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Positive and negative regulation of the master metabolic regulator mTORC1 by two families of *Legionella pneumophila* effectors

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Summary

All pathogens must acquire nutrients from their hosts. The intracellular bacterial pathogen *Legionella pneumophila,* the etiological agent of Legionnaires' disease, requires host amino acids for growth within cells. The mechanistic target of rapamycin complex 1 (mTORC1) is an evolutionarily conserved master regulator of host amino acid metabolism. Here we identify two families of translocated *L. pneumophila* effector proteins that exhibit opposing effects on mTORC1 activity. The *Legionella* glucosyltransferase (Lgt) effector family activates mTORC1, through inhibition of host translation, whereas the SidE/SdeABC (SidE) effector family acts as mTORC1 inhibitors. We demonstrate that a common activity of both effector families is to inhibit host translation. We propose that the Lgt and SidE families of effectors work in concert to liberate host amino acids for consumption by *L. pneumophila*.

eTOC blurb

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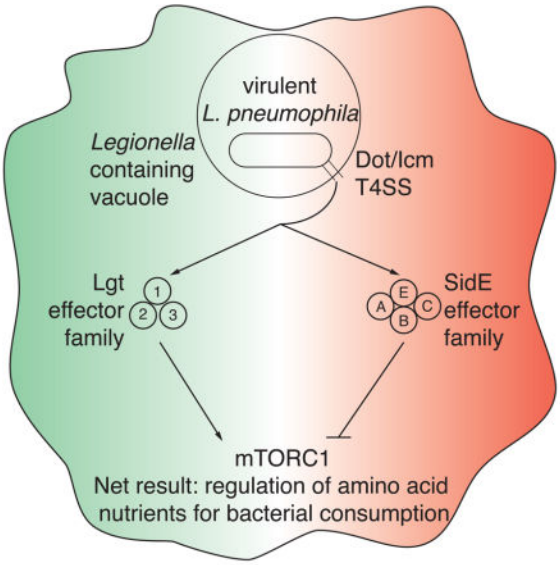
Pathogens manipulate host metabolism in order to acquire nutrients during infection. De Leon *et al.* show how the bacterial pathogen *Legionella pneumophila* targets mTORC1, a key nutrient signaling hub in host cells, by secreting two families of effectors that act via distinct mechanisms.



Keywords

mTORC1; *Legionella pneumophila*; effector; amino acids; T4SS

Introduction

All bacterial pathogens encode mechanisms to acquire nutrients and macromolecules from their hosts. *Legionella pneumophila* is an intracellular bacterial pathogen whose natural host cells are diverse species of freshwater amoebae (Fields et al., 2002). Upon inadvertent inhalation by humans, *L. pneumophila* can also replicate within alveolar macrophages to cause a severe pneumonia called Legionnaires' Disease (Copenhaver et al., 2014). Given the diversity of its host species, success as a pathogen requires *L. pneumophila* to target and modulate conserved host processes. To do this, *L. pneumophila* employs its Dot/Icm type IV secretion system to deliver more than 300 bacterial effector proteins into the host cell cytosol (Qiu and Luo, 2017). Because of functional redundancy among effectors, genetic deletion of individual effector genes rarely imparts a significant growth defect, but loss of a functional Dot/Icm system renders *L. pneumophila* avirulent and unable to replicate intracellularly (Ensminger, 2016). Numerous translocated *L. pneumophila* effectors target highly conserved host processes to establish the *Legionella* containing vacuole, a replicative niche for the bacterium (Isberg et al., 2009). Additional effectors target other conserved host processes. For example, as many as seven effectors have been identified that inhibit host protein synthesis (Barry et al., 2013; Belyi et al., 2008; Fontana et al., 2011; Shen et al., 2009). However, a *L. pneumophila* strain (Δ7) that lacks these seven effectors still inhibits host translation initiation via a Dot/Icm-dependent mechanism (Barry et al., 2017). Thus, *L.*

pneumophila likely encodes additional effectors that target conserved host signaling pathways that regulate translation initiation.

Although it has not been extensively studied, *L. pneumophila* also likely encodes effectors that promote acquisition of host nutrients, particularly amino acids. *L. pneumophila* is auxotrophic for several amino acids and requires host-derived amino acids for intracellular replication (Eylert et al., 2010; Sauer et al., 2005). Amino acid levels are tightly controlled in host cells. The mechanistic target of rapamycin complex 1 (mTORC1), a conserved protein complex consisting of the mTOR kinase and several regulatory proteins, is a critical regulator of the growth state of cells in response to the availability of amino acids and other nutrients (Efeyan et al., 2012). Active mTORC1 represses autophagy and lysosome biosynthesis and stimulates translation initiation (Mohr and Sonenberg, 2012). *L. pneumophila* has previously been reported to modulate mTORC1 activity in infected cells, but no effectors responsible for this modulation have been identified (Abshire et al., 2016; Ivanov and Roy, 2013).

