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Mixed Signals: Regulation of host metabolism by an intracellular bacterial pathogen

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Regulation of host metabolism by an intracellular bacterial pathogen

By

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Abstract

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A hallmark of all pathogens is an ability to acquire nutrients from hosts. In order to do this, pathogens must overcome both the tight regulation of host nutrients and the host defense mechanisms deployed to protect these nutrients.

In the introductory chapter of this dissertation, I begin with a discussion of innate immunity. I discuss the now appreciated view of the innate immune system as a sensor of patterns of pathogenesis. I then argue, based on our studies in *Legionella pneumophila*, that nutrient acquisition by microbes is a pattern of pathogenesis.

In chapter two, I provide experimental evidence that nutrient acquisition is a pattern of pathogenesis. I discuss my work describing how *L. pneumophila* alters a key metabolic signaling pathway, the mechanistic target of rapamycin complex 1 (mTORC1), in order to free amino acids for nutrition. Using an effector screen, I identified two groups of *L. pneumophila* Dot-dependent substrates that have opposing consequences on mTORC1. I showed that a family of *Legionella* glucosyltranferases, Lgt1-3, activate mTORC1 via translation inhibition, resulting in release of host amino acids. These amino acids activate mTORC1 but do so in a counterproductive way: stimulation of mTORC1 leads to the initiation of translation, which consumes the amino acids meant for *L. pneumophila*. To counter this, *L. pneumophila* also secretes the SidE family, which inhibits mTORC1 by directly inhibiting the Rag small-GTPases that are required for mTORC1-dependent amino acid sensing. The SidE effectors blind mTORC1 to the amino acids newly freed by the Lgt family. The net result of this battery of SidE and Lgt effectors is to enable *L. pneumophila* to manipulate the host into liberating amino acids for bacterial consumption.

I then close with a perspective chapter that describes the additional questions my work has given rise to and the state of the bacterial molecular pathogenicity field as a whole.

To Mallory Kathleen Richards,
because.

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Chapter One: Introduction

1.1 An overview of innate immunity

The fundamental role of the immune system is to distinguish between self and infectious nonself. This role can be exemplified by the innate immune system, which has the important role of sensing and responding to pathogens while simultaneously remaining inert to non-pathogens. In vertebrates, the innate immune system primes an adaptive immune response that encompasses various tissues. However, the innate immune system traces its origins to phylogenetically older organisms and is the primary basis of cell-intrinsic host defense.

Our current paradigm of the molecular underpinnings of innate immune recognition may have been consolidated with the thought-piece written by Charles Janeway, Jr in 1989 (Janeway, 1989). In this review, Janeway argued for the existence of an innate immune system that activates a second stimulatory signal necessary for antigen recognition, referred to as co-stimulation. He argued that the innate immune possesses germline-encoded receptors that recognize “pathogen associated molecular patterns” (PAMPs). These PAMPs are sensed by “pattern recognition receptors” (PRRs). Though the PAMP:PRR model argued by Janeway in 1989 provided a conceptual framework on which modern molecular innate immunity is based, molecular investigations about the innate immune system began long before that. Innate immunity studies may have begun with the work on what was referred to as “natural immunity” by Metchnikoff (Gordon, 2008)

The experimental evidence supporting the PAMP:PRR model began with the identification of Toll, a receptor in *D. melanogaster* that controls an anti-fungal response (Lemaitre et al., 1996). The human homologue of Toll was later identified and importantly, its activation was shown to lead to the expression of B7, the co-stimulatory molecule proposed by Janeway and mentioned above (Medzhitov et al., 1997). Human Toll, now referred to as Toll-like receptor 4 (TLR4) and later shown to sense lipopolysaccharide (LPS), a constituent of the Gram-negative outer membrane (Poltorak et al., 1998) is the classic example of a now broad distribution of pattern recognition receptors. Additional TLRs have been identified and shown to sense a variety of microbe-associated molecules ranging from bacterial flagellin (TLR5), bacterial lipoprotein (TLR2), as well as different nucleic acids (TLR3, TLR7, TLR8, and TLR9) (O'Neill et al., 2013).

In addition to the TLRs, other classes of PRRs exist, including the nucleotide binding domain, leucine rich repeat containing proteins (NLRs). Engagement of some NLRs by their cognate ligands stimulates the activation of a signaling platform referred to as the inflammasome. The inflammasome stimulates the production of bioactive species of cytokines IL-18 and IL-1β, thereby inducing inflammation, and an inflammatory form of lytic cell death referred to as pyroptosis (Fink and Cookson, 2006). Due to the

localization of NLRs to the cytosol of cells, the inflammasome is considered a cytosolic PRR.

The PAMP:PRR model of innate immunity coined by Janeway is an important advancement in how we think about immunology. However, one often-cited criticism of this model is that all microbes, including non-pathogenic microbes, possess PAMPs. Indeed, it has been proposed that PAMPs should be re-named microbe-associated molecular patterns (MAMPs). Additionally, many microbes are able to alter their PAMPs in order to prevent recognition by their cognate PRR. *Yersinia pestis* changes the acyl-structure of its LPS at 37°C that prevents it from being recognized by TLR4 (Montminy et al., 2006). Many bacterial pathogens down-regulate flagellin expression in order to prevent sensing by either TLR5 (Smith et al., 2003) or the Naip inflammasome. In some regards, even homeostatic iterations of certain PAMPs do not stimulate their cognate PRR. For example, *Legionella pneumophila* LPS does not stimulate TLR4 (Werts et al., 2001). Despite these means to avoid PRR sensing, pathogens are still sensed nonetheless. Perhaps the sensing of pathogens is more elaborate than simply which PAMPs a microbe may possess.

The problems with the PAMP:PRR model discussed above led to its expansion that argues that the innate immune system, in addition to sensing PAMPs, also senses "patterns of pathogenesis" (Vance et al., 2009) Moreover, the innate immune system senses pathogenic activity, examples of which are growth within host cells, cytosolic access, and disruption of the host cytoskeleton. The Naip5 inflammasome senses bacterial flagellin (Kofoed and Vance, 2011; Zhao et al., 2011). However, the patterns of pathogenesis model argues that Naip5 is not just simply sensing the PAMP flagellin but rather a pathogenic activity. In this case, Naip5 is sensing cytosolic access by a microbe, an activity restricted only to pathogens.

The patterns of pathogenesis model is a recent conceptual advancement in metazoan immunity but similar models have previously been extensively discussed in the plant immunity literature and referred to as the 'guard' strategy. This strategy consists of monitoring the status of host cellular processes that pathogens may target (Jones et al., 2016). Interestingly, plant immune receptors that mediate this process are similar in domain architecture to the NLRs used by the inflammasome (Jones et al., 2016).

Though it has been argued that the innate immune system helps prime an adaptive immune response orchestrated by lymphocytes, the innate immune system is also the primary mediator of cell-intrinsic immunity. The cell possesses a number of effector pathways that are antimicrobial and are induced by PRR engagement. The lysosome is an organelle that contains degradative enzymes and is perhaps the first cell intrinsic host defense mechanism pathogens must overcome. Some pathogens such *Legionella pneumophila* avoid the lysosome by preventing lysosomal fusion to the vacuole the pathogen resides in (Berger et al., 1994). Other pathogens such us *Listeria monocytogenes,* escape the vacuole before it can fuse with the lysosome (Portnoy et

al., 2002). *Salmonella enterica* resides in a lysosomal-like compartment but can survive this niche by preventing the recruitment of lysosomal degradative enzymes, thereby attenuating lysosomal function (McGourty et al., 2012). PRR engagement can stimulate lysosome activity (Sanjuan et al., 2009). Reactive oxygen and reactive nitrogen are directly antimicrobial and are also induced by PRR engagement (Nathan and Cunningham-Bussel, 2013).

Autophagy refers to a number of different processes that target cellular contents for lysosomal-dependent degradation and is reviewed in (Mitchell and Isberg, 2017). Though the molecular players involved between the differing types of autophagy, the basic mechanism involves the targeting of the cellular content to be degradative, the inclusion of the targeted content into a membrane bound body, and the fusion of the membrane body with the lysosome for degradation. Autophagy is a response to starvation but can also be used for cell-intrinsic defense. Referred to as 'xenophagy' this process involves the tagging of bacteria by ubiquitin, which are recognized by a group of adapter proteins. These adapter proteins link the targeted microbe to LC3-containing autophagosomal membranes, which are then fused to the lysosome. Membrane bound vacuoles containing bacteria can also ubiquitylated in the same manner, perhaps by recognition of the damaged vacuoles. Vacuoles containing bacteria can also be targeted by LC3-associated phagocytosis (LAP). LAP is a process in which bacteria-containing vacuoles are targeted with LC3, which marks the vacuoles for lysosomal fusion and ultimately lysosomal-mediated destruction. Both LAP and xenophagy are induced by TLR stimulation (Into et al., 2012; Martinez et al., 2015).

Host cell death can also be a form of cell-intrinsic host defense. Activation of the inflammasome leads to a lytic form of cell death referred to as pyroptosis, which removes the pathogen out of a replicative niche while simultaneously triggering an immune response (Moltke et al., 2013). Engagement of STING by *Chlamydia trachomatis* also leads to cell death independent of caspases (Sixt et al., 2017). Cell death may be protective to the host because it can remove pathogens from their replicative niche.

The sophistication of the adaptive immune system is a metazoan innovation. Meanwhile, components of the innate immune system can trace their origins to phylogenetically older species. The aforementioned Toll-like receptors were initially found in *Drosophila* (Lemaitre et al., 1996). The discovery of Toll in *Drosophila* came after a string of other studies in insects, beginning with the identification of inducible antimicrobial peptides in the moth *Hyalophora cecropia* (Steiner et al., 1981). Signaling molecules downstream of TLR signaling, such as NF-kB, are not only found *Drosophila* but in *Caenorhabditis elegans* as well (Irazoqui et al., 2010). It has been recently appreciated that cyclic dinucleotide sensing mediated by STING predates the evolution of animals (Margolis et al., 2017). In fact, many of the signaling components and their outputs were found as far back as *Nematostella*, the starlet sea anemone.

1.2 An overview of the mechanisms of protein synthesis in eukaryotes

Translation is the cellular process by which proteins are synthesized using messenger RNA as a template. Translation can be broken into three main parts: initiation, elongation, and termination.

1.2.1 Mechanism of translation initiation

The goal of translation initiation is to bring together an mRNA transcript with the ribosome and the necessary eIFs (eukaryotic initiation factors), along with an initiator tRNA charged with methionine. This process is reviewed in (Hinnebusch and Lorsch, 2012). It begins with the formation of the 43S preinitiation complex, which consists of the 40S subunit of the eukaryotic ribosome, eIF3, eIF5, and the ternary complex (TC) containing eIF2α loaded with GTP and the initiator tRNA. The 43S preinitation complex then binds a processed mRNA that is capped at the 5' end, and bound by poly-A binding protein (PABP) at the polyadenlyated at the 3' end. The PABP is bound to the eIF4F complex contains eIF4E, which binds to the 5' cap of the mRNA and eIF4G, which is bound to PABP itself. When this complex is formed, the ribosome scans along the mRNA transcript looking for the first start codon (AUG). At this point, the eIFs are displaced by the 60S subunit of the ribosome and translation initiation is complete.

1.2.2 Mechanism of translation elongation and termination

The mechanism of translation elongation and termination is reviewed in (Dever and Green, 2012). Briefly, after the 60S ribosomal subunit is added to the translation complex, eEF1A, loaded with GTP, facilities the next charged tRNA to enter the A site of the ribosome. The ribosome catalyzes the peptide bond between the amino acid from this tRNA with that of methionine from the initiator tRNA. eIF2α then facilitates the entire complex to translocate one codon downstream the mRNA, exposing the ribosomal A site for a new charged tRNA. Translation ends once the ribosome encounters a stop codon on the mRNA.

1.2.3 Regulation of translation initiation

1.2.3.1 Regulation of translation initiation by eIF2α

eIF2α has the important function of bringing in the initiator tRNA during translation initiation (Hinnebusch and Lorsch, 2012). This function makes it an obvious target for the cell to regulate mRNA translation. Phosphorylation of eIF2α at serine 51 inhibits its ability to stimulate translation initiation and is regulated by four main kinases, all of which sense some form of cellular stress. dsRNA, a PAMP possessed by viruses, stimulates the dsRNA-dependent kinase PKR (Walsh et al., 2013). GCN2 is activated by UV stress or amino acid deprivation (Deng et al., 2002). HRI senses oxidative stress,

osmotic stress, and heme deprivation (Lu et al., 2001). ER stress activates PERK (Ron and Walter, 2007).

