mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy

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Growth factors and nutrients enhance protein synthesis and suppress overall protein degradation by activating the protein kinase mammalian target of rapamycin (mTOR). Conversely, nutrient or serum deprivation inhibits mTOR and stimulates protein breakdown by inducing autophagy, which provides the starved cells with amino acids for protein synthesis and energy production. However, it is unclear whether proteolysis by the ubiquitin proteasome system (UPS), which catalyzes most protein degradation in mammalian cells, also increases when mTOR activity decreases. Here we show that inhibiting mTOR with rapamycin or Torin1 rapidly increases the degradation of long-lived cell proteins, but not short-lived ones, by stimulating proteolysis by proteasomes, in addition to autophagy. This enhanced proteasomal degradation required protein ubiquitination, and within 30 min after mTOR inhibition, the cellular content of K48-linked ubiquitinated proteins increased without any change in proteasome content or activity. This rapid increase in UPS-mediated proteolysis continued for many hours and resulted primarily from inhibition of mTORC1 (not mTORC2), but did not require new protein synthesis or key mTOR targets: S6Ks, 4E-BPs, or Ulks. These findings do not support the recent report that mTORC1 inhibition reduces proteolysis by suppressing proteasome expression [Zhang Y, et al. (2014) Nature 513(7518):440–443]. Several growth-related proteins were identified that were ubiquitinated and degraded more rapidly after mTOR inhibition, including HMG-CoA synthase, whose enhanced degradation probably limits cholesterol biosynthesis upon insulin deficiency. Thus, mTOR inhibition coordinately activates the UPS and autophagy, which provide essential amino acids and, together with the enhanced ubiquitination of anabolic proteins, help slow growth.

mTOR | proteasome | ubiquitination | autophagy

The balance between overall rates of protein synthesis and degradation determines whether a cell grows or atrophies. It has long been proposed that overall rates of protein synthesis and degradation can be coordinately regulated (1). For example, when hormones (e.g., insulin and insulin-like growth factor-1) and nutrients are plentiful, rates of protein synthesis are high and protein degradation is suppressed, whereas in starving cells, synthesis falls and overall degradation rises. One critical factor coordinating overall synthesis and degradation is the Ser/Thr protein kinase mammalian target of rapamycin (mTOR), which promotes protein translation (2) while suppressing autophagy (lysosomal proteolysis) (3). mTOR’s pleiotropic functions are catalyzed by two distinct kinase complexes: mTORC1, the key component of which is Raptor, and mTORC2, which instead contains Rictor (4, 5). Growth factors act through class I PI3K and Akt kinases to inhibit the tumor suppressor TSC2, and thereby activate mTORC1 (6). Adequate supply of amino acids, especially leucine, can also activate mTORC1, but through a distinct mechanism involving Rag GTPase and lysosomal recruitment of mTORC1 (4). Unlike mTORC1, mTORC2 is insensitive to amino acid supply but is activated by growth factors via mechanisms that remain unclear.

Rapamycin is a natural product that selectively inhibits mTORC1 but not mTORC2. Torin1 is a synthetic mTOR inhibitor that blocks ATP-binding to mTOR and thus inactivates both mTORC1 and mTORC2 (7). Both Torin1 and rapamycin inhibit overall protein synthesis, induce autophagosome formation, and thus mimic the effects of starvation. However, Torin1 is much more effective than rapamycin in affecting these two processes because rapamycin inhibits mTORC1 incompletely (7, 8). Because many types of cancer are associated with overactivation of mTOR, rapamycin and other novel mTOR inhibitors are useful in the treatment of certain cancers (9). Rapamycin is widely used in the clinic as an immune suppressor (10). Nevertheless, rapamycin extends lifespan in aged mice, perhaps by triggering similar changes as occur with dietary caloric restriction (11). Additionally, stimulating autophagy by rapamycin can help clear intracellular protein aggregates as they accumulate in many neurodegenerative diseases and reduce their toxicity (12).