In this study, we report that previously characterized substrates of the Dot/Icm type IV secretion system have additional functions in regulating mTORC1 activity. The *Legionella* glucosyltransferase (Lgt) family of effectors was originally identified as a family of enzymes that potently inhibits host protein synthesis (Belyi et al., 2006). Here we show that protein synthesis inhibition by the Lgt effectors results in activation of mTORC1. We also report that a distinct family of effectors, the SidE/SdeABC (SidE) family, negatively regulates mTORC1 by catalyzing the ubiquitylation of Rag small-GTPases that are important for mTORC1 amino acid sensing. We propose that a joint effect of the Lgt and SidE effector families is to promote liberation of host amino acids for bacterial consumption.

Results

An effector screen identifies Lgt effectors as activators of mTORC1

We sought to investigate mechanisms by which *L. pneumophila* might liberate host amino acids for its consumption. Given that mTORC1 is an important regulator of host amino acid metabolism, we decided to perform a qualitative screen to identify Dot/Icm effectors that activate mTORC1. To do this, we utilized a HEK 293T cell line stably expressing Transcription factor EB (TFEB) fused to enhanced Green Fluorescent Protein (293T-TFEB-eGFP) as a reporter of mTORC1 activity (Settembre et al., 2012). TFEB is a transcription factor that regulates lysosome biogenesis and is a target of mTORC1 (Settembre et al., 2012). In the presence of amino acids, mTORC1 is active and phosphorylates TFEB which is then retained in the cytosol. In the absence of amino acids, mTORC1 is inactive, and TFEB is hypophosphorylated and enters the nucleus to activate transcription of lysosome biogenesis genes (Figure 1A). We transfected the 293T-TFEB-eGFP reporter cells with 260 individual *L. pneumophila* Dot/Icm effectors and screened for effectors that prevented nuclear localization of TFEB upon amino acid withdrawal.

Reporter cells transfected with expression vectors encoding *lgt1, lgt2,* or *lgt3* exhibited constitutive TFEB cytosolic localization and mTORC1 activity, even under conditions of amino acid withdrawal (Figure 1A, B). To validate these results, we assessed mTORC1-

dependent phosphorylation of S6K1 at threonine 389 (T389), an mTORC1-specific substrate (Figure 1C). We observed that cells expressing Lgts showed robust T389 phosphorylation, even when starved of amino acids. Lgt-dependent phosphorylation of S6K1 T389 was mTORC1-dependent since it was inhibited by Torin1, an inhibitor of mTORC1 kinase activity.

The Lgt effectors are a family of *Legionella* glucosyltransferases that were previously shown to target host elongation factor 1A (eEF1A) and thereby inhibit translation (Belyi et al., 2006). Importantly, we found that mutant glucosyltransferase-dead Lgt effectors failed to activate mTORC1 (Figure S1A). Given that amino acids activate mTORC1, we reasoned that the Lgt family might indirectly activate mTORC1 by increasing the availability of intracellular amino acids via the inhibition of host protein synthesis. Consistent with this hypothesis, it has been shown previously that translation elongation inhibitors such as cycloheximide (CHX) can activate mTORC1, presumably through liberation of amino acids (Watanabe-Asano et al., 2014). We confirmed this result and further found that an inhibitor of translation initiation, bruceantin, also activates mTORC1 (Figure 1D). In addition, the phosphorylation of 4E-BP1, another mTORC1 substrate, was increased in cells expressing the Lgt family (Figure S1B). Thus, translation inhibition by diverse mechanisms activates mTORC1, suggesting that it is the block in protein synthesis and consequent liberation of amino acids, rather than another effect of the Lgts, that leads to mTORC1 activation.

To rule out the possibility that Lgt effectors activate mTORC1 via Akt, we also examined the phosphorylation state of Akt and saw no differences in cells transfected with Lgt1 or its glucosyltransferase-dead mutant (Figure S1C). In order to assess the effect of Lgts on mTORC1 in a more physiological setting, we infected bone marrow derived macrophages (BMMs) with a *L. pneumophila* strain that lacks the Lgt family and other known translation inhibitors (Δ7) (Barry et al., 2013; Fontana et al., 2011). In order to prevent the potentially confounding effects of flagellin-induced NAIP5 inflammasome-dependent macrophage cell death (Molofsky et al., 2006; Ren et al., 2006), we used a strain of *L. pneumophila* that lacks flagellin (Δ*flaA*) as the parental strain. Since TLR signaling is known also to activate mTORC1 (Abdel-Nour et al., 2014), we utilized BMMs from *Myd88*$^{-/-}$ mice that are defective for TLR signaling. A previous report demonstrated that *L. pneumophila* activates mTORC1 in a Dot-dependent manner in *Myd88*$^{-/-}$ macrophages (Abshire et al., 2016), but did not identify effectors responsible for mTORC1 activation. Remarkably, *Myd88*$^{-/-}$ BMMs infected with the Δ*flaA*Δ7 strain exhibit decreased mTORC1 activity (as measured by phospho-S6K1) compared to BMMs infected with Δ*flaA* (Figure 1E). mTORC1 activity was restored in cells infected with the Δ*flaA*Δ7 strain complemented with wild-type but not glucosyltransferase-dead Lgt2 and Lgt3. Thus, the Lgts appear to be the primary Dot/Icm-translocated effectors responsible for mTORC1 activation in infected macrophages. We were unable to observe a growth defect of the Δ*flaA*Δ7 strain during infection, even in amino acid limiting conditions ((Fontana et al., 2011) and data not shown). The lack of a growth phenotype is likely explained by the prior observation that the Δ*flaA*Δ7 strain appears to encode yet additional effectors that impose a (delayed) block on host protein synthesis (Barry et al., 2017). Nevertheless, taken together, our results indicate that *L. pneumophila* activates mTORC1 via secretion of Lgts, likely as an indirect effect of Lgt-dependent translation inhibition and the consequent liberation of host amino acids.