1.2.3.2 Regulation of translation initiation by the mechanistic target of rapamycin complex 1 (mTORC1).

The mechanistic target of rapamycin complex 1 (mTORC1) is a master regulator of metabolism in eukaryotes (Saxton and Sabatini, 2017). It senses a variety of nutrients and stimulates a number of cellular processes including translation initiation. It does this by phosphorylating eIF4E binding protein 1 (4E-BP1), which dissociates it from eIF4E (Choo et al., 2008). Freed eIF4E is then able to bind the mRNA cap, stimulating translation initiation (Hinnebusch and Lorsch, 2012). Activate mTORC1 also phosphorylates ribosomal S6 kinases 1 and 2 (S6K1/2) (Saitoh et al., 2002). In contrast to 4E-BP1, phosphorylated S6K1 is active, and can stimulate translation initiation via ribosomal protein S6 and eIF4B. Phosphorylated S6K1 derepresses eIF4A by inhibiting PDCD4 (Dorrello et al., 2006).

1.2.4 Regulation of translation elongation

AMP activated protein kinase (AMPK) is regulated by the AMP:ATP and ADP:ATP levels in the cell. When there is less ATP compared to AMP or ADP, as in the case during nutrient starvation, AMPK phosphorylates eEF2 kinase. eEF2 kinase then inhibits eEF2 and inhibits translation elongation (Browne et al., 2004). In addition to its role in regulating translation initiation, mTORC1 also regulates translation elongation by inhibiting eEF2 kinase, which derepresses eEF2 and stimulates translation elongation (Browne and Proud, 2004).

1.2.5 Methods deployed by pathogens to regulate host translation

1.2.5.1 Viral mechanisms of translation blockade

The ability to inhibit host protein synthesis is important for viruses because of their requirement to divert host translation machinery in order to produce virally encoded proteins. Viruses block translation at different stages of translation initiation. Picornaviruses block cap-dependent translation by preventing eIF4E from binding the 5' cap of mRNA (Walsh et al., 2013). ECMV promotes accumulation of eIF4E in the nucleus, sequestering it from the ribosome (Groppo et al., 2011). The micro RNA miR141, encoded by enterovirus 73, represses eIF4E expression (Ho et al., 2011).

Viruses can interfere with translation initiation by regulating eIF2α, reviewed in (Walsh and Mohr, 2011; Walsh et al., 2013). Viruses encode proteins that bind dsRNA, which prevent them binding to and activate PKR. These include E3L from vaccinia virus, Us11 from HSV, TRS1 and IRS1 from HCMV, SM from EBV, NS1 from influenza, m142/3 from MCMV and reovirus σ3 (Walsh and Mohr, 2011) Adenovirus, HCV, and EBV have

RNA decoys that antagonize PKR. Viruses also encode protein antagonists of PKR, such as C from Sendai virus, NS5A from HCV, E1b, 55k, and E4 orf6 from adenovirus, IE 180 from PRV and KSHV vIRF2.

eIF2α can be regulated directly from viruses, reviewed in (Walsh and Mohr, 2011; Walsh et al., 2013). This is exemplified by K3L from Vaccinia and vIF2 from Ranavirus, which are eIF2α pseudosubstrates. HSV, ASFV, and HPV interfere with the eIF2α phosphatase regulatory subunit as well. Other viruses can bypass eIF2 by stimulating eIF2-independent translation initiation, exemplified by CrPV, Sindbis virus, and poliovirus.

1.2.5.2 Bacterial mechanisms of translation blockade

Bacteria possess their own translation machinery and therefore do not need to hijack host translation machinery in order to synthesize their own proteins. Nevertheless, several bacterial pathogens inhibit host protein synthesis. A prominent class of bacteria-encoded toxins that inhibit host protein synthesis are a group of mono-ADP ribosyltransferases that target eEF2, of which diphtheria toxin from *Corynebacterium dipththeria* is the most classic example (Collier, 1975). ExoA from *Pseudomonas aeruginosa* and cholix toxin from *Vibrio cholerae* are other examples of this class of translation inhibitors (Jørgensen et al., 2008; Simon et al., 2014).

Bacteria inhibit translation through other means as well, many of which act through GCN2 to phosphorylate eIF2α. *Salmonella, Listeria*, and *Shigella* were all shown to induce GCN2 dependent phosphorylation of eIF2α (Tattoli et al., 2012; 2013). These same studies showed that these pathogens also inhibit mTORC1. The proposed common mechanism that enables these three different pathogens to regulate separate pathways that regulate host translation is the pore-forming activity of these pathogens. The authors purpose that these pores can lead to amino acid stress that inhibits mTORC1 and activates GCN2, though the mechanism of how this occurs is unknown. In two other studies, two additional pathogens were shown to inhibit host protein synthesis through GCN2-dependent phosphorylation of eIF2α. *Streptococcus pyogenes* and *Pseudomonas entomophila*, a pathogen of insects, were both shown activate GCN2 (Chakrabarti et al., 2012; Kloft et al., 2010; Liehl et al., 2006)

1.2.5.3 Inhibition of translation by parasites

Host translation inhibition is not restricted to viruses and bacteria. The protozoan parasite *Leishmania major*, the causative agent of leishmaniasis, encodes a mTORC1-specific protease GP63 (Jaramillo et al., 2011). In this study, GP63-competent *L. major* were able to inhibit host protein synthesis, which prevents a protective immune response. However, mice lacking 4E-BP1 and 4E-BP2, which is an inhibitor of translation in the absence of mTORC1-dependent phosphorylation, are protected from *L. major* infection.

1.3 *Legionella pneumophila* is a model to study bacterial inhibition of host protein synthesis

Legionella pneumophila is a Gram-negative facultative intracellular pathogen and the causative agent of Legionnaires' disease. The main reservoir for *L. pneumophila* is a variety of freshwater amoeba but *L. pneumophila* can cause disease in mammals due to their ability to replicate in alveolar macrophages (Copenhaver et al., 2014). This implies that in order to grow in both protozoa and mammalian cells, *L. pneumophila* must target conserved processes that exist in both of these phylogenetically distinct species. Replication within both of macrophages and protozoa occurs in an endoplasmic reticulum like vacuole referred to as the *Legionella* containing vacuole (LCV) (Tilney et al., 2001).

1.3.1 Molecular pathogenicity of *Legionella pneumophila*

A majority of the molecular mechanisms that govern *Legionella pneumophila* pathogenesis are due to the type IVB secretion system in encodes. This system is referred to as the Dot (deficient in organelle trafficking) or Icm (intracellular multiplication) (Berger and Isberg, 1993; Marra et al., 1992). As the name of these genes implies, *L. pneumophila* strains that lack certain Dot/Icm genes failed to prevent lysosomal fusion to the LCV and cannot replicate in cells (Berger and Isberg, 1993; Marra et al., 1992). Additional early observations with the Dot/Icm apparatus included a defect in macrophage killing in Dot/Icm-deficient *L. pneumophila* strains (Segal and Shuman, 1997). It is now appreciated that the Dot/Icm secretion system secretes as many as 300 effectors into the host cytosol (Hubber and Roy, 2010; Qiu and Luo, 2017) that possess a variety of activities and are described in the following sections.

1.3.1.1 Manipulation of host vesicular trafficking

In addition to its role in preventing lysosome fusion to the LCV, a large number of the effectors secreted by Dot facilitate the establishment of the LCV itself. Some of these effectors do this by hijacking vesicular trafficking through regulation of the Rab small-GTPases that govern where vesicles are destined. Rab1 is important for the trafficking of vesicles from the ER to the Golgi (Hutagalung and Novick, 2011). Inhibition of this arm of vesicular transport prevents the establishment of the LCV. The Dot-dependent effector SidM is a guanine exchange factor (GEF) specific for Rab1 (Murata et al., 2006). SidM also AMPylates Rab1, which prevents Rab1 from associating with GTPase activating proteins (GAPs) thereby keeping Rab1 in an active GTP-loaded state and maintained on the outer leaflet of the LCV membrane (Machner and Isberg, 2006). This facilitates the movement of ER vesicles to the LCV instead of the Golgi. To reverse this process, another Dot-dependent effector, SidD, deAMPylates Rab1, which removes it from the LCV exposes it to LepB, a GAP secreted by *L. pneumophila* to promote GTP hydrolysis by Rab1 (Ingmundson et al., 2007). The resulting GDP-loaded Rab1 can

then bind its inhibitor, guanosine nucleotide dissociation inhibitor (GDI) (Ullrich et al., 1993). Rab1 is targeted by another separate suite of enzymes that also have opposing consequences on its activity. AnxK is a novel phosphorylcholinase that targets Rab1 and maintains it in the *cis*-Golgi (Mukherjee et al., 2011). Lem3 reverses this modification (Tan et al., 2011). *L. pneumophila* also targets other Rab small-GTPases. Rab5, Rab21, and Rab22 were shown to be targeted by a protein encode by Lpg0393, another GEF (Sohn et al., 2015).

Regulation of Rab small-GTPases by *L. pneumophila* does not only occur by manipulation of their nucleotide loading state. Recently, a new family of effectors was shown to regulate Rab small-GTPases. The SidE family was shown to catalyze the E1 and E2-independent serine directed ubiquitylation of the Rab1, Rab6A, Rab30, Rab33B (Bhogaraju et al., 2016; Qiu et al., 2016). The SidE family does this by first ADP-ribosylating ubiqiuitin, the AMP is removed by the nucleotidase domain of SidE, and the resulting ribose-5-phosphate-ubiquitin is conjugated onto the target protein (Bhogaraju et al., 2016). In addition to their effects of vesicular trafficking, the SidE family also has drastic effects on global ubiquitin (Bhogaraju et al., 2016). It is believed that the modification the SidE family performs on ubiquitin prevents it from being used by host ubiquitin enzymes. The SidE family also targets Reticulon-4 (Rtn-4), an ER protein important for ER membrane curvature (Kotewicz et al., 2017). As opposed to the Rabs, in which SidE activity is inhibitory, Rtn-4 targeting by the SidE family appears to be a gain of function, leading to the formation of ER-tubules (Kotewicz et al., 2017). SidJ is a de-ubiquitinase the preferentially targets proteins ubiquitylated by the SidE family (Havey and Roy, 2015; Qiu et al., 2017).

Arf1 is another small-GTPase that is important for ER-Golgi transport and is targeted by *L. pneumophila* effectors. RalF is secreted by *L. pneumophila* and acts as a GEF for Arf1. GTP-loaded Arf1 is maintained on the LCV membrane where it is proposed to facilitate the recycling of membrane coat proteins from the *cis*-Golgi back to the ER, or from the LCV itself back to the ER.

1.3.1.2 Manipulation of host autophagy

Autophagy is a bona fide antimicrobial mechanism deployed by host cells in order to kill and degrade microbes (Huang and Brumell, 2014). *L. pneumophila* encodes two different effectors that inhibit autophagy. The *Legionella* sphingosine lyase LpSpl inhibits autophagy by degrading the sphingolipids that induce it (Rolando et al., 2016). In addition, *L. pneumophila* encodes RavZ, which is a potent LC3-protease (Choy et al., 2012). However, a *L. pneumophila* strain that lacks RavZ is still able to grow in macrophages, implying that *L. pneumophila* encodes additional effectors that inhibit autophagy. The intracellular replication of Δ*ravZ* Δ*lpspl* mutant in cells has not yet been tested.

1.3.1.3 Manipulation of host ubiquitin

Like many other pathogens, *L. pneumophila* encodes effectors that manipulate host ubiquitin. *L. pneumophila* encodes a number of E3 ligases such as LegU1, which targets SKP1 and BAT3, AnkB, which targets SKP1 and Parvin B, and LubX, which targets CLK1 (Ensminger and Isberg, 2010; Kubori et al., 2008; Lomma et al., 2010; Price et al., 2011). Recently, and as mentioned above, the SidE family was shown to catalyze the ubiquitylation through an unusual mechanism. The SidE family ubiquitylates target proteins at serine residues in a manner that is independent of E1 or E2 ligases. Instead, it uses an ADP-ribosylated ubiquitin as a reactive intermediate in a mono-ADP ribosyltransferase (mART) manner (Bhogaraju et al., 2016; Kotewicz et al., 2017; Qiu et al., 2016). The SidE family targets two distinct proteins, a group of Rab small-GTPases, which are important for vesicular trafficking, and an ER-resident protein Reticulon-4, which is important for ER membrane curvature. The unusual biochemistry of the SidE family exemplifies the treasure chest of interesting biochemical functions encoded by *L. pneumophila* effectors.

