

K63 Polyubiquitination and Activation of mTOR by the p62-TRAF6 Complex in Nutrient-Activated Cells

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SUMMARY

The ability of cells to respond to changes in nutrient availability is critical for an adequate control of metabolic homeostasis. Mammalian target of rapamycin complex 1 (mTORC1) is a central complex kinase in these processes. The signaling adaptor p62 binds raptor, and integral component of the mTORC1 pathway. p62 interacts with TNF receptor associated factor 6 (TRAF6) and is required for mTORC1 translocation to the lysosome and its subsequent activation. Here we show that TRAF6 is recruited to and activates mTORC1 through p62 in amino acid-stimulated cells. We also show that TRAF6 is necessary for the translocation of mTORC1 to the lysosomes and that the TRAF6-catalyzed K63 ubiquitination of mTOR regulates mTORC1 activation by amino acids. TRAF6, through its interaction with p62 and activation of mTORC1, modulates autophagy and is an important mediator in cancer cell proliferation. Interfering with the p62-TRAF6 interaction serves to modulate autophagy and nutrient sensing.

INTRODUCTION

Cell signaling is organized by adaptor proteins that act as hubs, conferring specificity and plasticity to the transmission of information inside the cell. Mammalian target of rapamycin (mTOR) is part of two signaling complexes termed mTORC1 and mTORC2 (Laplante and Sabatini, 2012). mTORC1 is a paradigmatic example of such a hub and is the primary nutrient-sensing mechanism. In addition to the mTOR kinase, the core components of mTORC1 include raptor and mLST8/GβL (Laplante and Sabatini, 2012). Recent findings have begun to shed light on the mechanisms connecting mTORC1 to nutrient availability. That is, the mTORC1 pathway is under the control of the Rag guanosine triphosphatases (GTPases), which play a critical role in the recruitment of mTORC1 to the lysosomal surface where it is activated (Durán and Hall, 2012; Sancak et al., 2008, 2010; Yuan et al., 2013). There are four mammalian Rag GTPases: RagA, RagB, RagC, and RagD, with RagA and RagB being

homologs to Gtr1, and RagC and RagD homologs to Gtr2 in budding yeast. These small GTPases form heterodimers consisting of RagA or RagB and RagC or RagD (Kim et al., 2008; Sancak et al., 2008; Sekiguchi et al., 2001). The active dimer interacts with raptor and modulates the translocation of mTOR to the lysosomal surface (Sancak et al., 2010). Identification of the protein complex termed Ragulator revealed additional complexity in the regulation of mTOR trafficking (Sancak et al., 2010). Ragulator is a lysosomal complex formed by MP1, p14, and p18 (Sancak et al., 2010). It also binds vacuolar adenosine triphosphatase (V-ATPase), is required for mTORC1 activation, and anchors the Rag GTPase complex to the lysosome (Zoncu et al., 2011a). Recently, two additional components of Ragulator have been identified. These are the proteins encoded by the HBXIP and C7orf59 genes, which form a pentameric complex empowered with guanine nucleotide exchange factor activity for RagA and RagB (Bar-Peled et al., 2012).

The protein known as p62, or sequestosome-1, is another example of a signal-organizing node. Initially identified by its ability to interact with the atypical protein kinase Cs (aPKCs) through their respective PB1 domains (Sanchez et al., 1998), it was later found to also bind other critical signaling intermediates such as TNF receptor associated factor 6 (TRAF6), which is essential to the regulation of NF-κB during osteoclastogenesis and bone homeostasis as well as in cancer (Duran et al., 2008; Durán et al., 2004; Guo et al., 2011; Ling et al., 2012; Moscat and Diaz-Meco, 2009a; Sanz et al., 2000; Starczynowski et al., 2011). p62 is also a substrate of autophagy, for which it needs to interact with microtubule-associated protein 1 light chain 3 (LC3) (Moscat and Diaz-Meco, 2009a, 2011). Moreover, we have recently shown that p62 binds raptor and that it is an integral part of the mTORC1 complex in response to nutrients (Duran et al., 2011). Consequently, p62 has emerged as a critical component of the mechanisms regulating mTORC1 in response to amino acid availability and as a negative regulator of autophagy (Duran et al., 2011). p62 is also required for translocation of the mTORC1 complex to the lysosomal surface by facilitating the interaction of mTOR with the Rag GTPases (Duran et al., 2011). Although p62 can bind both aPKCs through its PB1 domain, neither PKCζ nor PKCι are required for the activation of mTORC1 (Duran et al., 2011). In addition, NBR1, another protein interacting with p62 via PB1, was likewise dispensable for this pathway (Duran et al., 2011). These data suggest that different p62-containing complexes might have different

functions. In this regard, the potential for the p62-TRAF6 complex to act as an mTORC1 regulator has not yet been addressed. This could be of considerable relevance since TRAF6 has been shown to be amplified in human cancers and required for cell transformation (Beroukhi et al., 2010; Starczynowski et al., 2011). Although initially ascribed to its role as an E3 ubiquitin ligase for NF- κ B activation (Deng et al., 2000; Martin et al., 2006; Takayanagi et al., 2000), it is becoming apparent that scaffold proteins such as p62 may impose selectivity by directing TRAF6 selection of ubiquitination substrates (Jadhav et al., 2008; Nazio et al., 2013). Here we present compelling evidence demonstrating a role for the p62/TRAF6 complex in the amino acid-mediated activation of mTORC1 by K63-type polyubiquitination of mTOR. We have demonstrated that interfering with mTOR's ubiquitination or with the p62-TRAF6 interaction impairs mTORC1 activation in response to nutrients and has an important impact on the control of cell proliferation and transformation. This may open avenues for targeting mTORC1 activation in response to nutrients.

RESULTS

TRAF6 Is Recruited to the mTORC1 Complex through p62

To address the potential role of TRAF6 in p62-mediated mTORC1 activation, we first determined whether TRAF6 interacted with raptor, a major component of the mTORC1 complex (Hara et al., 2002). For this, we expressed hemagglutinin (HA)-tagged raptor along with Flag-TRAF6 in 293T cells, after which we immunoprecipitated ectopically expressed TRAF6 with an anti-Flag antibody and determined its association with raptor by immunoblotting with an anti-HA antibody. No interaction was detected between TRAF6 and raptor (Figure 1A). Since p62 interacts with both raptor and TRAF6, one possibility was that p62 could bridge TRAF6 to the mTORC1 complex. To test this, we expressed TRAF6 and raptor in the presence of p62. TRAF6 immunoprecipitated with ectopically expressed raptor only when p62 was cotransfected, but not in the absence of p62 (Figure 1B). No interaction was detected when raptor was expressed in a similar coimmunoprecipitation experiment (Figure S1A available online). These results demonstrate that p62 brings TRAF6 to the mTORC1 complex through its interaction with raptor. In addition, endogenous TRAF6 immunoprecipitated endogenous mTOR, raptor, and RagC, and the formation of this endogenous complex was enhanced by amino acids (Figure 1C). Notably, the endogenous interaction of TRAF6 with the mTORC1 complex was abolished in p62 knockout (KO) cells, indicating that p62 is required for TRAF6 recruitment to mTORC1 (Figure 1D).

TRAF6 Is Required for mTORC1 Activation

We next determined whether TRAF6 is actually required for the activation of mTORC1 in response to amino acids. We knocked down TRAF6 in 293 cells by lentiviral infection with short hairpin RNA (shRNA) specific for TRAF6 (shTRAF6), using nontargeting (shNT) lentivirus as control. Cells were starved of serum and amino acids and stimulated with amino acids for different durations. Notably, depletion of TRAF6 severely impaired S6K and

4EBP1 phosphorylation at the mTORC1 sites in response to amino acids (Figure 1E), suggesting that TRAF6 is required for amino acid-mediated mTORC1 activation. Expression of a shRNA-resistant TRAF6 complementary DNA (cDNA) rescued mTORC1 activation in TRAF6 knockdown cells (Figure 1F). In addition, TRAF6 overexpression was able to cooperate with amino acid stimulation to sustain a higher activation of the mTORC1 pathway (Figure 1G). Furthermore, consistent with the knockdown data of Figures 1E and 1F, embryo fibroblasts from TRAF6 KO mice showed a dramatic impairment in mTORC1 activation by amino acids (Figure 1H). Interestingly, growth factor, insulin, or energy-modulated mTORC1 activation was independent of TRAF6 (Figures S1B–S1E). These results demonstrate that TRAF6, like p62 (Duran et al., 2011), is an important modulator of mTORC1, selectively in the nutrient-response pathway. It is well established that amino acid deprivation results in the reduction of mTORC1 activity, which invariably leads to autophagy activation to generate intracellular nutrients that, in turn, reactivate mTORC1, even in the face of continuous deprivation of extracellular amino acids (Yu et al., 2010). Interestingly, the loss of TRAF6 or p62 also leads to the complete inhibition of the mTOR reactivation phase in response to intracellular nutrients generated by autophagy (Figures 1I and 1J). These results suggest that full activation of the mTORC1 pathway in response to amino acids depends on endogenous TRAF6 function.