Eukaryotic cells degrade proteins by both the autophagy-lysosome system and the ubiquitin proteasome system (UPS). Macrophagy delivers cytoplasmic proteins or organelles into autophagic vacuoles for degradation, and autophagosome formation is rapidly activated by starvation or mTOR inhibition (3). The UPS is responsible for the degradation of most cytosolic and nuclear proteins in mammalian cells, including the short-lived regulatory and misfolded proteins as well as the bulk of cell constituents, which are long-lived components (13, 14). Degradation by the UPS is highly selective, involving the...

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attachment of a ubiquitin (Ub) chain to the substrate through the sequential actions of a Ub-activating enzyme (E1), Ub-conjugating enzymes (E2s), and one of the cell’s many Ub ligases (E3s) (15). Proteins conjugated to Ub chains are rapidly degraded by the 26S proteasome, whereas the Ub molecules are recycled by proteasome-associated deubiquitinating enzymes (DUBs) (16). Although the degradation of specific short-lived proteins has been studied extensively, our understanding of the global mechanisms controlling proteasomal degradation of the bulk of cell constituents is very limited.

The best-characterized function of mTOR is to enhance protein translation through mTORC1-mediated phosphorylation of 4E-BPs and S6Ks (2). The simultaneous suppression of protein degradation is generally attributed to the ability of mTORC1 to inhibit autophagy, which seems to occur by the phosphorylation and inactivation of Atg1/ULKs (3). The goal of this study was to test whether the increased proteolysis upon mTOR inhibition may also occur through activation of the UPS. We demonstrate herein that mTOR inhibition not only enhances lysosomal proteolysis, but also rapidly stimulates the ubiquitination and proteasomal degradation of many proteins. We also investigated the mechanisms by which the UPS is activated, the role of mTORC1 or mTORC2, and the nature of the proteins whose stability is affected by mTOR.

Together with the enhancement of autophagy, this activation of proteolysis by the UPS upon mTOR inhibition appears to represent an important adaptation to starvation that helps slow growth and provide essential amino acids. A very different role of mTORC1 in regulating protein degradation was recently proposed by Zhang et al. (17), who surprisingly reported that rapamycin did not rapidly increase proteolysis, but after 16 h actually reduced overall proteolysis (16). Although the degradation of radiolabeled short-lived and more stable proteins in HEK293 cells was reproducibly by 1 h, although Torin1 consistently caused a larger increase (Fig. 1B). In contrast, neither agent increased the breakdown of short-lived proteins, which were degraded within 1 h after a brief labeling (20 min) (Fig. 1A). Therefore, subsequent studies focused on the degradation of long-lived proteins, which comprise the great majority of cell proteins.

Torin1 inactivates both mTORC1 and mTORC2 (7), as well as confirmed by the elimination of phosphorylated S6K (Thr389) and Akt (Ser473) (Figs. 2B, 3F, and 4C). However, rapamycin, which inhibits only mTORC1, abolished S6K phosphorylation, while causing hyperphosphorylation of Akt (Fig. 2B and SI Appendix, Fig. S6). Because rapamycin inhibits mTORC1 only partially (7), we mainly used Torin1 to clarify mTOR’s role, but also studied rapamycin because of its wide use in research and the clinic.

Inhibiting mTOR Stimulates both Autophagy and Proteasome-Mediated Proteolysis. To determine whether mTOR inhibitors enhance protein breakdown by the UPS, we measured their effects on the degradation mediated by proteasomes or lysosomes by analyzing the fraction of overall proteolysis sensitive to the proteasome inhibitor, bortezomib (BTZ), or to the inhibitor of lysosomal acidification, concanamycin A (CCA), as described previously (14). As expected, Torin1 and to a lesser extent rapamycin increased lysosomal (CCA-sensitive) proteolysis in both HEK293 cells and mouse embryonic fibroblasts (MEFs) (Fig. 1D). The larger stimulation of lysosomal proteolysis by
Torin1 is consistent with morphological evidence that Torin1 is more effective than rapamycin in inducing autophagosome formation (7). These increases in lysosomal degradation depend on autophagy, because Torin1 failed to stimulate this process in MEFs unable to form autophagosomes because of deficiency of Atg5 (Fig. 1D).