1.3.1.4 Manipulation of host cytoskeleton

Cited as a pattern of pathogenesis (Vance et al., 2009), many pathogens, *L. pneumophila* included, manipulate the host cytoskeleton. RavK is a metalloprotease that cleaves actin (Liu et al., 2017). VipA is an actin nucleator (Franco et al., 2012). LegK2 targets the ARP2/3 complex in order to prevent actin polymerization at the LCV (Michard et al., 2015).

1.3.1.5 Manipulation of other *L. pneumophila* effectors

Some effectors secreted by the Dot/Icm apparatus modulate other effectors. These effectors, termed “meta-effectors,” are important for their ability to tune down (or up) the effect of different Dot/Icm substrates. Some meta-effectors reverse biochemical changes of target proteins, evidenced by AnkX, which phosphocholinates Rab1, and is reversed by Lem3 (Mukherjee et al., 2011; Tan et al., 2011). SidJ is a de-ubiquitinase specific for SidE-dependent ubiquitylated proteins (Qiu et al., 2017). Urbanus *et al* took a non-biased approach to identify additional meta-effector interactions (Urbanus et al., 2016). Individual Dot/Icm substrates were expressed in pairwise fashion in yeast. If one of the substrates was a meta-effector for the other substrate, it should prevent effector mediated toxicity. One of the interactions they identified was the ability for LegL1 to block the active site of RavJ, a putative cysteine protease.

1.3.1.6 The difficulty in studying the molecular pathogenesis of *L. pneumophila*

L. pneumophila has been an interesting treasure chest for the discovery of the novel biochemical activities that microbes can employ against their hosts. Phenotypes for individual effectors are often observable in isolation, usually through studies with

recombinant protein or expression in HEK 293T cells or a similar cell type. However, phenotypes for effector mutants are seldom observed during infection, either in cell culture or *in vivo*. The often-cited culprit for the lack of infection phenotypes in mutant *L. pneumophila* strains is the redundancy between effectors. Often, a given effector will have additional paralogs encoded on the *L. pneumophila* genome. In one case, large deletions of the *L. pneumophila* genome failed to produce a growth phenotype in mouse macrophages (O'Connor et al., 2011).

1.3.2 *L. pneumophila* inhibits host translation

A number of Dot effectors have been shown to inhibit host translation. The first effectors shown to inhibit host translation are a family of glucosyltransferases, Lgt1-3 (Belyi et al., 2003; 2006; 2008). These effectors inhibit translation elongation by catalyzing the glucosylation of eEF1A. Two other effectors, SidI and SidL, also inhibit translation elongation. Though SidI and SidL were shown to bind to eEF1A, the mechanism by which SidI and SidL inhibit its function is still unknown (Fontana et al., 2011; Shen et al., 2009).

A *L. pneumophila* strain that lacks Lgt1-3, SidI, and SidL are still able to inhibit host protein synthesis. It was therefore hypothesized that *L. pneumophila* encodes additional effectors that inhibit host protein synthesis. Barry *et al* performed an effector screen and identified two other inhibitors of translation: Lpg1489, a protein of unknown function, a Pkn5, a predicted protein kinase (Barry et al., 2013). A strain that lacks these two effectors, as well as the five initially discovered translation inhibitors, still inhibits host protein synthesis (Barry et al., 2013). Interestingly, ribosome profiling experiments hinted that the remaining protein synthesis blockade in the Δ7 strain occurs at the level of translation initiation (Barry et al., 2017). Perhaps *L. pneumophila* is inhibiting translation initiation by targeting either eIF2α or mTORC1. I hypothesized that *L. pneumophila* inhibits host translation in order to prevent the host from consuming amino acids, which leads to an increased pool of amino acids that serve as nutrients for *L. pneumophila*

1.3.3 Nutritional requirements for *L. pneumophila* during infection

L. pneumophila is an auxotroph for several amino acids. Genomic studies revealed that *L. pneumophila* lacks genes for the synthesis of Cys, Arg, Leu, Ile, Met, Thr, and Val, all of which are essential for its growth (Brüggemann et al., 2006; Faucher et al., 2011; Price et al., 2014). A strain of *L. pneumophila* lacking the gene encoding a threonine transporter, *phtA,* is unable to grow in macrophages unless excess threonine is supplemented to the tissue culture media, similar results were reported with a strain lacking PhtJ, a valine transporter (Chen et al., 2008; Fonseca and Swanson, 2014; Sauer et al., 2005). *L. pneumophila* has been proposed to acquire amino acids through additional mechanisms. It was previously reported that the effector AnkB is required for the liberation of amino acids through ubiquitin-proteasome mediated degradation of

proteins, with the resulting peptides being used as amino acid nutrients (Price et al., 2011). A strain that lacks *ankB* was shown to suffer a growth defect (Price et al., 2011). However, other groups were unable to reproduce these results. *aroA* and *aroB*, which are involved in aromatic amino acid biosynthesis, are also required for growth (Jones et al., 2015). Like most bacteria, *L. pneumophila* requires iron for growth. In order to acquire iron, *L. pneumophila* secretes MavN facilitate iron transport through an unknown mechanism (Isaac et al., 2015; Portier et al., 2015)

1.3.4 Nutrient acquisition via translation inhibition is a pattern of pathogenesis

If translation inhibition is used as a means to acquire nutrients, then nutrient acquisition is a pattern of pathogenesis. Our laboratory has shown that a *L. pneumophila* strain lacking translation inhibitors still induces TLR-dependent responses but have impaired induction of specific cytokines such as IL-23, GM-CSF, and IL-1α, providing experimental evidence of the pattern of pathogenesis model (Barry et al., 2013; Fontana et al., 2011).

1.3.5 Methods deployed by other pathogens to acquire nutrients from hosts

Iron is required for the growth of most bacteria (Skaar, 2010). In order to scavenge iron, bacteria secrete a class of proteins that compete with host factors that also sequester iron. To counter this, host cells secrete siderocalins that bind bacterial siderophores in order to prevent them from binding their cognate receptor on the bacterial surface. Similar to *L. pneumophila*, both *Salmonella enterica* and *Bacillus anthracis*, the causative agent of anthrax, are amino acid auxotrophs. It is proposed that *S. enterica* obtains amino acids in host cells by diverting host vesicles containing amino acids to the *Salmonella* containing vacuole (Popp et al., 2015). Using a synthetic media devoid of amino acids, Terwilliger *et al* found that *B. anthracis* proteolytically cleaves host serum proteins in order to overcome amino acid auxotrophy (Terwilliger et al., 2015). In particular, they showed that the *B. anthracis* encoded InhA1 cleaves hemoglobin, the most abundant blood serum protein. *Chlamydia trachomatis* is an obligate intracellular pathogen and resides within a vacuole in host cells. This vacuole is rich in glycogen, a molecule commonly used for energy storage. In order to accumulate glycogen in the vacuole, *C. trachomatis* hijacks a host transporter SLC35D2 that imports UDP-glucose that is then imported into *de novo* glycogen synthesis (Gehre et al., 2016).

1.4 Dissertation overview

The methods by which intracellular bacterial pathogens acquire nutrients within host is not fully understood. In this dissertation, I provide molecular evidence that shows that *L. pneumophila*, regulates host metabolism via targeting of a conserved kinase complex called mTORC1. I hypothesize that *L. pneumophila* targets mTORC1 in order to free up host amino acids for bacterial consumption. I propose that these results provide further evidence that nutrient acquisition by bacteria is a pattern of pathogenesis and is sensed

by the innate immune system. In the concluding discussion chapter, I discuss the issues that arose during my dissertation, new questions that can be posed, and how we can address these questions. I close by offering my opinion on the state bacterial molecular pathogenesis field.

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

Portions of this chapter were adapted and/or reprinted with permission from "De Leon, J. A., Qiu, J., Nicolai, C. J., Counihan, J. L., Barry, K. C., Xu, L., et al. (2017). Positive and Negative Regulation of the Master Metabolic Regulator mTORC1 by Two Families of *Legionella pneumophila* Effectors. Cell Reports, 21(8), 2031–2038. http://doi.org/10.1016/j.celrep.2017.10.088"

2.1 Abstract

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*.

2.2 Introduction

All bacterial pathogens encode mechanisms to acquire nutrients from their hosts. For example, many bacteria utilize siderophores to acquire iron, which is normally sequestered by hosts using factors such as transferrin (Skaar, 2010). *Legionella pneumophila* (*L. pneumophila*) is an intracellular bacterial pathogen whose natural host cells are diverse species of freshwater amoebae (Fields et al., 2002). Upon inadvertent or accidental inhalation by humans, *L. pneumophila* can also replicate within alveolar macrophages to cause a severe pneumonia called Legionnaires' Disease (Brown et al., 2016; Copenhaver et al., 2014). Given the diversity of its host cells, success as a pathogen requires *L. pneumophila* to target and modulate conserved host processes. To accomplish this, *L. pneumophila* employs its Dot/Icm type IV secretion system to deliver more than 300 bacterial effector proteins into the host cell cytosol (Asrat et al., 2014; Hubber and Roy, 2010; Qiu and Luo, 2013). Because of functional redundancy among effectors, genetic deletion of individual effector genes rarely imparts a significant phenotype, but loss of a functional Dot/Icm system renders *L. pneumophila* avirulent and unable to replicate intracellularly (Ensminger, 2016). These effectors target numerous highly conserved host processes, including vesicular traffic and protein synthesis. A number of studies have identified as many as seven effectors that inhibit host protein synthesis. However, our results show that protein synthesis is still inhibited in bone marrow derived macrophages (BMDMs) infected with a *L. pneumophila* strain that lacks these seven effectors (Δ7) (Barry et al., 2013). Further analyses using

ribosome profiling reveal that this inhibition occurs at the level of translation initiation, indicating that additional *L. pneumophila* effectors target conserved host signaling pathways that regulate translation initiation (Barry et al., 2017).

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 essential amino acids and requires host-derived amino acids for intracellular replication (Eylert et al., 2010; Sauer et al., 2005). Intracellular amino acid levels are tightly controlled in host cells, with a number of pathways devoted to amino acid regulation. 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). Amino acids regulate mTORC1 activity via a number of sensors that converge on a family of small GTPases known as the Ras related GTPases (Rags) (Perera and Zoncu, 2016; Saxton and Sabatini, 2017). Rags exist as heterodimers of RagA or RagB (RagA/B) with RagC or RagD (RagC/D). A crucial property of the Rag GTPases is the modulation of their nucleotide state by amino acids. In particular, under high amino acid conditions RagA/B is believed to be GTP-loaded, whereas RagC/D is GDP-loaded. In this 'active' state, the Rag heterodimers physically bind to mTORC1 and recruit it to the cytosolic face of the lysosome where it can be activated by the Ras homologue enriched in brain (Rheb) small-GTPase. Rheb activation requires signaling through Akt, which is typically responsive to growth factor stimulation (Manning and Toker, 2017). Therefore, both Rag-dependent and Akt-dependent pathways are required for the activation of mTORC1, and mTORC1 thus serves as a central hub that promotes cellular growth in response to the availability of nutrients and growth factors. Active mTORC1 phosphorylates several target proteins, including ribosomal protein S6 kinase-1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), resulting in positive regulation of translation initiation (Mohr and Sonenberg, 2012). Active mTORC1 also inhibits lysosome biogenesis via inhibition of the TFEB transcription factor and autophagy via phosphorylation of Ulk-1 (Kim et al., 2011; Napolitano and Ballabio, 2016). *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.

2.3 Methods

2.3.1 Ethics code

These studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee at the University of California, Berkeley.

2.3.2 Bacterial strains

Unless otherwise noted, all *L. pneumophila* strains were derived from LP02, a streptomycin-resistant thymidine auxotroph derived from *L. pneumophila* LP01. The Δ*dotA*, Δ*flaA*, and Δ7 Δ*flaA* strains were generated on the LP02 background and have been described previously (Barry et al., 2013). Additional mutants were generated from by sequential in-frame deletion using the suicide plasmid pSR47S as described previously (Shen et al., 2009). Genetic complementation was performed as described previously (Fontana et al., 2011).

2.3.3 Infection and stimulation

For infections, bone marrow derived macrophages from male C57BL/6J mice aged 8-12 weeks were plated at varying numbers. The next day they were infected with *L. pneumophila* at the indicated MOI with spinfection (1200 RPM, 10 min, 23°C). One hour post infection, supernatants were replaced with fresh media to remove extracellular bacteria. Cells were stimulated with 50 uM cycloheximide (Sigma), 250 nM Torin1 (Cell Signaling Technologies), 1 uM LY294002 (Cell Signaling Technologies), or 50 nM bruceantin (MedChem Express). For amino acid withdrawal in HEK 293T cells, cell culture media was replaced with RPMI 1640 without amino acids (US Biological) supplemented with 5 mM glucose, and 10% dialyzed fetal bovine serum (Gibco). For amino acid withdrawal in BMMs, the same media was used but with 10% dialyzed supernatant from 3T3-M-CSF cells. aa-HI media consisted of RPMI 1640 (Gibco) supplemented with 2 mM L-glutamine (Gibco), 10% dialyzed serum and 10% dialyzed supernatant from 3T3–M- CSF cells. aa-LO media consisted of aa-HI media mixed 1:1 with amino acid withdrawal media for BMMs.

