Ragulator Is a GEF for the Rag GTPases that Signal Amino Acid Levels to mTORC1

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Ragulator Is a GEF for the Rag GTPases that Signal Amino Acid Levels to mTORC1

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SUMMARY

The mTOR Complex 1 (mTORC1) pathway regulates cell growth in response to numerous cues, including amino acids, which promote mTORC1 translocation to the lysosomal surface, its site of activation. The heterodimeric RagA/B-RagC/D GTPases, the Ragulator complex that tethers the Rags to the lysosome, and the v-ATPase form a signaling system that is necessary for amino acid sensing by mTORC1. Amino acids stimulate the binding of guanosine triphosphate to RagA and RagB but the factors that regulate Rag nucleotide loading are unknown. Here, we identify HBXIP and C7orf59 as two additional Ragulator components that are required for mTORC1 activation by amino acids. The expanded Ragulator has nucleotide exchange activity toward RagA and RagB and interacts with the Rag heterodimers in an amino acid- and v-ATPase-dependent fashion. Thus, we provide mechanistic insight into how mTORC1 senses amino acids by identifying Ragulator as a guanine nucleotide exchange factor (GEF) for the Rag GTPases.

INTRODUCTION

Mechanistic target of rapamycin complex 1 (mTORC1) is a master growth regulator that couples nutrient availability to the control of cell growth and proliferation. When active, mTORC1 stimulates anabolic processes, such as translation, transcription, lipid biosynthesis, and ribosome biogenesis and inhibits catabolic processes, such as autophagy (reviewed in Howell and Manning, 2011; Ma and Blenis, 2009; Zoncu et al., 2011b). Consistent with its growth-promoting function, many of the oncogenes and tumor suppressors that underlie familial tumor syndromes and sporadic cancers are upstream of mTORC1. mTORC1 responds to a variety of stimuli, including growth factors, oxygen availability, and energy levels, all of which impinge on mTORC1 through the tuberous sclerosis heterodimer (TSC1-TSC2). TSC1-TSC2 negatively regulates the mTORC1 pathway by acting as a GTPase-activating protein (GAP) for Rheb1, a small GTPase that when bound to guanosine triphosphate (GTP) is an essential activator of mTORC1 kinase activity.

One mTORC1 stimulus that does not funnel through the TSC1-TSC2-Rheb axis is amino acid sufficiency (Roccio et al., 2006; Smith et al., 2005). Recent findings indicate that amino acid signaling initiates within the lysosomal lumen (Zoncu et al., 2011a) and induces the translocation of mTORC1 to the lysosomal surface, where it comes in contact with Rheb and becomes activated. How mTORC1 moves to the lysosomal membrane is poorly understood, but another family of GTPases, known as the Rag GTPases, play an integral role (Kim et al., 2008; Sancak et al., 2008). Unique among the small GTPases, the Rags are obligate heterodimers: the highly related RagA and RagB are functionally redundant and bind to RagC or RagD, which are also very similar to each other (Hirose et al., 1998; Schürmann et al., 1995; Sekiguchi et al., 2001). The Rags localize to lysosomal membranes and bind to the raptor component of mTORC1, a process that depends on the binding of GTP to RagA or RagB. Amino acids regulate the binding of nucleotides to RagB, such that amino acid stimulation increases its GTP loading (Sancak et al., 2008). In cells expressing a RagA or RagB mutant that is constitutively bound to GTP, mTORC1 interacts with the Rags and localizes to the lysosome irrespective of amino acid levels, making the mTORC1 pathway immune to amino acid starvation (Kim et al., 2008; Sancak et al., 2008). Thus, a key event in the amino acid-dependent activation of mTORC1 is the conversion of RagA or RagB from a GDP-to-GTP-bound state, yet the putative guanine nucleotide exchange factors (GEFs) that mediate this transition have yet to be identified.

Unlike the many GTPases that rely on a lipid moiety for their subcellular localization, the Rags use the recently identified Ragulator complex as their tether to the lysosomal surface. Three proteins that localize to lysosomal membranes make up Ragulator: p18, p14, and MP1, which are encoded by the LAMTOR1, LAMTOR2, and LAMTOR3 genes, respectively. In cells depleted of these proteins, the Rags and mTORC1 no
longer reside at the lysosome, and, consequently, the mTORC1 pathway is inactive (Sancak et al., 2010).

The lysosomal v-ATPase is a recently characterized Ragulator-interacting complex and required for amino acid activation of mTORC1 (Zoncu et al., 2011a). The mechanisms through which the v-ATPase activates the mTORC1 pathway and whether or not Ragulator has additional regulatory functions remain unknown. Here, we identify two additional components of Ragulator, the proteins encoded by the HBXIP and C7orf59 genes. These proteins interact with the Rag GTPases and together with p18, p14, and MP1 form a pentameric Ragulator complex. HBXIP and C7orf59 are necessary for both Rag and mTOR lysosomal localization and mTORC1 activation. Surprisingly, the pentameric Ragulator, but not individual subunits or the trimeric Ragulator, has GEF activity toward RagA and RagB. Furthermore, modulation of the Ragulator-Rag interaction by amino acids requires the v-ATPase, suggesting that v-ATPase activity is upstream of the GEF activity of Ragulator.

