



Published in final edited form as:

Cell Rep. 2015 September 1; 12(9): 1445–1455. doi:10.1016/j.celrep.2015.07.052.

## Disruption of the Rag-Ragulator complex by c17orf59 inhibits mTORC1

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### SUMMARY

mTORC1 controls key processes that regulate cell growth, including mRNA translation, ribosome biogenesis and autophagy. Environmental amino acids activate mTORC1 by promoting its recruitment to the cytosolic surface of the lysosome, where its kinase is activated downstream of growth factor signaling. mTORC1 is brought to the lysosome by the Rag GTPases, which are tethered to the lysosomal membrane by Ragulator, a lysosome-bound scaffold. Here, we identify c17orf59 as a Ragulator-interacting protein that regulates mTORC1 activity through its interaction with Ragulator at the lysosome. The binding of c17orf59 to Ragulator prevents Ragulator interaction with the Rag GTPases, both in cells and *in vitro*, and decreases Rag GTPase lysosomal localization. Disruption of the Rag-Ragulator interaction by c17orf59 impairs mTORC1 activation by amino acids by preventing mTOR from reaching the lysosome. By disrupting the Rag-Ragulator interaction to inhibit mTORC1, c17orf59 expression may represent another mechanism to modulate nutrient sensing by mTORC1.

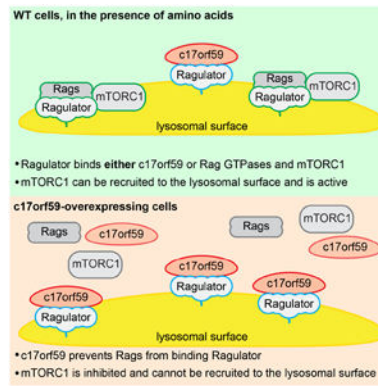
### Abstract

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#### AUTHOR CONTRIBUTIONS:

L.D.S. and D.M.S. designed the experiments. L.D.S. and W.C.C. carried out experiments. L.B-P. designed the initial strategy for cloning the c17orf59 cDNA. L.D.S. wrote and D.M.S. edited the manuscript.

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Amino acids induce recruitment of mTORC1 to the lysosomal surface through the Rag GTPases, which are tethered to the lysosomal membrane by Ragulator. c17orf59 interacts with Ragulator, and overexpression disrupts the Rag-Ragulator complex, preventing Rag lysosomal localization and leading to a failure to recruit mTORC1 in the presence of amino acids.

## INTRODUCTION

The mechanistic Target of Rapamycin Complex 1 (mTORC1) balances the metabolic state of the cell with environmental conditions. mTORC1 positively regulates anabolic processes to promote growth, and also controls the availability of the building blocks required for these processes by controlling catabolic pathways. The anabolic processes regulated by mTORC1 include mRNA translation, ribosomal biogenesis, and lipid and nucleotide biosynthesis. Autophagy and proteasomal degradation are the major catabolic degradation pathways influenced by mTORC1 activity (Laplante and Sabatini, 2012; Zhang et al., 2014).

Because mTORC1 activity promotes growth processes, which are energy intensive and vital for cell division and the functions of terminally differentiated cells, several aspects of cellular health control mTORC1 activation. Intracellular conditions, including low energy levels, DNA damage, and other stress signals regulate mTORC1, in addition to the environmental milieu. Environmental cues that control mTORC1 activity represent the general nutritional status of the cell or organism so that mTORC1 is activated by growth factors and nutrients, including amino acids and glucose, and is inhibited under hypoxia (Zoncu et al., 2011; Shimobayashi and Hall, 2014; Jewell and Guan, 2013; Gomes and Blenis, 2015; Zhang et al., 2014).

The mTORC1 pathway integrates different environmental signals by distinct mechanisms, well-exemplified by the contrast in pathway activation by growth factors as compared to nutrients. Growth factors activate mTORC1 downstream of canonical phosphatidylinositol-3-kinase (PI3K)/Akt signaling. Growth factor-stimulated Akt activates the small GTPase Rheb by inhibiting its GTPase activating protein (GAP), tuberous sclerosis complex (TSC). GTP-bound Rheb activates the mTOR kinase activity (Tee et al., 2003; Inoki et al., 2003; Inoki et al., 2002; Garami et al., 2003; Long et al., 2005; Manning et al., 2002; Zhang et al., 2003). However, in order for Rheb to control the kinase activity of mTORC1, it must

interact with the mTORC1 complex. This interaction is regulated independently of TSC/Rheb activity, and is controlled by intracellular amino acid and glucose availability (Sancak et al., 2008; Sancak et al., 2010; Efeyan et al., 2013; Long et al., 2005 (2); Kim et al., 2008; Smith et al., 2005; Hara et al., 1998; Lynch., 2000; Nobukuni et al., 2005). Specifically, Rheb resides at the cytosolic face of late endosomal and lysosomal membranes (Menon et al., 2014), and mTORC1 localizes to these organelles in an amino acid- and glucose-dependent manner in order to be activated.

The Rag GTPases regulate mTORC1 localization and recruitment to lysosomes in response to intracellular nutrient availability (Sancak et al., 2008; Kim et al., 2008; Sancak et al., 2010). The Rags are small GTPases encoded by four separate genes (RRAGA, -B, -C, and -D; coding for RagA, -B, -C, and -D), which form heterodimers in which a single RagA or RagB protein is paired with a single RagC or RagD. The Rags localize to the cytoplasmic surface of the lysosomal membrane (Sancak et al., 2008; Sancak et al., 2010). Evidence suggests that the nucleotide-bound state of each Rag protein in the dimer tends to be opposite that of its partner (Sancak et al., 2008). In the absence of amino acids, the RagA/B component of the dimer binds GDP and the RagC/D component binds GTP, and the dimer does not bind mTORC1 and therefore cannot recruit it to the lysosome. Within minutes of the addition of amino acids to the media of starved cells, the nucleotide-bound state is reversed such that RagA/B is GTP-bound and RagC/D is GDP-bound, leading to the binding and lysosomal recruitment of mTORC1. Expression of “active” Rag dimer mutants where RagA or B mimics the GTP-bound state (RagA/B<sup>GTP</sup>) is sufficient to constitutively localize mTORC1 to the lysosomal membrane and activate mTORC1, even in the absence of amino acids. Conversely, mTORC1 activity is inhibited by “inactive” Rag mutant dimers consisting of RagA/B<sup>GDP</sup> even when amino acids are present (Sancak et al., 2008; Kim et al., 2008).

The Ragulator complex maintains the Rags on the lysosome, and serves as a lysosomal docking site for the Rag dimers. Five proteins make up Ragulator: p18, p14, MP1, HBXIP, and c17orf59 (Wunderlich et al., 2001; Nada et al., 2009; Sancak et al., 2010; Bar-Peled et al., 2012). The complete Ragulator complex is required for mTORC1 activation by amino acids and for the appropriate lysosomal localization of the Rags and mTORC1. Loss of any component of Ragulator inhibits mTORC1 activation by amino acids and mis-localizes both the Rags and mTORC1 away from the lysosomes, even when amino acids are present (Sancak et al., 2010; Bar-Peled et al., 2012). Ragulator fulfills two major functions in mTORC1 activation: it acts as a scaffold and landing pad for the Rags and mTORC1 at the lysosome, and serves as the guanine nucleotide exchange factor (GEF) for RagA and RagB, activating the mTORC1 pathway in response to amino acids (Bar-Peled et al., 2012).

In recent years we have searched for novel regulators of the mTORC1 pathway, specifically of the amino acid sensing arm. Here, we describe the identification of c17orf59 as a Ragulator-interacting protein and its role in the regulation of mTORC1 activation. c17orf59 binds to Ragulator, but not the Rags, and inhibits the Rag-Ragulator interaction and mTORC1 activity when overexpressed. We find that c17orf59 is a component the pathway that regulates the nutrient-sensing arm of mTORC1 and represents an alternative mechanism

to inhibit mTORC1 via disruption of the interaction between the Rag GTPases and Ragulator.

## RESULTS

### c17orf59 interacts with Ragulator but not the Rag GTPases

In order to identify proteins that may play a role in the amino sensing pathway upstream of mTORC1, we used immunoprecipitation of epitope-tagged Ragulator components followed by mass spectrometry (IP/MS). Immunoprecipitation of the FLAG-tagged, stably-expressed Ragulator subunit HBXIP specifically yielded peptides corresponding to c17orf59 in addition to the Rag GTPases and other known members of Ragulator complex (Figure 1A). Consistent with this finding, mass spectrometric analysis of immunoprecipitates of stably-expressed, epitope-tagged c17orf59 contained peptides corresponding to all Ragulator subunits, including p18, p14, MP1, HBXIP, and c7orf59. Interestingly, no peptides were identified corresponding to any Rag protein, suggesting that c17orf59 only interacts with Ragulator (Figure 1A).