2.3.4 Cell Culture

HEK293T cells were grown in complete medium (Dulbecco's modified Eagle's medium (Gibco), 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 100 U/mL penicillin (Gibco), and 100 mg/mL streptomycin (Gibco)). Macrophages were derived from the bone marrow of C57BL/6J or *Myd88*$^{-/-}$ *Trif*$^{-/-}$ mice. Macrophages were derived by 8 d of culture in RPMI 1640 medium supplemented with 10% serum, 100 mM streptomycin,

100 U/ml penicillin, 2 mM L-glutamine and 10% supernatant from 3T3-M-CSF cells, with feeding on day 5.

2.3.5 Transfection

HEK 293T cells were seeded at a density of $1.5x10^5$ cells per well in 12-well tissue culture plates and transfected the next day using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

2.3.6 Immunoblotting

Cells were lysed 24 hours post transfection with radioimmunoprecipitation assay (RIPA) buffer supplemented with 2 mM $NaVO_3$, 50 mM b-Glycerophosphate, 50 mM NaF, 2 mM PMSF, and Complete Mini EDTA-free Protease Inhibititor (Roche). Proteins separated with denaturing PAGE and transferred to Immobilon-FL PVDF membranes (Millipore). Membranes were blocked with Li-Cor Odyssey blocking buffer. Primary antibodies used were: anti-S6K1 (49D7), anti-phospho-S6K1 (T389) (108D2), anti-Akt (11E7), anti-phospho-Akt (S473) (D9E), anti-phospho-Akt (T308) (244F9), all purchased from Cell Signaling Technologies. Secondary anti-rabbit IgG was conjugated to Alexa Fluor-680 (Invitrogen). Immunoblots were imaged using a Li-Cor fluorimeter.

2.3.7 Effector screen

A library of *L. pneumophila* Dot/Icm effectors was as previously described (Barry et al., 2013) and adapted from (Losick et al., 2010). $4x10^4$ 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.

2.3.8 ^{35}S Assays

For infections, $5x10^5$ BMMs were infected with the infection method above. At 4.5 h post infection, macrophages were treated with 25 μg/mL chloramphenicol to inhibit bacterial protein translation. 5 h post infection, 25 μCi/ml [^{35}S]methionine (Perkin Elmer) in RPMI 1640 medium without methionine supplemented with 10% serum, 2 mM l-glutamine, 25 μg/ml chloramphenicol. Cells were labeled for 1 h, washed three times with PBS, and then lysed with RIPA buffer. Lysates were cleared using centrifugation and spotted onto Whatman filters that were then placed in liquid scintillation reagent and radioactivity was measured using a liquid scintillation counter. For transfections, cells were labeled with radioactivity similarly 23 h post transfection and were lysed and analyzed similarly.2.3.9 Growth curve

$5x10^5$ BMMs were infected with various *L. pneumophila* strains at MOI 0.01 using the infection method above in 24-well format. 1 h post infection, supernatant was removed and replaced with fresh media. At 1, 24, 48, and 72 h post infection. BMMs were lysed with water and *L. pneumophila* growth was measured by enumerating colony forming units.

2.3.10 *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.

2.3.11 Amino acid measurements

$2x10^6$ BMMs from C57/BL6J mice were treated in quintuplicate with 10 ng/mL BLP for 4 h and with either: 250 nM Torin1 for 2 h, 10 uM cycloheximide for 4 h, or both. Cells were washed 3x with PBS and then pelleted using centrifugation. Amino acids from cell pellets were extracted in 40:40:20 acetonitrile:methanol:water with the inclusion of isotopic d3N15-serine as an internal standard. Samples were vortexed, sonicated, and centrifuged at 10,000 g, and an aliquot of the supernatant (20uL) was analyzed by selected reaction monitoring–based liquid chromatography– mass spectrometry (MS). Polar metabolite separation was achieved with a Luna normal-phase NH2 column (50 3 4.6 mm, with 5-mm–diameter particles; Phenomenex, Torrance, CA). Mobile phase A was composed of 100% acetonitrile, and mobile phase B consisted of water and acetonitrile in a 95:5 ratio. Solvent modifier 0.1% Formic Acid was used to assist ion formation and to improve the LC resolution in positive ionization mode. The gradient started at 0% B and increased linearly to 100% B over the course of 30 min with a flow rate of 0.7 ml/min. MS analysis was performed with an electrospray ionization (ESI) source on an 6460 Agilent QQQ-LC-MS/MS (Agilent Technologies, Santa Clara, CA). The capillary voltage was set to 3.0 kV, and the fragmentor voltage was set to 100 V. The drying gas temperature was 350 °C, the drying gas flow rate was 10 L/min, and the nebulizer pressure was 35 psi. Amino acids were quantified by selected reaction monitoring of the transition from precursor to product ions at associated optimized collision energies, and then via integrating the area under the curve normalized to internal standard values. Amino acid levels are expressed as relative abundances as compared to controls.

2.4 Results

2.4.1 An effector screen to identify mTORC1 regulators

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 supplemented amino acids, mTORC1 is active and phosphorylates TFEB which is then retained in the cytosol. In the absence of supplemented amino acids, mTORC1 is inactive, and TFEB is hypophosphorylated and enters the nucleus to activate transcription of lysosome biogenesis genes. 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.

2.4.2 Lgt family of effectors are mTORC1 activators

Although most effectors did not appear to modulate TFEB-eGFP localization, 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 and Figure 1B). To validate that the Lgt effectors activate mTORC1, 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 in the absence of amino acids, similar in magnitude to that seen with constitutively active Rags ($Rags^{CA}$), which potently activate mTORC1. Lgt-dependent phosphorylation of S6K1 T389 was indeed mTORC1-dependent since it was inhibited by Torin1, an inhibitor of mTORC1 kinase activity.

2.4.3 Lgt family activates mTORC1 via translation inhibition and consequential liberation of amino acids

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 point mutations that disrupt the glucosyltransferase activity of the Lgt effectors abrogated their ability to activate mTORC1 (Figure 2A). In addition, we ruled 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 2B)

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 (Watanabe-Asano et al., 2014). We confirmed this result and further found that an inhibitor of translation initiation, bruceantin, also activates mTORC1 (Figure 2C). Thus, translation inhibition by diverse mechanisms activates mTORC1, suggesting that it is the block in protein synthesis, rather than another effect of the Lgts, that leads to mTORC1 activation.

2.4.4 Characterization of Lgt effectors during infection

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. To assess the role of Lgt effectors on mTORC1 activation during infection, we measured S6K1 phosphorylation in BMMs infected with different *L. pneumophila* strains. 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 compared to BMMs infected with Δ*flaA* (Figure 3). mTORC1 activity was restored in cells infected with the Δ*flaA*Δ7 strain complemented with wild-type but not glucosyltransferase-dead Lgt2 or 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*Δ*7* 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.

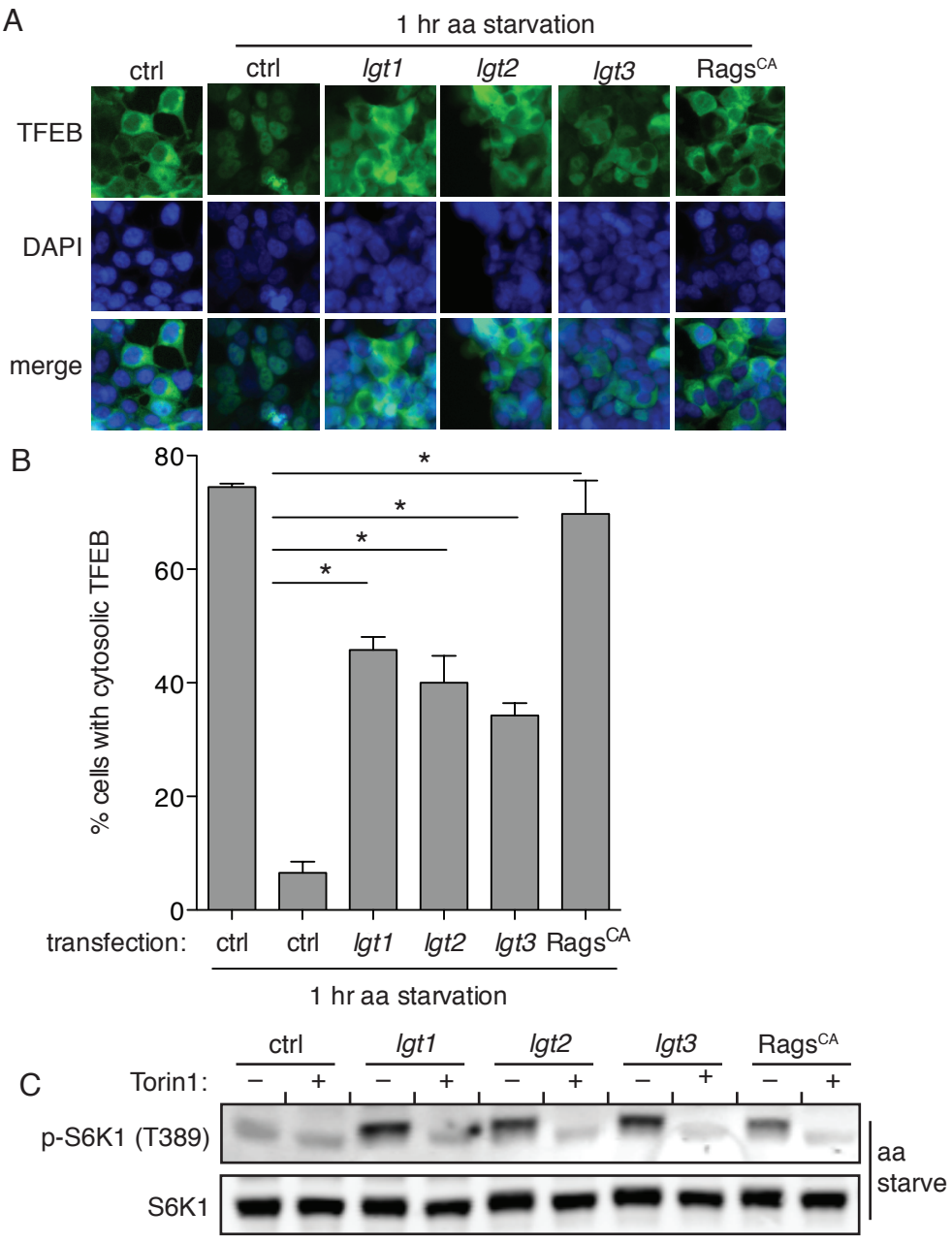

Figure 1: Lgt family of effectors activate mTORC1

A) Representative images of 293T-TFEB-eGFP reporter cells transfected with expression plasmids of the indicated effectors or with constitutively active Rags (RagsCA), 1 h prior to harvest, amino acids were withdrawn from the media. 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 RagsCA 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. *, $p < 0.001$; statistical test: unpaired t-test. (B) mean ± SD

