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Cell biology of infection by *Legionella pneumophila*

Li Xu¹ and Zhao-Qing Luo*

Department of Biological Sciences, Purdue University, 915 West State Street, West Lafayette, IN 47907

Abstract

Professional phagocytes digest internalized microorganisms by actively delivering them into the phagolysosomal compartment. Intravacuolar bacterial pathogens have evolved a variety of effective strategies to bypass the default pathway of phagosomal maturation to create a niche permissive for their survival and propagation. Here we discuss recent progress in our understanding of the sophisticated mechanisms used by *Legionella pneumophila* to survive in phagocytes.

Keywords

intracellular pathogens; vesicle trafficking; type IV secretion; effectors; host function subversion

1. Introduction

Legionella pneumophila, a Gram-negative opportunistic intracellular pathogen, is the causative agent of Legionnaires' disease, a severe form of pneumonia [81]. This disease was first described in 1976, when many attendees at the American Legion Convention in Philadelphia suffered from a sudden outbreak of pneumonia. A previously unrecognized bacterium was found to be responsible for the outbreak, and it was subsequently designated *Legionella pneumophila* [81]. Although more than 70 different serogroups *Legionella* species have since been described [86], most of the clinical cases (>90%) are caused by *L. pneumophila* serogroup 1 [86]. The symptoms of Legionnaires' disease resemble many other forms of pneumonia, including high fevers, chills, coughs and sometimes accompanied with headaches and muscle aches.

Legionnaires' disease emerged in the second half of the twentieth century partly due to the development of artificial water systems like air conditioning systems, cooling towers, showers, and other aerosolizing devices, which have allowed *Legionella* to gain direct contact with susceptible human populations. Contaminated aerosols inhaled by patients reach the alveoli of the lung where bacteria are engulfed by macrophages. Instead of being killed by macrophages, *L. pneumophila* survives and replicates in a protected niche within these phagocytes, leading to tissue damage and inflammation, thus the symptoms of Legionnaires' disease [86].

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*Correspondence: Zhao-Qing Luo, luoz@purdue.edu.

¹Current address: Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853

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L. pneumophila exists ubiquitously in natural fresh water environments such as lakes, ponds and wet soil, mostly as a parasite of protozoa, its natural hosts. Since person-to-person transmission of *L. pneumophila* infection has not yet been observed, the protozoan hosts are believed to have provided the primary evolutionary pressure from which the bacterium acquired and maintained its ability to overcome the defense mechanisms of phagocytes. In the past 15 years, scientists have made significant progress in the dissection of the strategies employed by *L. pneumophila* to exploit host functions. These discoveries have provided insights into not only the pathogenicity of this bacterium but also the intricacy of host cellular processes. Thus, *L. pneumophila* and its virulence factors have become invaluable tools and reagents in the study of host cell biology and signaling mechanisms, including the dissection of host innate immune surveillance mechanisms, a subject that has been discussed by several recent reviews [13,51], and thus will not be covered here.

2. The intracellular life cycle of *Legionella pneumophila*

Phagocytes eliminate engulfed microorganisms by delivering them into the lysosomal system. Bacteria not adapted for an intracellular life cycle are effectively digested within the phagolysosome, an acidic environment that contains various activated hydrolytic enzymes [24] (Fig. 1). However, phagocytosed *L. pneumophila* blazes an intracellular trail that exhibits striking differences from that of non-pathogens or inert particles (Fig. 1). Within minutes of internalization, the bacterial phagosome, also called the *Legionella*-containing vacuole (LCV), is covered by small smooth vesicles, which likely originate from the endoplasmic reticulum (ER) [15,18,63] (Fig. 1). The phagosomal membranes come to resemble those of the ER in thickness and protein composition and later become decorated with ribosomes [15]. Other host organelles such as mitochondria are also recruited to the proximity of the LCV [15] (Fig. 1). This change in membrane structure occurs concomitantly with the successful evasion of the endocytic pathways, as resident proteins of the late endosome and lysosome are absent from the LCV [71]. The LCV compartment not only protects *L. pneumophila* from being recognized by the cellular immune system but also provides the bacterium with nutrients for its replication.

3. The Dot/Icm transporter and its protein substrates

The ability of *L. pneumophila* to overcome the killing mechanisms of phagocytes entirely depends upon the Dot/Icm type IV secretion system (T4SS) [10,23]. Built of approximately 26 proteins, this specialized protein translocation system is a complex that presumably spans the two bacterial membranes as well as the phagosomal membranes [98]. The Dot/Icm T4SS is closely similar to the conjugation system found in some IncI plasmids and is a distantly homolog of the wide-spread machineries involved in plasmid conjugal transfer, of which the VirB type system from the plant pathogen *Agrobacterium tumefaciens* is arguably the best studied [98]. The latter group has been designated type IVA systems whereas the group exemplified by the Dot/Icm has been categorized as type IVB [98]. Most components of the Dot/Icm transporter are expressed constitutively [80], which is unsurprising given the fact that its activity is required for almost the entire intracellular growth cycle of the bacterium [56]. The Dot/Icm transporter is indispensable for the virulence of *L. pneumophila*, because it delivers substrates that directly engage various host processes, resulting in the biogenesis of a vacuole permissive for bacterial replication [66]. Because of their roles in the modulation of host functions, these protein substrates are also called effectors. The temporal regulation of host functions at different phase of infection can be achieved by controlling effector activity by mechanisms such as transcription, translocation, protein stability and regulation conferred by host factors and/or other effectors.