An effector screen identifies the SidE family as inhibitors of mTORC1

We also used the 293T-TFEB-eGFP reporter cells to screen for effectors that inhibit mTORC1. In this screen, reporter cells were transfected with constructs expressing individual effectors as before, but instead of withdrawing amino acids prior to imaging, we maintained the cells in complete media. Under these conditions, mTORC1 is active and TFEB-eGFP is cytosolic, unless an effector blocks mTORC1 activity. Most tested effectors did not block mTORC1, but we found that expression of *sidE, sdeA, sdeB,* or *sdeC* induced nuclear localization of TFEB (Figure 2A, B). These four paralogs, referred to here collectively as the SidE family, are a group of recently characterized effectors that catalyze the ubiquitylation of Rab small-GTPases and Reticulon-4 (Rtn4) via an unusual biochemical mechanism that does not require E1 or E2 enzymes (Bhogaraju et al., 2016; Kotewicz et al., 2017; Qiu et al., 2016). To confirm that the SidE effectors interfere with mTORC1 activity, we found that S6K1 phosphorylation was also inhibited in HEK 293T cells expressing the SidE family (Figure 2C). The inhibition by the SidE family required the mART (mono-ADP ribosyltransferase) motif required for catalyzing ubiquitylation. Importantly, inhibition of mTORC1 by SidE effectors was comparable in magnitude to the effect of dominant-negative RagB and RagD ($Rags^{DN}$) which inhibit mTORC1 (Han et al., 2012; Oshiro et al., 2014). We also observed decreased phosphorylation of 4E-BP1 in cells expressing the SidE family (Figure S1D). Moreover, the inhibitory effect of SidE did not appear to be due to modulation of Akt, as phosphorylation of Akt at T308 or S473 was unaffected by SdeA transfection (Figure S1E).

We next assessed the effects of SidE effectors during *L. pneumophila* infection of BMMs. We were unable to observe an effect on mTORC1 signaling in cells infected with a strain lacking the SidE family (ΔsidEs) when pulsed with amino acids (Figure S2A). This may be due to the presence of additional mTORC1 inhibitors. We were able to observe a modest growth defect during infection with strains lacking the SidE family (Figure S2B). This growth defect was further exacerbated in amino acid limiting conditions and partially rescued upon complementation with a plasmid expressing wild type but not mART-dead *sdeA* (Figure S2B). However, because SidE effectors have global effects on ubiquitylation, vesicular trafficking, and the tubular endoplasmic reticulum in host cells (Bhogaraju et al., 2016; Kotewicz et al., 2017; Qiu et al., 2016), the cause of this growth defect may not solely be due to the effects on mTORC1 signaling. In summary, our results suggest that mTORC1 inhibition is an additional function of the SidE family.

Inhibition of protein synthesis is a common effect of Lgt and SidE effectors

Initially we were puzzled as to why *L. pneumophila* would encode two families of effectors with opposing effects on mTORC1. However, this counterproductive behavior could be rationalized if both families of effectors had an underlying common purpose, namely, inhibition of host protein synthesis. The Lgts have already been shown to act as direct inhibitors of translation elongation (Belyi et al., 2006). To test whether negative regulation of mTORC1 by SidE effectors might also block host protein synthesis, we measured the incorporation of [^{35}S]-labeled methionine into effector-transfected cells. We observed that cells expressing *sidE* paralogs *sdeA-C*, but not the mART catalytic mutants of *sdeA-C*,

exhibited a decrease in protein synthesis (Figure 2D). These data suggest that inhibition of protein synthesis is a common downstream effect of both the Lgt and SidE effector families.