TRAF6 Interaction with p62 Is Important for mTORC1 Activation

Because p62 mediated the recruitment of TRAF6 to the mTORC1 complex, we next sought to identify the region of p62 responsible for this interaction and asked whether or not this region was necessary and sufficient for channeling mTORC1 activation. We previously determined that the region of p62 that interacts with TRAF6 encompasses amino acids 228–254 (TB domain, Figure 2A) (Sanz et al., 2000). This region includes a TRAF6-binding motif (PSEDP, amino acids 228–232) that conforms to the canonical TRAF6 consensus binding site (PXEXX) found in other bona fide TRAF6 partners (Geetha et al., 2005; Ye et al., 2002). To test whether the interaction of TRAF6 with p62 was important for mTORC1 activation, we deleted the TB domain (p62 Δ TB) or selectively mutated residues 228, 230, and 232 of the TRAF6-binding site to alanine, either individually or in combination (Figure 2A). We first determined the effect that these deletions and mutations in the TB sequence had on the interaction of TRAF6 with p62. Figure 2B demonstrates that deletion of the TB domain totally abolished p62 interaction with TRAF6 and that progressive mutation of the conserved sites in the TB motif increasingly impaired TRAF6 binding to p62. This indicates that the TB sequence is necessary for the p62-TRAF6 interaction and that residues 228, 230, and 232 are critical for binding. To test whether this p62 region is also sufficient for TRAF6 binding, we expressed the TB domain as a glutathione S-transferase (GST) fusion protein. Notably, the TB domain was sufficient to bind TRAF6 (Figure 2C). Furthermore, increasing amounts of GST-TB were also able to compete the TRAF6-p62 interaction in a dose-dependent manner, whereas the expression of a TB-AAA mutant with mutations in the TRAF6-binding motif had no effect (Figure 2D). In addition, this

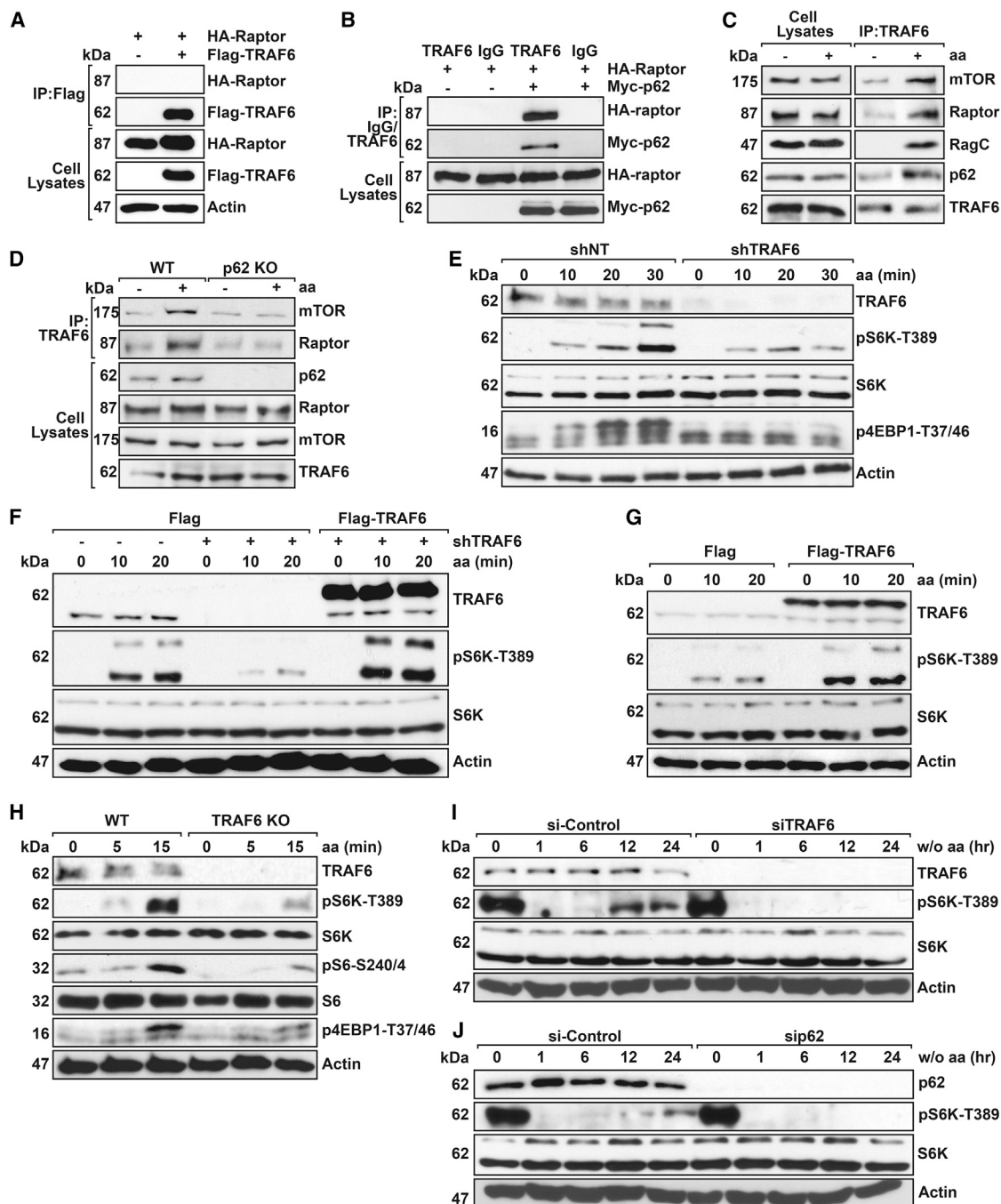


Figure 1. TRAF6 Is Required for mTORC1 Activation in Response to Amino Acids

(A and B) Human embryonic kidney (HEK) 293T cells were transfected with the indicated plasmids. Cell lysates, Flag (A), or TRAF6 immunoprecipitates (B) were analyzed by western blot.

(C and D) TRAF6 immunoprecipitates from HEK 293T cells (C) or WT and p62 KO mouse embryonic fibroblasts (MEFs) (D) were deprived of amino acids and serum for 50 min or 4 hr, respectively, and then stimulated with amino acids for 10 min. Cell lysates were analyzed by western blot.

(E) shNT or shTRAF6 HEK 293T cells were treated as in (C) and immunoblotted for the specified proteins.

(F) shNT or shTRAF6 HEK 293T cells were reconstituted with Flag-TRAF6 or control vector, treated as in (C), and immunoblotted as above.

(G) HEK 293T cells stably expressing Flag or Flag-TRAF6 were treated as in (C) and immunoblotted for the specified proteins.

(H) WT and TRAF6 KO MEFs were deprived of amino acids and serum for 4 hr and stimulated with amino acids for the indicated durations. Cell lysates were analyzed by western blot.

(I and J) A549 cells transfected with scramble siRNA, TRAF6 siRNA (I), or p62 siRNA (J) were deprived of amino acids and serum. Cell lysates were analyzed by western blot. These results are representative of three experiments. See also Figure S1.