The development of several sensitive protein translocation assays along with pre-screening strategies involving genetic, bioinformatic and cell biological methods has led to the identification of a surprisingly large number of proteins transported by Dot/Icm [1,2,22,50,59,69,75,94,108]. To date, there are at least 275 experimentally confirmed Dot/Icm substrates [2]. In addition to the apparently large number, which comprises close to 10% of the protein coding capacity of *L. pneumophila*, Dot/Icm substrates have several outstanding features: First, most of them at present are hypothetical proteins, with known homology to proteins of identified functions. Indeed, this category does not even harbor motifs suggestive of potential biochemical activity. However, some of these proteins do form distinct families, and in some cases with highly homologous members [50]. Third, deletion of a single effector gene alone rarely leads to defects in intracellular growth in standard laboratory conditions, which points to potential functional redundancy among these proteins [33,50,66].

4. Hijacking of host pathways by *Legionella* effectors

4.1 Interference with the endocytic pathway

Professional phagocytes such as fresh water amoebae and human macrophages destroy invading microorganisms or foreign particles by engulfing them into phagosomes. After the initial internalization event, the newly formed phagosome sequentially fuses with a series of compartments of the endocytic network, such as sorting endosome, late endosome and lysosome [24]. In this process, both the composition of its membrane and its luminal content continue to be modified. In the end, the original plasma membrane-bound vacuole is transformed into a phagolysosome with efficient digestive capacity characterized by low pH and the presence of hydrolytic enzymes, reactive oxygen species and bactericidal peptides [85]. The acidic environment of the phagolysosome is necessary for the activation of various hydrolytic enzymes and production of reactive oxygen species. The acidification of lysosome and many other organelles in the cell is mainly regulated by the vacuolar-ATPase (v-ATPase) machinery, a proton pump driven by ATP hydrolysis [83]. The v-ATPase is composed of two domains: the trans-membrane domain V_0 which is responsible for H^+ translocation across the lipid bilayer and the cytosolic V_1 domain, which hydrolyzes ATP to provide energy for proton translocation [83].

Successful intracellular bacterial pathogens have developed sophisticated strategies to evade the destruction in the phagolysosome [68]. The majority of LCVs containing wild type bacterium do not acquire early endosome markers such as Rab5 or the lysosome marker LAMP-1 during early phase of infection (<1 hour) [101]. However, phagosomes containing a Dot/Icm-deficient mutants do ultimately fuse with lysosomes [101], indicating that the interactions with endocytic pathway are blocked immediately by effectors introduced by the bacterium. Conversely, inactivation of genes encoding essential components of the Dot/Icm system such as *dotA*, *dotG* and *icmQ*, can each result in the acquisition of LAMP1 on the LCV membrane [66]. Consistent with these observations, *L. pneumophila* is able to maintain a neutral pH in its phagosome for at least 6 hours whereas vacuoles containing non-pathogenic bacteria or formalin killed *L. pneumophila* become acidified within 15 minutes after their formation [71].

A number of Dot/Icm substrates that potentially target the endocytic pathway have been identified. VipA, VipD and VipF were found based upon their ability to interfere with lysosomal protein trafficking [22]. VipA has been shown to function as an actin nucleator, which binds to and polymerizes actin microfilaments [82]. Thus, its role in hijacking the endocytic pathway may result from its engagement with the host cytoskeleton. VipD contains a motif conserved in members of the patatin-like phospholipases although such activity has not yet been documented [12,22]. Similarly, VipF harbors an N-

acetyltransferase domain, however, whether this motif confers the predicted biochemical activity remains unknown. These proteins likely support distinct bacterial strategies to alter the lysosomal protein trafficking.

Although *L. pneumophila* avoids the fusion of its vacuole with lysosome and maintains a neutral pH in its phagosomal lumen, proteomic analyses have revealed that the v-ATPase is present on the membrane of LCVs, including early phagosomes analyzed within one hour of formation [14]. This observation suggests that *L. pneumophila* can antagonize the activity of the v-ATPase, probably using specific effectors. Based on its ability to render yeast sensitive to neutral pH (a typical phenotype of yeast v-ATPase mutants), Xu et al identified SidK, a substrate of the Dot/Icm transporter that is capable of specifically binding to VatA, the catalytic subunit of the v-ATPase [5]. Such binding leads to the arrest of v-ATPase-mediated proton translocation. Moreover, macrophages loaded with SidK are impaired in phagosomal acidification and cannot carry out lysosomal killing of phagocytosed non-pathogenic bacteria [5]. Interestingly, expression of *sidK* in bacteriological medium decreases when the bacterium enters post exponential phase [5], which is consistent with the fact that the lumen of LCVs becomes acidified after the infection has proceeded for more than 10 hours [19]. Thus, *L. pneumophila* appears to manipulate the activity of v-ATPase to meet the need for the development of the LCV. Further study of SidK, particularly its structural complex with the proton pump and the potential biochemical effects it imposes on VatA, will allow more detailed understanding of its mechanism of action and regulation.

Bacterial factors beyond Dot/Icm effectors have been implicated in the evasion of the endocytic pathway. For examples, lipopolysaccharides (LPS)-rich outer membrane vesicles released by *L. pneumophila* can target to the LCV membrane, where components of these vesicles inhibit the fusion with the lysosomal compartment [87]. Similarly, the multifunctional chaperone HtpB exposed on the bacterial surface has been implicated in the phagosomal maturation process of *L. pneumophila* [78]. Beads coated with HtpB pass through an intracellular trafficking pathway similar to the early phase of *L. pneumophila* infection with features such as inhibition of lysosomal fusion and the recruitment of mitochondria [99]. Finally, *L. pneumophila* proteins containing Sel-1 repeat, such as LpnE, EnhC and LidL appear to inhibit the acquisition of lysosomal markers such as LAMP-1 by the LCVs [36]. Because the mechanisms of action of these proteins remain enigmatic, it is unclear whether they directly contribute the arrest of LCV maturation. It remains possible that the loss of one or more of these proteins alters the physiology of *L. pneumophila*, which impairs the fitness of the bacterium, leading to the observed defects in its interactions with the phagocytes.