The SidE effectors ubiquitylate Rag small-GTPases

Protein synthesis inhibition by the Lgt effectors results in mTORC1 activation (Figure 1). In order for SidE effectors to block translation without activating mTORC1, we hypothesized that the SidE effectors must act at the level of, or downstream of, the Rag small-GTPases that are required for mTORC1 responsiveness to amino acids. Otherwise, the liberated amino acids from SidE-mediated translation arrest would presumably activate mTORC1. Thus, to test whether SidE effectors act at the level or downstream of Rags, we co-expressed constitutively active Rags ($Rags^{CA}$), with *sdeA* or $sdeA^{mART}$. The activation of mTORC1 by $Rags^{CA}$ was abolished in the presence of *sdeA* but not in the presence of $sdeA^{mART}$ (Figure 2E). This result implies that SdeA blinds mTORC1 to elevated intracellular levels of amino acids, resulting in constitutive inhibition of mTORC1 even in the presence of elevated amino acid levels associated with protein synthesis inhibition.

Given these results, we wondered if the Rags could be directly targeted by SdeA. SdeA has been reported to catalyze the mART-dependent ubiquitylation of Rab small GTPases (Bhogaraju et al., 2016; Qiu et al., 2016). Indeed, we observed that co-transfection of SdeA with the small GTPases RagB or RagD resulted in a molecular weight shift consistent with monoubiquitylation (Figure 3A) (Bhogaraju et al., 2016; Kotewicz et al., 2017; Qiu et al., 2016). The molecular weight shift required the mART motif in SdeA, similar to what has previously been observed upon SdeA-dependent modification of the Rab small GTPases and Rtn4 (Figure 3A). *In vitro* reactions with recombinant purified proteins show that the SdeA-dependent modification of the Rags depends on the presence of NAD and Ubiquitin (Figure 3B). This suggests that SdeA inhibits mTORC1 by directly inhibiting the Rag small-GTPases.

If the SidE effectors inhibit Rag-dependent amino acid sensing by mTORC1, we reasoned that they should be able to dominantly abolish the ability of Lgt or other translation inhibitors to activate mTORC1. However, when we attempted to co-express Lgts with SidE effectors in transfected 293T cells, we observed that the Lgts blocked SidE effector expression (presumably via inhibition of translation). To circumvent this technical difficulty, we mimicked the effect of the Lgts by adding chemical translation inhibitors (cycloheximide or bruceantin) after transfected SidE effectors were expressed. In line with our hypothesis, the activation of mTORC1 by these translation inhibitors was abrogated in the presence of catalytically active SdeA (Figure 3C). Based on these results, we hypothesize that a role of the SidE family is to blind mTORC1 to the amino acids liberated by the Lgt effectors and other translation inhibitors.

Taken together our results suggest a model in which protein synthesis inhibition and mTORC1 inhibition might synergistically elevate the pools of intracellular amino acids in cells. Indeed, we found that cells treated with a protein synthesis inhibitor (cycloheximide) and an mTORC1 inhibitor (Torin1) displayed elevated levels of several amino acids (Figure S3). Not all amino acid levels were increased, however, underlining the complexity of amino acid metabolism in cells. Importantly, though, several of the increased amino acids (e.g.,

isoleucine, arginine, and phenylalanine) are ones for which *L. pneumophila* is reported to be an auxotroph (Eylert et al., 2010).

L. pneumophila strain that lacks all known translation inhibitors still inhibit host protein synthesis

Given the above results, we asked if *L. pneumophila* strains that lack all known translation inhibitors are still able to inhibit host protein synthesis. Indeed, expression of Lgt3 combined with chemical inhibition of mTORC1 led to synergistic inhibition of protein synthesis (Figure 4A). The Δ*flaA*Δ7 strain, which lacks Lgts and other effectors, still inhibits translation (Barry et al., 2017; 2013). We therefore tested whether deletion of the SidE family in the Δ*flaA*Δ7 background restores host protein synthesis. We infected BMMs with strains of *L. pneumophila* lacking a varying number of translation inhibitors. A strain lacking the seven known translation inhibitors as well as the four members of the SidE family (Δ*flaA*Δ11) still inhibited translation (Figure 4B), while the Δ*flaA* Δ*dotA* strain does not inhibit translation. This indicates that *L. pneumophila* may possess still additional inhibitors of host protein synthesis and/or perhaps additional effectors that inhibit mTORC1. The high level of redundancy demonstrated by these results suggests that translation inhibition is important for *L. pneumophila* fitness during infection.

Discussion

L. pneumophila is an amino acid auxotroph and must therefore target conserved host processes in order to obtain amino acids. Our data lead us to propose a speculative model for how *L. pneumophila* employs multiple effectors to inhibit mTORC1 and host protein synthesis—and thereby liberate host amino acids for bacterial consumption—without engaging autophagic responses that might restrict bacterial replication (Figure S4A). Of course, the effectors we identified have been shown to have diverse effects on cells, and it is therefore likely that mTORC1 modulation may only be a part of the complex biological roles of these effectors (Bhogaraju et al., 2016; Hempstead and Isberg, 2015; Kotewicz et al., 2017; Qiu et al., 2016; Treacy-Abarca and Mukherjee, 2015).