RESULTS

HBXIP and C7orf59 Encode Components of an Expanded Ragulator Complex

We previously identified p14, MP1, and p18, collectively named Ragulator, as proteins that interact with the Rag GTPases within cells (Sancak et al., 2010). However, in cell-free assays, Rag heterodimers interact relatively weakly with purified, recombiantly produced Ragulator (Sancak et al., 2010), suggesting that proteins responsible for stabilizing the interaction within cells are missing from our in vitro preparations. To identify such proteins, we used a purification strategy involving immunoprecipitation followed by mass spectrometry that previously led to the discovery of other mTORC1 pathway components (see Experimental Procedures). Immunoprecipitates prepared from HEK293T cells stably expressing FLAG-tagged p18, p14, or RagB, but not the Metap2 control, consistently contained HBXIP and the protein product of the C7orf59 gene, hereafter called C7orf59. Several studies implicate HBXIP in the regulation of cell cycle progression, proliferation, apoptosis, and Hepatitis B virus replication (Fuji et al., 2006; Marusawa et al., 2003; Melegari et al., 1998; Wang et al., 2007; Wen et al., 2008), while C7orf59 has no described functions.

Orthologs of HBXIP and C7orf59 exist in mammals besides humans and in Drosophila (Figure S1A available online). Like other Ragulator components, HBXIP and C7orf59 lack protein sequence homology with any fission or budding yeast proteins, including Ego1p and Ego3p, which tether the yeast orthologs of the Rag GTPases to the vacuole (Binda et al., 2009; Dubouloz et al., 2005; Gao and Kaiser, 2006). High-resolution crystal structures of MP1 and p14 reveal the presence of a roadblock domain in each (Kurzbauer et al., 2004; Lunin et al., 2004), and secondary structure predictions suggest that the C-terminal regions of RagB and RagC also contain this domain (Gong et al., 2011) (Figure S1B). While the function of the domain is unknown, it is interesting to note that HBXIP also contains a roadblock domain (Garcia-Saez et al., 2011), and our secondary structure analyses predict the same for C7orf59 (Figure S1B). Thus, the Rag-Ragulator complex is likely to contain six roadblock domains.

Experiments in cells and in cell-free systems indicate that HBXIP and C7orf59 are bona fide Ragulator components. When expressed in HEK293T cells, FLAG-tagged HBXIP or C7orf59, but not Rap2a, coimmunoprecipitated endogenous RagA, which is highly similar but far more abundant than RagB (Figures S1D and S1E), RagC, p18, and MP1 at similar levels as FLAG-p14 (Figures 1A and 1B). Gratifyingly, endogenous HBXIP and C7orf59 coimmunoprecipitated with an antibody to endogenous p18, but not a control protein (Figure 1C). When coexpressed along with Ragulator proteins in HEK293T cells, HBXIP and C7orf59 colocalized with p18 (Figure 1D), consistent with the lysosomal localization of other Ragulator components (Nada et al., 2009; Sancak et al., 2010; Wunderlich et al., 2001). In an in vitro binding assay, HBXIP bound to C7orf59 in the absence of other proteins, and the HBXIP-C7orf59 heterodimer, but neither protein alone, bound the established Ragulator components (MP1, p14, and p18) (Figure 1E). These results indicate that Ragulator is a pentameric complex in which HBXIP and C7orf59 form a heterodimer that interacts, through p18, with the MP1-p14 heterodimer (Figure 1F).

Consistent with our initial hypothesis that the original Ragulator lacked components required to bind strongly to the Rag GTPases, in HEK293T cells the pentameric Ragulator interacted to a much greater degree with the Rags than the trimeric one (Figures 1G and S1C). Likewise, in an in vitro binding assay, Rags interacted with an intact pentameric Ragulator, but not one lacking p18 (Figure 1H). It is likely that in previous work, these additional Ragulator components were present in binding experiments in substoichiometric amounts, explaining the weaker interactions we had observed (Sancak et al., 2010). Collectively, our results show that HBXIP and C7orf59 are part of an expanded Ragulator that requires all its subunits to bind strongly to the Rag GTPases.

HBXIP and C7orf59 Are Necessary for TORC1 Activation by Amino Acids in Mammalian and Drosophila Cells

We next examined the functions of HBXIP and C7orf59 in mTORC1 signaling. In HEK293T cells, RNA interference (RNAi)-mediated reductions in HBXIP or C7orf59 expression blunted mTORC1 activation by amino acids, as detected by S6K1 phosphorylation, to similar extents as knockdowns of the established Ragulator proteins p18 and p14 (Figure 2A). As expected for positive regulators of the growth-promoting mTORC1 pathway (Fingar et al., 2002; Kim et al., 2008; Sancak et al., 2008, 2010; Stocker et al., 2003), reductions in HBXIP and C7orf59 levels also decreased the size of HEK293T cells (Figure 2B). As the components of obligate heterodimers often behave (Cortez et al., 2001; Sancak et al., 2008), loss of either HBXIP or C7orf59 reduced the expression of its partner, but not of p14 (Figure 2A). Finally, consistent with the conserved functions of the Rag and Ragulator proteins in Drosophila (Kim et al., 2008; Sancak et al., 2008, 2010), treatment of S2 cells with dsRNAs targeting the HBXIP (CG14812) or C7orf59 (CG14977) fly orthologs strongly inhibited dTORC1 activation by amino acids (Figure 2C). These results establish that HBXIP and C7orf59 are positive components in mammalian and Drosophila cells of the amino acid sensing branch of the TORC1 pathway.
Upon amino acid stimulation, the Rag GTPases recruit mTORC1 to the lysosomal surface (Sancak et al., 2010). In the absence of Ragulator, the Rags detach from the lysosome and cannot target mTORC1 to this organelle. The inability of amino acids to activate mTORC1 in cells depleted of HBXIP and C7orf59 suggested that HBXIP and C7orf59, like p14, MP1, and p18, might also localize the Rags, and thus mTORC1, to the lysosome. Indeed, in HEK293T cells treated with small interfering RNAs