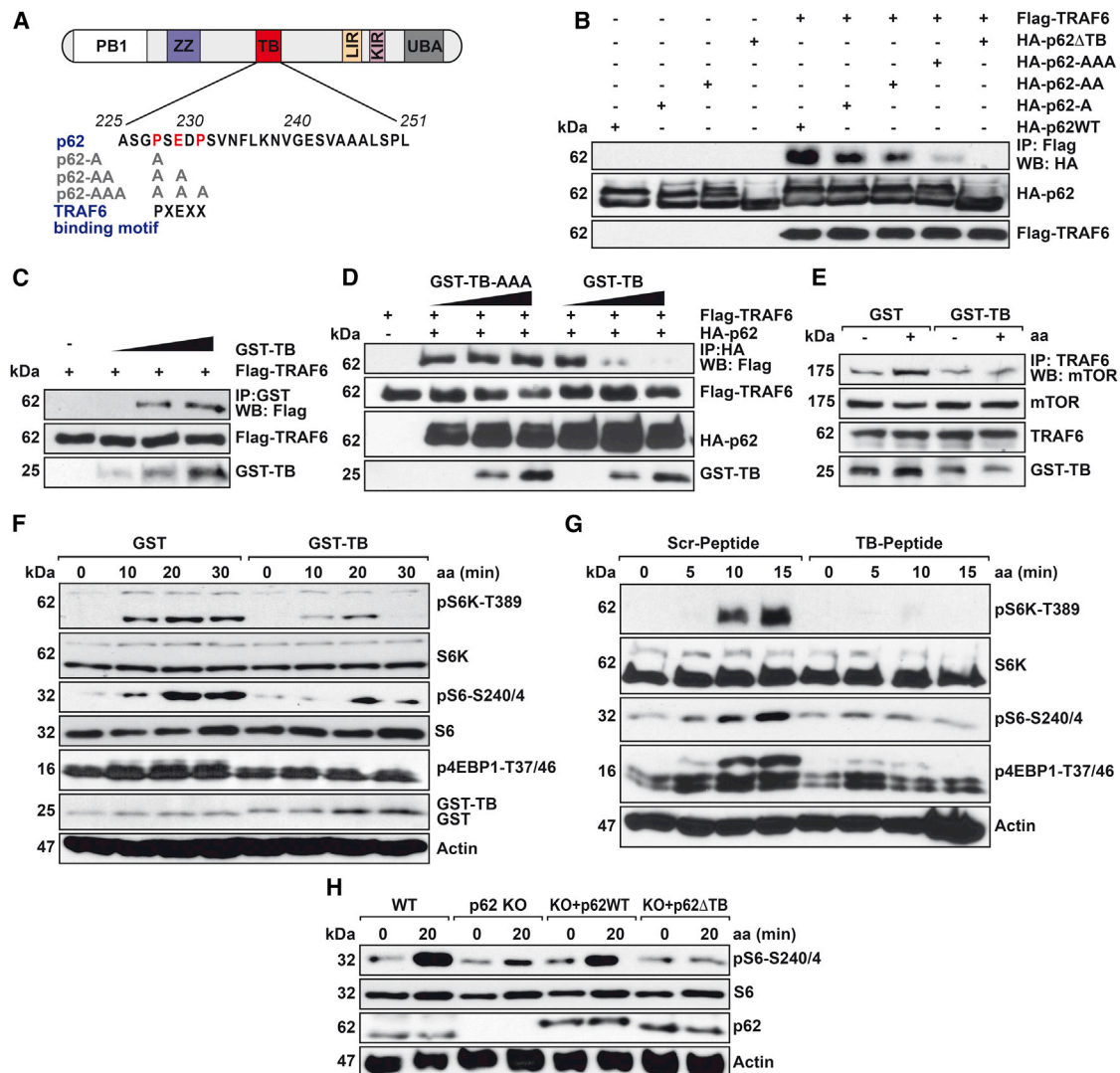


Figure 2. The TRAF6-p62 Interaction Is Necessary for mTOR Activation upon Amino Acid Stimulation

(A) Domain organization of p62. PB1, Phox, and Bem1p-1 oligomerization domain; ZZ, zinc finger; TB, TRAF6-binding sequence; LIR, LC3-interacting region; KIR, Keap1-interacting region. p62 mutants in the TRAF6-binding motif are depicted.

(B–D) HEK 293T cells were transfected with the indicated plasmids, cell lysates were prepared, and lysates and Flag (B), GST (C), or HA (D) immunoprecipitates were analyzed by western blot.

(E) HEK 293 cells transfected with GST or GST-TB were deprived of amino acids and serum for 50 min followed by amino acid stimulation for 10 min. Cell lysates and TRAF6 immunoprecipitates were analyzed for the indicated proteins.

(F) HEK 293T cells transfected with GST or GST-TB plasmids were deprived of amino acids and serum for 50 min and then stimulated with amino acids for the indicated durations. Cell lysates were analyzed by western blot.

(G) Cap2 cells were deprived of amino acids and serum for 4 hr and stimulated with amino acids in the presence of a cell-permeable TB peptide or a scramble peptide as a control. Cell lysates were analyzed by western blot.

(H) WT and p62 KO MEFs reconstituted with p62WT or p62ΔTB were deprived of amino acids and serum for 4 hr and stimulated with amino acids. Cell lysates were analyzed by immunoblot. These results are representative of three experiments. See also Figure S2.

approach is selective for TRAF6-p62 interaction since the expression of TB or TB-AAA mutant did not disrupt the interaction of TRAF6 with receptor activator of NF- κ B (RANK), a binding partner of TRAF6 (Ye et al., 2002) (Figure S2A). More importantly, overexpression of GST-TB abolished TRAF6 recruitment to mTORC1 (Figure 2E). Thus, TB expression disrupted the endogenous interaction of TRAF6 and mTOR in response to amino

acids, consistent with p62 bridging TRAF6 to mTOR. This is functionally relevant since overexpression of GST-TB impaired mTORC1 activation in response to amino acids (Figure 2F). This effect was selective for amino acid-induced mTORC1 activation since TB expression did not affect its activation in response to insulin (Figure S2B). In addition, we tested a cell-permeable peptide (TB peptide) encompassing amino acids

228–249 of p62 linked to the HIV-1 Tat protein that confers cell permeability or with a corresponding scrambled peptide used as a control. Interestingly, this peptide inhibited mTORC1 activation in response to amino acids, as measured by phosphorylation of S6K and 4EBP1 (Figure 2G). Furthermore, reconstitution of p62 KO embryonic fibroblasts (EFs) with a p62 mutant lacking the TB sequence (p62 Δ TB) was unable to rescue the defective activation of mTORC1 in p62 KO cells, whereas the expression of wild-type p62 efficiently rescued mTORC1 activation (Figure 2H). These results demonstrate that TRAF6 is a critical component of the mTORC1 activation cascade triggered by amino acids and that its interaction with p62 is necessary and sufficient for that function.

TRAF6 Translocation to the Lysosomes by Amino Acid Stimulation

Cell stimulation by amino acids triggers the translocation of the mTORC1 complex from the cytoplasm to lysosomal-associated membrane protein 2 (LAMP2)-positive intracellular structures identified as lysosomes (Sancak et al., 2008). Since TRAF6 is required for mTORC1 activation and binds p62, which regulates mTORC1 recruitment to the lysosome, we investigated the subcellular localization of TRAF6 by confocal immunofluorescence in both starved and amino acid-stimulated cells. Notably, in contrast to p62, which constitutively colocalized with LAMP2-positive lysosomes (Figures 3A and S3A) (Duran et al., 2011), TRAF6 was translocated to LAMP2-positive structures upon amino acid stimulation (Figures 3B and S3B) to an extent similar to that observed for mTOR (Figure 3C), as demonstrated in the panels showing quantification of colocalized pixels (Figures 3B and 3C). Consistent with this, TRAF6 colocalization with mTOR was enhanced by amino acid stimulation (Figure 3D). TRAF6 also colocalized with p18, a subunit of the Ragulator complex (Figure 3E), and with RagC (Figure 3F). This TRAF6 colocalization was also regulated by amino acids (Figures 3E and 3F). These results reinforce the notion that TRAF6 is part of a functionally relevant lysosomal mTORC1 complex. Consistent with this, TRAF6 and p62 colocalization was significantly enhanced upon amino acid stimulation (Figure 3G). More importantly, the translocation of mTOR to LAMP2-positive lysosomes required TRAF6. Thus, knockdown of TRAF6 significantly impaired mTOR colocalization with LAMP2 in response to amino acids (Figure 3H). Collectively, these results demonstrate that TRAF6 is an important regulator in the recruitment of mTOR to the lysosome and its subsequent activation.

K63 Ubiquitination of mTOR in Response to Amino Acids Is Mediated by the p62-TRAF6 Complex

TRAF6 is an E3 ubiquitin ligase that undergoes K63 autopolyubiquitination upon activation (Chen, 2012; Deng et al., 2000) and is required for mTORC1 stimulation by amino acids. Thus, we next tested whether amino acid stimulation could modulate TRAF6 autoubiquitination and activation. We found a rapid and transient increase in TRAF6 polyubiquitination in response to amino acids, as detected in immunoprecipitates of endogenous TRAF6 (Figure 4A). This suggests that TRAF6 E3 ubiquitin ligase activity could be essential for TRAF6's role in sensing nutrients as part of the regulation of mTORC1 activation. To test whether