A previous report suggested that *L. pneumophila* requires an effector called AnkB to liberate host amino acids via ubiquitin-proteasome-mediated degradation of host proteins (Price et al., 2011). In this report, *ankB* mutants in the AA100 strain background were found to be severely attenuated for intracellular growth. However, in our experiments, we could not detect any significant defects in intracellular replication of *ankB* mutants (Figure S4B). We cannot offer an explanation for this discrepancy, but it would not be surprising if *L. pneumophila* encodes multiple redundant strategies to acquire host amino acids.

Our results provide insights into the long-standing question of how *L. pneumophila* obtains amino acids in order to replicate in its intracellular niche. Our data are consistent with emerging evidence that mTORC1 is a key signaling hub in numerous bacterial infections (Jaramillo et al., 2011; Lu et al., 2015; Tattoli et al., 2012). Indeed, mTORC1 regulates known antimicrobial factors such as autophagy and lysosomes. In addition, the importance of mTORC1 as a key regulator of host nutrients makes it a lucrative target for pathogens.

Future studies will likely identify additional mTORC1 regulators in other intracellular pathogens.

Experimental Procedures

Effector library screen

A library of *L. pneumophila* Dot/Icm effectors was as previously described (Barry et al., 2013) and adapted from (Losick et al., 2010). 4×10^4 HEK 293T TFEB-eGFP cells were reverse transfected with 100 ng of each effector with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Cells were plated on clear bottom 96-well imaging plates (EK-Scientific) seeded with fibronectin (Corning). 24 h post transfection, cells were fixed and stained with DAPI. GFP and DAPI were imaged using a Molecular Devices ImageXpress Micro.

In vitro ubiquitylation assay

To purify Flag-RagB or Flag-RagD from mammalian cells, 293T cells transfected with the indicated plasmids for 24 h were lysed with RIPA buffer. ANTI-FLAG M2 Affinity Gel was added to cleared lysates obtained by centrifugation at 12,000*g* for 10 min. The mixtures were incubated at 4°C with agitation for 4 h. Unbound proteins were removed by washing the beads three times with RIPA buffer and the Flag-tagged proteins were eluted with 450 μg/ml 3×Flag peptide (Sigma). A ubiquitylation assay was performed at 37°C for 2 h in a reaction buffer containing 50 mM Tris-HCl (pH=7.5), 0.4 mM β-nicotinamide adenine dinucleotide (β-NAD) (Sigma-Aldrich) and 1mM DTT. Each 50-μl reaction contains 10 μg ubiquitin, 5 μg SdeA or $SdeA^{mART}$, and 5 μg Flag-RagB or Flag-RagD. Reactions were terminated by adding 5×SDS loading buffer. Samples were subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining or Western blot with Flag antibody.

Bacterial strains

For detailed experimental procedures, please see the Supplemental Experimental Procedures.

Cell culture

For detailed experimental procedures, please see the Supplemental Experimental Procedures.

Infection and stimulation

For detailed experimental procedures, please see the Supplemental Experimental Procedures.

Transfection and immunoblotting

For detailed experimental procedures, please see the Supplemental Experimental Procedures.

Growth curve

For detailed experimental procedures, please see the Supplemental Experimental Procedures.

[^{35}S] Metabolic labeling

For detailed experimental procedures, please see the Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The human pathogen *L. pneumophila* secretes effectors that modulate mTORC1
- The Lgt effector family activates mTORC1 upstream of the Rag small-GTPases
- The SidE family inhibits mTORC1 via direct ubiquitylation of Rag small-GTPases
- Lgt and SidE families work in concert to free amino acids for bacterial consumption