In addition, Torin1 and rapamycin increased proteasomal degradation, measured by either of two approaches: the CCA-resistant proteolysis in HEK293 cells and MEFs (Fig. 1C, Left) or the BTZ-sensitive proteolysis in Atg5−/− MEFS (Fig. 1C, Right). In autophagy-competent cells, the CCA-resistant component was used to measure proteasomal degradation (Fig. 1C, Left) because we found that although BTZ does not directly inhibit lysosomal function (19), it partially reduced the activation of autophagy by Torin1 (SI Appendix, Fig. S1) (probably by blocking the degradation of an inhibitor of autophagy). Torin1 treatment similarly increased proteasomal proteolysis in both undifferentiated and differentiated C2C12 cells (SI Appendix, Fig. S2). Therefore, mTOR inhibition in many cell types stimulates overall proteolysis independently of autophagy.

In several cell types analyzed, proteasomes accounted for two-thirds or more of the degradation of long-lived proteins, and lysosomes for nearly all of the remainder (SI Appendix, Table S1). Upon mTOR inhibition, although the relative increases in lysosomal proteolysis are much larger than that by the UPS (especially with Torin1), proteasomes still accounted for a greater or equal fraction of the total proteolysis than lysosomes (Fig. 1 C and D).

mTOR Inhibition Rapidly Stimulates K48-Linked Ub Conjugation to Proteins in Cells and in Vivo, Which Leads to Increased Proteolysis.

Activation of Proteasomal Degradation upon mTOR Inhibition Does Not Require Protein Synthesis. Although mTOR inhibition alters rates of translation (8) and transcription (20, 21), it rapidly stimulates autophagy independently of protein synthesis (3). To learn whether the enhancement of proteasomal degradation by mTOR inhibitors requires protein synthesis, we pretreated HEK293 cells with cycloheximide for 1 h before Torin1 addition. Although cycloheximide by itself reduced proteasomal proteolysis (probably by enhancing mTOR activity (22)) after cycloheximide treatment, Torin1 caused a similar or greater increase in proteasomal proteolysis (Fig. 2A). Thus, this rapid stimulation by mTOR inhibitors does not require new protein or mRNA synthesis.
for at least 3 h (SI Appendix, Fig. S3B). Similar increases in Ub conjugate levels were seen in HEK293 cells using three different Ub antibodies (FL-76, FK2, P4D1) (SI Appendix, Fig. S3A) and no change was observed in the levels of SUMO2/3 conjugates (Fig. 2B and SI Appendix, Fig. S3C).

Ub chains are formed through isopeptide linkages between the C-terminal glycine of Ub and one of the lysines on the preceding Ub. Although K48-linked Ub chains target proteins to 26S proteasomes, K63-linked chains do not (23). Using antibodies specific for these two linkage types, we found that rapamycin and Torin1 increased the cellular content of K48-linked Ub chains, but not of K63-linked chains (23). Using antibodies specific for these two linkage types, we found that rapamycin and Torin1 increased the cellular content of K48-linked Ub conjugates. MEFs were treated with the Ube1 inhibitor or vehicle for 1 h before lysis, and the levels of Ub conjugates was measured by Western blot with anti-Ub (FK2). (C) The increase in proteasomal proteolysis by Torin1 was independent of S6ks or 4EBPs. Proteasomal proteolysis was determined in wild-type MEFs and MEFs lacking 4EBP1 and 2 or S6K1 and 2. (D) Overexpression of Ulk1 or Ulk2 enhanced lysosomal but not proteasomal degradation. Proteolysis was determined in HEK293 cells after 20 h of transfection with vectors expressing Ulk1, Ulk2, or an inactive mutant of Ulk1 (ULK1-K46N). (E) Unlike Torin1, the Akt inhibitor, Akti-1/2, (2 μM) did not increase proteasomal proteolysis in MEFs. (F) Akt inhibition did not induce the degradation of HMGCS1 and SUPT6H. The contents of these proteins were measured by Western blot after treatments for 6 h in the presence of cycloheximide. Phospho-Pras40 was measured to confirm the efficacy of Akti-1/2. (G) Torin1 increased proteasomal degradation in mTORC2-deficient (Rictor-null) MEFs. (H) Rictor-null MEFs showed no mTORC2 activity, reduced mTORC1 activity, and similar levels of HMGCS1, SUPT6H, and α-taxilin proteins compared with wild-type MEFs. (I) Selective activation or inhibition of mTORC2 did not affect proteasomal degradation in HEK293 cells. Proteasomal proteolysis was measured as described in Methods. For EBSS treatment, cells were washed once with EBSS and incubated with EBSS plus or minus insulin for 30 min, and then Torin1 was added for 1 h before measuring proteolysis. *P < 0.05; n.s., not significant.