TRAF6 E3 ubiquitin ligase activity is indeed required for its role in the mTORC1 cascade, we used a TRAF6 dominant-negative mutant that had a defective really interesting new gene (RING) finger (C70A) and was therefore devoid of ligase activity (Lamothe et al., 2007). Interestingly, unlike cells expressing TRAF6 wild-type (WT), mTORC1 was not activated in response to amino acids in 293T cells expressing the TRAF6 C70A mutant (Figure 4B). These results demonstrate that the E3 ligase activity of TRAF6 is necessary for its role in the mTORC1 nutrient pathway. Based on this finding, we reasoned that TRAF6 might control mTORC1 activity via a mechanism involving direct polyubiquitination of components of the mTORC1 complex. To test this hypothesis, we used a previously identified consensus sequence for ubiquitination substrates of p62-bound TRAF6 and screened for proteins in the nutrient-sensing pathway that conformed to these criteria (Jadhav et al., 2008). Following this approach, we identified this consensus sequence in mTOR, which, importantly, was conserved across species (Figures 4C and S4A). To test whether mTOR is a p62-TRAF6 ubiquitination substrate, we used immunopurified Myc-tagged mTOR as a substrate in an *in vitro* ubiquitination assay with Ube1 (E1) and Ubch7 (E2) in the presence or absence of TRAF6. Notably, mTOR was indeed found to be a substrate of the E3 ligase activity of TRAF6 in an *in vitro* assay (Figure 4D). Therefore, we next determined whether mTOR was ubiquitinated *in vivo* and whether or not its ubiquitination could be modulated by amino acids. To test this, we expressed Myc-tagged mTOR with HA-ubiquitin with or without Flag-tagged TRAF6 in 293T cells, which were then treated with amino acids. Importantly, amino acid stimulation promoted the polyubiquitination of mTOR (Figure 4E), suggesting that this posttranslational modification could be a mechanism for sensing nutrient availability. Furthermore, TRAF6 overexpression induced mTOR polyubiquitination even in the absence of amino acids and to the same extent as that observed in amino acid-stimulated cells in the absence of TRAF6 (Figure 4E), indicating that TRAF6 is most likely the E3 ubiquitin ligase responsible for mTOR ubiquitination.

Ubiquitination through K48 of the ubiquitin chain generally targets proteins for degradation, whereas K63 ubiquitination often regulates signaling activation and trafficking (Clague and Urbé, 2010). Expression of a K63R ubiquitin mutant (unable to form K63-linked chains) impaired mTOR polyubiquitination, whereas a K48R mutant (unable to form K48-linked chains) did not (Figure 4F). These results demonstrate that the polyubiquitination of mTOR occurred through K63, but not through K48. Because TRAF6 expression was able to induce mTOR ubiquitination, we next addressed whether it was required for this process. To do this, we selectively depleted TRAF6 by small interfering RNA (siRNA) and measured mTOR ubiquitination in response to amino acids. Figure 4G shows that knockdown of TRAF6 provoked a significant reduction in the amino acid-mediated polyubiquitination of ectopically expressed mTOR. Furthermore, when endogenous mTOR polyubiquitination was determined in cells expressing lentiviruses for TRAF6 (shTRAF6) or a nontargeted control (shNT) in response to amino acid stimulation, a robust and transient induction of mTOR ubiquitination was observed in control cells, and this response was blunted in shTRAF6 cells (Figure 4H). No endogenous mTOR ubiquitination

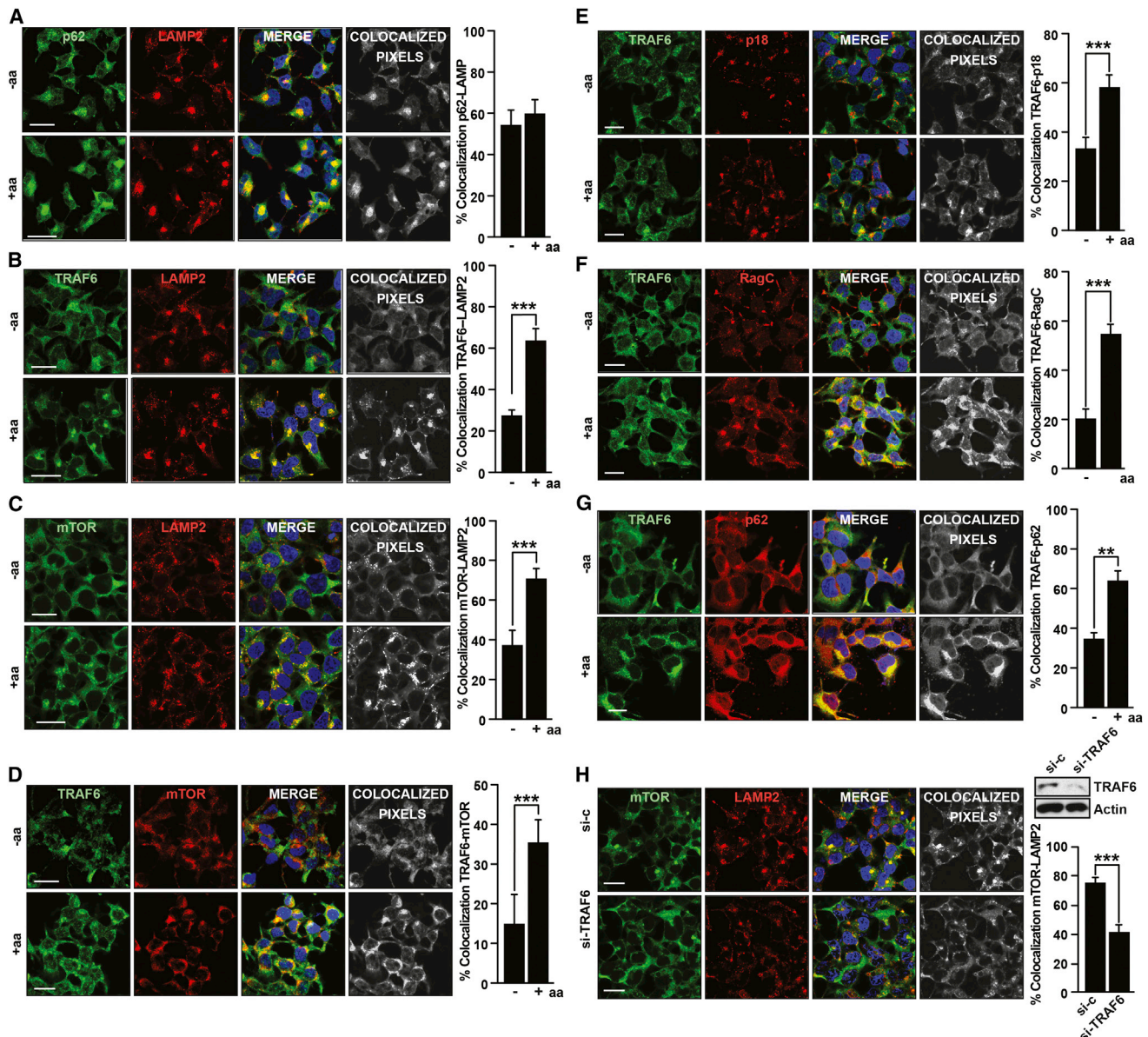


Figure 3. TRAF6 Regulates mTOR Translocation to the Lysosomal Compartment

(A–G) Images of HEK 293T cells coimmunostained for p62 and LAMP2 (A); TRAF6 and LAMP2 (B); mTOR and LAMP2 (C); TRAF6 and mTOR (D); TRAF6 and p18 (E); TRAF6 and RagC (F); and TRAF6 and p62 (G).

(H) Shown are images of HEK 293T cells transfected with si-TRAF6 or si-C, as control, coimmunostained for mTOR and LAMP2. Cells were starved for 50 min and stimulated with amino acids for 10 min before processing. In all images, graphs show the areas of staining overlaps (merge). Scale bars = 10 μ m. The quantification of colocalization was carried out on at least 15 cells per condition from 2 independent experiments. Results are shown as means \pm SEM (** p < 0.01, *** p < 0.001). See also Figure S3.

was detected in response to insulin or serum stimulation (Figure S4B). Collectively, these results demonstrate that mTOR is selectively ubiquitinated in response to amino acids in a TRAF6-dependent manner. Because TRAF6's function in mTORC1 activation is mediated through its interaction with p62, we asked whether this interaction could also be critical for TRAF6-mediated mTOR polyubiquitination. To answer this question, endogenous mTOR polyubiquitination was deter-

mined in cells expressing GST-TB, which blocked p62-TRAF6 interaction and mTORC1 activation (Figures 2D–2F). Results in Figure 4I demonstrate that endogenous mTOR ubiquitination in response to amino acids was severely inhibited by expression of GST-TB, indicating that the p62-TRAF6 interaction is indeed required for amino acid-induced mTOR ubiquitination. Collectively, these results demonstrate that TRAF6 is a bona fide E3 ubiquitin ligase for mTOR in the nutrient-sensing pathway.