To confirm the mass spectrometry data, we analyzed immunoprecipitates of c17orf59, Ragulator, and the Rags by western blotting. Stably-expressed, epitope-tagged c17orf59 co-immunoprecipitated all subunits of Ragulator, to levels comparable with epitope-tagged p14 (Figure 1B). While, as expected, p14 interacted with RagA and RagC, c17orf59 did not co-immunoprecipitate either Rag GTPase (Figure 1B). In reciprocal immunoprecipitations, epitope-tagged RagB co-immunoprecipitated Ragulator subunits, but not c17orf59 (Figure 1C).

We used *in vitro* binding assays to test whether c17orf59 binds directly to Ragulator, as opposed to an indirect interaction that depends on other proteins or components of the cell. In these assays, purified Ragulator bound c17orf59 (Figure 1D). Purified Rags failed to interact with purified c17orf59, even when Ragulator was present (Figure 1D). This confirms that c17orf59 likely interacts directly with members of Ragulator and that the Rags do not interact with c17orf59.

### c17orf59 localizes to the lysosome along with Ragulator

Ragulator localizes to lysosomes and late endosomes by virtue of lipid modifications and targeting sequences on the N-terminus of p18 (Sancak et al., 2010, Nada et al., 2009). Consistent with its interaction with Ragulator, HA-tagged c17orf59 co-localizes with the lysosomal marker LAMP2 (Figure 2A) indicating its presence at lysosomes. To determine the extent of co-localization between c17orf59 and Ragulator, we re-expressed the cDNA for p18 in p18-null MEFs and examined the localization of c17orf59 and p18. Cells expressing HA-tagged c17orf59 display a highly significant co-localization with p18 (Figure 2B, Supplemental Figure 1A and 1B). c17orf59 also co-localizes with another Ragulator subunit, LAMTOR4, in a p18-dependent manner (Supplemental Figure 1C), further supporting the existence of a c17orf59-Ragulator interaction. The subcellular localization of c17orf59 was unaffected by the presence or absence of amino acids or insulin (Figure 2C and 2D).

### **c17orf59 loss does not alter mTORC1 activity in response to amino acids or insulin**

To examine the effects of loss of c17orf59 on mTORC1 activation, we generated c17orf59-null HEK-293E and HeLa cells using the sgRNA/Cas9 system and reconstituted c17orf59 with expression of its cDNA driven by the c17orf59 promoter. The c17orf59-null cells showed no signaling defects in response to amino acid or serum starvation and re-stimulation, as compared to non-targeting sgRNA or c17orf59-null cells reconstituted with c17orf59 (Figure 3A and 3B, Supplemental Figure 2A and 2B), even when intermediate doses of either amino acids or insulin were added back to cells (Supplemental Figure 2C and 2D). In addition, c17orf59 null cells had no alterations in mTORC1 signaling in response to cholesterol deprivation, the only known stimulus that alters c17orf59 expression (Bartz et al., 2009) (Supplemental Figure 2E). Despite its interaction with Ragulator, loss of c17orf59 does not cause alterations in mTORC1 activation by amino acids or insulin. This lack of signaling phenotype was consistent across multiple clones of c17orf59-null cells using multiple guides in both HEK-293E and HeLa cells, as well as using shRNA-mediated knockdown of c17orf59 (data not shown), so we are confident that the results are not the product of re-wiring in the single cell clones that became the c17orf59-null cells. Based on these results, we tested the effects of c17orf59 overexpression on Ragulator function and mTORC1 activity.

### **c17orf59 disrupts the Rag-Ragulator interaction in cells and *in vitro***

The interaction between c17orf59 and Ragulator, but not the Rags, implies that there is a subset of the cellular pool of Ragulator that does not interact with the Rags. As some of this pool interacts with c17orf59, it is possible that increasing the amount of the c17orf59-Ragulator complex could lead to the loss of the Rag-Ragulator interaction by titrating away Ragulator from the Rags. We tested this hypothesis by overexpressing c17orf59 transiently in cells expressing either FLAG-tagged p14 or RagB.

As expected, Ragulator subunits p18 and p14 both co-immunoprecipitated with purified FLAG-tagged RagA when a control protein (Methionine aminopeptidase 2, Metap2) was overexpressed. As expected, Raptor, the defining subunit of mTORC1, also co-immunoprecipitated with FLAG-RagA. However, overexpression of c17orf59 disrupted the Rag-Ragulator complex so that RagA no longer co-immunoprecipitated p18, p14, or Raptor to the same extent, indicating a loss of binding to both Ragulator and mTORC1 (Figure 4A). Conversely, immunoprecipitated Ragulator interacted with RagA and RagC, as well as Raptor, but these interactions diminished upon c17orf59 overexpression (Figure 4B).

To examine if c17orf59 can alter the Rag-Ragulator interaction in a cell-free setting, we used *in vitro* binding assays with purified proteins. Much like in cells, when purified c17orf59 was pre-incubated with the GST-Ragulator, there was a dose-dependent decrease in the amount of purified Rags that bound to immobilized Ragulator (Figure 4C). In a similar experiment using immobilized, GST-tagged RagB with RagC, increasing amounts of purified c17orf59 decreased Ragulator binding to the Rags (Figure 4D).

Because Ragulator is required for the lysosomal localization of the Rag GTPases, it is possible that the disruption of the Rag-Ragulator interaction due to c17orf59 overexpression

results in a mis-localization of the Rag GTPases away from the lysosome. To test this, we transiently expressed FLAG-tagged c17orf59 in HEK-293T cells, marking cells that were transfected using GFP driven by an internal ribosome entry sequence (IRES) downstream of the c17orf59 cDNA. The amount of RagC that co-localizes with the lysosomal marker LAMP1 decreases in cells that overexpress c17orf59, but not FLAG-tagged GFP alone (Figure 4E, GFP-positive cells). Importantly, overexpression of c17orf59 does not alter the localization of Ragulator component LAMTOR4 (Supplemental Figure 3A) indicating that Ragulator is still intact and present at lysosomes.

In summary, c17orf59 disrupts the Rag-Ragulator complex in cells as well as *in vitro* resulting in a reduction in the lysosomal localization of the Rag GTPases. *In vitro* binding of either Rags or c17orf59 to Ragulator implies that c17orf59 has the ability to directly compete with the Rags for binding to Ragulator, producing a Rag- and mTORC1-free Ragulator-c17orf59 complex.

### c17orf59 overexpression inhibits mTORC1

We hypothesized that because c17orf59 can disrupt the Rag-Ragulator interaction and RagC localization, c17orf59 overexpression would inhibit mTORC1 activation by amino acids. Indeed, overexpression of c17orf59 produced a dose-dependent inhibition of mTORC1 activation in response to amino acid stimulation, to a level comparable to the inhibition produced by dominant-negative RagB<sup>GDP</sup> expression (Figure 5A).

To confirm that overexpression of c17orf59 inhibits mTORC1 through the nutrient sensing machinery and through the disruption of the Rag-Ragulator interaction, we examined mTOR localization in cells that overexpress c17orf59. Using the FLAG-c17orf59-IRES-GFP construct described above, mTOR remained largely diffuse upon stimulation with amino acids in c17orf59-expressing HEK-293T cells (Figure 5B, GFP-positive cells in the top panel). mTOR localizes to lysosomes in control cells that either were not transfected or expressed FLAG-GFP alone (Figure 5B, GFP-negative cells in the top panel or GFP-positive cells in the bottom panel).

If the c17orf59 overexpression-mediated disruption of the Rag-Ragulator interaction represents the mechanism of mTORC1 inhibition by c17orf59, overexpression of c17orf59 should inhibit mTORC1 even in cells that express the dominant-active RagB<sup>GTP</sup> mutant. Under normal conditions, dominant-active RagB<sup>GTP</sup> constitutively recruits mTORC1 to the lysosome. The interaction between Rags and Ragulator is required to bring mTORC1 to the lysosome; disruption of the Rag-Ragulator complex by c17orf59 would act in an epistatic nature to the normally activating effect of RagB<sup>GTP</sup>. Without Rag binding to Ragulator, the Rag mTORC1 will not be recruited to the lysosome.