4.2 Hijacking the secretory pathway

The secretory pathway in eukaryotic cells transports proteins synthesized in the ER to the Golgi complex and finally to downstream cellular destinations or the extracellular environment. The secretory network is a continuous endomembrane system that functions sequentially in protein synthesis, modification, sorting and secretion [47]. Cargo trafficking in this pathway occurs via the generation of several types of coated vesicles, including COPI, COPII and clathrin coated vesicles. Newly synthesized polypeptides are packaged into COPII coated vesicles formed at the ER exit site, which are uncoated and delivered to its next station, the Golgi apparatus [64]. The proteins are modified mostly by glycosylation when transported from the *cis*-Golgi through a series of cisternae and sorted at the *trans*-Golgi network to their final destinations via clathrin-coated vesicles. The retrograde transport mediated by COPI vesicles ferries back to the components of the COPII transport machinery as well as ER resident proteins that have been mis-sorted to the Golgi apparatus [64].

It has been well established that one of the most important features of the intracellular life cycle of *L. pneumophila* is its ability to hijack vesicles trafficking between the ER and the Golgi apparatus. Interception of vesicles originating from the ER facilitates the conversion of the plasma membrane of the LCV into membranes with ER characteristics [63,92]. These vesicles also provide the membrane materials to support the expansion of the LCV necessitated by bacterial replication. The presence of ER resident proteins on the LCV was first revealed in a study that attempted to determine the overall identity of membranes of the bacterial phagosome [18]. Inhibition of ER-Golgi trafficking by chemical or genetic manipulation has been shown consistently to block the development of the LCV and the conversion of its membranes into ER-like membranes [63,90]. It is notable that all of these cell biological changes associated with the LCV require a functional Dot/Icm transporter [62,92], suggesting the involvement of Dot/Icm substrates in this process.

Small GTPases, also called Ras superfamily GTPases, are a form of G protein that binds and hydrolyzes guanosine triphosphate (GTP). These proteins regulate diverse aspects of intracellular signaling by serving as molecular switches [8]. Binding to GTP allows small GTPases to expose their conserved switch regions, which facilitates the recruitment of downstream effectors critical for the signaling events or for the execution of relevant physiological processes. Their intrinsic GTPase activity leads to generation of the inactive GDP bound form, concomitant with the completion of the signaling event. The exchange between the GDP and GTP-bound forms by GTPases is tightly regulated by several groups of regulatory enzymes. Under resting conditions, GDP-bound small GTPases are sequestered by guanine nucleotide dissociation inhibitors (GDIs), which keep them in the inactive status. When the cell receives proper signals, GDI displacement factors (GDFs) stimulate the release of GDI from GDP-bound GTPases, leading to the exposure of the hydrophobic tail and its subsequent insertion into the membrane of the appropriate compartment. The membrane associated GTPases are activated by guanine nucleotide exchange factors (GEFs), which induce the exchange of GDP with GTP. Finally, GTPases return to the inactive GDP-bound form by the function of GTPase activating proteins (GAPs), which stimulate the intrinsic GTPase activity to produce GDP-bound molecules. The GDP-GTPase complex is then sequestered by cognate GDIs with high binding affinity [46].

Members of the Arf (ADP-ribosylation factor), Rab and Sar families of small GTPases are primary regulators of vesicle trafficking [95]. Arf1 regulates COPI-coated retrograde trafficking from the *cis*-Golgi compartment to the ER [95]. This small GTPase is recruited and enriched on the LCV membrane in a Dot/Icm-dependent manner [40]. Bioinformatics analysis has revealed that *L. pneumophila* codes for a protein with a Sec7 domain, which is conserved in all GEFs of Arf small GTPases [40]. Further study found that this Sec7 domain protein is required for the recruitment of Arf1 by the LCV and was appropriately designated recruitment of Arf1 to *Legionella* vacuole factor (RalF). As expected, RalF is a GEF for Arf1, which induces the loading of GTP to the small GTPase [40]. Interestingly, the structure of RalF shows that its C-terminal domain forms a cap over the Sec7 domain active site, suggesting that the activity of RalF is regulated [107]. However, the mechanism of such regulation remains unknown.

The Rab family of small GTPases constitutes the largest branch of the small GTPase superfamily and its members are essential for vesicle tethering and fusion to the recipient membrane [20]. Among more than 60 members, Rab1 in particular play important roles in several aspects of vesicle trafficking between the ER and the Golgi apparatus [20]. Like Arf1, Rab1 is recruited to the LCV in a process that requires the Dot/Icm transporter [62,92]. Using beads coated with GDP-Rab1, Machner and Isberg identified the Dot/Icm substrate SidM/DrrA that interacts with GDP-Rab1 with high affinity [49]. They further

showed that SidM/DrrA is a GEF factor for Rab1 and is required for the recruitment of this small GTPase by the LCV [49]. Similar results were obtained in a study in which SidM/DrrA was identified by screening for *L. pneumophila* mutants that failed to recruit Rab1 [41]. The SidM/DrrA-mediated Rab1 recruitment is enhanced by LidA, another Dot/Icm substrate; LidA interacts with several different Rab proteins including Rab1, irrespective of their nucleotide binding status [38,49]. The recruitment of Rab1 by SidM/DrrA directly contributes to the fusion of ER-derived vesicles to the LCVs [105].

In addition to its GEF activity, SidM/DrrA is important for the displacement of Rab1 from its GDI. It had been suggested that a discrete domain on SidM/DrrA confers a GDF function that extracts Rab1 from the GDI [48]. However, another study proposed that instead of catalyzing an independent reaction, GDI displacement by SidM/DrrA is a direct consequence of its GEF activity [25]. Furthermore, structural analysis has revealed that SidM/DrrA contains a G-X₁₁-D-X-D motif similar to the one responsible for the adenylyl transferase activity in the *E. coli* glutamine synthetase (GS-ATase) [42]. Biochemical experiments have established that SidM/DrrA catalyzes adenylation (AMPylation) tyrosine 77 of Rab1 within the switch II region. This posttranslational modification locks GTP-loaded Rab1 in the active form by restricting the access of GAPs [42](Fig. 2). Such multi-layer regulation apparently is to ensure that the activity of Rab1 is not interfered with by factors such as host GAP proteins.