This rapid increase in K48-linked Ub conjugates can account for the enhanced degradation by proteasomes after mTOR inhibition and must reflect increased ubiquitination of many cell proteins. Accordingly, treating MEFs with a specific inhibitor of the major Ub-activating enzyme Ube1 (24) depleted the MEFs of nearly all Ub conjugates in 1 h (Fig. 3B) and reduced proteasomal proteolysis by at least 60% (Fig. 3I). Importantly, inhibition of Ube1 completely eliminated the enhancement of proteasomal degradation by Torin1 (Fig. 3J). Although blocking ubiquitination also reduced slightly the increase in lysosomal proteolysis by Torin1, about 70% of the Torin1-induced activation of lysosomal degradation was independent of ubiquitination (Fig. 3J). Thus, the increased protein ubiquitination after mTOR inhibition is required for the accelerated proteasomal degradation of long-lived proteins. We also tested for a possible additional effect of Torin1 in stimulating proteasomal activity, but could detect no change in 26S proteasomal peptidase activity in HEK293 extracts or after purification of proteasomes from the treated cells (Fig. 2F). In addition, we did not detect any effect of mTOR inhibitors on overall deubiquitinating activity in HEK293 extracts, measured using the model substrate, Ub-aran (SI Appendix, Fig. S4I), nor did we observe any change in the activities of many individual DUBs measured by their modification with the active site probe, HA-Ub-vinyl sulfone (SI Appendix, Fig. S4B).

mTOR Inhibition Increases Ubiquitination and Degradation of Growth-Related Proteins. To identify some of the long-lived proteins whose ubiquitination is stimulated by mTOR inhibition, we pretreated HEK293 cells with cycloheximide and then with Torin1 or vehicle for 1 h. We then isolated ubiquitinated proteins from the lysates using immobilized tandem-repeated Ub-binding entities (25) and analyzed the conjugates by mass spectrometry (Fig. 4A). Many proteins were identified but showed little or no change in their...
ubiquitination following mTOR inhibition, as determined by counts of peptides recovered (SI Appendix, Table S2). However, we identified 55 proteins for which at least two peptides were detected (see examples in Fig. 4B; SI Appendix, Table S3) that showed at least a 50% increase in ubiquitination after Torin1 treatment in two independent experiments and we tested whether mTOR inhibition also enhances their degradation. Antibodies were available for 12 of the proteins that showed increased ubiquitination, and Torin1 treatment in the presence of cycloheximide reproducibly decreased the levels of four proteins, including HMGCS1 (cytoplasmic HMG-CoA synthase), SUPT6H, α-taxilin, and Myst2 (Fig. 4C). Their accelerated degradation was mediated by proteasomes because these decreases were blocked by BTZ, but not by CCA (Fig. 4C). For the other eight proteins tested, mTOR inhibition did not significantly alter their degradation within 6 h (SI Appendix, Fig. S5).

Among these four proteins, the degradation of HMGCS1 was stimulated to the largest extent by Torin1, which decreased its half-life from >10 h to about 2.5 h (SI Appendix, Fig. S6). In the mevalonate pathway for synthesis of cholesterol and isoprenoids, HMGCS1 precedes HMG-CoA reductase (26). Inhibition of mTORC1 reduces the transcription of HMGCS1 and other enzymes in this pathway through SREBP2 (sterol regulatory element-binding protein 2) (20, 27, 28), and we found that Torin1 also rapidly decreased the synthesis of HMGCS1 by 25% (SI Appendix, Fig. S7). Therefore, mTOR inhibition reduces levels of HMGCS1 by three mechanisms: decreased transcription and translation, which affect enzyme levels only slowly (because of its long half-life), and by accelerated degradation, which allows enzyme levels to change much faster. Accordingly, we observed a marked reduction in HMGCS1 levels upon Torin1 treatment or deprivation of growth factors and amino acids (EBSS buffer) for 3–6 h that could only occur through enhanced degradation, not by reduced synthesis. In fact, BTZ completely prevented this fall in HMGCS1 content by Torin1 or EBSS buffer (Fig. 4D). This finding of accelerated degradation of HMGCS1 after mTOR inhibition represents a new mechanism for rapid inhibition of the biosynthesis of cholesterol and isoprenoids upon nutrient or insulin deficiency.