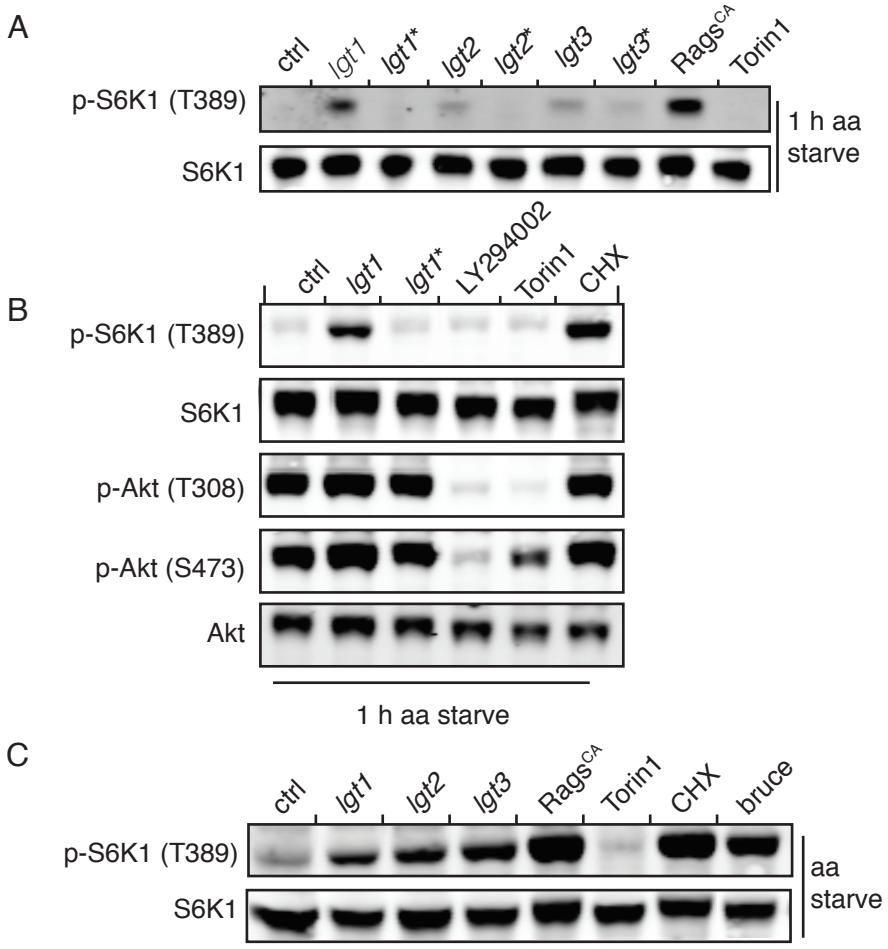

Figure 2: Lgt family of effectors activates mTORC1 via translation inhibition and consequential release of amino acids.
A) HEK 293Ts were transfected with wild-type or glucosyltransferase dead (Lgt*) Lgt effectors. Lysates were harvest 24 hours post transfection. 1 h prior to harvest, amino acids were with drawn. 24 h, the cells were lysed and mTORC1 activity was measured via western blots probing for phosphorylated and total S6 Kinase 1 (S6K1), an mTORC1 substrate. **B)** HEK 293T cells were transfected with the indicated constructs or treated with 1 μM LY294002, 250 nM Torin1, or 10 uM cycloheximide (CHX). 1 h prior to harvest, amino acids were withdrawn (B) or amino acids were withdrawn for 50 min and replenished for 10 min (C). 24 h post transfection, the cells were lysed and mTORC1 activity was measured as in (A). Akt activation was measured via immunoblotting for phosphorylated and total AKT. **C)** HEK 293T cells were transfected with Lgt effectors or RagsCA or were treated with Torin1, cycloheximide, or bruceantin as indicated.

2.4.5 SidE family are mTORC1 inhibitors

Although *L. pneumophila* blocks host protein synthesis via the action of Lgts and other effectors, the resulting activation of mTORC1 described above is known to stimulate host protein synthesis. We reasoned that increased protein synthesis might be counterproductive for *L. pneumophila* because it would consume the host amino acids that we hypothesize *L. pneumophila* seeks to liberate for its consumption. We therefore suspected that *L. pneumophila* might encode effectors that block mTORC1. We decided to use the 293T-TFEB-eGFP reporter cells to screen for such effectors. In this screen,

reporter cells were transfected with mammalian expression 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 4A and Figure 4B). 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 4C). The inhibition by the SidE family required the mART (mono-ADP ribosyltransferase) motif in each of the effectors, a motif that is also 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).

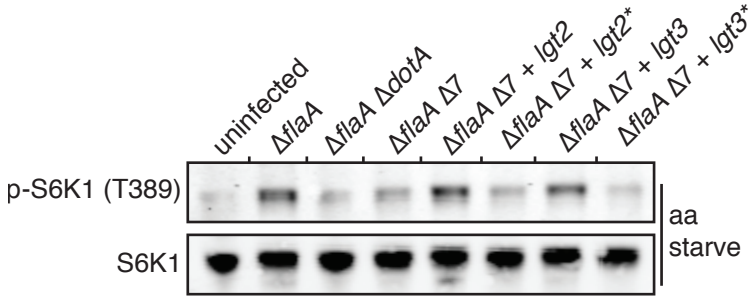

Figure 3: L. pneumophila strain that lacks translation inhibitors fails to elicit mTORC1 activity in bone marrow derived macrophages.
Bone marrow derived macrophages from *Myd88*$^{-/-}$ mice were infected with the indicated strains at MOI 3. 9 h post infection, amino acids were withdrawn from the media for 1 h. 10 h post infection, cell lysates were harvested and mTORC1 activity was measured via western blots probing for phosphorylated and total S6 Kinase 1 (S6K1), an mTORC1 substrate.

2.4.6 Characterization of SidE family during infection

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 5). 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 6). 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 6). However, because SidE effectors have global effects on ubiquitylation, vesicular trafficking, and the tubular endoplasmic reticulum in host cells

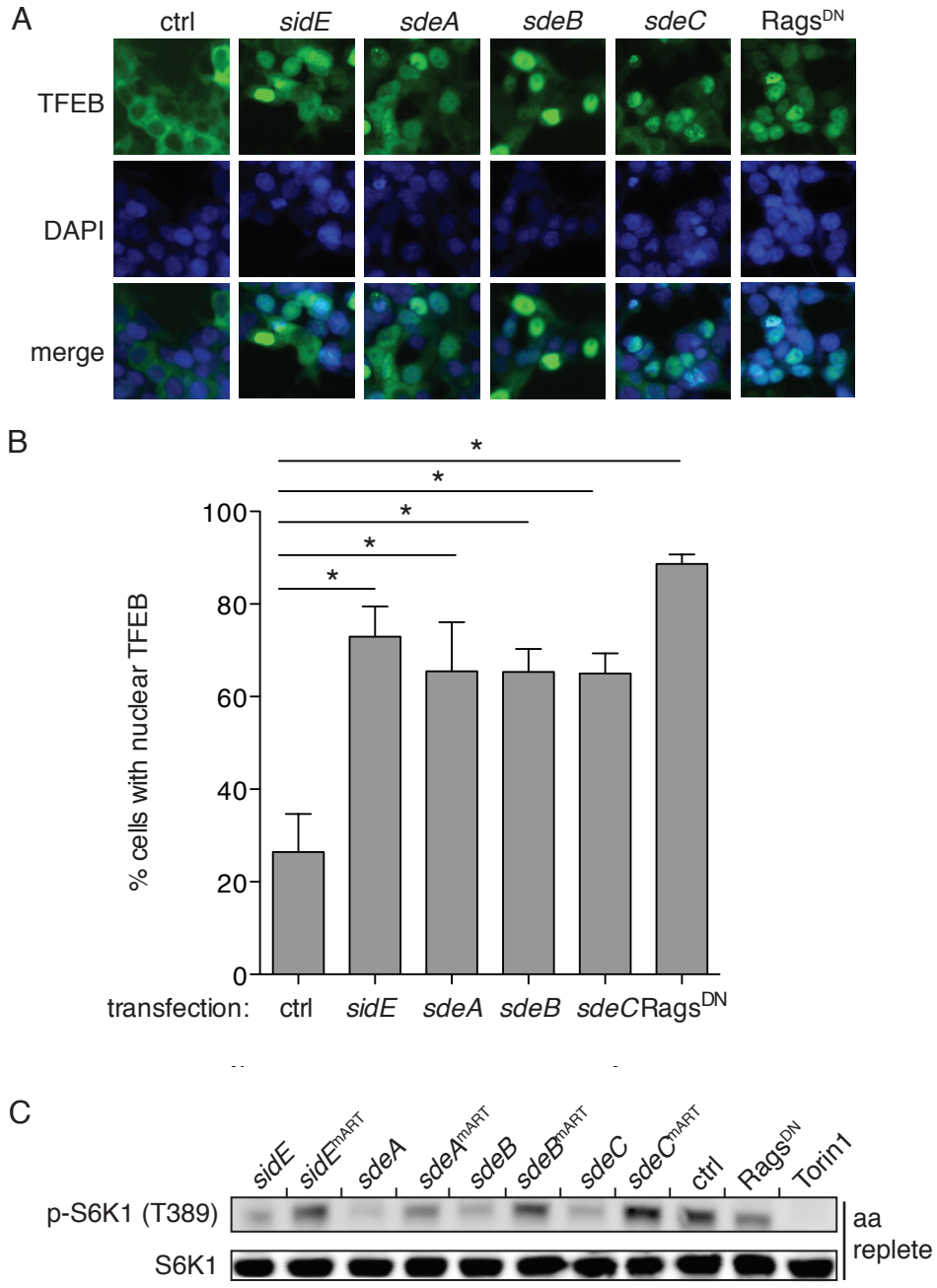

Figure 4: SidE family of effectors inhibits mTORC1

A) Representative images of 293T-TFEB-eGFP reporter cells transfected with expression plasmids of the indicated effectors or with dominant negative RagB and RagD (RagsDN: RagBT54L and RagDQ121L) and retained in complete media. **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 RagsDN 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. *, $p < 0.01$; statistical test: unpaired t-test. (B) mean ± SD

(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.

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

We were curious about how the SidE family inhibits mTORC1. 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 7). Initially we were puzzled as to why *L. pneumophila* would encode two families of effectors with opposing effects on mTORC1. However, this apparently 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. 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 8). These data suggest that inhibition of protein synthesis is a common downstream effect of both the Lgt and SidE effector families.

2.4.8 The SidE effectors ubiquitylate Rag small-GTPases

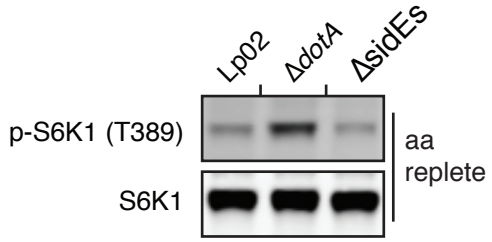

Figure 5: *L. pneumophila* strain that lacks SidE family still inhibits mTORC1 in bone marrow derived macrophages.
Bone marrow derived macrophages from C57BL/6J mice were infected with the indicated Lp02 strains at MOI 3. At 1 h post infection. Amino acids were withdrawn. 50 min later, amino acids were replenished. 2 h post infection, cells were lysed and mTORC1 activity was measured via western blots probing for phosphorylated and total S6 Kinase 1 (S6K1), an mTORC1 substrate.

Protein synthesis inhibition by the Lgt effectors results in mTORC1 activation (Figure 2). 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

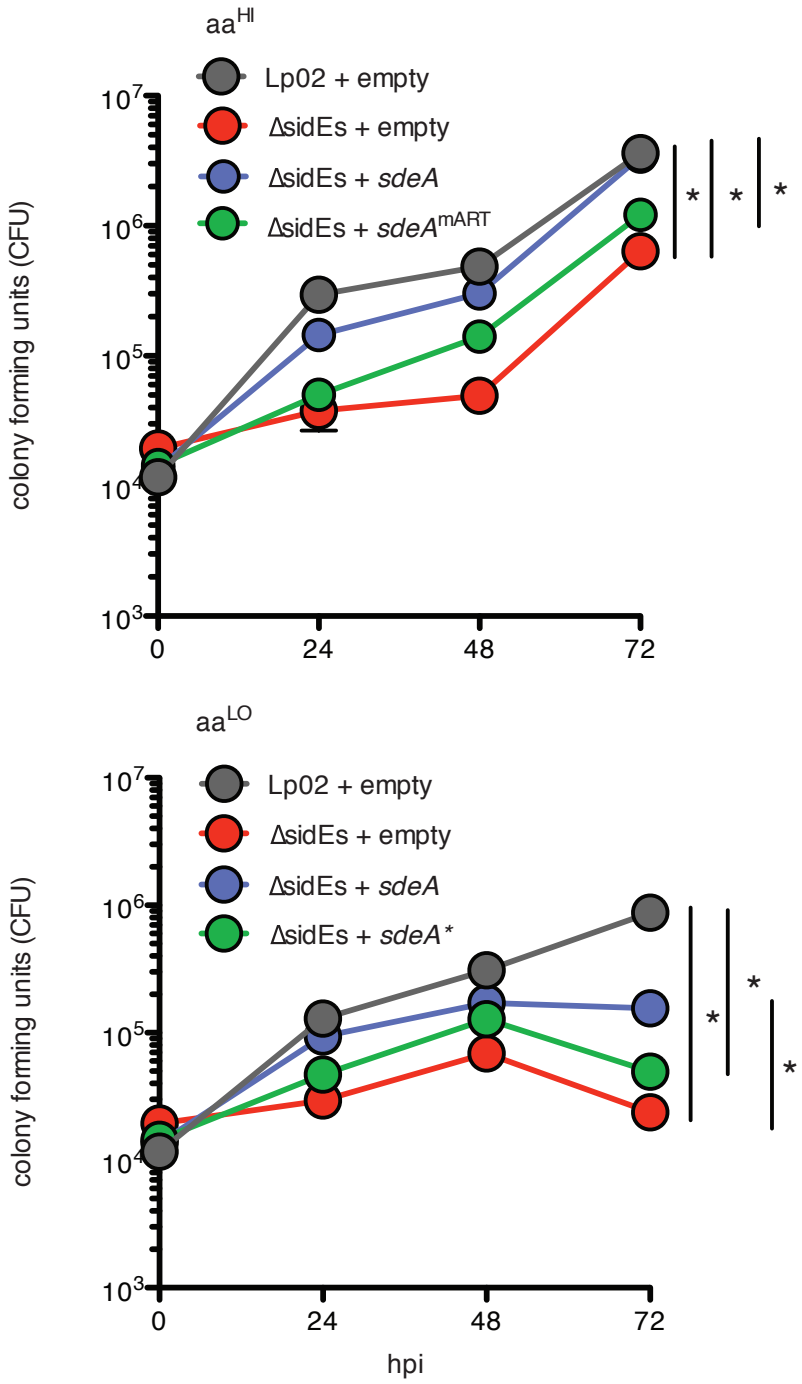

Figure 6: *L. pneumophila* strain that lacks SidE family suffers growth defect in bone marrow derived macrophages that is exacerbated when amino acids are limited.
Bone marrow derived macrophages from C57BL/6J mice were infected with the indicated Lp02 strains at MOI 3. At 1 h post infection. Amino acids were withdrawn. 50 min later, amino acids were replenished. 2 h post infection, cells were lysed and mTORC1 activity was measured via western blots probing for phosphorylated and total S6 Kinase 1 (S6K1), an mTORC1 substrate.