See also Figure S1.

**Figure 1. HBXIP and C7orf59 Are Components of an Expanded Ragulator Complex**

(A) Recombinant epitope-tagged HBXIP communoprecipitates endogenous MP1, p18, RagA, and RagC. Anti-FLAG immunoprecipitates were prepared from HEK293T cells transfected with the indicated cDNAs in expression vectors. Cell lysates and immunoprecipitates were analyzed by immunoblotting for levels of indicated proteins.

(B) Recombinant C7orf59 communoprecipitates endogenous MP1, p18, RagA, and RagC. HEK293T cells were transfected with the indicated cDNAs in expression vectors and analyzed as in (A).

(C) Endogenous p18 communoprecipitates endogenous p14, MP1, RagA, RagC, HBXIP, and C7orf59. Anti-p18 immunoprecipitates were prepared from HEK293T cells transfected with the indicated cDNAs and analyzed for the levels of the indicated proteins.

(D) Images of HEK293T cells coimmunostained for p18 (green) and FLAG-HBXIP (red) or FLAG-C7orf59 (red). Cells were cotransfected with cDNAs encoding HBXIP: p14, p18, and either FLAG-HBXIP or FLAG-C7orf59 and processed for immunostaining and imaging. In all images, insets show selected fields that were magnified five times and their overlays. Scale bar represents 10 μm.

(E) Schematic summarizing intra-Ragulator interactions: p18 bridges MP1-p14 with HBXIP-C7orf59.

(F) The pentameric Ragulator complex communoprecipitates recombinant RagB and RagC. HEK293T cells were cotransfected with the indicated cDNAs in expression vectors and analyzed as in (A).

(H) Requirement for a pentameric Ragulator complex to interact with Rags. In vitro binding assay in which recombinant HA-GST Ragulator with or without p18 was incubated with purified FLAG-RagB-RagC and analyzed as in (E).

See also Figure S1.

**Localization of the Rag GTPases and mTOR to the Lysosomal Surface Requires HBXIP and C7orf59**

Upon amino acid stimulation, the Rag GTPases recruit mTORC1 to the lysosomal surface (Sancak et al., 2010). In the absence of Ragulator, the Rags detach from the lysosome and cannot target mTORC1 to this organelle. The inability of amino acids to activate mTORC1 in cells depleted of HBXIP and C7orf59 suggested that HBXIP and C7orf59, like p14, MP1, and p18, might also localize the Rags, and thus mTORC1, to the lysosome. Indeed, in HEK293T cells treated with small interfering RNAs
Amino Acids Regulate the Rag-Ragulator Interaction

Multimeric signaling complexes often engage in regulated interactions as a mechanism to control downstream signaling events (Good et al., 2011). Because the Rag GTPases interact with mTORC1 in an amino acid-dependent manner, we wondered if the binding of Ragulator to the Rags might also be amino acid sensitive. In order to detect the endogenous Rag-Ragulator interaction using the antibodies available in the past, we had found it necessary to use crosslinked conditions that would have prevented detection of a regulated interaction (Sancak et al., 2010). Using optimized cell lysis conditions and improved antibodies, we find that amino acid starvation strengthens the interaction between endogenous Rags and the Ragulator isolated through p14, p18, HBXIP, or C7orf59 (Figures 3A, 3B, S3D, and S3E). Similarly, amino acid stimulation decreased the amounts of endogenous Ragulator that coimmunoprecipitated with RagB (Figures 3C and S3F). Leucine is necessary for mTORC1 activation (Hara et al., 1998) and the Rag-Ragulator interaction as well as the binding of recombinant RagB or RagC is the dominant determinant of the interaction between Rag heterodimers and Ragulator. To address this issue, we generated two different classes of Rag nucleotide binding mutants (Figure 4D). In the first, a critical Thr/Ser that is necessary for stabilizing magnesium was changed to Asn, resulting in mutants (RagB<sub>Q99L</sub> and RagC<sub>Q120L</sub>) that bind negligible amounts of nucleotides (Figure S4C). The corresponding H-Ras mutant (H-Ras<sub>S17N</sub>) also binds nucleotides poorly, but, interestingly, interacts with GEFs to a greater extent than the wild-type protein (Feig, 1999; Feig and Cooper, 1988; John et al., 1993). Mutants in the second class are homologous to H-Ras<sub>G12L</sub> and are constitutively bound to GTP because they lack GTPase activity (RagB<sub>G99L</sub> and RagC<sub>C120L</sub>) (Frech et al., 1994; Krangel et al., 1990). Within cells the heterodimer of nucleotide-free RagB and wild-type RagC (RagB<sub>T54N</sub>-RagC<sub>C120L</sub>) interacted with Ragulator at levels 4- to 6-fold greater than the heterodimer of wild-type RagB and nucleotide-free RagC (RagB<sub>W</sub>RagC<sub>Q75N</sub>) (Figures 4E and 4F), suggesting that the presence or absence of nucleotide on RagB largely controls the Rag-Ragulator interaction. Consistent with this interpretation, a heterodimer of GTP-bound RagB and nucleotide-free RagC (RagB<sub>G99L</sub>-RagC<sub>C75N</sub>) interacted with Ragulator much more weakly than a heterodimer with the opposite properties (RagB<sub>B</sub>RagC<sub>T54N</sub>) (Figure S4E).