The other three proteins also seem likely to have functions downstream of nutrient and growth factor signaling. Their high levels are elevated in several tumors (29–32) and some proliferating normal cells (33, 34). However, we could only examine the stabilities of 12 proteins, and many ubiquitinated proteins must have been missed by this approach because of their low abundance, rapid degradation, or incomplete isolation. Most likely, these four represent only a small fraction of the proteins whose ubiquitination and degradation are suppressed by mTOR. Stabilizing key anabolic proteins along with the reduced ubiquitination of cell
proteins generally seem to represent additional new mechanisms by which mTOR promotes growth.

The Enhancement in Proteasomal Proteolysis Does Not Involve mTOR Targets 4E-BPs, S6Ks, Ulks, or Akt. Because the increases in proteasomal proteolysis and autophagy upon mTOR inhibition occur simultaneously with the decrease in protein synthesis, we investigated whether the mTOR targets that activate translation or inhibit autophagy might also regulate proteolysis by the UPS, S6Ks and 4E-BPs mediate the stimulation of translation by mTORC1 (2). However, neither appears to be important for effects on proteasomal proteolysis, because Torin1 treatment of MEFs lacking S6K1 and S6K2 or lacking 4E-BP1 and 4E-BP2 increased proteasomal proteolysis as in wild-type MEFs (Fig. 3C). Ulk1 and Ulk2 are critical for the suppression of autophagy by mTORC1 (3). Although overexpression of either Ulk1 or Ulk2, but not the catalytically inactive mutant Ulk1-K46N, stimulated proteolysis by lysosomes, these treatments did not affect proteasomal degradation (Fig. 3D). Because Akts are substrates of mTORC2 and activate many growth-related processes, we tested if Akts are also important in suppressing proteasomal proteolysis. However, inhibition of Akts with Akti-1/2, as confirmed by the loss of phosphorylated Pras40 (Thr246) (Fig. 3F), did not increase proteasomal proteolysis generally (Fig. 3E), nor the degradation of HMGC51 and SPT541 (Fig. 3F). Although the simultaneous inhibition of Akt by mTOR of TSC and the inhibition of autophagy and proteasomal proteolysis are coordinated responses that together promote protein accumulation, they are signaled by distinct mechanisms.

The Increase in Proteasomal Degradation Results Mainly from Inhibition of mTORC1. To determine whether the enhancement of proteasomal proteolysis by Torin1 is caused by an inhibition of mTORC1 or mTORC2, we performed two types of experiments. First, in Rictor-null MEFs that lack mTORC2 activity (Fig. 3H), Torin1 treatment still increased overall proteasomal degradation (Fig. 3G) and enhanced HMGC51 degradation as in wild-type MEFs (SI Appendix, Fig. 8S). Thus, mTORC1 alone stabilizes many cell proteins. Second, when HEK293 cells were deprived of both serum and amino acids, which inactivated completely mTORC1 activity and reduced significantly mTORC2 activity, protein degradation was increased (Fig. 3I). However, activation of insulin, which activated strongly mTORC2 but not mTORC1, did not reduce the enhancement of proteasomal proteolysis, and inhibiting this hyperactivation of mTORC2 by Torin1 did not enhance it either (Fig. 3J). Taken together, the ability of Torin1 to activate proteasomal proteolysis in the absence of mTORC2 (Fig. 3G) and the lack of effect of mTORC2 activation or inhibition alone (Fig. 3I) indicate that in HEK293 cells and MEFs, mTORC1 plays the major role in suppressing overall proteolysis by the UPS.