We overexpressed c17orf59 in cells stably expressing RagB<sup>Q99L</sup>, the RagB<sup>GTP</sup> mutant, or a control protein, Metap2. Overexpression of c17orf59 diminished phosphorylation of S6K1 in a dose-dependent manner in both constitutively active, RagB<sup>GTP</sup>-expressing and control cells (Figure 5C), indicating that c17orf59 overexpression inhibits mTORC1 signaling even in the presence of the active Rags, likely due to the loss of lysosomal Rag localization.

## DISCUSSION

We find that c17orf59 is a Ragulator-interacting protein. c17orf59 interacts with Ragulator in a manner that competes with the Rag GTPases for binding, both in cells and *in vitro*. Loss of the Rag-Ragulator interaction due to c17orf59 overexpression decreases binding between the Rags and mTORC1 as well as Ragulator and mTORC1 and prevents both the Rags and mTORC1 from binding to the lysosomal membrane. Concomitant to the loss of the Ragulator-Rag-mTORC1 interactions, overexpression of c17orf59 inhibits mTORC1 activity in response to amino acid availability. RagA<sup>GTP</sup> expression, which normally induces constitutively active amino acid signaling by maintaining mTORC1 on the lysosome, is not sufficient to rescue the signaling defect downstream of c17orf59 overexpression, indicating that high levels of c17orf59 prevent productive interactions between the Rags, Ragulator and mTORC1 in a Rag nucleotide state-independent manner, likely by preventing the Rags from localizing to the lysosome.

Identifying and characterizing proteins that interact with Ragulator or other components of the amino acid-sensitive mTORC1 signaling pathway is important for understanding how eukaryotic cells sense amino acids and other nutrients that regulate mTORC1. Ragulator was originally described as the lysosomal scaffold for the Rags (Sancak et al., 2010) and upon identification of additional components of the Ragulator (HBXIP and c7orf59) we found that the scaffold also acts as a GEF for the Rags (Bar-Peled et al., 2012). It is likely that as more Ragulator-interacting proteins are uncovered, additional ways that Ragulator can control mTORC1 will be found; here c17orf59 competes with the Rags to bind Ragulator.

c17orf59-mediated inhibition of the Rag-Ragulator interaction represents an alternative mechanism of mTORC1 inhibition. Potential negative regulators of the amino acid sensing pathway have been reported to act directly upon the Rag GTPases or their regulatory proteins to alter their nucleotide-bound state (e.g. GATOR [Bar-Peled et al., 2013; Panchaud et al., 2013], Sestrins [Parmigiani et al., 2014; Chantranupong et al., 2014; Peng et al., 2014], Leucyl-tRNA synthetase [Han et al., 2012; Bonfils et al., 2012], SH3BP4 [Kim et al., 2012]). Interestingly, the mechanism of mTORC1 inhibition by c17orf59 is independent of the Rag nucleotide-bound state, implying that regardless of the presence of nutrients, c17orf59 can inhibit the Rag-mTORC1 interaction and prevent mTORC1 from being activated by Rheb.

The disruption of the Rag-Ragulator interaction by c17orf59 also suggests the possibility for the design of a new mTORC1 inhibitor. That c17orf59 can prevent the Rags from binding to Ragulator *in vitro*, as well as in cells, implies that the binding site between the two complexes is an accessible surface that could be the target of a small molecule or peptide. A molecule that mimics the presence of c17orf59 could provide an mTORC1-specific inhibitor that inhibits mTORC1 completely without affecting mTORC2. An inhibitor of only mTORC1 would be a useful research tool to determine the distinct functions of the complex in cells. Currently, mTOR kinase inhibitors target both mTORC1 and mTORC2 by virtue of the shared kinase between the two complexes (Thoreen et al., 2009; Feldman and Shokat, 2010) and rapamycin, which was originally thought to be mTORC1-specific, is a compromised tool to interrogate mTORC1 activity because it only partially inhibits

mTORC1 and can inhibit mTORC2 assembly upon prolonged treatment (Thoreen et al., 2009; Feldman et al., 2009; Sarbassov et al., 2006). Peptides derived from c17orf59 could potentially inhibit mTORC1; alternatively a small molecule that binds to the same, accessible surface on the structure of Ragulator that c17orf59 and, likely, the Rags bind to could be an effective mTORC1 inhibitor.

The binding of c17orf59 to the Ragulator in a Rag-independent manner and the inhibition of mTORC1 activity by c17orf59 overexpression suggest that c17orf59 is a negative regulator of the nutrient-sensing pathway. However, loss of c17orf59 did not result in detectable defects in mTORC1 signaling in response to starvation of or re-stimulation by amino acids or insulin in HEK-293E and HeLa cell lines. c17orf59-null cells also displayed no alterations in the inhibition of mTORC1 by cholesterol deprivation, the only reported regulator of c17orf59 expression. It is possible that other conditions may inhibit mTORC1 in a manner that depends on either an increase in c17orf59 expression or promotes c17orf59 binding to Ragulator.

c17orf59 is present in and binds to Ragulator in the cell lines used in this work, however, it is possible that there are cell types that express c17orf59 at a higher level, which would allow for greater levels of binding and inhibition of mTORC1 and might manifest stronger response to c17orf59 loss or perturbation.

It is also possible that c17orf59 is not a negative regulator of the mTORC1 pathway, but rather that the c17orf59-Ragulator complex has mTORC1-independent functions. c17orf59 could have a role in a different, unidentified pathway that also utilizes Ragulator. If this were the case, Ragulator would have mTORC1-independent functions when bound to c17orf59 and not the Rags. This would not be completely unprecedented, as other components of the nutrient-sensing pathway serve other functions in the cell. We have previously shown that the v-ATPase is a Ragulator-binding complex that regulates mTORC1 signaling but also functions as a major proton pump to maintain the acidic nature of the lysosome as well as other organelles, and that the maintenance of pH may be decoupled from the role of the v-ATPase in mTORC1 activation (Zoncu et al., 2011). Similarly, GATOR2, which acts is upstream of the GAP for RagA and B (GATOR1) and is required for amino acid signaling to mTORC1, includes Sec13 and Seh1L (Bar-Peled et al., 2013), which serve multiple other functions in the cell. Sec13 is a COPII component required for the generation of vesicles that traffic from the endoplasmic reticulum and Golgi and also participates in the nuclear pore complex (Devos et al., 2004; Gurkan et al., 2006; Brohawn et al., 2009). Seh1L is also a component of the nuclear pore complex, where it binds directly to Sec13 (Brohawn et al., 2009).

One potential function for the c17orf59-Ragulator complex could be the recently described BLOC-1-related complex (BORC), which contains c17orf59 and controls lysosomal positioning (Pu et al., 2015). It is unclear how the assembly of BORC is regulated within the cell or if this complex regulates mTORC1, but the authors imply that Ragulator interacts with BORC subunits (Pu et al., 2015), which would indicate a role for the c17orf59-Ragulator complex in lysosomal positioning as a part of BORC. This is consistent with literature that Ragulator components are important for maintaining endolysosomal



morphology and biogenesis (Teis et al., 2006; Nada et al., 2009; Takahashi et al., 2012; Vogel et al., 2015) and consistent with our observed alterations in lysosomal staining in cells that overexpress c17orf59 (Figure 4E and Figure 5B). It is possible that Ragulator binds to c17orf59 and BORC in a Rag- and mTORC1-independent manner to control endosome and lysosome morphology.

Cholesterol deprivation has been shown to cause a mild increase in c17orf59 expression in the only previously reported regulation or function of c17orf59 (Bartz et al., 2009). When we deprived cells of cholesterol, we saw a mild upregulation of c17orf59 at the protein level, in accordance with the previously reported regulation of c17orf59, potentially by the Sterol Responsive Element Binding Protein (SREBP) (Bartz et al., 2009).

It remains to be determined if c17orf59 is indeed a negative regulator of the nutrient-sensing pathway, or if it is a component of a Ragulator-containing pathway that acts independently of mTORC1. Regardless of the physiological role of c17orf59 in the cell, we have uncovered an additional cellular mechanism for mTORC1-specific inhibition, namely by altering the Rag-Ragulator interaction.