Ragulator Preferentially Interacts with Nucleotide-free Rag GTPases

Regulation of the Rag nucleotide-binding state is not understood, but is key for amino acid signaling to mTORC1. The amino acid-sensitive interaction between Rags and Ragulator prompted us to examine whether Ragulator might also regulate nucleotide binding to the Rags. Intriguingly, many proteins that regulate nucleoside triphosphatase (NTPases) have roadblock domains (Bowman et al., 1999; Koonin and Aravind, 2000; Miertzschke et al., 2011; Wanschers et al., 2008), which four of the five Ragulator components are likely to contain. Preliminary experiments indicated that Ragulator does not have GAP activity toward the Rag GTPases, and so we examined whether it might have the activity of a GEF. A characteristic of GEFs is their strong preference for binding nucleotide-free over nucleotide-loaded GTPases (Bos et al., 2007; Feig, 1999). Incubation with buffers containing EDTA, which chelates the magnesium ion necessary for nucleotide binding, is a common way to generate largely nucleotide-free GTPases (Wang et al., 2000). Interestingly, the presence of EDTA in the cell lysis buffer significantly increased the interaction of recombinant RagB and endogenous Ragulator proteins (Figures 4A and S4A) as well as the binding of recombinant p18 to endogenous RagA and RagC (Figure 4B). In vitro binding assays proved useful in dissecting the effects of nucleotides on the Rag-Ragulator interaction. Ragulator readily bound to the Rags in vitro, likely by displacing their nucleotides (see below), but the addition of GTP significantly weakened the interaction (Figure S4B). In a complementary experiment, highly purified Ragulator had a clear preference for interacting with a recombinant Rag heterodimer stripped of its nucleotides rather than nucleotide bound, indicating that both in cells and in vitro Ragulator prefers binding to nucleotide-free Rags (Figure 4C).

To study a potential regulatory function for Ragulator, it was necessary to first determine if the nucleotide binding state of RagB or RagC is the dominant determinant of the interaction between Rag heterodimers and Ragulator. To address this issue, we generated two different classes of Rag nucleotide binding mutants (Figure 4D). In the first, a critical Thr/Ser that is necessary for stabilizing magnesium was changed to Asn, resulting in mutants (RagB<sub>Q99L</sub> and RagC<sub>Q120L</sub>) that bind negligible amounts of nucleotides (Figure S4C). The corresponding H-Ras mutant (H-Ras<sub>S17N</sub>) also binds nucleotides poorly, but, interestingly, interacts with GEFs to a greater extent than the wild-type protein (Feig, 1999; Feig and Cooper, 1988; John et al., 1993). Mutants in the second class are homologous to H-Ras<sub>G12L</sub> and are constitutively bound to GTP because they lack GTPase activity (RagB<sub>G99L</sub> and RagC<sub>C120L</sub>) (Frech et al., 1994; Krangel et al., 1990). Within cells the heterodimer of nucleotide-free RagB and wild-type RagC (RagB<sub>T54N</sub>-RagC<sub>C120L</sub>) interacted with Ragulator at levels 4- to 6-fold greater than the heterodimer of wild-type RagB and nucleotide-free RagC (RagB<sub>W</sub>RagC<sub>Q75N</sub>) (Figures 4E and 4F), suggesting that the presence or absence of nucleotide on RagB largely controls the Rag-Ragulator interaction. Consistent with this interpretation, a heterodimer of GTP-bound RagB and nucleotide-free RagC (RagB<sub>G99L</sub>-RagC<sub>C75N</sub>) interacted with Ragulator much more weakly than a heterodimer with the opposite properties (RagB<sub>B</sub>RagC<sub>T54N</sub>) (Figure S4E). Thus, the nucleotide binding state of RagB is the major determinant of the strength of the interaction between Rag heterodimers and Ragulator.
Figure 2. HBXIP and C7orf59 Are Necessary for TORC1 Activation by Amino Acids and Localization of the Rag GTPases and mTOR to the Lysosomal Surface

(A) C7orf59 and HBXIP are necessary for the activation of the mTORC1 pathway by amino acids. HEK293T cells, treated with siRNAs targeting the mRNAs for the indicated proteins, were starved of amino acids for 50 min, or starved and stimulated with amino acids for 10 min. Immunoblot analyses were used to measure the levels of the indicated proteins and phosphorylation states.