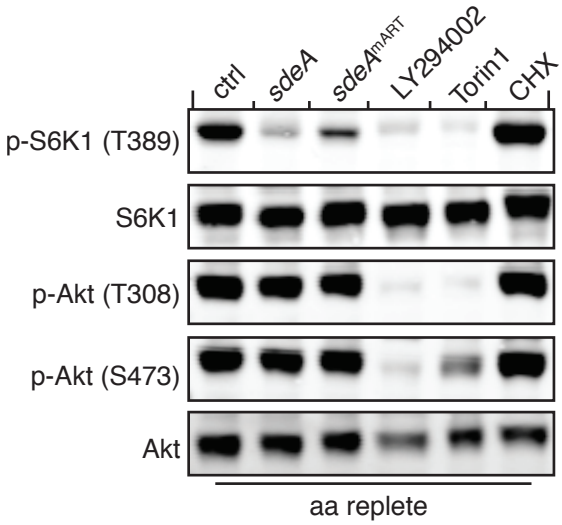

Figure 7: SidE family of effectors does not inhibit mTORC1 via Akt.
HEK 293T cells were transfected with the indicated constructs or treated with 1 μM LY294002, 250 nM Torin1, or 10 uM cycloheximide (CHX). 1 h prior to harvest, r amino acids were withdrawn for 50 min and replenished for 10 min (C). 24 h post transfection, the cells were lysed and mTORC1 activity was measured via immunoblotting for phosphorylated and total S6 Kinase 1 (S6K1), an mTORC1 substrate. Akt activation was measured via immunoblotting for phosphorylated and total AKT.

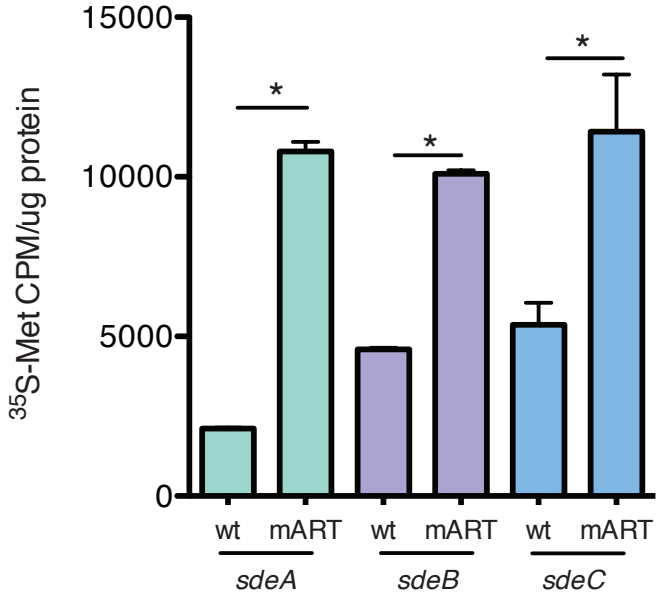

Figure 8: SidE family of effectors inhibits translation.
HEK 293T cells were transfected with the indicated constructs and protein synthesis was assessed by measuring [^{35}S]-methionine incorporation. *, $p < 0.05$; **, statistical test: unpaired t-test. Mean ± SD

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 9A). 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 9B) (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 9B). *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 9C). This suggests that SdeA inhibits mTORC1 by directly inhibiting the Rag small-GTPases.

2.4.9 SidE family dominates effects of Lgt family

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. Unfortunately, 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 transfection of SidE. In line with our hypothesis, the activation of mTORC1 by these translation inhibitors was abrogated in the presence of catalytically active SdeA (Figure 10). 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 family and other translation inhibitors.

2.4.10 *L. pneumophila* strain that lacks all known translation inhibitors still inhibit host protein synthesis, indicating the presence of additional mTORC1 regulators

Given the above results, I 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 11A). The Δ*flaA*Δ7 strain, which lacks Lgts and other effectors, still inhibits translation (Barry et al., 2013; 2017). We therefore tested whether deletion of the SidE family in the Δ*flaA*Δ7 background restores host protein synthesis. We infected BMMs from C57BL/6J mice 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 11B). 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

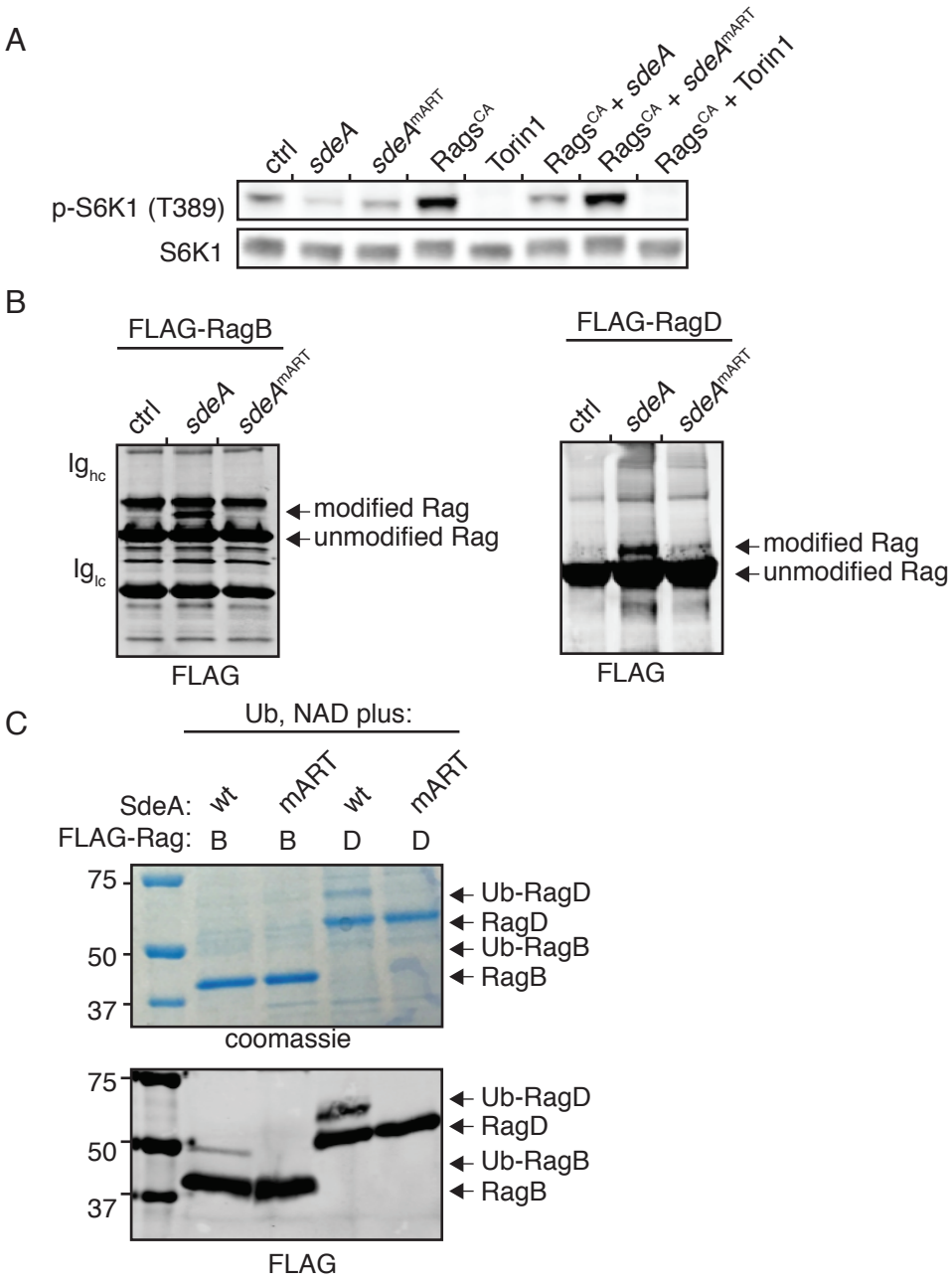

Figure 9: SidE family of effectors inhibits mTORC1 by inhibiting the Rag small-GTPases. HEK 293T cells were transfected with the indicated constructs and treated with 250 nM Torin1 4 h prior to harvest as indicated in amino acid replete conditions. 24 h post transfection, the cells were lysed and mTORC1 activity was measured via immunoblotting for phosphorylated and total S6 Kinase 1 (S6K1), an mTORC1 substrate. B) 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. **C)** Flag-RagB or Flag-RagD purified from transfected 293T cells were incubate with SdeA or SdeAmART and ubiquitin in the presence of β-NAD at 37°C for 2 h. Ubiquitylation of RagB and RagD was probed by Coomassie staining (top) or by immunoblotting (bottom) with antibodies specific for FLAG. The experiments in Figure 9B and Figure 9C were performed by Jiazhang Qiu and Zhao-Qing Luo.

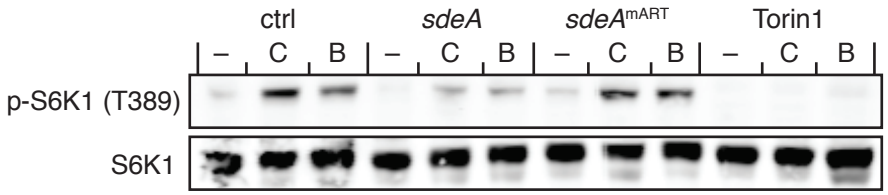

Figure 10: Effects of SidE family of effectors dominate that of Lgt family of effectors. HEK 293T cells were transfected with the indicated constructs or treated with 250 nM Torin1. In addition, cells were treated with cycloheximide (C), or bruceantin (B) for 4 h prior to harvest. 1 h prior to harvest, 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.

redundancy demonstrated by these results suggests that translation inhibition is important for *L. pneumophila* fitness during infection. These data support a model that propose that the SidE family, by inhibiting mTORC1 amino acid sensing, blinds mTORC1 to the amino acids liberated by the Lgt family. The net result is an increase in amino acid availability for bacterial consumption.

2.4.11 Simultaneous inhibition of translation and mTORC1 leads to synergistic increases in certain amino acids.

I propose that the Lgt family and SidE family work in conjunction to liberate host amino acids for bacterial consumption. To test this I treated BMMs with small molecules that mimic the effects of either the Lgt family or the SidE family. I used cycloheximide as a proxy for Lgt and Torin1 as a proxy for SidE. Simultaneous administration of both cycloheximide and Torin1 lead to an increase in isoleucine, arginine, lysine, and phenylalanine (Figure 12). Interestingly, these are amino acids for which *L. pneumophila* is an auxotroph for (Eylert et al., 2010).

2.5 Conclusions

Acquisition of host nutrients and macromolecules is a fundamental challenge for all pathogens. Intracellular pathogens, in particular, must devise strategies to liberate nutrients from their host cells. *L. pneumophila* is an intracellular bacterial pathogen that is auxotrophic for several essential amino acids. Here we sought to determine a mechanism by which *L. pneumophila* might obtain necessary amino acids from its host cells. Since *L. pneumophila* evolves in freshwater amoebae, and is not believed to be transmitted among animals, the success of *L. pneumophila* in vertebrates depends on targeting of deeply evolutionarily conserved pathways. The mTOR pathway regulates amino acid metabolism in all eukaryotes, from yeast to humans. Previous work has established that mTORC1 activity is indeed altered in *L. pneumophila*-infected cells though the underlying molecular mechanisms have remained unclear (Abshire et al., 2016; Ivanov and Roy, 2013).