Discussion

This New General Mechanism Controlling Protein Turnover and the Conflicting Report. The present studies demonstrate that in nutrient-rich environments and during growth, mTOR (primarily mTORC1) decreases overall protein degradation by the UPS and stabilizes many normally long-lived proteins by suppressing their ubiquitination. Under these conditions, mTOR does not seem to affect the breakdown of short-lived proteins (i.e., misfolded proteins or highly regulated proteins, whose ubiquitination has been studied most extensively). When mTOR levels fall, upon lack of nutrients or insulin, this mechanism can rapidly enhance (within 30–60 min) the supply of amino acids without requiring alterations in transcription or translation.

As we were preparing this report, opposite findings were reported by Zhang et al. (17), who concluded that rapamycin treatment for 16 h or longer reduces overall proteolysis by decreasing proteasome expression through decreases in the expression of the transcription factor, Nrf1 (17). Surprisingly, no change in rates of proteolysis was observed until 16 h of rapamycin treatment, in sharp contrast to the rapid increase in proteasomal degradation described here and the rapid activation of autophagy observed by many prior investigators (3). Their proposed mechanisms are also inconsistent with a number of prior findings about the UPS. Proteasomes are long-lived cell constituents (subunit half-lives of 40–200 h) (35). Thus, simply decreasing proteasome gene transcription should not reduce their content within a short period. In fact, after rapamycin treatment for 24 h, we did not detect any significant decrease in the levels of several proteasome subunits (SI Appendix, Fig. S9) or in 26S activity (peptidase and Ub conjugate degradation) (Fig. 2F). Furthermore, proteasomes appear to be present in excess in cells (36), and their amount is usually not rate-limiting for proteolysis. In fact, we demonstrated recently that a modest (~30%) reduction of 26S proteasome content by knocking down PSM2 has no effect on overall protein degradation by proteasomes (37). Most importantly, using the same cells they studied, we did not find any decrease in proteolysis, but only that mTOR inhibition activates overall proteolysis rapidly and for at least 28 h (18).

As discussed elsewhere (18), both the pulse-chase analysis and experimental design of Zhang et al. (17) appear potentially misleading. In our experiments, we always maintained cells in complete medium and compared the effects of mTOR inhibition on degradation of the exact same pool of radiolabeled proteins. However, their experiments compared the breakdown of proteins labeled in TSC2-null, TSC2-expressing, or rapamycin-treated MEFs, where patterns of transcription and translation differ as a result of differences in mTORC1 activity (17), and thus, they have compared the degradation of distinct sets of proteins and not just studied mTORC1’s effects on the proteolytic machinery. Additionally, Zhang et al.’s experiments involved depriving TSC2-null MEFs of serum for extended periods (up to ~66 h) (17) to study mTORC1’s actions separately from mTORC2’s. However, this approach is problematic for studies of proteolysis because serum deprivation by itself stimulates proteolysis in all cells, and based on the present findings should do so more in wild-type cells than in TSC2-null MEFs. In fact, when we measured the breakdown of the same set of proteins in TSC2-null MEFs cells after serum deprivation for 17 h, the subsequent inhibition of mTORC1 by rapamycin or Torin1 still increased overall proteolysis (SI Appendix, Fig. S10), as we consistently found in complete medium. Furthermore, long-term mTOR inhibition or serum deprivation may trigger secondary responses. In fact, although serum deprivation acutely activates autophagy, long-term serum deprivation or sustained inactivation of insulin-like growth factor 1 signaling reduces autophagy (38). Therefore, we focused in this study on the effects of mTOR inhibition for minutes or several hours.