(B) Cell number and cell diameter distributions were determined for cells treated with scrambled or HBXIP siRNA.

(C) dsRNA targeting dS6K and dHBXIP were used to inhibit the activity of these proteins.

(D) Immunofluorescence images of RagA and Lamp2 localization with antibodies against RagA and Lamp2 before and after treatment.

(E) Immunofluorescence images of mTOR and Lamp2 localization with antibodies against mTOR and Lamp2 before and after treatment.

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(E) Immunofluorescence images of mTOR and Lamp2 localization with antibodies against mTOR and Lamp2 before and after treatment.
Ragulator Is a GEF for RagA and RagB

The binding properties of Ragulator are highly consistent with it having GEF activity toward RagB. To test this possibility, it was necessary to develop a way to measure GDP dissociation from one Rag and not the other. To this end, we mutated the conserved Asp to Asn in the Rag “NKxD motif” (RagBD163N and RagCD181N). This mutation changes the base specificity of a GTPase from guanine to xanthosine nucleotides (Hoffenberg...
Figure 4. Ragulator Preferentially Interacts with Nucleotide-free RagB

(A) EDTA increases the interaction between endogenous Ragulator and FLAG-RagB. HEK293T cells stably expressing Flag-RagB were lysed in the absence or presence of EDTA and cell lysates and anti-FLAG immunoprecipitates analyzed by immunoblotting for the levels of the indicated proteins.

(B) FLAG-p18 coimmunoprecipitates more endogenous Rags in the presence of EDTA. HEK293T cells stably expressing FLAG-p18 were treated and analyzed as in (A).

(C) Ragulator preferentially interacts with nucleotide-free Rags. In vitro binding assay in which immobilized HA-GST-Ragulator was incubated with nucleotide-free FLAG-RagB-RagC or Rag heterodimers loaded with GTP. HA-GST precipitates were analyzed for the levels of the indicated proteins.

(D) Table summarizing Rag mutants used in this study.

(E) The RagB<sup>T54N</sup> mutant preferentially interacts with endogenous Ragulator. Anti-FLAG immunoprecipitates were prepared from HEK293T cells transfected with the indicated cDNAs in expression vectors and analyzed as in (A).

(F) Quantification of endogenous MP1 and p18 binding to RagB<sup>T54N</sup>-RagC and RagB-RagC<sup>S75N</sup>. Each value represents the normalized mean ± SD for n = 3. See also Figure S4.
et al., 1995; Schmidt et al., 1996), and we denote these mutants as RagBX or RagCX both of which bind less than 2% of the guanine nucleotides than their wild-type counterparts (Figure S4D). Therefore, when we load RagBX-RagC or RagB-RagCX with GDP or GTP in vitro, we know which of the Rag GTPases in the heterodimer is bound to the guanine nucleotide.

In vitro many GEFs displace GDP and GTP from their cognate GTPases (Klebe et al., 1995; Lenzen et al., 1998; Zhang et al., 2005). Thus, we loaded RagBX-RagC or RagB-RagCX heterodimers with labeled GDP or GTP and tested the effects of Ragulator on their dissociation. Ragulator did not affect GDP or GTP dissociation from the Rap2a control GTPase (Figure 5A). When tested on RagC within the RagBX-RagC heterodimer, Ragulator modestly increased the release of GDP but not that of GTP (Figure 5B). In contrast, Ragulator greatly accelerated both GDP and GTP dissociation from RagB in the RagB-RagCX heterodimer (Figures 5C and S5A) and did so in a dose-dependent manner (Figure S5C). As expected from the very high level of homology between RagA and RagB (Figure S1D), Ragulator also greatly increased guanine nucleotide dissociation from RagA in the RagA-RagCX heterodimer (Figure 5D).

Consistent with its function as a GEF, in a GTP binding assay in which we prebound RagB-RagCX or RagA-RagCX with unlabeled GDP and then incubated it with labeled GTP, Ragulator significantly increased GTP binding to RagB and RagA (Figures 5E, 5F, and S5B).

Because the Rags function as a heterodimer, we wondered whether the nucleotide binding state of RagC might alter the function of Ragulator toward RagB. When the RagB-RagCX heterodimer was coloaded with either XDP or XTP in addition to GDP or GTP (Figures S5D and S5E), there was no difference in Ragulator-mediated GDP or GTP dissociation from RagB, suggesting that the nucleotide binding state of RagC does not alter Ragulator GEF activity toward RagB.

To determine if the exchange activity of Ragulator depends on a particular subunit, we tested p14-MP1, HBXIP-C7orf59, and

![Figure 5. Ragulator Is a GEF for RagA and RagB](image-url)
p18 separately in the GDP exchange assay. Unlike the pentameric complex, none of these subassemblies increased GDP dissociation from RagB (Figure 5G). Likewise, trimeric Ragulators composed of either p14-MP1-p18 or HBXIP-C7orf59-p18 were no more effective at accelerating GTP dissociation from RagB than a control protein (Figure 5H). These results indicate that Ragulator is a GEF for RagA and RagB and that a pentameric Ragulator is required for this activity.