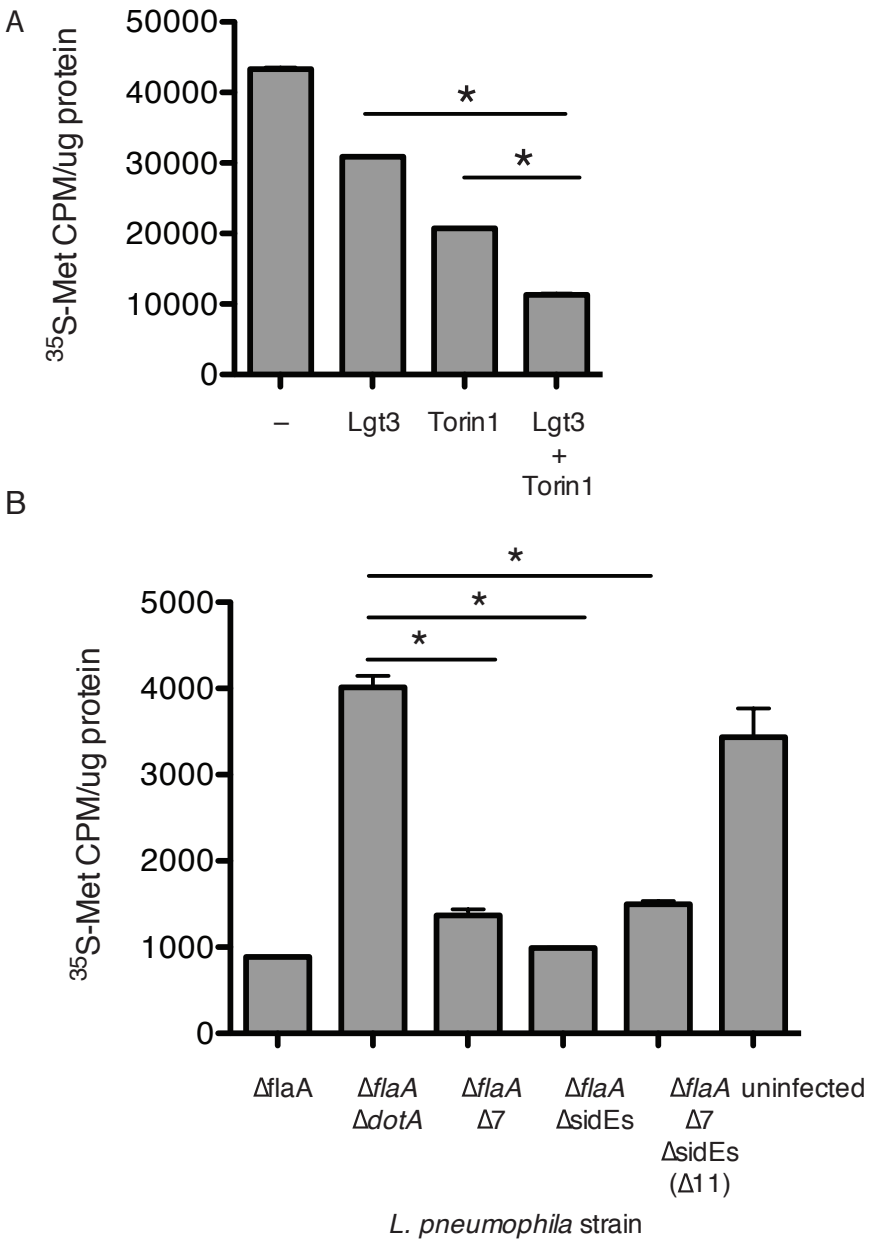

Figure 11: *L. pneumophila* strain that lacks all known translation inhibitors maintains protein synthesis blockade.
HEK 293T cells were transfected with the indicated constructs or treated with 250 nM Torin1. Protein synthesis was assessed by measuring [35S]-methionine incorporation. **B)** BMMs from C57BL/6J mice were infected with the indicated L. pneumophila ΔflaA strains. 5 h post infection cells were labeled with [35S]-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

Here we provide evidence that *L. pneumophila* secretes at least two families of effectors that modulate mTORC1. Our data lead us to propose a speculative model for how these

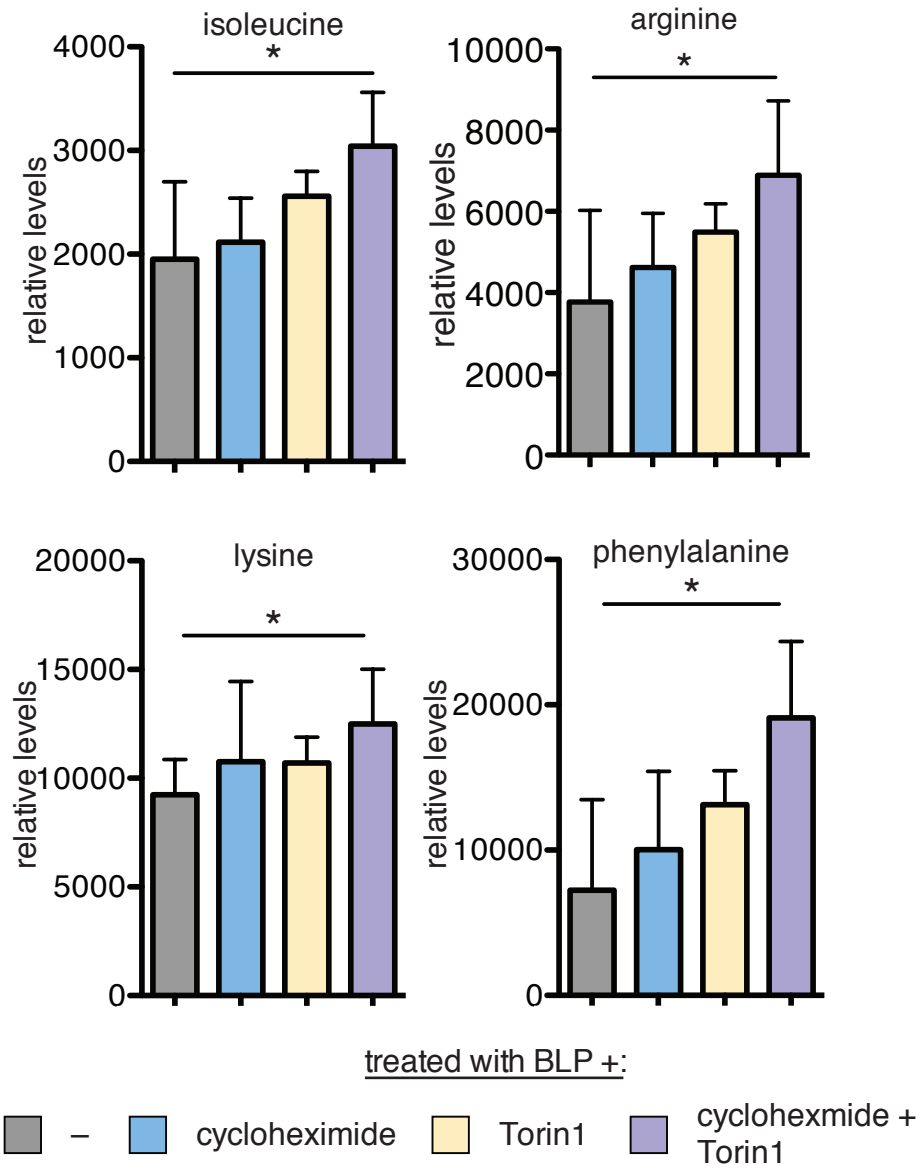

Figure 12: Synergistic increases in amino acids during simultaneous inhibition and activation of mTORC1.
BMMs from C57BL/6J mice were treated with 10 uM CHX for 4 h, 250 nM Torin1 for 2 h, or both. Cell lysates were harvested and amino acids were measured using flow cytometry. The experiment in Figure 12 was performed by Jessica Counihan and Dan Nomura.

effectors act to promote bacterial replication (Figure 13). In this model, *L. pneumophila* secretes the Lgt effectors, which directly inhibit protein translation. The block in host protein synthesis limits host cell consumption of amino acids and thereby liberates amino acids for bacterial consumption. Although *L. pneumophila* could utilize these amino acids for growth, they also have the indirect effect of activating mTORC1. Stimulation of mTORC1 promotes translation initiation, which might counteract protein synthesis inhibition by the Lgts and restore host (rather than bacterial) consumption of

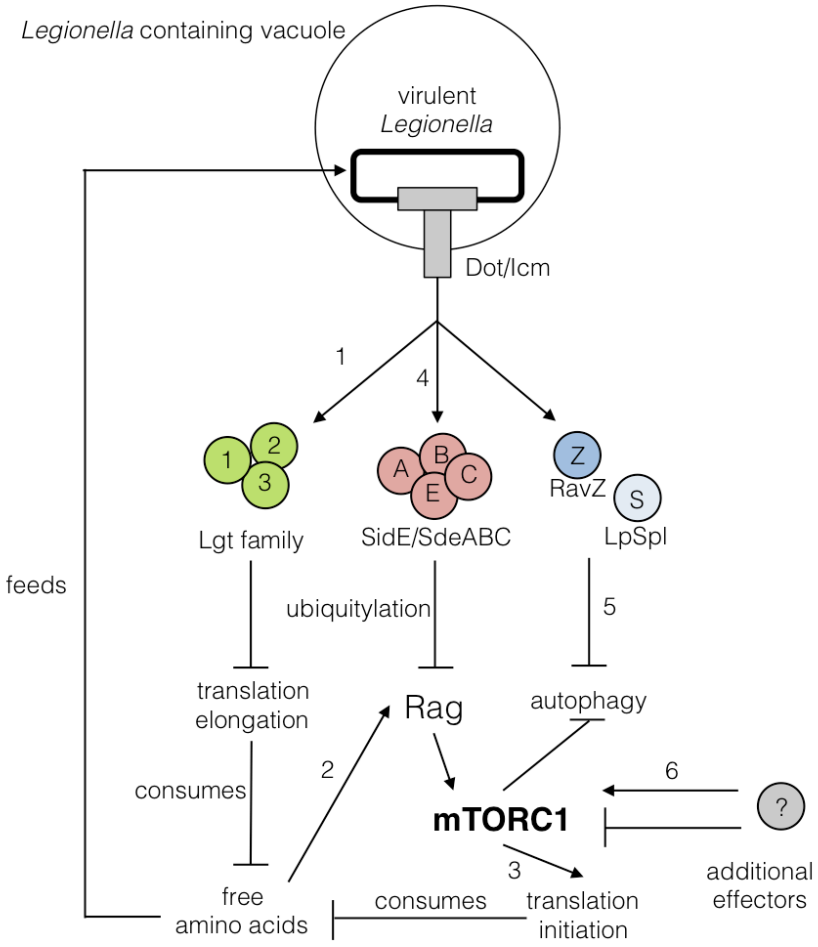

Figure 13: Speculative model depicting how the Lgt and SidE families regulate mTORC1 in order to free amino acids

1) *L. pneumophila* secretes Lgt1-3 which inhibits host translation elongation and frees host amino acids to serve as nutrients for *L. pneumophila*. 2) Free amino acids signal through Rag small GTPases to active mTORC1, which 3) stimulates host translation initiation and consumes host amino acids away from Legionella. 4) To counteract this, *L. pneumophila* secretes SidE/SdeABC that ubiquitylates Rag small GTPases and blinds mTORC1 to amino acids. 5) Inhibition of mTORC1 induces autophagy. However, *L. pneumophila* secretes RavZ and LpSpl, which inhibit autophagy. 6) Additional effectors may exist.

amino acids. In part to counter this, we propose *L. pneumophila* secretes the SidE family, which inhibits the Rag small-GTPases and effectively blinds mTORC1 to the amino acids liberated by the Lgts. However, since mTORC1 is known to negatively regulate autophagy, an unintended possible consequence of strong inhibition of mTORC1 might be the derepression of autophagy. Autophagy is known to restrict replication of numerous intracellular bacterial pathogens (Huang and Brumell, 2014). Indeed, the inadvertent activation of autophagy as a result of mTORC1 inhibition may be one reason why *L. pneumophila* secretes RavZ, an ATG8-specific protease and a potent inhibitor of autophagy, as well as LpSpl, a sphingosine-1 phosphate lyase, another autophagy inhibitor (Choy et al., 2012; Rolando et al., 2016). In sum, we propose that the net effect of *L. pneumophila* modulation of translation, mTORC1, and

autophagy is to enable *L. pneumophila* to obtain essential host amino acids without negative consequences. Of course, the effectors we identified have been shown to have diverse effects on cells, and it should therefore be acknowledged that mTORC1 modulation may only be a part of the complex biological roles of these effectors (Bhogaraju et al., 2016; Hempstead and Isberg, 2015; 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 derived on the AA100 background (data not shown)

The coordinated assault on a single host target protein by multiple *L. pneumophila* effectors is not unique to mTORC1. Rab1 is another host target on which *L. pneuomphila* effectors have opposing effects. The effector protein SidM activates Rab1 via activity while LepB inhibits Rab1 via its GAP activity (Ingmundson et al., 2007; Machner and Isberg, 2006)

In addition, AnkX catalyzes the phosphocholination of Rab1, which is removed by Lem3 (Mukherjee et al., 2011; Tan et al., 2011). It therefore appears that *L. pneumophila* employs the “Goldilocks principle” to tune key host signaling hubs to promote virulence. Along these lines, it is interesting that several reports have identified SidJ as an effector that reverses the effects of the SidE family (Havey and Roy, 2015; Jeong et al., 2015; Qiu et al., 2017). Thus, complex effector and meta-effector regulation appears to be a common theme among *L. pneumophila* effectors.