## MATERIALS AND METHODS

### Reagents

The following antibodies were obtained from Cell Signaling Technologies (CST): antibodies against Phospho-T389-S6K (#9206 and #9205), S6K1 (9202 and 2708), mTOR (#293), c17orf59/LAMTOR4 (#12284 for blotting; #13140 for immunofluorescence), p18 (#8975), MP1 (#8168), p14 (#8145), HBXIP (#14633), RagA (#4357), RagC (#3360), FLAG epitope (2368), and HA epitope (#2367 and #3724). Antibodies against Raptor (Millipore #09-217), FLAG (Sigma #F1804), LAMP1 (Developmental Studies Hybridoma Bank at the University of Iowa # 1D4B), LAMP2 (Santa Cruz Biotechnology #18822) and Actin (Sigma #A5441) were obtained elsewhere. The antibody to c17orf59 was a generous gift from Jianxin Xie at CST.

Inactivated Fetal Bovine Serum (IFS), RPMI, and DMEM were obtained from US Biologicals. Amino acids for re-stimulation experiments were purchased from Sigma and dissolved in water to a concentration of 10x compared to the RPMI amino acid concentrations, as described previously (Sancak et al., 2008).

### Cell lysis and immunoprecipitation

Cells were washed once with ice-cold PBS and lysed with CHAPS lysis buffer (0.3% CHAPS [Sigma #C3023], 10 mM beta-glycerol phosphate [Sigma #G9422], 10mM sodium pyrophosphate [Sigma #221368], 40mM HEPES [pH 7.4], and 2.5 mM MgCl<sub>2</sub>) with one tablet of EDTA-free protease inhibitor (Roche #11 873 580 001) per 25 ml. Lysates were cleared by centrifugation at 13,000 rpm in a table-top microcentrifuge for 10 minutes. For anti-FLAG immunoprecipitations, cleared lysates were normalized for protein content by Bradford assay (Bio-Rad #500-0006) and incubated for 1.5-3 hours with 30  $\mu$ l of 50% slurry of FLAG-M2 affinity gel (Sigma #A2220) that had been washed three times in lysis buffer. After incubation, beads were washed three times with lysis buffer containing 150 mM NaCl.

Immunoprecipitated proteins were denatured by the addition of 35  $\mu$ l of sample buffer and boiling for 5 minutes. In experiments using only cell lysates, without immunoprecipitation, 1% Triton X-100 (Sigma T9284) was substituted for CHAPS in the lysis buffer.

In transfection experiments in which epitope-tagged proteins were immunoprecipitated, 2 million HEK-293T cells were seeded in 10 cm culture dishes. 24 hours later 500 ng of each pRK5-based plasmid was transfected using X-tremeGENE9 (Roche #06 365 809 001) according to the manufacturer's protocol: FLAG-Metap2, FLAG-p14/HA-MP1, FLAG-c17orf59, FLAG-RagB/HA-RagC. Empty pRK5 vector was used to normalize total plasmid amount to 2  $\mu$ g per 10 cm culture dish. Thirty-six hours after transfection, cells were lysed in 1 ml lysis buffer as described above.

For experiments in which c17orf59 was expressed with Rag or Ragulator components, 2 million HEK-293T cells were seeded in 10 cm culture dishes. Twenty-four hours later, the following pRK5-based plasmids were transfected using X-tremeGENE9: 100 ng of each FLAG-Metap2, FLAG-p14 with HA-MP1, FLAG-RagB with HA-RagC co-transfected with 1  $\mu$ g of either HA-c17orf59 or HA-Metap2. Thirty-six hours after transfection, cells were lysed in CHAPS lysis buffer as described above.

For experiments where cells were starved and re-stimulated with amino acids or insulin, 400,000 HEK-293E cells were seeded in 6-well plates coated with fibronectin (Millipore #341635). Forty-eight hours later, cells were washed once with PBS or starvation media and incubated in RPMI lacking all amino acids (US Biological # R9010) supplemented with 5% dialyzed IFS for one hour or in DMEM without serum for 3 hours and stimulated with amino acids or insulin (100 ng/ml; Sigma # I2643) for 10 minutes. Cells were lysed as described above in 130  $\mu$ l Triton lysis buffer. Alternatively, 500,000 HeLa cells were seeded in 6-well plates and starved, re-stimulated and lysed as described above after culturing overnight.

For dialysis, 100 ml IFS was dialyzed in SnakeSkin Dialysis Tubing with a 3.5 kDa cutoff (Thermo #PI88244) against approximately 3L PBS at 4°C for 48 hours, replacing with fresh PBS after 24 hours.

### **c17orf59 overexpression in signaling experiments**

In experiments where mTORC1 activity was assessed upon overexpression of c17orf59, FLAG-S6K1 co-expression and immunoprecipitation was used as described previously (Bar-Peled et al., 2012). Briefly, 2 million HEK-293T cells were seeded in 10 cm culture dishes. 24 hours later, 2  $\mu$ g of the cDNA for a control protein, HA-Metap2, 200 ng HA-RagB-T54N (RagB<sup>GDP</sup>) or between 500 ng and 2  $\mu$ g of the cDNA for HA-c17orf59 was co-transfected into HEK-293T cells with 2 ng of the cDNA for FLAG-S6K1. Empty pRK5 vector was used to normalize total plasmid amount to 2  $\mu$ g per 10 cm plate. 36 hours post-transfection, cells were starved of amino acids, stimulated with amino acids and lysed and FLAG-S6K1 was immunoprecipitated as described above.

## Mass spectrometry

Immunoprecipitates from nearly confluent 15 cm culture dishes containing HEK-293T cells stably expressing FLAG-Metap2, FLAG-HBXIP, or FLAG-c17orf59 were prepared using CHAPS lysis as described above, except 50  $\mu$ l of FLAG-M2 affinity gel was used for immunoprecipitations and beads were washed 6 times in lysis buffer containing 150mM NaCl. Bound proteins were eluted from the FLAG-M2 affinity gel by incubation in 50 $\mu$ l 1mg/ml FLAG-peptide (sequence DYKDDDK) for 45 minutes on ice and denatured by addition of loading buffer and boiling for 5 minutes. Samples were resolved on 4-12% NuPage gels (Life Technologies) and stained with SimplyBlue SafeStain (Life Technologies LC6065). Each lane was cut into 10 pieces and digested in trypsin overnight. Resulting digests were analyzed at the Whitehead Institute Proteomics core using a Thermo Fisher LTQ with Waters NanoAcuity UPLC mass spectrometer. Data were analyzed using Scaffold Free Viewer (Proteome Software).

## In vitro binding

Purification of recombinant proteins and in vitro binding assays using GST-tagged Rag GTPases or Ragulator were done as described (Bar Peled et al., 2012). Briefly, 4 million HEK-293T cells were seeded in 15cm culture dishes. Forty-eight hours after seeding, cells were transfected with cDNA for the following genes using PEI (Polysciences #23966; 3  $\mu$ l PEI at 1 mg/ml per  $\mu$ g DNA): for HA-GST-Ragulator: 4  $\mu$ g HA-GST-p14, 8  $\mu$ g HA-MP1, 8  $\mu$ g p18G2A-FLAG (a lipidation defective mutant), 8  $\mu$ g HA-HBXIP, and 8  $\mu$ g HA-c7orf59; for FLAG-Ragulator: 4  $\mu$ g p18G2A-FLAG, 8  $\mu$ g HA-MP1, 8  $\mu$ g HA-p14, 8  $\mu$ g HA-HBXIP, and 8  $\mu$ g c7orf59; for HA-GST-Rag GTPases: 8  $\mu$ g HA-GST-RagB, 16  $\mu$ g HA-RagC; for FLAG-Rag GTPases: 8  $\mu$ g FLAG-RagB, 16  $\mu$ g HA-RagC; for individual proteins: 10  $\mu$ g FLAG-c17orf59; 10  $\mu$ g FLAG-Rap2a; 10  $\mu$ g HA-GST-Rap2a.

Thirty-six hours after transfection, cells were lysed as described above, using 750  $\mu$ l Triton lysis buffer per dish. After clearing the lysates, 50  $\mu$ l of 50% slurry of FLAG-M2 affinity resin in lysis buffer or 200  $\mu$ l of 50% slurry of immobilized glutathione affinity resin (Thermo #15160) in lysis buffer were added to lysates expressing FLAG- or GST-tagged proteins, respectively. Recombinant proteins were incubated with affinity resin for 2 hours at 4°C with rotation. Each sample was washed once in lysis buffer, five times in wash buffer (0.3% CHAPS, 2.5 mM MgCl<sub>2</sub>, 40 mM HEPES, 500 mM NaCl) and three times in binding buffer (0.3% CHAPS, 2.5 mM MgCl<sub>2</sub>, 40 mM HEPES). GST-tagged samples were re-suspended in 160  $\mu$ l binding buffer and FLAG-tagged samples were eluted in 50  $\mu$ l FLAG-peptide in binding buffer. When necessary, eluates were concentrated using centrifugal filters with a 10 kDa cutoff (Millipore #UFC501024).