The Potential Mechanisms by Which mTORC1 Suppresses Proteasomal Degradation. Our various experiments indicate that the activation by mTOR inhibition of protein breakdown by the UPS results primarily from inhibition of mTORC1 and is driven by increased ubiquitination without any demonstrable activation of the proteasomes. In several prior reports, mTOR-mediated phosphorylation of proteins was shown to influence their ubiquitination and stability. For example, Gribel, a negative regulator of growth factor signaling, was reported to be stabilized by mTORC1 (39, 40). Among the four proteins we identified that show accelerated degradation upon mTOR inhibition, SPT541 was reported to be a potential substrate of mTOR. Among the 55 proteins we identified that show increased ubiquitination after mTORC1 inhibition, 9 were reported to be tentative mTOR substrates (39, 40). Therefore, mTORC1-mediated phosphorylation of certain proteins may lead to their stabilization by inhibiting their ubiquitination, and this mechanism may contribute to the general increases in Ub-dependent proteolysis upon mTORC1 inhibition. Another possibility is that mTOR directly inhibits certain ubiquitinating enzymes or stimulates DUBs, and thus influences the degradation of some proteins. However, we have not succeeded in finding such enzyme
candidates. Possibly, multiple mechanisms downstream of mTORC1 (e.g., phosphorylation of substrates, Ub ligases, or DUBs) together account for the increases in ubiquitination and proteasomal proteolysis upon mTOR inhibition. It will clearly be important to learn which Ub ligases are responsible for the increased ubiquitination of the mTOR-regulated proteins, such as HMGCS1. A large fraction of the Ub ligases in the human genome belong to cullin-based protein complexes, many of which modify phosphorylated proteins selectively. However, these cullin-based Ub ligases appear not to be involved, because exposing cells to the selective neddylation inhibitor MLN4924 (41), which inhibits their activities, did not inhibit the degradation of these four proteins (Fig. 4C), nor the increase in overall proteasomal proteolysis by Torin1 (Fig. 4E). Thus, the responsible UB ligases belong to the monomeric RING or HECT family.

Although cells contain a family of proteins that bind ubiquitinated proteins and can target them to autophagic vacuoles for degradation (42), it seems most likely that a great majority of proteins showing increased ubiquitination upon mTOR inhibition are degraded by the 26S proteasome. As shown in Fig. 3A, the Torin1-induced increase in lysosomal proteolysis was largely unaffected by blocking Ub conjugation, whereas the increase in proteasomal proteolysis required ubiquitination. In addition, the four proteins identified us undergoing accelerated ubiquitination upon Torin1 treatment were all degraded by proteasomes and not lysosomes (Fig. 4C). Thus, the stimulation of autophagy and proteasomal degradation differs in their dependence upon ubiquitination, and the increase in protein ubiquitination probably drives the enhanced proteasomal proteolysis.

Although the degradation of many individual proteins by the UPS has been extensively studied, global regulation of proteolysis by this pathway has received little attention, even though a general stimulation of degradation by the UPS is critical in catabolic states, such as muscle atrophy (43). Recently, a very different type of global regulation of the UPS has been described in which proteasome function is activated by cAMP and PKA-dependent phosphorylation, where proteolysis rises without any increase in total ubiquitination (44, 45). It is noteworthy that these two very different biochemical mechanisms also have distinct physiological roles and substrates. The PKA-mediated phosphorylation of proteasomes stimulates selectively the degradation of short-lived regulatory or misfolded proteins (e.g., aggregation-prone proteins causing neurodegenerative diseases) (44, 45), whereas mTOR affects the breakdown by the UPS of long-lived components, which comprise the bulk of cell proteins and thus must be important in growth regulation and adaptation to starvation.

Coordinate Regulation of the UPS and Autophagy in Growing and Starving Cells. Traditionally, the UPS and the lysosomal pathway have been assumed to function independently, and autophagy activation has been viewed as a compensatory mechanism when proteasome function is inhibited (46). However, our prior studies demonstrated that in fasting, after denervation and in other catabolic states, the loss of muscle protein (atrophy) involves coordinate activation of these two degradation systems through the activation of FoxO transcription factors (14, 47). Unlike mTOR inhibition, which enhances proteolysis via the UPS and autophagy within minutes, the FoxO-mediated stimulation requires hours for transcription and translation, but can lead to prolonged activation of these two degradative systems (14, 47). Most likely, upon starvation in vivo these complementary mechanisms for activating proteolysis, by mTOR inhibition and FoxO activation, function sequentially to enable cells to mobilize essential amino acids for the synthesis of proteins necessary for cell survival or energy production. Conversely, when nutrients and growth factors are ample, mTOR is activated, and FoxOs inactivated, the coordinate reduction in both proteolytic systems synergizes with the enhancement of protein synthesis to promote protein accumulation. Although these three anabolic actions of mTORC1 are simultaneous and linked, they involve distinct downstream mechanisms, because mTORC1’s suppression of the UPS is independent of its targets that control translation or autophagy. This rapid activation of the UPS may also synergize with the activation of autophagy in helping eliminate abnormal toxic proteins and thus contribute to the beneficial effects of rapamycin in models of neurodegenerative disease (12) and in extending lifespan (11).