The association of Rab1 with the LCV lasts only for the first 6 hours of *L. pneumophila* infection [67], suggesting that the entire Rab cycle is completed in that time on the bacterial phagosome. Further, it has been shown that GTP hydrolysis by Rab1 is specifically catalyzed by the bacterial GAP, LepB [67]. The insensitivity of AMP-GTP-Rab1 to GAPs such as LepB suggested the existence of bacterial enzyme(s) that function to remove the AMP moiety from the small GTPase (Fig. 2), and this was subsequently identified by two independent studies. Neunuebel et al identified the deAMPylase SidD by its syntenic relationship with SidM/DrrA [39], whereas Tan and Luo found SidD by its ability to suppress the cytotoxicity of SidM/DrrA in yeast [16]. Consistent with its biochemical activity, deletion of *sidD* led to extended association of Rab1 on the LCV, but only for 2 extra hours [16,39]. The completion of the Rab1 activity cycle in the absence of SidD may result from the function of host deAMPylases, the decrease of SidM/DrrA activity level or some combination of the two.

Adding to the above-mentioned complexity of the modulation of Rab1 activity by *L. pneumophila* is the discovery of AnkX, another Dot/Icm substrate that targets Rab1 by phosphorylcholine (PC) [17,43]. AnkX catalyzes covalent attachment of the PC moiety to serine 76 of Rab1, which is adjacent to the tyrosine residue AMPylated by SidM/DrrA. Interestingly, the PC transferase activity of AnkX requires a filamentation induced by cAMP (Fic) domain [17,43], which had been shown previously to catalyze the AMPylation reaction [4]. Furthermore, *L. pneumophila* is able to directly regulate AnkX activity by Lem3, which is a de-phosphorylcholinase that reverses the effects of AnkX on Rab1 [17]. Whereas the role of SidD in the AMPylation of Rab1 is well appreciated, the importance for the PCylation of this small GTPase is not fully understood. Tan et al proposed that PCylation of Rab1 (and possibly other proteins such as USO1, p115 in mammalian cells) that are involved in the fusion of ER-derived vesicles to the *cis*-Golgi compartment, helps to divert the vesicles to the LCV. In this scenario, the function of Lem3 is to prevent unintended inhibition of the fusion on the bacterial phagosome [17]. Alternatively, because PCylated Rab1 has a lower affinity for GDI, Oesterlin et al suggested that this modification expedites the activation of the small GTPase, a mechanism that may be utilized by some cell types under normal physiological conditions [32].

AMPylation has been recognized as a universal posttranslational modification (PTM) catalyzed mostly by Fic domain proteins first, which were found in some bacterial virulence factors and later in some eukaryotes, including fruit flies and humans [60]. The fact that proteins with the G-X₁₁-D-X-D motif also modify their targets by AMPylation potentially increases the number of proteins with this biochemical activity. The identification of SidD revealed that AMPylation is a reversible PTM catalyzed by distinct enzymes. Further, the discovery that the Fic domain in AnkX is able to catalyze phosphorylcholine expands the biochemical capacity of this motif [17,43]. Interestingly, the diversity of Fic domain biochemistry was further broadened by the identification of the uridylyl transferase AvrAC from *Xanthomonas spp.*, which modifies and blocks conserved phosphorylation sites on BIK1 and RIPK, two kinases important for plant immunity [88]. These results once again underscore the usefulness of using bacterial pathogenic factors as reagents and research tools to unveil novel host cell signaling processes.

The importance of the small GTPase Sar1 in intracellular replication of *L. pneumophila* has been well documented. Expression of dominant negative mutants of Sar1 led to repression of intracellular bacterial growth and diminished tethering of vesicles to the LCV [63] [26]. Similarly, RNA interference analysis showed that Sar1 is required for the optimal intracellular growth of *L. pneumophila* [90]. These observations indicate an important role of the Sar1/COPII system in the intracellular life cycle of *L. pneumophila*, raising the possibility that targeting COPII components is a strategy used by the bacterium to hijack the host vesicle trafficking pathway. It will not be surprising if one or more *L. pneumophila* Dot/Icm substrates were found to target the Sar1/COPII core of the secretory pathway.

The regulation of vesicle trafficking in the secretory pathway is apparently more complicated than previously appreciated. Many *L. pneumophila* effectors have been implied in the interference of this process by yet undefined mechanisms. For example, ectopic expression of a large number of Dot/Icm substrates causes growth defects in yeast. Some of these, such as SetA, Ceg19 and Ceg9 appear to interfere with the secretory pathway [75]. Similarly, the effector SidC and its paralog SdcA have been also shown to be required for the recruitment of ER vesicles to the LCVs [27]. The challenge will be to reveal the biochemical functions of these proteins and their contributions to the modulation of vesicle trafficking in *L. pneumophila* infection.

Although investigation of the hijacking of regulatory molecules involved in vesicle trafficking has yielded some appreciable progress, our understanding of the bacterial factors that mediate regulation of the subsequent events in this pathway remains relatively scarce. *L. pneumophila* appears to take advantage of host SNARE (soluble NSF attached protein receptor)-mediated membrane fusion. It has been long observed that ER-derived v-SNARE protein Sec22b is associated with the LCV membrane in a Dot/Icm dependent manner [62,92]. Recent studies showed that *L. pneumophila* induces the non-canonical pairing of the plasma membrane t-SNARE syntaxins with Sec22b on the LCV membrane in a process that is simulated by the function of SidM/DrrA [105] [106]. Sorting out the minimal cohort of Dot/Icm substrates involved in the modulation of the host secretory pathway and elucidating their roles in bacterial replication will surely lead to a better understanding of this essential cellular process.