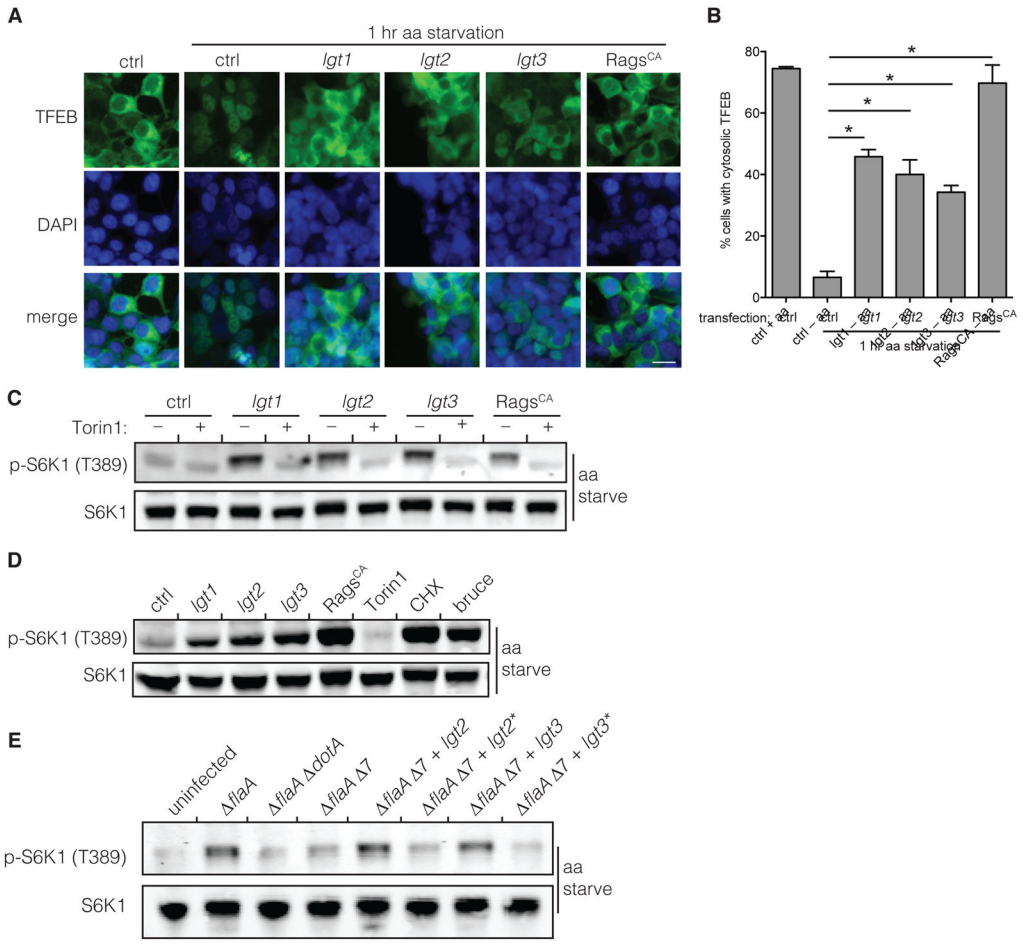

Figure 1. Lgt1-3 activates mTORC1 through host translation arrest and resulting liberation of amino acids

A) Representative images of 293T-TFEB-eGFP reporter cells transfected with expression plasmids of the indicated effectors or with constitutively active Rags (Rags^{CA}), 1 h prior to harvest, amino acids were withdrawn from the media. Scale bar represents 10 μm.

B) Quantification of percent of cells with cytosolic TFEB in 293T-TFEB-eGFP cells treated as in (A).

C) HEK 293T cells were transfected with empty vector, Lgt effectors, or Rags^{CA} and then were either left untreated or treated with 250 nM Torin1 for 4 h. 1 h prior to harvest, amino acids were withdrawn for 1 h. 24 h post transfection, the cells were lysed and mTORC1 activity was measured via western blots probing for phosphorylated and total S6 Kinase 1 (S6K1), an mTORC1 substrate.

D) HEK 293T cells were transfected with Lgt effectors or Rags^{CA} or were treated with 250 nM Torin1, 10 μM cycloheximide (CHX), or 50 nM bruceantin (bruce) for 4 h. 24 h post transfection, cells were harvested and probed as in (C).

E) Bone marrow derived macrophages from *Myd88*$^{-/-}$ mice were infected with the indicated strains at MOI 3. 9 h post infection, amino acids were withdrawn from the media for 1 h. 10 h post infection, cell lysates were harvested and probed as in (C). Asterisk indicates catalytically inactive alleles.

(B) mean ± S.D., (C–E) data shown are representative of least three independent experiments.

*, $p < 0.001$; statistical test: unpaired t-test

See also Figure S1.