The SidE family has previously been shown to target other host proteins including Rab small-GTPases and Reticulon-4 (Bhogaraju et al., 2016; Kotewicz et al., 2017; Qiu et al., 2016). Targeting of Rabs and Rags by SidE effectors can be rationalized as both targets are small GTPases and may share some commonalities that allow for their targeting (Helmreich, 2004). However, given recent evidence that the non-GTPase Reticulon-4 is also a substrate of SidE effectors, the mechanisms that regulate the target specificity of the SidE family are unclear (Kotewicz et al., 2017). Perhaps the enzymatic activity of these effectors is promiscuous and the localization of SidE on the cytosolic portion of the *Legionella* containing vacuole enables these effectors to sample these targets (Jeong et al., 2015). Along these lines, it would be interesting to determine the subcellular localization of mTORC1 in *L. pneumophila* infected cells. Unfortunately, our attempts to address this question using immunofluorescence in macrophages have thus far been inconclusive.

Our results provide insights into the long-standing question of how *L. pneumophila* obtains amino acids in order to replicate in its intracellular niche. The balancing act that

L. pneumophila deploys to target mTORC1 is 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 provides a lucrative target for pathogens.

Chapter Three: Questions and Perspectives

4.1 Remaining questions

4.1.1 Are there other effectors that inhibit translation and mTORC1?

Our laboratory has performed two separate screens to identify additional bacterially encoded translation inhibitors. We performed a screen that looked for direct inhibitors of translation and identified Pkn5 and Lpg1489 as translation inhibitors (Barry et al., 2013). However, a strain that lacks these two effectors in a background that lacked all known (at that time) translation inhibitors, the Δ*flaA* Δ7 strain, still maintained the ability to inhibit translation. This prompted us to consider mTORC1 as a possible hub *L. pneumophila* could be inhibiting in order to inhibit protein synthesis, especially given by results in our group showing that the Δ*flaA* Δ7 strain inhibits protein synthesis at the level of translation initiation. Through this screen, as described in Chapter Two, we found the SidE family. Deletions of these four SidE effectors in the Δ*flaA* Δ7 background (Δ*flaA* Δ11) had no effect on translation inhibition.

The remaining block in translation caused by the Δ*flaA* Δ11 strain may indicate that *L. pneumophila* encodes additional effectors that inhibit translation. These additional effectors could inhibit translation directly. However, given the remaining inhibition of mTORC1 in macrophages infected with the ΔsidEs strain, additional effectors can inhibit translation indirectly by inhibiting mTORC1-dependent translation initiation. Indeed, it was shown recently that the Dot/Icm substrate WipB interacts with components of the amino acid sensing portion of mTORC1 (Prevost et al., 2017) but no effects on mTORC1 signaling were measured. In our mTORC1 inhibitor screen, we identified SdbA as an inhibitor of mTORC1 but follow up experiments revealed no reproducible effects. Preliminary results showed that AnkX also inhibits mTORC1. However, I find it difficult to rationalize how AnkX could be exerting its effect on mTORC1 outside its known role of regulating vesicular trafficking (Mukherjee et al., 2011)

It is likely that the screen performed in Barry et al., 2013 and the screen performed in this dissertation may have produced a number of false negatives that need to be validated. Despite the fruitfulness of these two screens, one caveat is that by transfecting each effector individually, an effector that has a fast and potent effect on translation could inhibit its own expression and score as a false negative. Looking at the expression of each effector would be useful quality control.

Now that we have a strain that lacks 11 effectors that inhibit host translation, perhaps this is a more sensitive background in which to perform a transposon screen. I have experimented with using flow cytometry-based screen to identify *L. pneumophila* mutants that cannot inhibit host translation. In this screen, cells expressing doxycycline-inducible GFP are infected with a transposon mutant library of dsRed-tagged *L. pneumophila*. If a cell is infected with a *L. pneumophila* strain that has a mutation in a

gene encoding an inhibitor of host translation, that cell should be GFP-positive in the presence of doxycycline. These cells should also be dsRed positive because they are infected. These GFP and dsRed double-positive cells can then be sorted, lysed in hypotonic solution to liberate the bacteria, and the transposon insertion site can be determined using arbitrary PCR. However, there are a number of technical issues that arose in devising this screen. First, the fluorescent strains of *L. pneumophila* used to pilot this screen were filamentous when grown in broth. It could be that overexpression of dsRed is toxic, and it is unclear whether these filamentous bacteria are infectious or fit. In addition, in pilot sorts testing if I can enrich a *dotA*-deficient mutant (which does not inhibit translation) using this GFP-positive sort approach, I showed that I can enrich for the mutant only seven-fold. This may be due to the dimness of the GFP, making it difficult to sort truly GFP-positive cells. It was difficult in determining the optimal doxycycline treatment time in conjunction with how long post infection to measure GFP fluorescence. One may argue that I should instead constitutively express GFP, however it would be difficult to assay changes in translation if there is already a large pool of GFP present within the cell. I also developed an immortalized macrophage cell line stably expressing firefly luciferase, the activity of which can be used to measure translation. Preliminary experiments show that there is a large difference in luciferase activity between cells infected with wild-type versus *dotA*-deficient *L. pneumophila*. This cell line can be used to screen transposon mutants individually.

4.1.2 Is the remaining block in translation a host induced response instead?

Perhaps there is a host derived response that inhibits host protein synthesis. Previous unpublished work in our lab show that there are no changes in eIF2α phosphorylation between macrophages infected with wild-type or Dot-deficient *L.pneumophila*, thus ruling out the involvement of eIF2α. These experiments should be revisited and reconfirmed. Additionally, currently unidentified regulators of eIF2α could be leveraged by *L. pneumophila* in order to inhibit protein synthesis.

An unbiased approach may be more suitable in identifying novel host processes that inhibit protein synthesis in response to *L. pneumophila* infection. Perhaps we can compare global changes in phosphorylated or ubiquitylated proteins between cells infected with wild-type or Dot-deficient *L. pneumophila.* These analyses may reveal a novel signature that can point to a specific host pathway that may be responsible for the remaining block in translation.

4.1.3 Is there a main function for effectors with multiple targets?

The SidE family and the Lgt family have other functions in addition to those described here. The SidE family was originally shown to ubiquitylate both Rab-small GTPases and Reticulon-4 (Bhogaraju et al., 2016; Kotewicz et al., 2017; Qiu et al., 2016), while this work shows that they also inhibit mTORC1 signaling by inhibiting mTORC1. Meanwhile, the Lgt family was also shown to inhibit the unfolded protein response (Hempstead and

Isberg, 2015; Treacy-Abarca and Mukherjee, 2015), in addition to their ability to inhibit translation which I show activates mTORC1 as a consequence. Why do *L. pneumophila* effectors have multiple targets case? Does this inform us about *L. pneumophila* virulence strategies as a whole? Perhaps *L. pneumophila* effectors have been selected to target different pathways simultaneously in order to enable *L. pneumophila* to survive in a variety of different environments or amoebae hosts.

4.2 Final perspectives

4.2.1 The headache of redundancy

This dissertation is perhaps another victim of the main issue of studying *L. pneumophila* molecular pathogenicity. This issue is the redundancy behind each effector. There are approximately 300 translocated substrates of the Dot/Icm secretion system (Qiu and Luo, 2017). A large number of these effectors are encoded as part of multi-gene paralogous families. In some cases, different families of effectors may have the same outcome for a given host process. This redundancy makes it difficult to observe phenotypes in deletion strains, exemplified by Figure 5 and Figure 11B. In some cases, a *L. pneumophila* mutant may grow fine in mouse macrophages but suffers a growth defect in amoebae (Fontana et al., 2011; Kotewicz et al., 2017; Qiu et al., 2016) Indeed, in some studies, deletion of an effector does not impart a growth defect in mouse macrophages (Choy et al., 2012; Nagai et al., 2002), despite the interesting biochemistry exhibited by the effector.

Indeed, as exemplified in this work, studying individual effectors in isolation (i.e. transfection of plasmids encoding each effector in HEK 293T cells) remains a powerful discovery tool. At the same time, deletion strains fail to impart a fitness cost during infection. This can raise a philosophical dilemma. If there is no fitness cost to the bacterium during infection, is it really that important? Are phenotypes observed in HEK 293Ts or *in vitro* merely artifacts?

4.2.2 *L. pneumophila* as a model for intracellular pathogens

Despite the issue of redundancy, *L. pneumophila* remains a popular model for those interested in host-pathogen interactions. Perhaps researchers have remained interested in *L. pneumophila* because it has been treasure chest of interesting biochemical activity encoded by its effectors. The SidE effectors studied here exemplify this with their ability to catalyzed serine-linked ubiquitylation independent of E1 or E2 proteins (Bhogaraju et al., 2016; Kotewicz et al., 2017; Qiu et al., 2016). However, seldom have these interesting biochemical activities been found in other pathogens so it makes me wonder what does *L. pneumophila* teach us about intracellular pathogens as a whole?

Studies with *L. pneumophila* have also been critical in understanding the host response to infection (Vance, 2010). This is exemplified by the discovery of the Naip

inflammasome and its sensing of bacterial flagellin (Kofoed and Vance, 2011; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). In addition, infections with *L. pneumophila* were used to argue that cytosolic DNA is sensed by the host to elicit a type I interferon response (Stetson and Medzhitov, 2006). This study by Stetson and Medzhitov initiated studies to identify the PRR that senses the PAMP of cytosolic DNA, eventually leading to the discovery cyclic GMP-AMP synthase (cGAS), which is widely accepted as a cytosolic DNA sensor that stimulates type I interferon (Sun et al., 2013).

Why have *L. pneumophila* studies been so fruitful for immunology? Despite being touted as a sophisticated cell biologist, *L. pneumophila* is considered a poor immunologist (Vance, 2010). This means that, *L. pneumophila* has not co-evolved with the sophistication of metazoan immunity and thus has not been selected to suppress immune effector mechanisms. Therefore, *L. pneumophila* has been useful in elucidating the mechanisms by which cells detect infection. More professional pathogens, such as *Yersinia pestis,* encode a variety of methods to prevent immune sensing (Montminy et al., 2006; Mukherjee et al., 2006).

Going forward, what else can *L. pneumophila* teach us about biology? I think there is an interesting molecular evolutionary biology question that can be addressed by examining the genetics of *L. pneumophila* effectors. As exemplified by the work in this dissertation, many *L. pneumophila* effectors have paralogs encoded in the genome as well. What were the selective pressures that enabled *L. pneumophila* to not only acquire these paralogs, but to retain them in the genome? It has been proposed that different effector paralogs are required for a different amoeba host in the wild (Ensminger, 2016). An approach employed by Shames *et al* may be useful in providing experimental evidence that demonstrate the requirement of different paralogs for different hosts (Shames et al., 2017). In this work, pools of transposon mutants of *L. pneumophila* effectors were used to infect different hosts. In this case, mice, mouse macrophages and *Acanthamoeba castellani* were infected and the surviving pool of transposon mutants was compared to the input pool. A mutant that was lost during the infection is proposed to be required for virulence. Perhaps this work can be expanded to other amoebae species such as *Hartmannella, Echinamoeba, Tetrahymena, Naegleria,* or *Vahlkampfia* (Ensminger, 2016). Despite the issue of redundancy in *L. pneumophila*, I believe that it will continue to provide new insights about biology.

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