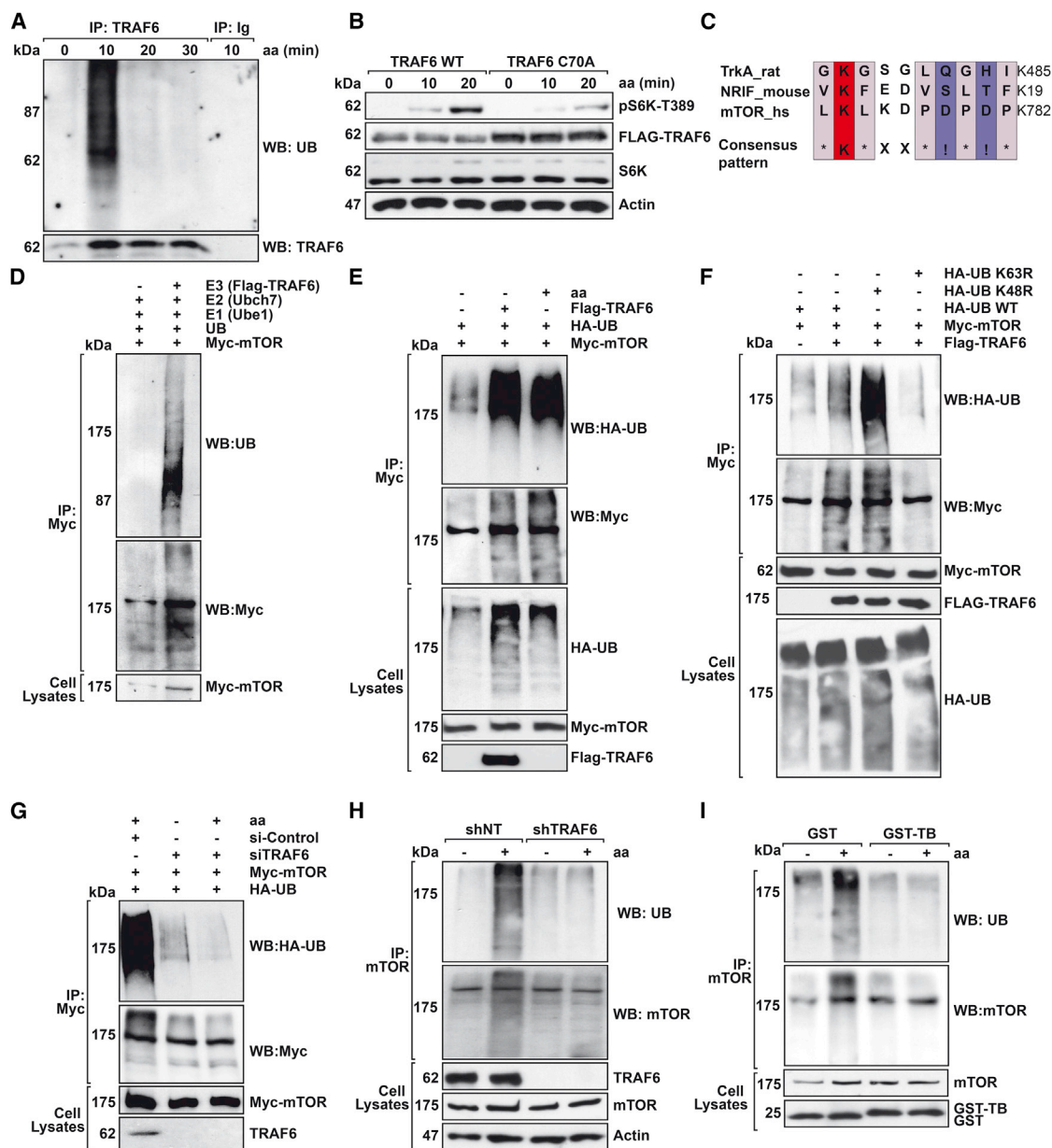


Figure 4. TRAF6 Ubiquitinates mTOR in Response to Amino Acids

(A) TRAF6 immunoprecipitates from HEK 293T cells deprived of amino acids and serum for 50 min and stimulated with amino acids were immunoblotted for ubiquitin.

(B) HEK 293T cells were transfected with the indicated plasmids and treated as in (A). Cell lysates were analyzed by western blot.

(C) Conserved sequences flanking the TRAF6/p62 ubiquitin acceptor site. Amino acids of the same type are marked as hydrophobic (*), polar (!), any amino acid residue (X), and the acceptor lysine residue (K).

(D) HEK 293T cells were transfected with the indicated plasmids. Myc-tagged immunoprecipitates were included in an in vitro ubiquitination assay in the presence or absence of TRAF6.

(E) HEK 293T cells transfected with the indicated plasmids were treated as in (A). Myc-tagged immunoprecipitates were analyzed by immunoblot.

(F) HEK 293T cells transfected with the indicated plasmids were treated as in (A). Myc-tagged immunoprecipitates were analyzed by immunoblot.

(G) HEK 293T cells treated as in (A) and expressing si-Control or siTRAF6 were transfected with the indicated cDNAs, and Myc-tagged immunoprecipitates were analyzed by immunoblot.

(H) shNT or shTRAF6 HEK 293T cells were treated as in (A), and mTOR immunoprecipitates were analyzed by immunoblot.

(I) HEK 293T cells treated as in (A) were transfected with the indicated plasmids, and mTOR immunoprecipitates were analyzed by immunoblot. See also Figure S4.

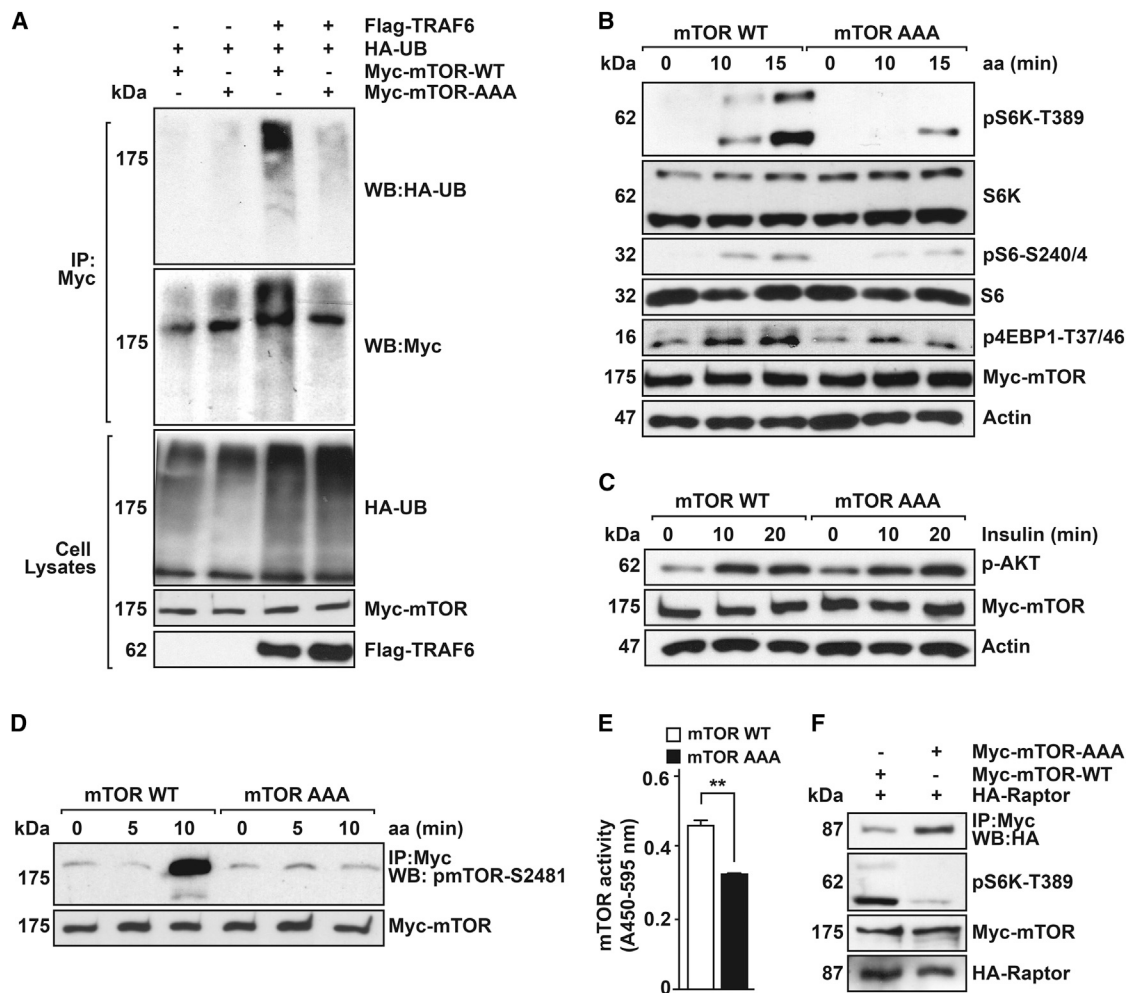


Figure 5. TRAF6 Regulates mTOR Ubiquitination and Activation

(A) HEK 293T cells were transfected with the indicated plasmids, deprived of amino acids and serum for 50 min, and stimulated with amino acids for 10 min. Cell lysates and Myc-tagged immunoprecipitates were analyzed by immunoblot.