4.3 Modulation of the ubiquitination machine

Ubiquitination is a PTM that regulates the activity, half-life and the cellular localization of a wide variety of proteins. This eukaryotic cell-specific PTM is involved in diverse cellular processes such as proteasomal degradation, signaling cascade and DNA repair [61]. The process of ubiquitination requires at least three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). The attachment of a

single ubiquitin molecule to the substrate is defined as monoubiquitination, which is a regulatory signal for endocytosis, sorting events as well as DNA epigenetics [61]. Polyubiquitination occurs when additional ubiquitin molecules are attached to a lysine residue of the previous ubiquitin and form a ubiquitin chain (usually >4). Although all seven lysine residues on ubiquitin are capable of mediating the formation of polyubiquitin chains, Lys 48- and Lys 63-linked chains are the most abundant. Lys 48-linked chains are well-established regulators of protein degradation by serving as the recognition signal for the 26S proteasome, whereas Lys 63-linked chains are involved in nonproteolytic events, such as DNA repair [61].

Ubiquitination plays many essential roles in eukaryotic cells. From the host's perspective, this process is an important component of its immune system, which functions to recognize and contain pathogens by activating the host immune system, degrading bacterial effectors or by delivering the bacteria escaped into the cytoplasm back into the autophagosomes [97]. Given the importance of ubiquitination in host cell function, it is not surprising that many pathogens have evolved the ability to hijack this process to facilitate their colonization, mostly by using effectors that mimic host E3 ligases or adaptor proteins of the ubiquitination system [73].

The importance of the ubiquitination machinery in *L. pneumophila* infection clearly demonstrated by the enrichment of ubiquitinated proteins on its phagosome as well as the fact that pharmaceutical inhibition of proteasomal degradation causes arrest in intracellular bacterial growth [90]. Ubiquitin conjugation mediated by K63 has also been found accumulated on the membrane of LCV containing wild type *L. pneumophila* [65], suggesting that some *L. pneumophila* effectors participate in cellular processes independent of proteasomal degradation, possibly by assuming roles in regulatory circuits.

At least 6 Dot/Icm substrates bear features associated with typical eukaryotic E3 ubiquitin ligases. Among these, LubX is a U-box protein, which appears to have targets from both the host cell and the pathogen itself. This protein participates in the ubiquitination of the cell cycle related kinase Clk1, but the cellular consequence of this modification is unknown [59]. More recently, LubX was found to target SidH, another *L. pneumophila* effector for proteasomal degradation [58]. Importantly, expression of *lubX* does not become apparent until after 2 hrs post bacterial internalization, which suggests a requirement for SidH early in *L. pneumophila* infection [58]. Interestingly, similar to the positioning *sidM/drrA-sidD* and *AnkX-lem3*, the gene pairs with clear functional relevance [17,39], *lubX* localizes next to *sidH* on the *L. pneumophila* chromosome [58] (Fig. 3). The close positioning of genes of opposite activity may allow their simultaneous acquisition by new recipient (bacterial) hosts or ensure proper regulation of their expression. Interestingly, clustering of two or more Dot/Icm substrate genes occurs in many cases, which are conserved in almost all sequenced *L. pneumophila* strains [2,76]. Such arrangements may be exploited in the study of the function of these proteins.

L. pneumophila strain Philadelphia-1 codes for five Dot/Icm effectors with an F-box motif [89]. The F-box motif proteins are one component of the SCF (Skp1-Cullin-F-box) ubiquitin ligase complex, which is involved in the degradation of diverse substrates [7]. In this ligase complex, the F-box protein binds to Skp1 via the conserved F-box motif. The F-box protein also dictates the substrate specificity of the complex by directly interacting with the substrate. Three of the five *L. pneumophila* F-box proteins, LegU1, AnkB (LegAU13), and LicA have been shown to interact with Skp1 [30,89]. In particular, LegU1 specifically interacts with and ubiquitinates BAT3, a host chaperone associated with apoptosis and ER stress response [89], which may contribute to cell death regulation or the homeostasis of the ER. However, the identities of the substrates of other F-box proteins remain elusive.

The *Legionella* effector AnkB is an F-Box protein with two ankyrin repeats. Although the ankyrin repeat domain, it is prevalent in F-box-like proteins from the poxvirus family [21], often involved in protein-protein interactions and has never been reported in eukaryotic F-box proteins. Thus, this domain in AnkB is likely a novel adaptor important for substrate recruitment. *AnkB* deletion mutants have been shown to exhibit severe defects in intracellular bacterial growth in some studies [28,109] but are negligible in others [55,89]. Such discrepancy may result from the variations among different *L. pneumophila* strains and/or host cells used in the studies. AnkB has been reported to interact with Parvin B, a host protein present in focal adhesions and in lamellipodia [55], but the biological significance of such interactions is not clear. Interestingly, a recent report suggests that AnkB is involved in the production of amino acids to provide nutrients for *L. pneumophila* inside the LCV [28].

4.4 Interference with host cell death pathways

Apoptosis is a process of programmed cell death prevalent in multicellular organisms and critical for various important cellular events, such as development, regulation of the immune response and removal of defective cells. Elimination of the replication niche by apoptosis is an effective mechanism for defense against infections, particularly those caused by intracellular pathogens. Thus, it is not surprising that many intracellular pathogens, such as *Chlamydia trachomatis* and *Coxiella burnetii*, have evolved effective mechanisms to suppress the apoptotic process and thus ensure host cell survival until the completion of their intracellular growth cycle [104].