Recently, Vam6 was shown to act as a GEF for Gtr1p, the yeast ortholog of RagA and RagB, and to be necessary for the activation of the TORC1 pathway in yeast (Binda et al., 2009). However, we found that VPS39, the mammalian ortholog of VAM6, not only failed to interact with endogenous RagA (Figure S5F) but also did not stimulate GDP or GTP dissociation from RagB (Figure S5G). These findings suggest that VPS39 is not a GEF for RagA or RagB and that the amino acid sensing mechanisms of yeast and higher eukaryotes have diverged.

The v-ATPase Controls Ragulator Function in Cells

The v-ATPase is a positive regulator of the mTORC1 signaling pathway that acts downstream of amino acids and upstream of the Rags (Zoncu et al., 2011a). The v-ATPase consists of V0 and V1 domains, two multisubunit complexes (Forgac, 2007), both of which interact with Ragulator (Zoncu et al., 2011a). Interestingly, amino acid starvation and restimulation strengthens and weakens the V1-Ragulator interaction, respectively, while v-ATPase inhibition prevents regulation of the interaction by locking it in the amino acid-free state. Because the v-ATPase, unlike Ragulator, is not required to maintain the Rag GTPases on the lysosomal surface (Zoncu et al., 2011a), it must have an important function distinct from the control of Rag localization. Given its regulated interaction with Ragulator, it seemed likely that the v-ATPase might regulate the GEF activity of Ragulator. To test this possibility, we used the amino acid-sensitive interaction between the Rag heterodimers and Ragulator as a marker of Ragulator GEF activity in cells. Consistent with the nucleotide loaded state of RagB determining the Rag-Ragulator interaction, in cells stably expressing the GTP-bound RagB<sup>C99L</sup> mutant, the interaction between Ragulator and RagB<sup>C99L</sup> was no longer regulated by amino acids and resembled the weak Rag-Ragulator interaction observed in amino acid stimulated cells (Figure 6A). Interestingly, pretreatment of cells with the v-ATPase inhibitors Salicylhalamide A (SalA) (Xie et al., 2004) or Concanamycin A (ConA) (Bowman et al., 2004), prevented amino acid stimulation from weakening the Rag-Ragulator interaction, which remained at the strong level observed in the absence of amino acids (Figures 6A, 6B, and S6A). Importantly, v-ATPase inhibition did not affect the

Figure 6. The v-ATPase Controls the Function of Ragulator

(A) The v-ATPase functions upstream of the regulated binding between Rags and Ragulator. HEK293T cells stably expressing FLAG-RagB or FLAG-RagB<sup>C99L</sup> were starved for 2 hr or starved and stimulated with amino acids for 15 min in the absence or presence of the v-ATPase inhibitor SalA. Cell lysates and anti-FLAG immunoprecipitates were analyzed by immunoblotting for the levels of the indicated proteins.

(B) Inactivation of the v-ATPase blocks the amino acid dependent Rag-Ragulator interaction. HEK293T cells stably expressing FLAG-p18 were treated as in (B). Anti-FLAG immunoprecipitates were analyzed for the levels of the indicated proteins.

(C) Model for amino acid induced mTORC1 activation. In the absence of amino acids the v-ATPase, Ragulator, and Rags exist in a tightly bound super complex and mTORC1 cannot associate with the lysosomal surface and remains inactive. Amino acid accumulation in the lysosomal lumen generates an activating signal that is transmitted in a v-ATPase-dependent fashion to activate the GEF activity of Ragulator toward RagA. Upon RagA-GTP loading, the Rag-Ragulator interaction weakens and mTORC1 is recruited to the lysosomal surface where it interacts with Rheb and becomes activated. Rheb is not shown. See also Figure S6.
already weak interaction between the RagB\textsuperscript{Q99L} mutant and Ragulator (Figure 6A). Thus, regulation of the Rag-Ragulator interaction depends on the v-ATPase, which is necessary to transmit the amino acid signal to the GEF activity of Ragulator.

**DISCUSSION**

In this study, we identify HBXIP and C7orf59 as two additional components of the mTORC1 pathway. In association with known Ragulator proteins (p18, p14, and MP1), HBXIP and C7orf59 form a pentameric complex that is essential for localizing the Rag GTPases to the lysosomal surface and activating mTORC1 in response to amino acids. In addition to being a scaffold, Ragulator promotes nucleotide exchange of RagB and of the highly related RagA. Thus, we identify a key link in the signaling cascade that converts a signal emanating from amino acids into the nucleotide loading of the Rags and ultimately the recruitment of mTORC1 to the lysosomal surface. We suggest that C7orf59 and HBXIP be renamed LAMTOR4 and LAMTOR5, respectively, to reflect their critical roles in regulating the mTORC1 pathway and to be consistent with the naming convention of other Ragulator components.

Our in vitro binding results and secondary structure predictions, combined with available structural data, support the following molecular architecture for pentameric Ragulator: p18 is a lysosome-associated scaffold protein that binds two roadblock-containing heterodimers, p14-MP1 and HBXIP-C7orf59, and thereby tethers them to the lysosome. In vitro and in vivo data suggest that all five members of Ragulator must be present to efficiently interact with the Rag heterodimers, although the stoichiometry between the two complexes is unknown. The recently reported crystal structure of a Gtr1p-Gtr2p heterodimer, the yeast orthologs of mammalian Rags, reveals the presence of roadblock domains in the C-terminal portion of both GTPases, a structural feature that the C-terminal domains of the Rags are also predicted to have (Gong et al., 2011). Thus, the roadblock domain may represent the basic architectural element of the Ragulator-Rag complex.