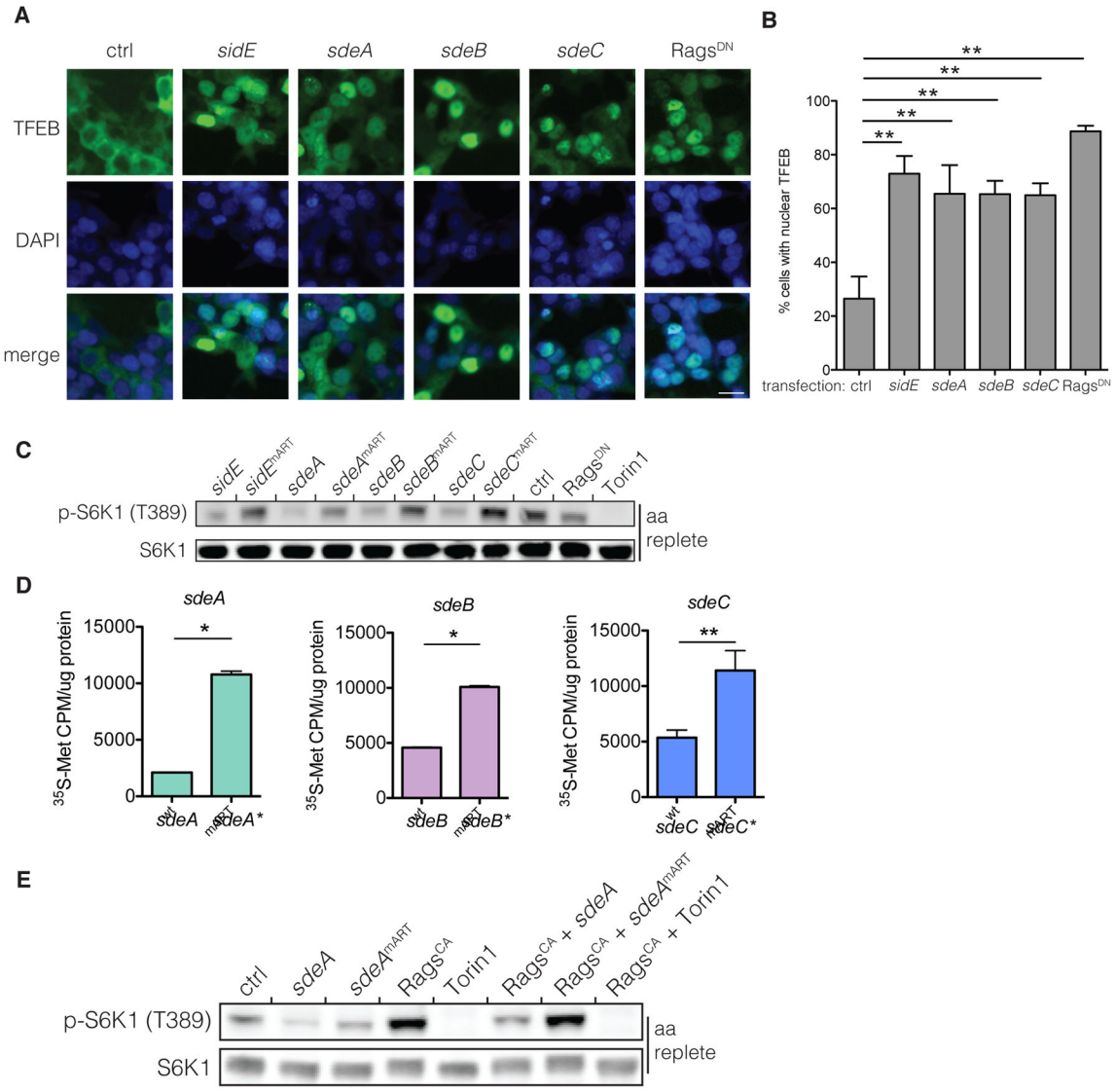

Figure 2. SidE family of effectors inhibits mTORC1 downstream or at the level of Rag small-GTPases

A) Representative images of 293T-TFEB-eGFP reporter cells transfected with expression plasmids of the indicated effectors or with dominant negative RagB and RagD ($Rags^{DN}$: $RagB^{T54L}$ and $RagD^{Q121L}$) and retained in complete media. Scale bar represents 10 μm.

B) Quantification of percent of cells with nuclear TFEB in 293T-TFEB-eGFP cells treated as in (A).

C) HEK 293Ts were transfected with empty vector, wild type, or mART-dead SidE family effectors. As a positive control, cells were also transfected with $Rags^{DN}$ or treated with 250 nM Torin1. 1 h prior to harvest, amino acids were withdrawn for 50 min and then replenished for 10 min. 24 h post transfection, the cells were lysed and mTORC1 activity was measured via western blots probing for phosphorylated and total S6 Kinase 1 (S6K1), an mTORC1 substrate.

D) HEK 293T cells were transfected with the indicated constructs and protein synthesis was assessed by measuring [^{35}S]-methionine incorporation.

E) HEK 293T cells were transfected with the indicated constructs and treated with 250 nM Torin1 4 h prior to harvest as indicated. 1 h prior to harvest, amino acids were withdrawn for 50 min and then replenished for 10 min. 24 h post transfection, the cells were lysed and probed as in (C).

*, $p < 0.05$; **, $p < 0.01$; statistical test: unpaired t-test

(B) and (D) mean ± SD, (C) and (E), data shown are representative of least three independent experiments.

See also Figure S1 and Figure S2.