For the initial binding reactions, 20  $\mu$ l of glutathione affinity resin containing immobilized HA-GST-tagged proteins was incubated in binding buffer supplemented with 2 mM DTT and 1 mg/mL BSA (NEB #B9000) with 745 ng (20 pmol) FLAG-c17orf59 or 1.5  $\mu$ g (20 pmol) FLAG-Ragulator to a final volume of 50  $\mu$ l for 90 minutes on ice. To terminate binding assays, samples were washed three times with 1 ml of ice-cold binding buffer supplemented with 150 mM NaCl followed by the addition of 50  $\mu$ l sample buffer.

Binding assays in which increasing amounts of c17orf59 were added to GST-tagged Ragulator or Rag GTPases were done as described above, except that FLAG-c17orf59 was incubated with FLAG-Ragulator for 30 minutes prior to the incubation with other proteins on ice. For experiments where FLAG-c17orf59 was added to GST-Ragulator, 100 ng-10 µg (2.7 pmol-270 pmol) was incubated with Ragulator and 2 µg (25 pmol) Flag Rags were added to the reaction for 90 minutes. For experiments where FLAG-c17orf59 was added to GST-Rags, 100 ng-2 µg (2.7 pmol-270 pmol) was incubated with 1 µg (14 pmol) FLAG-Ragulator for 30 minutes prior to addition to GST-Rag GTPases.

### Immunofluorescence

1.5 million p53-null MEFs were transfected with 20 ng HA-c17orf59 and 1 µg empty pRK5 using the MEF Nucleofector kit 1 (Lonza #VPD-1004) using a nucleofector (Lonza) and seeded onto fibronectin-coated glass cover slips. Two wells of a 6-well culture dish were seeded per 1.5 million MEFs transfected. The following day, cells were either left untreated or starved and re-stimulated of amino acids or insulin as described above. Slides were fixed and processed as described previously (Zoncu et al., 2011). Briefly the slides were rinsed once with PBS and fixed using 4% paraformaldehyde in PBS for 15 minutes. Slides were washed twice with PBS and permeabilized with 0.05% Triton X-100 in PBS for 15 minutes. Slides were washed three times in PBS and incubated with primary antibody in 5% normal donkey serum (Jackson #017-000-121) for 1 hr at room temperature (1:300 for anti-LAMP1 antibody and 1:150 for anti-HA antibody), washed three times with PBS, and incubated with secondary antibodies produced in donkey (diluted 1:500 in 5% normal donkey serum) for 45 min at room temperature in the dark, and washed three times with PBS. Slides were mounted on glass coverslips using ProLong Diamond Antifade Mountant (Molecular Probes #P36966) and imaged on a spinning disk confocal system (Perkin Elmer).

p18<sup>-/-</sup> MEFs were transfected, treated and fixed as above. Samples were washed three times in PBS and blocked for 1 hour in 5% normal donkey serum in PBS containing 0.3% Triton. Samples were incubated in primary antibodies diluted in 1% BSA in PBS containing 0.3% Triton overnight in a humidified chamber at 4degC. Primary antibodies used were as follows: anti-LAMP1 (diluted 1:200), anti-HA (diluted 1:100), p18 (diluted 1:100) and LAMTOR4 (diluted 1:800) The next day samples were washed with PBS three times and incubated for 1-2 hours at room temperature with Alexa Fluor-conjugated secondary antibodies (Molecular Probes, diluted 1:500). Samples were then washed two times with PBS and incubated with Hoechst 33342 (Molecular probes H3570, 1:10,000) in PBS for 15 seconds and washed twice in PBS. Cover slips were mounted on glass slides with Vectashield Mounting Media and cured overnight at room temperature and then imaged as described above.

Alternatively, HEK-293T cells were transfected with 1 µg FLAG-c17orf59-IRES-GFP or 1 µg FLAG-GFP with 1 µg empty pRK5 in 10 cm culture plates as described above for signaling experiments. 24 hours after transfection, 300,000 cells were seeded per well on fibronectin-coated cover slips in 6-well plates. The following day cells were processed as described above and stained using anti-LAMP2 antibody at 1:400, anti-mTOR at 1:200, anti-RagC at 1:100, or anti-LAMTOR4 at 1:800.

Images are max projections of 0.5  $\mu\text{m}$  z-stacks. For MEF images, 5 slices were used; 10 slices were used for HEK-293T images. Pearson's correlation was calculated for the p18-c17orf59 quantitation using ImageJ Coloc 2 plugin and analyzed using a Students two-tailed t-test.

### Generation of CRISPR/Cas9 knockout cells

To generate c17orf59-null cells, guide RNAs were cloned into the pLentiCRISPR vector (Addgene) that was cut with BbsI (NEB #R0539). GuideRNAs were generated by annealing the following pairs of oligonucleotides and ligated into pLentiCRISPR:

Guide 1 fwd: caccGGGGCGGCCCGGGCCCGAGA

Guide 1 rev: aaacTCTCGGGCCCGGGCCCGCCC

Guide 2 fwd: caccgCAAAGTGGGTAAGGTCGCCG

Guide 2 rev: aaacCGGCGACCTTACCCACTTTGc

One million HEK-293E or 500,000 HeLa cells were seeded in 10cm culture dishes. 24 hours later, cells were transfected with 500 ng guideRNA plasmid with X-tremeGENE9. The following day, transfected cells were selected using puromycin. 48 hours after puromycin selection started, cells were re-transfected with guide RNA plasmid and allowed to grow to near confluence. 90% confluent dishes were trypsinized and single-cell sorted with a flow cytometer into the wells of a 96-well plate containing 150  $\mu\text{l}$  of DMEM supplemented with 30% IFS. Cells were grown until visible colonies were present, and the resultant colonies were trypsinized and expanded. Clones were validated for loss of the c17orf59 via immunoblotting.

### Cholesterol depletion

Cells were depleted of cholesterol using a protocol modified from that described previously (Bartz et al., 2009). Briefly, 3 million HeLa cells were seeded in 10cm culture dishes. The following day, cells were washed twice in PBS and media was changed to either full DMEM with 10% IFS or DMEM containing 0.5% lipid depleted serum (LDS, Intracel #RP-056). Twenty-four hours later, cells were incubated with DMEM with 10% IFS, DMEM with 0.5% LDS, or 2% 2-Hydroxypropyl)- $\beta$ -cyclodextrin in DMEM with 0.5% LDS for 5 hours. Cells were lysed in 1ml Triton lysis buffer as described above.

### c17orf59 cDNA cloning

c17orf59 cDNA was PCR amplified from HEK-293T cDNA using PlatinumTaq HIFI (Life Technology) the below gene-specific primers which have SalI and BamHI sites added at the 5' and 3' ends, respectively. The PCR product was purified from a 1% agarose gel and digested with SalI and BamHI restriction endonucleases (NEB #R0138 and #R0136) overnight. The restriction product was gel purified and ligated into previously SalI-BamHI digested pRK5. For further subcloning, a NotI site present in the c17orf59 cDNA was mutated using overlapping PCR with the following primers, digested with SalI and NotI (NEB #R0189), and ligated into SalI-NotI-digested pRK5.

To amplify c17orf59 cDNA from HEK-293T, the following primers were used:

Fwd: ACGCGTCGACGATGGAGTCGTCTCGGGGGCGG

Rev: CGCGGATCCTCACTTGCACAGGGCCTCAA

To remove the internal NotI site and add Sall-NotI subcloning sites, the following primers were used:

Fwd1: caagtcgtcgacgATGGAGTCGTCTCGGGGGC

SDM1 rev: CGAGGAGGCTGCAGCGGGCAGC

SDM2 fwd: GCTGCCCCGCTGCAGCCTCCTCG

Rev2: catgatcggcccgTCCTTGCACAGGGCCTCC

The 1000 nucleotides upstream of the c17orf59 gene locus were used as its endogenous promoter and amplified by PCR from genomic DNA from HEK-293T cells with the below primers and ligated to c17orf59 and pLEX-TRC202 plasmid cut with XmaI and BsrGI (NEB #R0180 and #R0575) by Gibson assembly (NEB #E5510). The ligation maintains the XmaI site upstream of the promoter, but eliminates the BsrGI site after the gene of interest.