Accumulating evidence indicates that *L. pneumophila* maintains a balance between induction and inhibition in its manipulation of host cell death processes [52]. Earlier studies found that infection by *L. pneumophila* in permissive cells caused activation of the executioner caspase, caspase-3 [79]. Active caspase-3 may contribute to intracellular bacterial replication by its involvement of vesicle trafficking but not cell death [45]. However, such effects, if any, appear to be cell type specific because mouse macrophages deficient in caspase-3 still support full bacterial replication [3]. The importance of caspase-3 activation becomes more apparent in dendritic cells, which undergo rapid cell death upon being challenged by *L. pneumophila* [34]. Interestingly, dendritic cells from caspase-3 knockout mice support productive but not optimal bacterial replication; more complete abrogation of the cell death pathway by deleting Bax and Bak, the two major upstream regulators of mitochondrial apoptosis, allows maximal intracellular bacterial growth [34]. Thus, a cell death process is initiated in response to *L. pneumophila* infection, but the ultimate outcome of such induction largely is dependent upon the host cell type.

Permissive macrophages infected by wild type *L. pneumophila* exhibit strong resistance to exogenous cell death stimuli such as staurosporine, a potent inhibitor of protein kinases [110]. Such resistance is achieved by reprogramming host cell death pathways at both the transcriptional and posttranslational levels and bacterial proteins that potentially engage in host cell death pathways have been identified. For example, SidF directly inhibits host cell death by targeting pro-apoptotic proteins [103]. Further, the interference with host phosphoinositide signaling by this protein may also contribute to such inhibition [70]. On the other hand, in primary mouse macrophages, SdhA is essential for the protection of cell death caused by type I interferon by maintaining the integrity of the LCVs [44,57,96]. The function of these proteins may buy the time necessary for the mechanisms potentiated by modulation of transcription to take effect.

Challenge by virulent *L. pneumophila* induces expression of a array of host genes that promote cell survival, including pro-survival members of the Bcl2 family and CIAP2 [53,111]. The induction of these genes is essential for the cell death resistance phenotype

because inactivation of a single such gene, the plasminogen activator inhibitor-2, causes more cell death in infected cells and less intracellular bacterial growth [53]. Such induction mainly is achieved by the activation of NF κ B, the major regulator of cell survival and immune response [53]. In agreement with this notion, several Dot/Icm substrates, including LegK1, LnaB, SdbA and LubX have been shown to be involved in the NF κ B activation [54,77,102]. With the exception of LegK1, which is a Ser/Thr kinase that targets NF κ B signaling by directly phosphorylating its inhibitor I κ B α [77], the biochemical basis for the mechanism of action of most of these proteins is unknown. It has been suggested that the collective effects of multiple Dot/Icm substrates also contribute to NF κ B activation, probably by non-canonical mechanisms [52]. This notion was supported by the discovery that deletion of 5 Dot/Icm substrate genes involved in protein synthesis inhibition causes defects in NF κ B activation [84]. These effectors block efficient translation of I κ B, the labile inhibitor for NF κ B, leading to extended activation of the transcriptional activator [84]. Further study of the modes of action of Dot/Icm substrates involved in NF κ B activation will reveal not only the strategies of host function manipulation by pathogens, but also potentially novel immune surveillance mechanisms in host cells.

4.5 Exploitation of host lipid metabolism and signaling

Lipids play essential roles in many diverse cellular processes such as signaling, vesicle trafficking and organelle definition. Some Dot/Icm substrates contain motifs predicted to affect lipid metabolism. For example, members of the VipD family harbor a lipase motif present in the patatin-like family of lipase [12,22]. A motif involved in similar activity is found in members of the SidB family [50]. However, the enzymatic activity of these effectors has yet not been demonstrated, nor have their natural substrates. Moreover, some Dot/Icm substrates exploit the host lipidation process by conjugating a farnesyl group to the CAAX motif (C: cysteine residue and A: aliphatic amino acid) found in their C-terminal ends [29,65]. Down regulation of farnesylation causes defects in intracellular bacterial replication [29] and one role of this modification is to target the effector to the proper cellular compartment. For example, farnesylation of AnkB promotes the insertion of AnkB into LCV membrane during *L. pneumophila* infection [29].

Phosphoinositides (PIs) are a critical determinant in a broad spectrum of cellular processes, including defining intracellular organelle identity, cell signaling, proliferation, cytoskeleton organization, and membrane trafficking [91]. These lipids are substrates for a large number of kinases and phosphatases, which are essential in PI metabolism where they catalyze reversible phosphorylation at the 3', 4', and 5' positions of the inositol headgroup. The PIs are important for defining different cellular compartments. For example, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] predominantly localizes on the plasma membrane; phosphatidylinositol 3-phosphate [PI(3)P] is enriched in early endocytic pathway and phosphatidylinositol 4-phosphate [PI(4)P] is abundant in the Golgi apparatus [91]. Many intracellular pathogens manipulate host PI metabolism to alter signaling or lipid composition in certain compartments for their benefit [31]. For instance, *Mycobacterium tuberculosis* secretes SapM, a PI(3)P phosphatase, which participates in the inhibition of phagosomal maturation [11]. PI phosphatases are also used by *Salmonella* spp. and *Shigella* spp. to promote bacterial entry into non-phagocytic cells [35,74].

