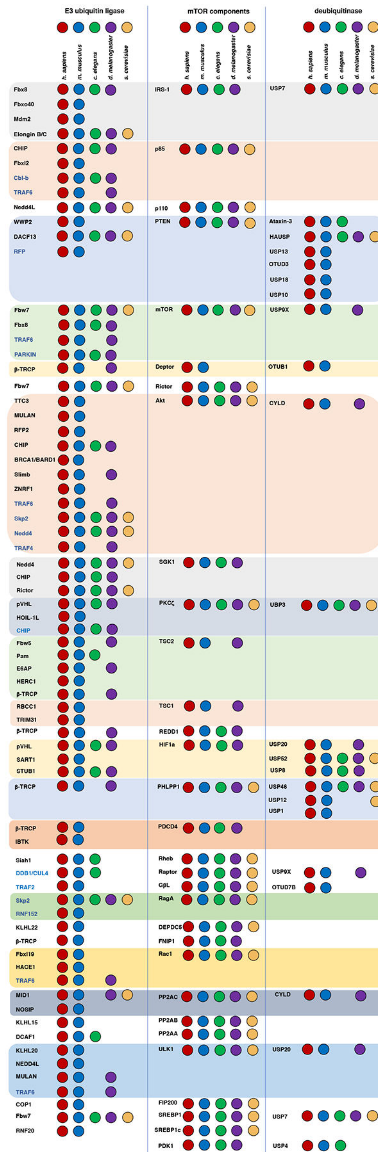

Figure 2. Evolutionary conservation of E3 ligases and DUBs regulating mTOR signaling.

The presence of mTOR signaling components (middle), identified regulating E3 ligases (left) and DUBs (right) in indicated four species (human, mouse, c. elegans and fruit fly) are indicated by dots with distinct colors. Conservation information is obtained from NCBI-homologene, genecards, KEGG pathway, uniport and literature search. Absence of any dot means either not conserved or never examined and reported.

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