To amplify the c17orf59 promoter the following primers were used:

Fwd: ccggctcgagggggcccgCTTTCCAATGTCGCTGCACCATTGCATTAG

Rev: catttccatACTGCAGGTGGGGGCCG

To amplify FLAG-c17orf59 the following primers were used:

Fwd: cctgcagtATGGAAATGGACTACAAGGATGAC

Rev: gtctcgagtttagactTCACTTGCACAGGGCCTC

To generate the FLAG-c17orf59-IRES-GFP construct, FLAG-c17orf59 cDNA was generated by PCR using a forward primer adding an NheI site and Kozak sequence to the FLAG-tag and the NotI-Rev2 primer described above, purified, and digested using NheI and NotI restriction endonucleases (NEB #R0131 and #R0189). Digested PCR product was ligated into pCAGGS-PSAML141F, Y115F:GlyR-IRES-GFP (Addgene #32480) that had been digested with NheI and NotI to remove the insert.

To amplify FLAG-c17orf59 for cloning into pCAGGS-IRES-GFP the following primers were used:

Fwd: caagtcctagcggccaccATGGAAATGGACTACAAGGATG

Rev: catgatcggcccgTCCTTGCACAGGGCCTCC

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We would like to thank the entire Sabatini lab for helpful suggestions and input as well as critical reading of the manuscript. This work was supported by grants from the NIH (R01 CA103866, R01 CA129105, R37 AI047389), Department of Defense (W81XWH-07-0448), and grants from the David H. Koch Institute and the Ellison Medical

Foundation to D.M.S., fellowship support from the NIH to L.D.S. (F31 CA167872), and an award from the David H. Koch Graduate Fellowship Fund to L.B-P. W.C.C. is supported by an American Cancer Society - Ellison Foundation postdoctoral fellowship (PF-13-356-01-TBE). D.M.S. is an investigator of the Howard Hughes Medical Institute.

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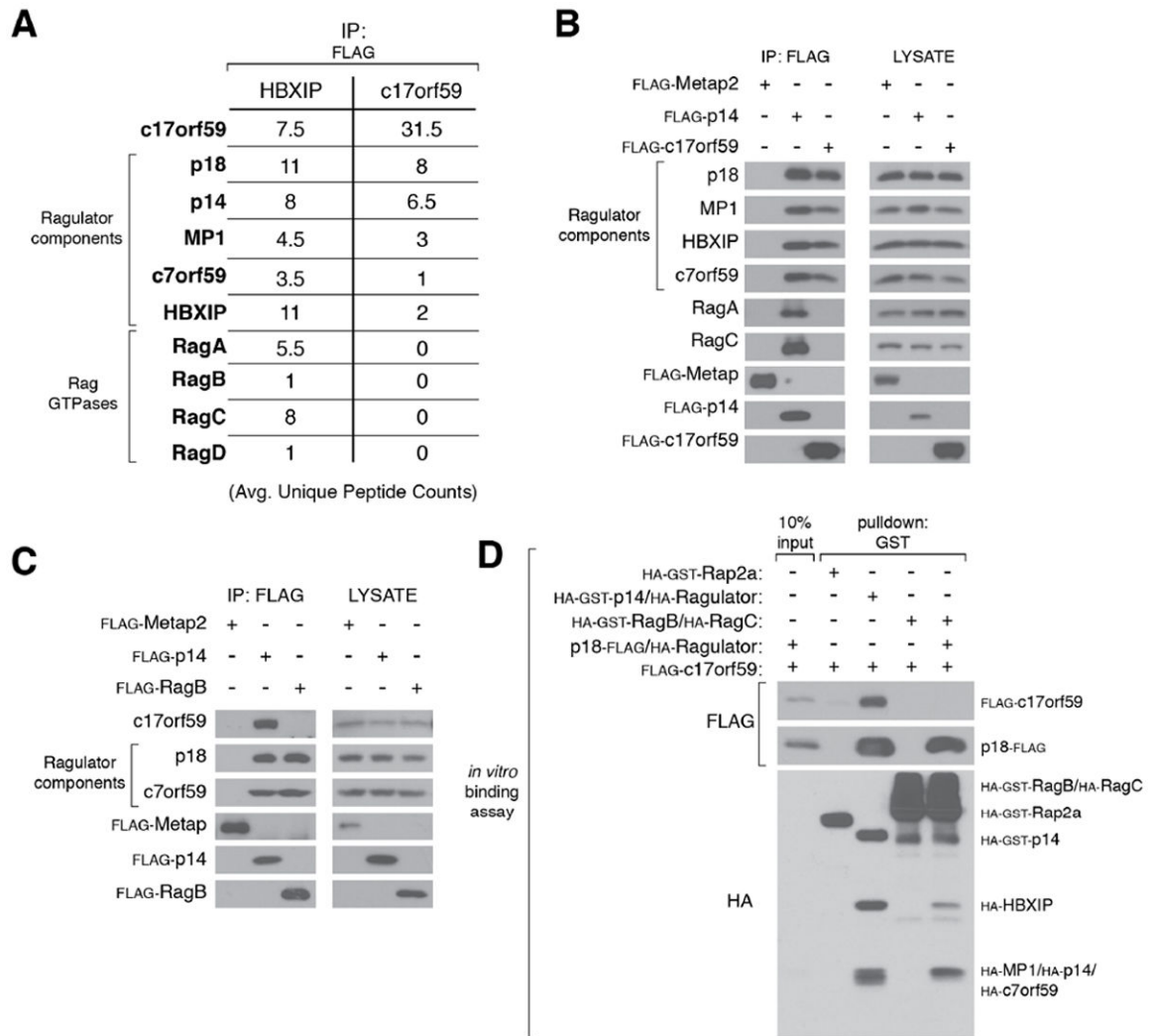
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- c17orf59 interacts with Ragulator, but not the Rag GTPases
- c17orf59 disrupts the Rag-Ragulator interaction in cells and *in vitro*
- c17orf59 overexpression inhibits mTORC1 and prevents its lysosomal recruitment



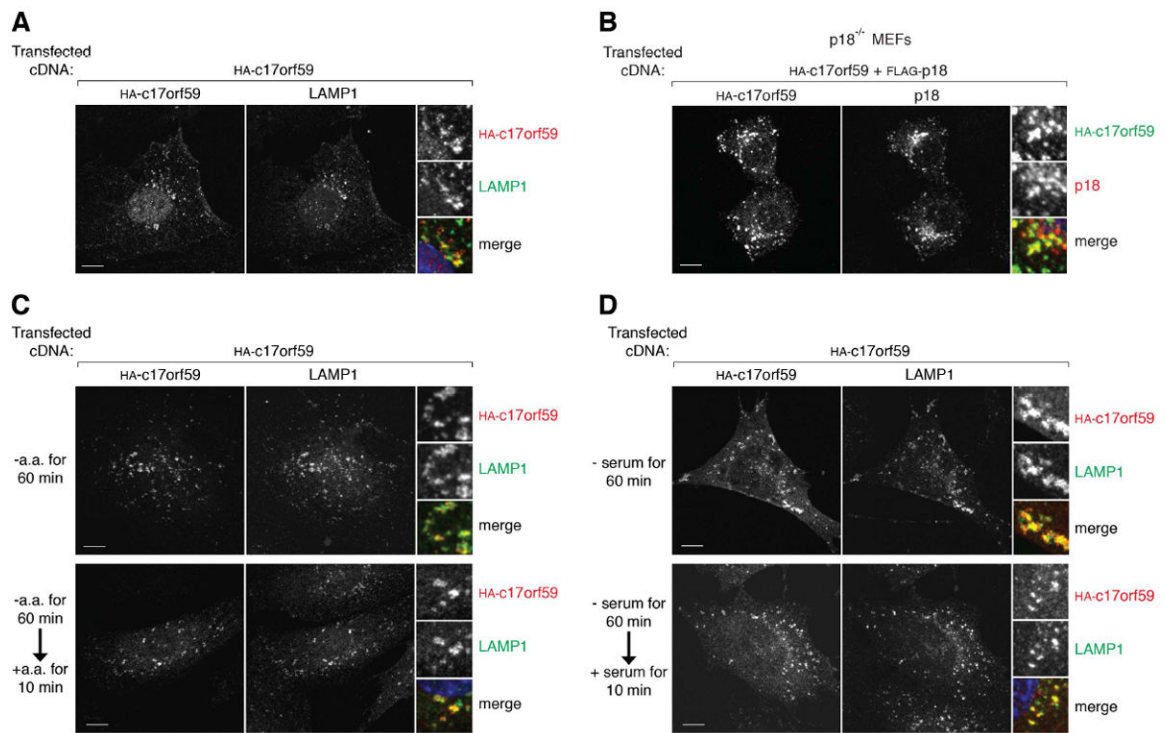
20pmol purified, FLAG-tagged c17orf59. In lane 5, HA-GST-tagged Rag dimer was incubated with 20pmol FLAG-tagged Ragulator in addition to 20pmol FLAG-tagged c17orf59. Precipitates from glutathione affinity resin were immunoblotted for the indicated epitope tags. GST-Ragulator was expressed as HA-GST-p14, HA-MP1, -HBXIP, -c7orf59 and p18-FLAG. FLAG-Ragulator was expressed as p18-FLAG, HA-p14, -MP1, -HBXIP, and -c7orf59.