Three Dot/Icm substrates, SidC, SdcA and SidM/DrrA are capable of binding PI(4)P [72]. Furthermore, these proteins are concentrated on the surface of LCV, suggesting the enrichment of PI(4)P on membranes of this organelle [72]. One mechanism underlying the enrichment of PI(4)P on the LCV membrane was recently revealed by the discovery of the *Legionella* phosphoinositide phosphatase SidF, which dephosphorylates the D3 position of the inositol ring on PI(3,4)P₂ and PI(3,4,5)P₃ to produce PI(4)P and PI(4,5)P₂, respectively [70]. During *L. pneumophila* infection, SidF is associated with the LCV membrane [70,103],

probably via the two transmembrane domains localized in its C-terminal end. Because the oculocerebrorenal syndrome of Lowe 1 (OCRL1) enzyme, a 5-phosphatase that can only hydrolyze the D5 phosphate of PI(4,5)P₂ or PI(3,4,5)P₃, is associated with the LCV [9], it is likely that the final lipid product also is PI(4)P when PI(3,4,5)P₃ is used as substrate by SidF (Fig. 4). Consistent with its role in the generation of PI(4)P, deletion of *sidF* reduced the rates of SidC association with the LCV [70]. Proteins from host, the bacterium or both may contribute to the remaining PI(4)P that is responsible for the SidC retention seen in vacuoles containing the *sidF* mutant. In addition to anchoring effectors, the enrichment of PI(4)P on the LCV clearly will benefit the bacterium by causing its membranes to mimic those of the *cis*-Golgi compartment, which will serve as a better recipient site for the fusion of ER-derived vesicles. It will be interesting to determine how complete depletion of PI(4)P from the LCV affects intracellular *L. pneumophila* growth.

PI(3)P has been found to interact with *L. pneumophila* effectors, including LidA, which binds multiple Rab proteins [38], and LpnE (the Dot/Icm-dependent translocation of which has not yet been established). The latter protein contains a Sel-1 repeat domain and is required for the entry of *L. pneumophila* into host cell [36,37]. Because one of the roles played by PI(3)P is to promote fusion between endosomes and a newly formed phagosome [93], it is important to determine whether PI(3)P is enriched on the LCV, and if so how one can reconcile the effects of such enrichment and its role in promoting phagolysosomal fusion. Elucidation of the roles of host proteins and potential bacterial proteins involved in PI(3)P metabolism will certainly shed light on this conundrum. Finally, given the pleiotropic effects of PIs [91], perturbation of their cellular concentrations either globally or locally by bacterial effectors will surely lead to considerable alterations in the host cell. Sorting out the nature of these changes and determining how such changes benefit or restrain *L. pneumophila* infection will be a great challenge. Nevertheless, the availability of specific enzymes involved in inducing these alternations will be invaluable tools in our probe of the roles of these lipids in normal host cell biology.

5. Concluding remarks

L. pneumophila devotes close to 10% of its protein coding capacity for functions that are likely involved in direct interactions with host cellular machineries [2,76]. Progress in our understanding of the roles of these proteins in bacterial infection has revealed some highly sophisticated mechanisms in the hijacking of host function. To some extent, the effects of these proteins on eukaryotic cells are similar to the introduction of specific mutations that induce readily detectable phenotypes, which can be used to study the function of the bacterial proteins as well as the host processes involved. The study of these proteins is hindered, however, by several outstanding problems.

First, in most cases, deletion of one or more effectors does not result in defects in standard assays used to evaluate the virulence of *L. pneumophila*. Functional redundancy among effectors may contribute to the lack of phenotype in mutants missing some genes. With the development of cluster deletion strains in which up to 31% of the chromosome had been deleted [33], further deletions aiming at eliminating additional effector genes may eventually lead to detectable phenotypes in standard experimental conditions. Alternatively, it is possible that some of these effectors are not involved in intracellular replication, the most frequently examined phenotype. For example, effectors may participate in the response of the host cell to fluctuations in the environment such as starvation, shift in temperature and the presence of other invaders. It will be useful to establish alternative assays to evaluate the fitness of *L. pneumophila* mutants in various host systems under different conditions.

Second, the progress of biochemical characterization of Dot/Icm substrates without homology to proteins of known functions by current technologies is far from ideal. Methods that allow identification of potential host targets of large number of proteins are in great need. One possibility is to exploit the yeast mutant library [6] by systems biology technologies or to use sensitive protein arrays [100]. In the coming years, exciting discoveries are likely to be driven by the entry into this field of investigators from biochemistry, cell biology and structural biology, with expertise and approaches that are highly complementary to those of the traditional microbiology and microbial pathogenesis.

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References

1. Zusman T, Degtyar E, Segal G. Identification of a hypervariable region containing new *Legionella pneumophila* Icm/Dot translocated substrates by using the conserved icmQ regulatory signature. *Infect Immun*. 2008; 76:4581–4591. [PubMed: 18694969]
2. Zhu W, Banga S, Tan Y, Zheng C, Stephenson R, Gately J, Luo ZQ. Comprehensive identification of protein substrates of the Dot/Icm type IV transporter of *Legionella pneumophila*. *PLoS One*. 2011; 6:e17638. [PubMed: 21408005]
3. Zamboni DS, Kobayashi KS, Kohlsdorf T, Ogura Y, Long EM, Vance RE, Kuida K, Mariathasan S, Dixit VM, Flavell RA, Dietrich WF, Roy CR. The Bir1c1 cytosolic pattern-recognition receptor contributes to the detection and control of *Legionella pneumophila* infection. *Nat Immunol*. 2006; 7:318–325. [PubMed: 16444259]
4. Yarbrough ML, Li Y, Kinch LN, Grishin NV, Ball HL, Orth K. AMPylation of Rho GTPases by *Vibrio* VopS disrupts effector binding and downstream signaling. *Science*. 2009; 323:269–272. [PubMed: 19039103]
5. Xu L, Shen X, Bryan A, Banga S, Swanson MS, Luo ZQ. Inhibition of host vacuolar H⁺-ATPase activity by a *Legionella pneumophila* effector. *PLoS Pathog*. 2010; 6:e1000822. [PubMed: 20333253]
6. Winzler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke JD, Bussey H, Chu AM, Connelly C, Davis K, Dietrich F, Dow SW, El Bakkoury M, Foury F, Friend SH, Gentale E, Giaever G, Hegemann JH, Jones T, Laub M, Liao H, Liebundguth N, Lockhart DJ, Lucau-Danila A, Lussier M, M'Rabet N, Menard P, Mittmann M, Pai C, Rebischung C, Revuelta JL, Riles L, Roberts CJ, Ross-MacDonald P, Scherens B, Snyder M, Sookhai-Mahadeo S, Storms RK, Veronneau S, Voet M, Volckaert G, Ward TR, Wysocki R, Yen GS, Yu K, Zimmermann K, Philippsen P, Johnston M, Davis RW. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science*. 1999; 285:901–906. [PubMed: 10436161]
7. Willems AR, Schwab M, Tyers M. A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. *Biochim Biophys Acta*. 2004; 1695:133–170. [PubMed: 15571813]
8. Wennerberg K, Rossman KL, Der CJ. The Ras superfamily at a glance. *J Cell Sci*. 2005; 118:843–846. [PubMed: 15731001]
9. Weber SS, Ragaz C, Hilbi H. Pathogen trafficking pathways and host phosphoinositide metabolism. *Mol Microbiol*. 2009; 71:1341–1352. [PubMed: 19208094]
10. Vogel JP, Andrews HL, Wong SK, Isberg RR. Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science*. 1998; 279:873–876. [PubMed: 9452389]
11. Vergne I, Chua J, Lee HH, Lucas M, Belisle J, Deretic V. Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A*. 2005; 102:4033–4038. [PubMed: 15753315]