(B) HEK 293T cells transfected with mTOR WT or mTOR AAA mutant were treated as in (A). Cell lysates were analyzed by western blot.

(C) HEK 293T cells transfected with mTOR WT or mTOR AAA mutant deprived of serum for 24 hr and stimulated with insulin were analyzed as in (B).

(D) HEK 293T cells transfected with mTOR WT or mTOR AAA mutant were treated as in (A), and cell lysates and Myc-tagged immunoprecipitates were analyzed by immunoblotting.

(E) In vitro kinase activity of Myc-tagged immunoprecipitates from HEK 293T cells transfected with Myc-mTOR WT or Myc-mTOR AAA. Results are shown as means \pm SEM ($n = 3$).

(F) HEK 293T cells were transfected with the indicated plasmids and cell lysates, and Myc-tagged immunoprecipitates were analyzed by immunoblotting. These results are representative of three experiments. See also Figure S5.

mTOR Polyubiquitination Is Required for mTORC1 Activation

As K63 ubiquitination is a frequent posttranslational modification that is known to influence signaling activation, we next investigated how mTOR function is affected by polyubiquitination. To address this question, we first mapped the residues accounting for its TRAF6-mediated ubiquitination. Based on the consensus motif for TRAF6/p62 ubiquitination shown in Figure 4C, we mutated K782 in mTOR to alanine and then determined polyubiquitination in response to TRAF6. We found that the K782A mTOR mutant displayed levels of polyubiquitination similar to mTOR WT (not shown). Since there are two flanking lysines sur-

rounding K782, we reasoned that K777 and K784 could be polyubiquitinated in the K782A mutant. Therefore, we generated a triple mutant K777/782/784A (mTOR AAA) and tested its polyubiquitination. Data shown in Figure 5A demonstrate that TRAF6-induced mTOR polyubiquitination was severely reduced in the mTOR AAA mutant, indicating that this mutant functions in a dominant-negative manner. Therefore, we next determined the functional repercussions that expression of the mTOR ubiquitination-defective mutant had in mTORC1 activation. Importantly, expression of the mTOR AAA mutant in cells activated by amino acids severely diminished the phosphorylation of S6K and 4EBP1 (Figure 5B) as well as the phosphorylation of a

transfected S6K reporter construct (Figure S5A). As a control, we found that there was no effect on the activation of Akt by insulin in cells expressing the mTOR AAA mutant (Figure 5C), indicating that mTOR polyubiquitination selectively affects mTORC1, but not mTORC2, activity. Furthermore, mTORC1's intrinsic kinase activity was reduced in the mTOR AAA mutant as monitored by mTOR S2481 autophosphorylation and in vitro kinase assay (Figures 5D and 5E). In addition, amino acid-induced mTOR ubiquitination was not affected by rapamycin or torin treatment, indicating that this is a process relevant for mTOR activation and not its consequence (Figure S5B). Consistent with this, the interaction of raptor with mTOR was increased when mTOR could not be ubiquitinated (Figure 5F). These results are consistent with the fact that the K782 ubiquitination site lies in the N-terminal region of mTOR, which includes the HEAT motifs and mediates mTOR's interaction with raptor (Kim et al., 2002). Moreover, the stronger interaction of the mTOR AAA mutant with raptor is in keeping with the fact that association of raptor with mTOR also negatively regulates mTOR kinase activity (Kim et al., 2002). Together, these results demonstrate that mTOR polyubiquitination by TRAF6 modulates its activation in response to amino acids and point to a role for K63 ubiquitin modification as a sensor of nutrient status.

TRAF6 Contributes to Cell Proliferation through mTORC1 Activation

Based on the facts that mTORC1 is a central kinase complex that integrates upstream signals to control protein synthesis and cell proliferation and that it is frequently deregulated in cancer, we next sought to investigate how TRAF6-mediated mTOR polyubiquitination influences cell proliferation. Interestingly, expression of the mTOR ubiquitination-defective mutant (mTOR AAA) significantly reduced cell proliferation under normal growing conditions (Figure 6A). Consistent with this, TRAF6 KO EFs transformed by the K-Ras V12 oncogene also displayed cell growth inhibition (Figure 6B) with a parallel blockade in mTORC1 activation (Figure 6C). Furthermore, rapamycin impaired growth in WT cells to levels comparable to those of TRAF6 KO cells, with no further effect on the growth of TRAF6 KO cells (Figures S6A and S6B). These results are consistent with the notion that the impaired cell growth observed in TRAF6 KO cells is most probably accounted for by mTORC1 inhibition due to TRAF6 deficiency. Likewise, knockdown of TRAF6 in the CaP2 PTEN null prostate cancer cell line, as well as in the human lung cancer cell line A549, reduced cell proliferation (Figures 6D and 6E) and focus-forming activity (Figure 6F) in both cell types. These results suggest that TRAF6 is important for the full proliferative and transforming properties of cancer cells. To test the functional contribution of mTORC1 activation in the oncogenic role of TRAF6, we expressed a constitutively active mutant of RagB, an activator of mTORC1 downstream of p62 (Duran et al., 2011; Kim et al., 2008; Sancak et al., 2008), into TRAF6-deficient cells and determined whether this was sufficient to rescue the defects in cell proliferation and transformation observed in TRAF6 knockdown cells (Figure 6D). Notably, expression of the active mutant of RagB was sufficient to rescue mTORC1 activation in the TRAF6 knockdown cells (Figure 6G). Moreover, its expression also partially reversed cell growth (Fig-

ure 6E) and the focus-forming activity of TRAF6 knockdown cells (Figure 6F), which supports the notion that mTORC1 is an important component of TRAF6-mediated oncogenic signaling in cancer cells. Interestingly, Cap2 cells treated with a cell-permeable peptide based on the TB sequence, which has previously been shown to block the interaction of TRAF6 with p62 (Geetha et al., 2005), displayed reduced proliferation as compared to cells treated with a control peptide (Figure 6H). These results demonstrate that the interaction of TRAF6 with p62 is important for cancer cell proliferation through the activation of mTORC1 and support the notion that TRAF6 functions in this pathway are mediated through p62.

TRAF6 Modulates Autophagy in Response to Nutrients

As mTORC1 negatively regulates autophagy (Alers et al., 2012; Jewell et al., 2013), we next asked whether TRAF6 deletion would activate autophagy in response to nutrient deprivation. As shown in Figures 7A and 7B, amino acid deprivation in A549 shTRAF6 cells induced autophagy as monitored by accumulation of the lipidated form of LC3, LC3-II. Notably, TRAF6 knockdown cells had higher basal levels of LC3-II, which was further increased in the presence of bafilomycin A1, an inhibitor of autophagosomal and lysosomal fusion, indicating enhanced autophagic flux (Figures 7A and 7B). Similar results were obtained when autophagy was measured by assessing the occurrence of autophagosomes visualized as LC3-positive dots (Figures 7C and 7D). Interestingly, electron microscopy of TRAF6 KO EFs under basal or amino acid starvation conditions showed an increase in autophagosomes, as compared to WT controls (Figure 7E). Treatment of these cells with lysosomal inhibitors ammonium chloride and leupeptin during leucine starvation also increased accumulation of LC3-II, consistent with enhanced autophagic flux in TRAF6 KO cells (Figures 7F and 7G). Importantly, increased LC3-II accumulation, along with mTORC1 inhibition as measured by pS6K, was also detected in livers of liver-specific TRAF6 KO mice (TRAF6^{fl/fl} Alb-cre⁺) as compared to WT control mice (TRAF6^{fl/fl} Alb-cre⁻) (Figures 7H and 7I). Altogether, these results suggest that TRAF6 modulates autophagy in response to nutrient starvation, consistent with its role in the regulation of mTORC1 activation. Since p62 is essential for TRAF6 function in the mTORC1 cascade, we next tested whether TRAF6-p62 interaction was also required for its role in autophagy. Interestingly, the results in Figure 7J demonstrate that expression of the GST-TB construct, which disrupts the TRAF6-p62 interaction, was able to induce autophagy as measured by LC3-II accumulation. These results suggest that p62 acts as a scaffold for TRAF6 that modulates its E3 ubiquitin ligase activity and confers specificity in response to nutrients.