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**Figure 2.**

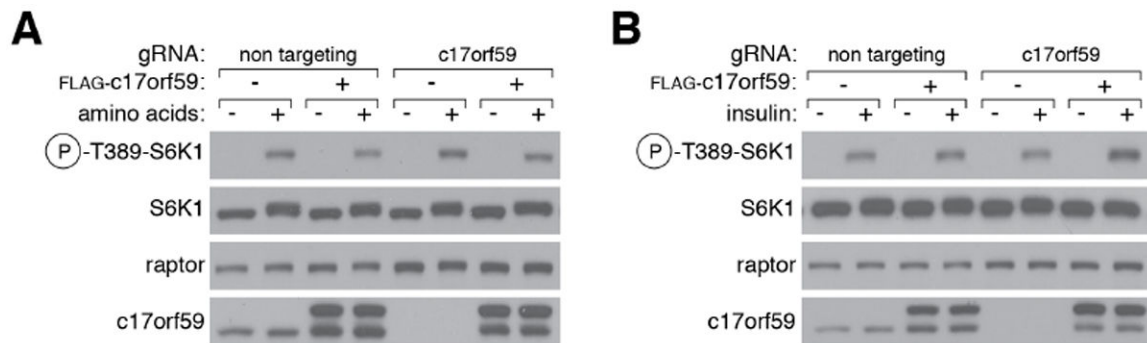
c17orf59 localizes to lysosomes with Ragulator

A) Recombinant c17orf59 localizes to lysosomes. p53-null MEFs were transfected with the HA-c17orf59 cDNA, immunostained with antibodies against LAMP1 (pseudocolored green) and the HA epitope tag (red), and imaged using confocal microscopy. Insets represent selected fields that have been magnified as well as the overlay of the fields. Scale bar represents 10 micrometers.

B) Recombinant c17orf59 co-localizes with p18. p18-null MEFs were transfected with the HA-c17orf59 and FLAG-p18 cDNA and processed and imaged as in (A), with p18 pseudocolored red and the HA epitope tag pseudocolored green. Insets represent selected fields that have been magnified as well as the overlay of the fields. Scale bar represents 10 micrometers.

C) Recombinant c17orf59 co-localizes with lysosomes in an amino acid-insensitive manner. p53-null MEFs were transfected with the HA-c17orf59 cDNA, starved for amino acids for 60 minutes and stimulated with amino acids for 10 minutes. Cells were processed and imaged as in (A) and with antibodies against LAMP1 (pseudocolored green) and the HA epitope tag (red). Insets represent selected fields that have been magnified as well as the overlay of the fields. Scale bar represents 10 micrometers.

D) Recombinant c17orf59 co-localizes with lysosomes in serum-insensitive manner. p53-null MEFs were transfected with the HA-c17orf59 cDNA, starved for amino acids for 60 minutes and stimulated with amino acids for 10 minutes. Cells were processed and imaged as in (A). Insets represent selected fields that have been magnified as well as the overlay of the fields. Scale bar represents 10 micrometers.



**Figure 3.**

Loss of *c17orf59* does not alter mTORC1 signaling in response to amino acids or insulin

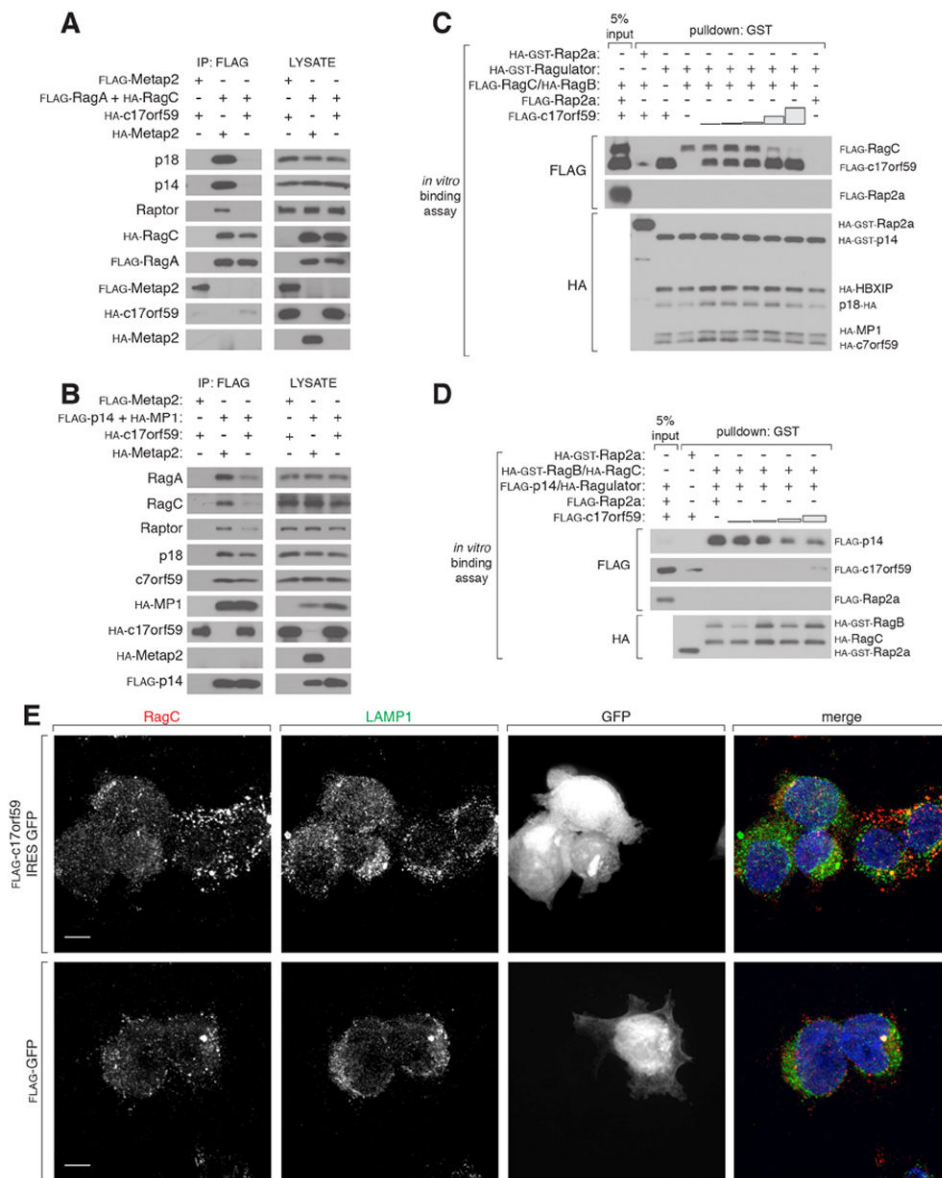
A) *c17orf59*-null cells do not have alterations in amino acid-sensitive mTORC1 activity.

*c17orf59*-null HEK-293E cells were generated by CRISPR/Cas9 technology. Cells were starved of amino acids for one hour and re-stimulated with amino acids for 10 minutes.

Lysates were analyzed by immunoblotting for the indicated proteins.

B) *c17orf59*-null cells do not have alterations in growth factor-sensitive mTORC1 activity.

*c17orf59*-null HEK-293E cells were starved of serum for three hours and re-stimulated with insulin for 10 minutes. Lysates were analyzed as in (A)



**Figure 4.**  
c17orf59 disrupts the Rag-Ragulator interaction

A) c17orf59 overexpression decreases the amount of Ragulator and Raptor recovered with immunoprecipitated Rags. Anti-FLAG immunoprecipitates and whole cell lysates from HEK-293T cells transfected with 100ng of the cDNAs encoding a FLAG-RagA/HA-RagC dimer or a control protein along with 1 $\mu$ g of HA-c17orf59 or a control protein were analyzed by immunoblotting for the indicated proteins.