Several proteins that interact with NTPases have a roadblock domain, suggesting that it may have regulatory functions as well as structural ones (Koonin and Aravind, 2000). The recently solved crystal structure of the bacterial GAP, MglB, shows that it contains a roadblock domain and may promote GTP hydrolysis through stabilization of the catalytic machinery of its cognate GTPase (Miertzschke et al., 2011). Consistent with a possible regulatory role for proteins with this domain, Ragulator prefers to bind to nucleotide-free rather than nucleotide-bound RagB. These binding properties are characteristic of other GEF-GTPase interactions and suggested that Ragulator might be a GEF for RagA and RagB. To test this hypothesis, it was necessary to develop a system for monitoring the nucleotide bound to an individual Rag in a heterodimer containing two of them. To this end, we made use of Rag complexes containing a wild-type Rag that binds guanine nucleotides and a Rag\textsuperscript{x} mutant that cannot. We suggest that the Rag\textsuperscript{x} mutants may be useful reagents for the identification of other factors that control the nucleotide-loading state of the Rags. Ragulator greatly increases both GDP and GTP dissociation from RagB and RagA but not RagC. The preferential GEF activity of Ragulator for RagB and RagA likely stems from differences between the RagA/B and the RagC switch I/II regions, which are known to serve as a critical recognition motif on a GTPase for its cognate GEF (Fiegen et al., 2006; Goldberg, 1998). In addition, the intrinsic rapid GDP dissociation capacity of RagC suggests that a GEF might not be necessary for it. Rather, other regulators, namely guanine dissociation inhibitors, which block GDP dissociation (Garcia-Mata et al., 2011; Jennings and Pavitt, 2010), might have a more prominent role in the regulation of GTP binding by RagC.

Protein scaffolds encompass one of the most diverse sets of signaling molecules in cells. Recent studies have suggested that in addition to bringing multiple proteins together, some scaffolds also have catalytic functions. *Escherichia coli* uses the catalytic scaffold EspG to inhibit host intracellular trafficking by bringing together the Arf1 GTPase and Pak2 kinase as well as blocking Arf1-GAP assisted-GTP hydrolysis and activating Pak2 kinase activity (Selyunin et al., 2011). Similarly, by binding to both Rags and the v-ATPase, Ragulator not only physically connects two major regulators of mTORC1 but also transmits the amino acid signal from the v-ATPase to the Rags through its GEF activity.

Our inability to detect GEF activity in partial assemblies of Ragulator implies that multiple surfaces, which exist only on the pentameric Ragulator are required to endow it with exchange activity. Recently, TRAPPI, a multiprotein tethering complex was identified as a GEF for YPT1 (Jones et al., 2000; Wang et al., 2000). Like Ragulator, the GEF activity of the TRAPPI complex is not contained in one subunit, but requires the presence of multiple components (Cai et al., 2008).

The likely presence of roadblock domains in the C-terminal regions of all four Rags raises the tantalizing possibility that one Rag may directly regulate the nucleotide cycle of the other. The solution of a Rag-Ragulator structure will greatly enhance our understanding of the function of the roadblock domain in this system and the precise mechanism by which Ragulator activates RagA and RagB. While Ragulator is a GEF that directly regulates Rag nucleotide binding, we anticipate the identification of other Rag regulatory proteins such as GAPs that will help explain how amino acid starvation inactivates the Rags and by extension the mTORC1 pathway.

The regulated interaction between Rags and Ragulator depends on amino acids and the nucleotide binding state of RagA and RagB and provides an in-cell output for the activity of Ragulator and the pathway downstream of it. Using this assay, we find that inhibition of the v-ATPase inactivates Ragulator. The fact that the v-ATPase is required for mTORC1 activation, functions downstream of amino acids but upstream of RagA/B nucleotide loading, and interacts with Ragulator, suggests a model in which the v-ATPase links an amino acid-generated signal to the activation of the Ragulator GEF activity (Figure 6C).

There are many possible functions for the regulated interaction between the Rags and Ragulator. In one model, the Rag-Ragulator complex exists in two conformations that are determined by amino acid availability: a tightly bound state, which cannot interact with mTORC1, and an open one that favors mTORC1 recruitment to the lysosomal surface. Upon amino acid stimulation, Ragulator promotes GTP loading of RagA/B, leading to
a weakening of Ragulator-Rag binding and a conformation that may expose an mTORC1-binding surface on the Rag GTPases. A precedent for such a nutrient-dependent conformational change exists within mTORC1. Conditions that inhibit the mTORC1 pathway result in a stronger association between mTOR and raptor with a concomitant decrease in in vitro kinase activity, and conditions that activate result in a weaker interaction and greater in vitro activity (Kim et al., 2002).