DISCUSSION

How cells coordinate growth with nutrient availability is a fundamental issue in cell biology. Cells need to adapt and respond to changes in nutrient status to maintain cell growth and metabolic homeostasis and to cope with stress. Deregulation of the cellular response to nutrients influences tumorigenesis as well as metabolic diseases (Laplanche and Sabatini, 2012; Yuan et al., 2013; Zoncu et al., 2011b). mTORC1 is a pivotal nutrient-sensitive

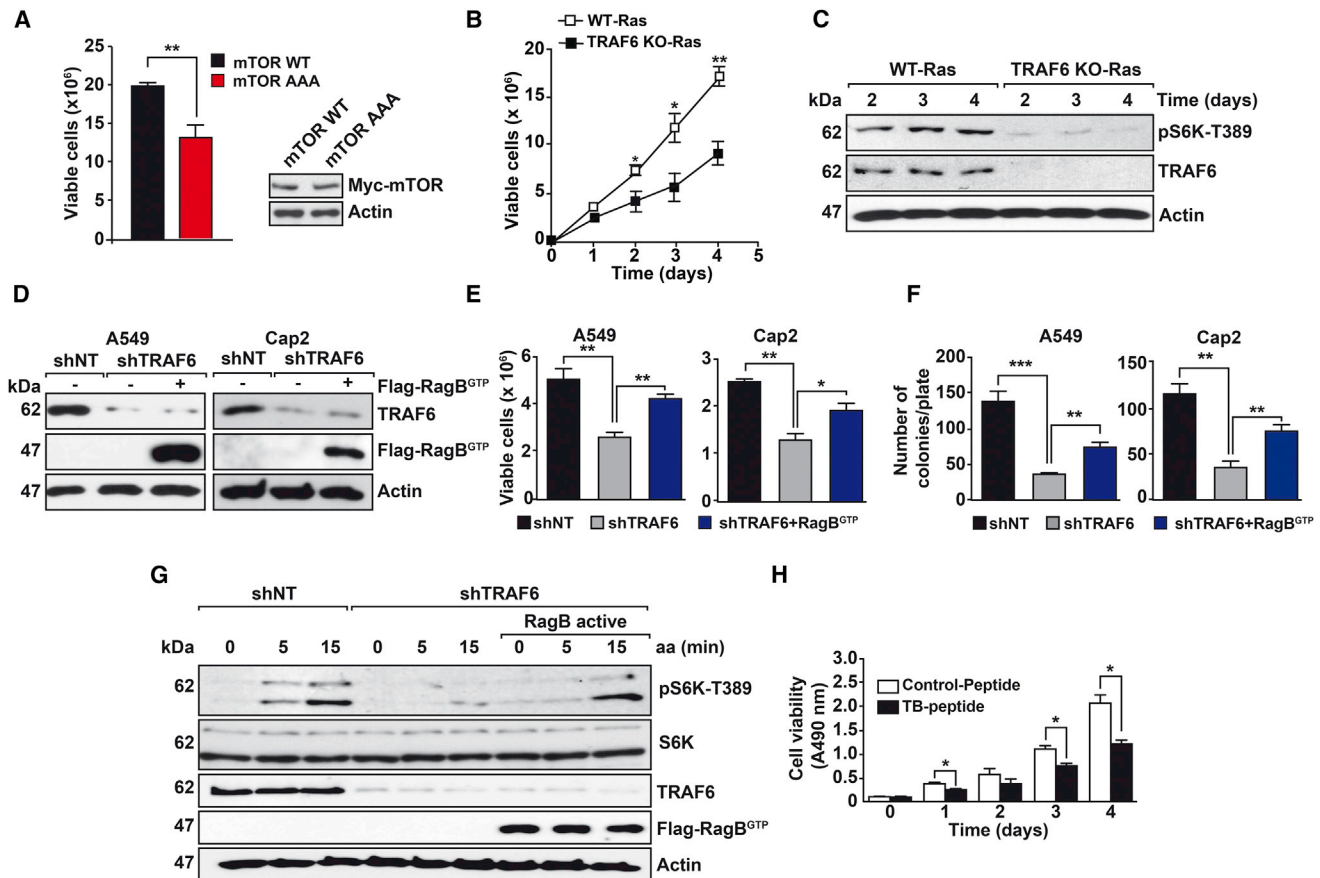


Figure 6. TRAF6 Drives Cell Proliferation through Activation of the mTORC1 Pathway

(A) HEK 293T cells transfected with the indicated plasmids were cultured under normal growing conditions, and cell viability was determined by trypan blue exclusion assay. Results are shown as means \pm SEM ($n = 3$).

(B) Proliferation curve of WT and TRAF6 KO MEFs infected with HA-RASV12 was determined as above. Results are shown as means \pm SEM ($n = 3$).

(C) Cell lysates from (B) were analyzed by western blot.

(D–F) Cap2 and A549 cells stably expressing Flag-RagB^{GTP} were infected with shNT or shTRAF6 lentiviral vectors. (D–F) Cell lysates were analyzed by western blot (D), cell viability was determined (E), and colony formation assays were performed (F). Results are shown as means \pm SEM ($n = 3$).

(G) A549 cells expressing Flag-RagB^{GTP} and infected with shNT or shTRAF6 lentiviral vectors were deprived of amino acids and serum for 4 hr and stimulated with amino acids. Cell lysates were analyzed by western blot.

(H) Cap2 cells were treated with a control or TRAF6 inhibitory peptide (150 μ M). Cell viability under normal growing conditions was determined by MTT assay. Results are shown as means \pm SEM ($n = 4$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See also Figure S6.

kinase complex that responds to glucose, energy, growth factors, and amino acids (Howell and Manning, 2011; Laplante and Sabatini, 2012). In this regard, we have previously shown that p62 is a critical component of mTORC1's response to amino acids (Duran et al., 2011; Moscat and Diaz-Meco, 2011). p62 interacts with raptor and the Rags, favoring Rag complex formation and, ultimately, activation of mTORC1 at the lysosome (Duran et al., 2011). Because p62 acts as a cell signaling hub (Moscat and Diaz-Meco, 2009a), it was important to determine whether any of the p62 domain-specific complexes participated in the regulation of mTORC1. Our previous results demonstrated that the PB1-mediated p62 partners, including the aPKCs (PKC ζ or PKC λ/ι) and NBR1, were dispensable for amino acid-induced mTORC1 activation (Duran et al., 2011). Therefore, we investigated here the potential role of another critical p62 partner, TRAF6, in sensing amino acids.

p62 binds to TRAF6, which is important for p62's role in the activation of NF- κ B in different in vitro and in vivo systems (Duran et al., 2008; Durán et al., 2004; Martin et al., 2006; Sanz et al., 2000; Wooten et al., 2005). These studies underscored the importance of p62 as a scaffold for TRAF6-dependent polyubiquitination (Moscat et al., 2007). Here we show that the interaction of TRAF6 with p62 is a key step in activation of the mTORC1 complex. Furthermore, amino acids promote, in a TRAF6-dependent manner, the K63-mediated polyubiquitination of mTOR, which is instrumental in its activation and function. In this regard, previous reports have pointed to the role of ubiquitin in the mTOR cascade, both upstream and downstream of mTOR, but these examples all involved K48 ubiquitination, which controls protein stability and degradation of the different components of the mTOR pathway. These components included mTOR itself, which undergoes ubiquitin-mediated degradation by