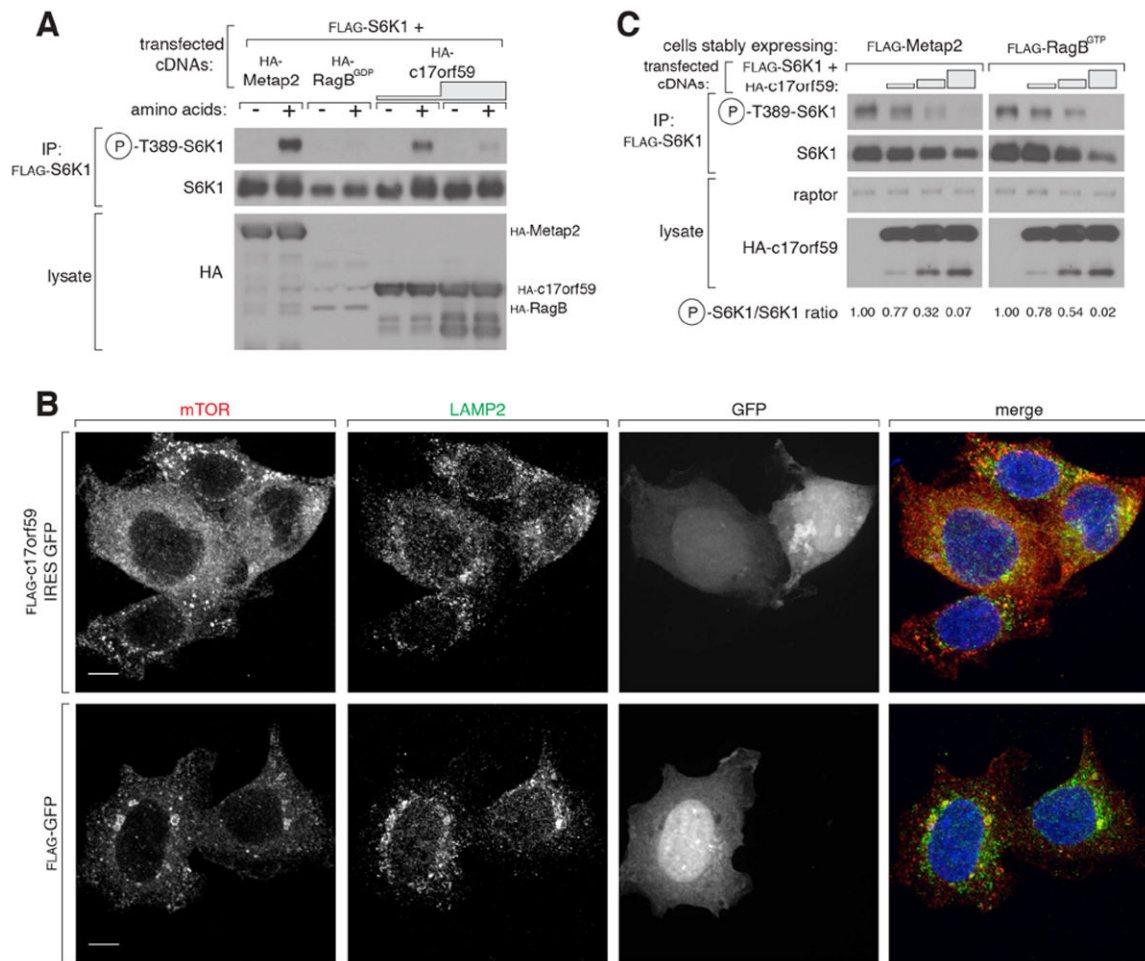
B) c17orf59 overexpression decreases the amount of Rag and Raptor, but not Ragulator subunits, that interact with p14. Anti-FLAG immunoprecipitates and whole cell lysates from HEK-293T cells transfected with 100ng of the cDNAs encoding FLAG-p14 and HA-MP1 or a control protein along with 1 $\mu$ g HA-c17orf59 or a control protein were analyzed as in (A).

C) Purified c17orf59 binds Ragulator in vitro and inhibits the Rag-Ragulator interaction. In vitro binding assay in which recombinant HA-GST Ragulator was pre-incubated with increasing quantities of purified FLAG-c17orf59 and followed by an incubation with purified FLAG-RagB/HA-RagC. For all samples in which FLAG-RagC/HA-RagB were used, 2 $\mu$ g (25 pmol) of Rag protein was added (lane 2, lanes 4-9). Similarly, 2 $\mu$ g of FLAG-Rap2a protein as added in each marked sample (lane 10). The amount of FLAG-c17orf59 protein added was as follows: 100ng (2.7 pmol, lane 5), 500ng (13.5 pmol, lane 6), 1 $\mu$ g (27 pmol, lane 7), 5 $\mu$ g (135pmol, lane 8), and 10 $\mu$ g (270 pmol, lane 9 and all lanes marked with a "+"). The bar above each lane is to scale for c17orf59 protein added. Precipitates using glutathione affinity resin were analyzed by immunoblotting for indicated tagged proteins.

D) c17orf59 decreases the amount of Ragulator bound to Rags in vitro. In vitro binding assay in which purified HA-GST-RagB/HA-RagC was incubated with purified FLAG-Ragulator and increasing amounts of purified FLAG-c17orf59. For all samples in which FLAG-Ragulator were used, 1 $\mu$ g (14 pmol) of Ragulator protein was added (lanes 3-7). Similarly, 2 $\mu$ g of FLAG-Rap2a protein as added in each marked sample (lane 3). The amount of FLAG-c17orf59 protein added was as follows: 100ng (2.7pmol, lane 5), 500ng (13.5pmol, lane 6), 1 $\mu$ g (27pmol, lane 7), 2 $\mu$ g (135pmol, lane 8 and all lanes marked with a "+"). The bar above each lane is to scale for c17orf59 protein added. Precipitates using glutathione affinity resin precipitates were analyzed as in (C).

E) c17orf59 overexpression decreases the amount of lysosomal RagC. HEK-293T cells were transfected with 1 $\mu$ g of the cDNA encoding FLAG-c17orf59-IRES-GFP (top panels) or FLAG-GFP controls (bottom panels). Cells were starved of amino acids for one hour and re-stimulated with amino acids for 10 minutes, and immunostained with antibodies against RagC (pseudocolored red) and the LAMP1 (green) and imaged for GFP (white) using confocal microscopy. GFP-positive cells (third column) represent transfected cells.



**Figure 5.**

Overexpression of c17orf59 inhibits mTORC1

A) Overexpression of c17orf59 inhibits mTORC1 activity in cells. HEK-293T cells were transfected with 2 ng of the cDNA for FLAG-S6K1 as well as the increasing amounts of HA-c17orf59, HA-RagB-54L (GDP-bound mimetic), or control protein cDNAs. The amounts of HA-c17orf59 cDNA transfected are as follows: 500ng (lanes 5 and 6), and 2 $\mu$ g (lanes 7 and 8). FLAG immunoprecipitates and whole cell lysates were analyzed by immunoblotting for the indicated proteins.

B) c17orf59 overexpression decreases the amount of lysosomal mTOR. HEK-293T cells were transfected with 1 $\mu$ g of the cDNA encoding FLAG-c17orf59-IRES-GFP (top panels) or FLAG-GFP controls (bottom panels). Cells were starved of amino acids for one hour and re-stimulated with amino acids for 10 minutes, and immunostained with antibodies against mTOR (pseudocolored red) and the LAMP1 (green) and imaged for GFP (white) using confocal microscopy. GFP-positive cells (third column) represent transfected cells.

C) c17orf59 inhibits mTORC1 in cells with amino acid-insensitive mTORC1 signaling. HEK-293T cells stably expressing the RagB Q99L mutant (GTPase deficient; GTP-bound) or a control protein were transfected with a cDNA for FLAG-S6K1 and increasing amounts of the cDNA for HA-c17orf59 as in (A). The amounts of HA-c17orf59 cDNA transfected

are as follows: 500ng (lane 2), 1 $\mu$ g (lane 3) and 2 $\mu$ g (lane 4). The bar above each lane is to scale for c17orf59 cDNA added. Samples were analyzed as in (A).

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