Alternatively, or in addition to the first model, the regulated interaction might be necessary for the Rags to reversibly leave the lysosomal surface. During starvation conditions, Ragulator would hold Rags at the lysosome. Upon amino acid stimulation, Rags may dissociate from Ragulator when RagA/B binds GTP, capture mTORC1 in the cytoplasm, and then shuttle it back to the lysosome by reassociating with Ragulator. Many GTpases are known to cycle on and off membranes in a nucleotide dependent manner (Hutagalung and Novick, 2011), and Rag cycling may provide a physical means to ferry mTORC1 to the lysosome. Future work combining structural studies and dynamic live cell imaging will clarify the mechanistic aspects of the regulation of Rag-mTORC1 binding, and how mTORC1 is ferried to the lysosome.

**EXPERIMENTAL PROCEDURES**

**Cell Lysis and Immunoprecipitation**

Cells were rinsed once with ice-cold PBS and lysed with Chaps lysis buffer (0.3% Chaps, 10 mM β-glycerol phosphate, 10 mM pyrophosphate, 40 mM HEPES [pH 7.4], 2.5 mM MgCl₂, and one tablet of EDTA-free protease inhibitor [Roche] per 25 ml). Where specified in the figures, Chaps lysis buffer was supplemented with 12.5 mM EDTA. When only cell lysates were required (i.e., no immunoprecipitation was to be performed), 1% Triton X-100 was substituted for Chaps. When the interaction between Ragulator and mTORC1 was interrogated, in cell crosslinking with DSP was preformed as described in (Sancak et al., 2008) prior to cell lysis. The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm in a microcentrifuge for 10 min. For immuno-precipitations, primary antibodies were added to the cleared lysates and incubated with rotatorion for 1.5 hr at 4°C. 60 μl of a 50% slurry of protein G-Sepharose was then added and the incubation continued for an additional 1 hr. Immunoprecipitates were washed three times with lysis buffer containing 150 mM NaCl. Immunoprecipitated proteins were denatured by the addition of 20 μl of sample buffer and boiling for 5 min, resolved by 8%–16% SDS-PAGE, and analyzed by immunoblotting as described above. For the binding reactions, 20 μl of sample buffer containing immobilized HA-GST-tagged proteins were incubated in binding buffer (1% Triton X-100, 2.5 mM MgCl₂, 40 mM HEPES [pH 7.4], 2 mM DTT, and 1 mg/ml BSA) with 2 μg of FLAG-tagged proteins in a total volume of 50 μl for 1 hr and 30 min at 4°C. In binding assays where HA-GST-Ragulator was used, HA-GST-p14-MP1 was prebound to FLAG-HBXIP-HA-C7orf59 and FLAG-p18 for 5 min at 4°C prior to the addition of other FLAG-tagged proteins. In experiments where the Flag-RagB-HA-RagC heterodimer was loaded with nucleotide, 2 μg of FLAG-RagB-HA-RagC was incubated at 25°C for 10 min in Rag loading buffer (0.3% Chaps, 40 mM HEPES [pH 7.4], 5 mM EDTA, 2 mM DTT, and 1 mg/ml BSA) supplemented with 1 μM GDP·S in a total volume of 10 μl. The Rag-nucleotide complex was stabilized by the addition of 20 mM MgCl₂ and incubated for an additional 5 min at 25°C. In assays with nucleotide free Rags, 2 μg of FLAG-RagB-HA-RagC was added to the binding assay with 3 μl of Calf alkaline phosphatase (NEB). Binding assays in which Ragulator was incubated with nucleotide-loaded or -free Rags were conducted at 4°C for 45 min. For the nucleotide competition assay, 2 μg FLAG-RagB-HA-RagC was prebound to Ragulator proteins for 30 min followed by the addition of 1 μM GDP·S and further incubated for 1 hr and 30 min at 4°C. To terminate all 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 μl of sample buffer.

**Nucleotide Exchange Assays**

40 pmols of FLAG-RagB163N-HA-RagC, FLAG-RagB181N-HA-RagB, Flag-RagCD181N-HA-RagA, or Flag-Rag2a were loaded with either 2 μM of [γ-32P]GDP (25–50 Ci/mmol), 10 μCi of [35S]GTP·S (1,250 Ci/mmol), 2 mM GDP (for GTP binding assays), or coloaded with guanine nucleotides and either 50 nM of XTP·S or 50 nM XDP (Ragulator GEF activity was maintained between a range of 5–500 nM xanthine nucleotide) in a total volume of 100 μl of Rag loading buffer as described above. The GTPase-[3H]GDP-XDP/XTP·S or GTPase-[35S]GTP·S-XDP/XTP·S and GTPase-GDP complexes were stabilized by addition of 20 mM MgCl₂, followed by a further incubation at 4°C for 2 hr or 25°C for 5 min. To terminate the GEF assay, 40 pmols of pentameric Ragulator, the indicated Ragulator subcomplexes or a control (FLAG-Metap2, Flag-VPS39, or Flag-HBXIP-HA-C7orf59) were added...
along with 200 μM GTPγS or 5 μCi of [35S]GTPγS (for GTP binding assays) and incubated at 25 °C. Samples were taken every 2 min and spotted on nitrocellulose filters, which were washed with 2 ml of wash buffer (40 mM HEPES [pH 7.4], 150 mM NaCl, and 5 mM MgCl2). Filter-associated radioactivity was measured using a TriCarb scintillation counter (PerkinElmer).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.07.032.

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