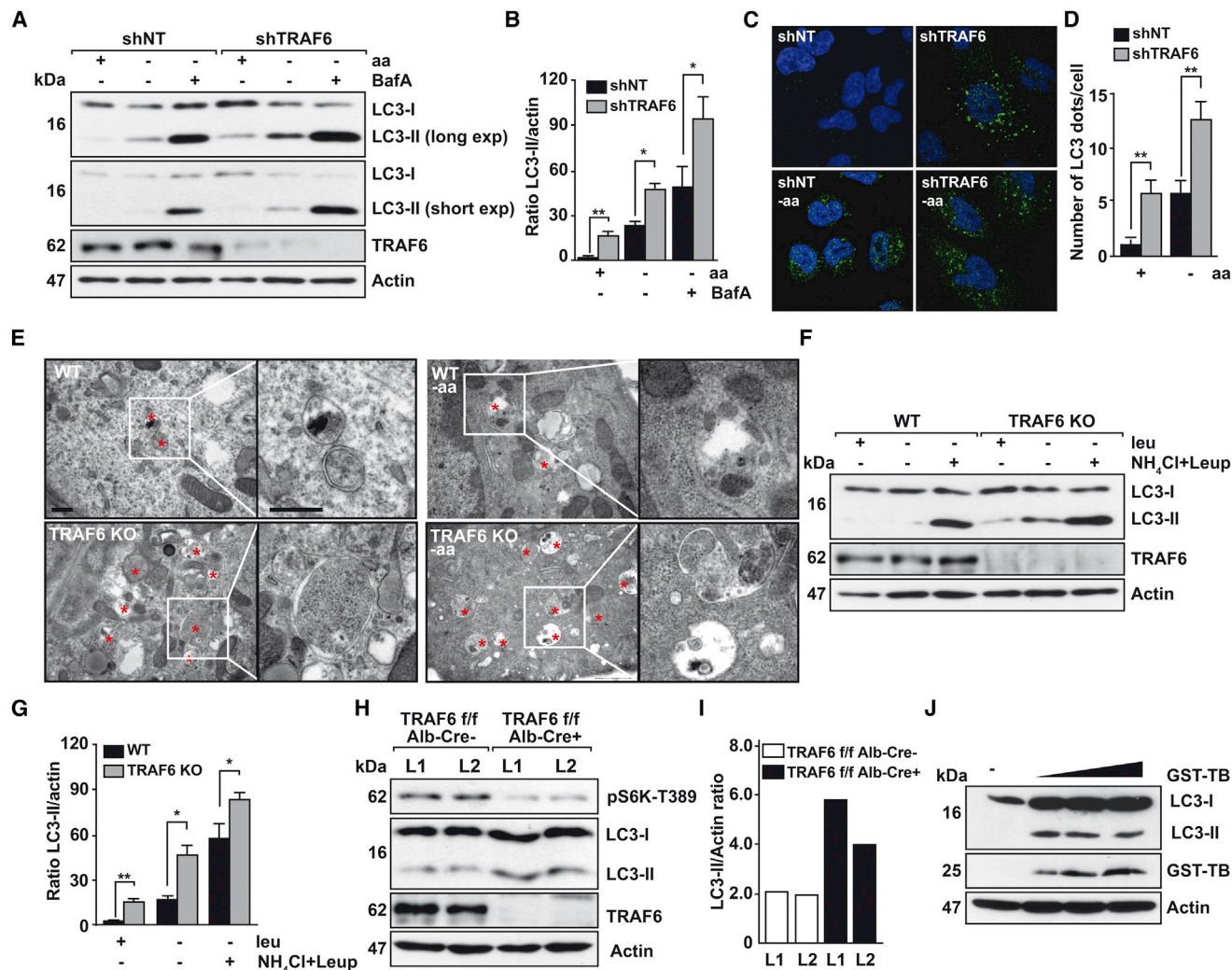


Figure 7. TRAF6 Influences Autophagy in Response to Nutrients

(A) A549 cells were deprived of amino acids and serum for 4 hr in the absence or presence of bafilomycin. Cell lysates were analyzed by western blot.

(B) Graph represents LC3-II/actin ratio by densitometry and shows means \pm SEM (n = 2).

(C) Images of A549 cells treated as in (A) and coimmunostained for LC3 and DAPI.

(D) Quantification of the number of vesicles/cell is shown. The graphs show means \pm SEM (n = 10).

(E) Representative transmission electron micrographs of WT and TRAF6 KO MEFs under basal or amino acid-starved conditions. Asterisk indicates autophagosome. Scale bar = 0.5 μ m.

(F) WT and TRAF6 KO MEFs were deprived of leucine and serum for 4 hr in the absence or presence of NH₄Cl and leupeptin. Cell lysates were analyzed by immunoblot.

(G) Graph represents LC3-II/actin ratios by densitometry and shows means \pm SEM (n = 2). *p < 0.05, **p < 0.01.

(H) Protein extracts of livers of 24 hr fasted liver-specific TRAF6 KO mice (TRAF6^{f/f}Alb-cre⁻) and WT control mice (TRAF6^{f/f}Alb-cre⁺) were analyzed by immunoblot.

(I) Graph represents the LC3-II/actin ratios of (H) by densitometry.

(J) HEK 293T cells were transfected with increasing amounts of GST-TB. Cell lysates were analyzed by immunoblotting to determine levels of the specified proteins.

Cul1-Skp1-Fbw7 E3 ligase, and also TSC2, DEPTOR, REDD1, and IRS1 (Gao et al., 2011; Hu et al., 2008; Katiyar et al., 2009; Mao et al., 2008; Xu et al., 2008; Zhao et al., 2011). However, by utilizing TRAF6, p62 modulates K63-linked ubiquitination of mTOR to regulate its kinase activity, but not its levels. This provides evidence of a mechanism whereby mTOR senses the cell's nutrient status, specifically with regard to amino acids.

Importantly, mTOR ubiquitination by the p62-TRAF6 complex is central to regulating cell growth, tumor transformation, and autophagy. In this regard, p62 is constitutively degraded by autophagy (Bjorkoy et al., 2005; Pankiv et al., 2007). This may be an important mechanism of quality control to eliminate misfolded proteins or damaged mitochondria or a way to keep p62 levels low under conditions of nutrient scarcity

(Moscat and Diaz-Meco, 2009a, 2009b). Notably, recent data show that in cells undergoing nutrient deprivation, TRAF6 in complex with the autophagy protein AMBRA1 serves to promote K63 polyubiquitination and activation of ULK1, a critical positive regulator of autophagy that is inactivated by mTOR-induced phosphorylation in conditions of plentiful nutrients (Nazio et al., 2013). However, under normal nutrient conditions, mTORC1 phosphorylates AMBRA and prevents its involvement in the TRAF6-mediated activation of ULK1 (Nazio et al., 2013). Therefore, TRAF6 plays dual and opposite roles in autophagy. On the one hand, through p62 and K63 polyubiquitination of mTOR, TRAF6 activates mTORC1 and inhibits autophagy when amino acids are available. On the other hand, under conditions of nutrient stress, the AMBRA1-TRAF6 complex promotes K63-mediated polyubiquitination and activation of ULK1 and consequently induces autophagy. Thus, TRAF6 serves as the catalytic regulator of the two opposite ubiquitination processes under different nutritional conditions. Interestingly, TRAF6 overexpression has been shown to induce autophagy, likely through AMBRA, as well as through the direct polyubiquitination of beclin 1 (Shi and Kehrl, 2010). These data are apparently contradictory to the fact that increased autophagy was observed in TRAF6-deficient cells under basal nutrient-proficient conditions. A potential explanation could be that overexpression of TRAF6 could bypass the blockade of autophagy imposed by mTORC1 basal activity by directly targeting beclin 1, irrespective of the state of the upstream step controlled by ULK1. In contrast, TRAF6 deficiency will trigger autophagy by inhibiting mTORC1. This could mean that under conditions of mTORC1 inactivation by TRAF6 deficiency, autophagy can proceed even in the absence of TRAF6, suggesting compensation by other potential pathways.

Collectively, these observations unveiled a role for TRAF6 in metabolism in addition to its better-known functions in inflammation (Chen, 2012). Interestingly, other mTORC1-independent metabolic connections have also been reported for TRAF6. That is, TRAF6 is a direct E3 ligase for Akt, being essential for Akt ubiquitination, membrane recruitment, and phosphorylation upon growth factor stimulation (Yang et al., 2009). Therefore, TRAF6 acts upstream of mTORC1 in two ways: through p62 during nutrient sensing or through the Akt-TSC pathway in response to insulin and growth factors. The TRAF6 adaptor for the Akt pathway remains to be identified.

Finally, by mapping the domain of p62 responsible for TRAF6 binding, we identified a functional region that discriminates the different functions of p62 and thus opened avenues for selectively blocking mTORC1 signaling in response to amino acids. Interestingly, expression of this targeted domain is sufficient to block mTORC1 and to induce autophagy. In this regard, the development of autophagy-inducing peptides as therapeutic candidates has recently been shown to be an attractive strategy to exploit autophagy in the treatment of pathologies such as cancer and infectious diseases (Shoji-Kawata et al., 2013). Intervention at the level of p62-TRAF6 with such peptides, or drugs based on these peptides, may have the potential to treat diseases in which mTORC1 has been shown to play a relevant role.

EXPERIMENTAL PROCEDURES

Mice

Liver-specific TRAF6 KO mice (TRAF6^{fl/fl}Alb-cre⁺) and littermate control mice (TRAF6^{fl/fl}Alb-cre⁻), all 8 weeks old, were fasted for 24 hr. Animal handling and experimental procedures conformed to institutional guidelines (Institutional Animal Care and Use Committee, SBMRI). See also [Supplemental Experimental Procedures](#).

Materials, Plasmids, Cell Culture, Cell Lysis, and Immunoprecipitations

See the [Supplemental Experimental Procedures](#).

Ubiquitin Detection and In Vitro Ubiquitination Assay

Detection of endogenous in vivo mTOR ubiquitination was performed in mTOR immunoprecipitates as described in detail in the [Supplemental Experimental Procedures](#).

In Vitro mTOR Activity

In vitro kinase mTOR activity was determined in cell extracts using the K-LISA mTOR Activity Kit. See also [Supplemental Experimental Procedures](#).

Statistical Analysis

Significant differences between groups were determined using Student's *t* test. Significant differences are as follows: **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. All experiments were performed at least two or three times.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.molcel.2013.06.020>.

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