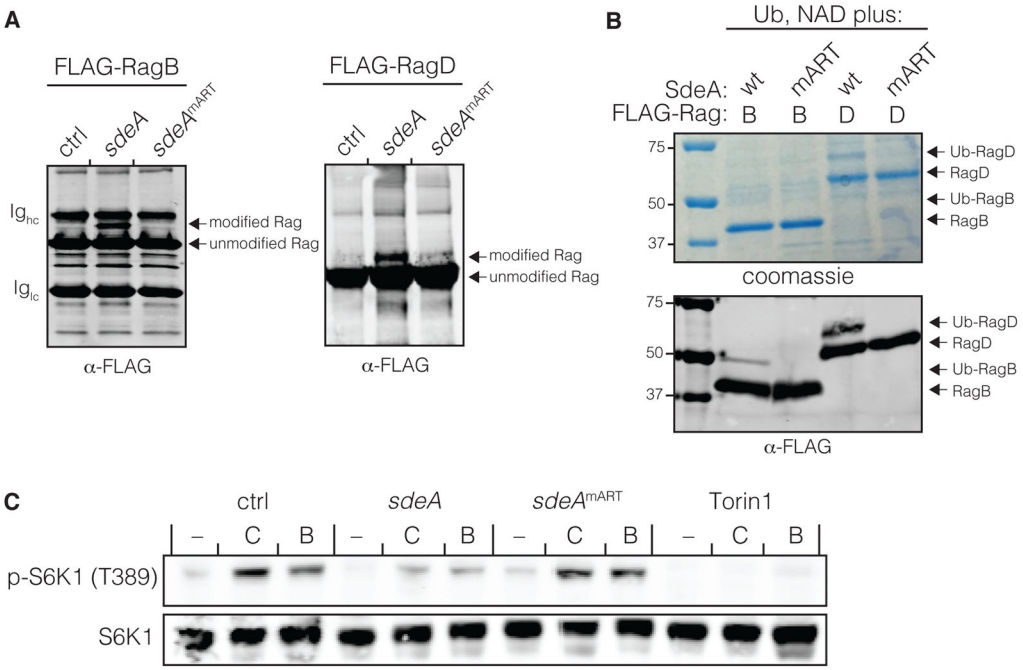

Figure 3. SidE family inhibits mTORC1 amino acid sensing by direct inhibition of Rag small-GTPases

A) HEK 293T cells were transfected with FLAG-RagD and with either wild-type SdeA or SdeA^{mART}. FLAG immunoprecipitation was performed on cell lysates and then probed for FLAG via western blot.

B) Flag-RagB or Flag-RagD purified from transfected 293T cells were incubate with SdeA or SdeA^{mART} and ubiquitin in the presence of β-NAD at 37°C for 2 h. After termination by 5×SDS loading buffer and separation by SDS-PAGE, ubiquitylation of RagB and RagD were probed by Coomassie staining (top) or by immunoblotting (bottom) with antibodies specific for FLAG.

C) HEK 293T cells were transfected with the indicated constructs or treated with 250 nM Torin1. In addition, cells were treated with cycloheximide (C), or bruceantin (B) for 4 h prior to harvest. 1 h prior to harvest, amino acids were withdrawn for 50 min and then replenished for 10 min. 24 h post transfection, the cells were lysed and mTORC1 activity was measured via western blots probing for phosphorylated and total S6 Kinase 1 (S6K1), an mTORC1 substrate.

(A–C), data shown are representative of least three independent experiments.

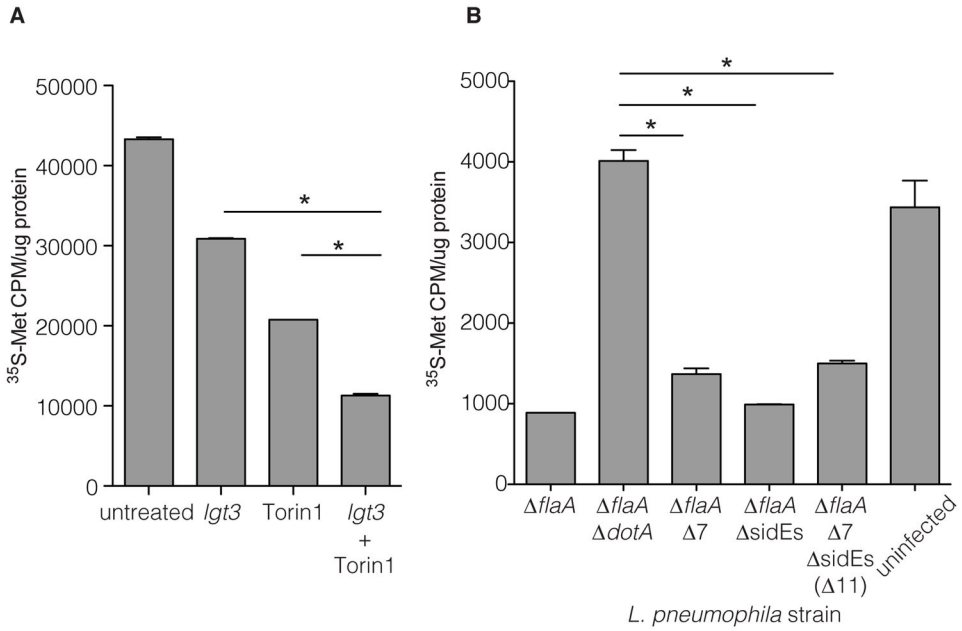

Figure 4. Examination of the synergistic effects of mTORC1 effectors on translation and their role in translation inhibition during infection

A) HEK 293T cells were transfected with the indicated constructs or treated with 250 nM Torin1. Protein synthesis was assessed by measuring [^{35}S]-methionine incorporation

B) BMMs from C57BL/6J mice were infected with the indicated *L. pneumophila* Δ*flaA* strains. 5 h post infection cells were labeled with [^{35}S]-methionine. 1 h later, the cells were lysed and radioactivity was measured using liquid scintillation counting.

*, $p < 0.05$; statistical analysis: unpaired t-test

(A) and (B) mean ± SD

See also Figure S3 and S4.