Methods

Cell Culture and Materials. HEK293, MEFs, and C2C12 myoblasts were maintained in DMEM with 10% (vol/vol) FBS in 5% (vol/vol) CO₂ at 37 °C. C2C12 myotubes were obtained by incubating C2C12 myoblasts with differentiation medium (DMEM with 2% (vol/vol) horse serum) for 6 d in 8% (vol/vol) CO₂ at 37 °C. Rapamycin and Akt1/2 were purchased from Calbiochem, concanamycin A from Santa Cruz, and HA-Ub-vinyl sulfone from Boston Biochem. All mouse experiments were performed with the approval of the Harvard Medical School Institutional Animal Care and Use Committee and are in accordance with the Guide for the Care and Use of Laboratory Animals (48).

Determination of Total, Proteasomal, and Lysosomal Protein Degradation. This approach was described in detail and validated previously (14). Briefly, cells were incubated with H–Phe (5 μCi/mL) in standard culture medium for 20 h to label the long-lived cell proteins before switching the chase medium for 2 h that contains 2 mM nonradioactive Phe to prevent reincorporation of released radioactive amino acids. The medium was replaced with fresh chase medium containing the vehicle, BTZ (1 μM), or CCA (100-200 nM). One hour later, the mTOR inhibitors were added for another 1 h. Then multiple samples of the medium were collected at different times (up to 2 h) and mixed with trichloroacetic acid (TCA, final 10% [vol/vol]) to precipitate proteins. The TCA-soluble radioactivity in the medium at different times reflects the amount of prelabeled, long-lived proteins degraded and was expressed relative to the total radioactivity initially incorporated into protein. We generally used CCA-sensitive proteolysis to evaluate lysosomal degradation and CCA-resistant proteolysis to evaluate proteasome-mediated protein breakdown. All measurements were performed at least in triplicate, and the calculated rates of proteolysis are shown as “mean ± SEM.” P-values were determined by two-tailed Student’s t test.

Western Blotting. Cell proteins were extracted in lysis buffer (1% Triton X-100, 10 mM Tris pH 7.6, 150 mM NaCl, 30 mM Na pyrophosphate, 50 mM NaF, 5 mM EDTA, 0.1 mM NaVO₃, and protease inhibitor mixture (Roche)). When measuring the levels of Ub conjugates, N-ethylmaleimide (10 mM) was added to the lysis buffer to inactivate most DUBs. Thirty micrograms of total proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was done using the ECL method (Amersham). We used antibodies against Akt, P-S6K (Thr389), P-Akt (ser473), P-Pras40 (T246), β-actin, α-tubulin, and Rpn11 from Cell Signaling; HMGCS1, P4D1, FL-76, and HMGCS1 from Proteintech; and FK2, Rpt5, α-subunits from Bioworld. Western blot results were quantified by using ImageJ.

Isolation and Mass Spectrometry Analysis of Ubiquitinated Proteins. Twelve 150-mm dishes of HEK293 cells were pretreated with cycloheximide for 1 h and then with either vehicle or Torin1 for another 1 h. After cell lysis in the presence of 10 mM N-ethylmaleimide to block deubiquitination, 800 μg of GST-UBA proteins were added to the lysates and incubated for 4 h and then with glutathione-agarose for another 2 h. The immobilized proteins were extensively washed with PBS, digested with PreScission protease overnight to release the ubiquitinated proteins together with the UBA domains from the GST-resin. These soluble proteins were then separated by SDS/PAGE, digested with trypsin, and analyzed by nanoscale-microcapillary reversed-phase liquid chromatography tandem mass spectrometry (LC-MS/MS) (49).

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