12. VanRheenen SM, Luo ZQ, O'Connor T, Isberg RR. Members of a *Legionella pneumophila* family of proteins with ExoU (phospholipase A) active sites are translocated to target cells. *Infect Immun*. 2006; 74:3597–3606. [PubMed: 16714592]
13. Vance RE. Immunology taught by bacteria. *J Clin Immunol*. 2010; 30:507–511. [PubMed: 20373001]
14. Urwyler S, Nyfeler Y, Ragaz C, Lee H, Mueller LN, Aebersold R, Hilbi H. Proteome analysis of *Legionella* vacuoles purified by magnetic immunoseparation reveals secretory and endosomal GTPases. *Traffic*. 2009; 10:76–87. [PubMed: 18980612]
15. Tilney LG, Harb OS, Connelly PS, Robinson CG, Roy CR. How the parasitic bacterium *Legionella pneumophila* modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. *J Cell Sci*. 2001; 114:4637–4650. [PubMed: 11792828]
16. Tan Y, Luo ZQ. *Legionella pneumophila* SidD is a deAMPyase that modifies Rab1. *Nature*. 2011; 475:506–509. [PubMed: 21734656]
17. Tan Y, Arnold RJ, Luo ZQ. *Legionella pneumophila* regulates the small GTPase Rab1 activity by reversible phosphorylcholine. *Proc Natl Acad Sci U S A*. 2011; 108:21212–21217. [PubMed: 22158903]
18. Swanson MS, Isberg RR. Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. *Infect Immun*. 1995; 63:3609–3620. [PubMed: 7642298]
19. Sturgill-Koszycki S, Swanson MS. *Legionella pneumophila* replication vacuoles mature into acidic, endocytic organelles. *J Exp Med*. 2000; 192:1261–1272. [PubMed: 11067875]
20. Stenmark H. Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol*. 2009; 10:513–525. [PubMed: 19603039]
21. Sonnberg S, Seet BT, Pawson T, Fleming SB, Mercer AA. Poxvirus ankyrin repeat proteins are a unique class of F-box proteins that associate with cellular SCF1 ubiquitin ligase complexes. *Proc Natl Acad Sci U S A*. 2008; 105:10955–10960. [PubMed: 18667692]
22. Shohdy N, Efe JA, Emr SD, Shuman HA. Pathogen effector protein screening in yeast identifies *Legionella* factors that interfere with membrane trafficking. *Proc Natl Acad Sci U S A*. 2005; 102:4866–4871. [PubMed: 15781869]
23. Segal G, Purcell M, Shuman HA. Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. *Proc Natl Acad Sci U S A*. 1998; 95:1669–1674. [PubMed: 9465074]
24. Scott CC, Botelho RJ, Grinstein S. Phagosome maturation: a few bugs in the system. *J Membr Biol*. 2003; 193:137–152. [PubMed: 12962275]
25. Schoebel S, Oesterlin LK, Blankenfeldt W, Goody RS, Itzen A. RabGDI displacement by DrrA from *Legionella* is a consequence of its guanine nucleotide exchange activity. *Mol Cell*. 2009; 36:1060–1072. [PubMed: 20064470]
26. Robinson CG, Roy CR. Attachment and fusion of endoplasmic reticulum with vacuoles containing *Legionella pneumophila*. *Cell Microbiol*. 2006; 8:793–805. [PubMed: 16611228]
27. Ragaz C, Pietsch H, Urwyler S, Tiaden A, Weber SS, Hilbi H. The *Legionella pneumophila* phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole. *Cell Microbiol*. 2008; 10:2416–2433. [PubMed: 18673369]
28. Price CT, Al-Quadan T, Santic M, Rosenshine I, Abu Kwaik Y. Host proteasomal degradation generates amino acids essential for intracellular bacterial growth. *Science*. 2011; 334:1553–1557. [PubMed: 22096100]
29. Price CT, Al-Quadan T, Santic M, Jones SC, Abu Kwaik Y. Exploitation of conserved eukaryotic host cell farnesylation machinery by an F-box effector of *Legionella pneumophila*. *J Exp Med*. 2010; 207:1713–1726. [PubMed: 20660614]
30. Price CT, Al-Khodori S, Al-Quadan T, Santic M, Habyarimana F, Kalia A, Kwaik YA. Molecular mimicry by an F-box effector of *Legionella pneumophila* hijacks a conserved polyubiquitination machinery within macrophages and protozoa. *PLoS Pathog*. 2009; 5:e1000704. [PubMed: 20041211]
31. Payraastre B, Gaits-Iacovoni F, Sansonetti P, Tronchere H. Phosphoinositides and cellular pathogens. *Subcell Biochem*. 2012; 59:363–388. [PubMed: 22374097]

32. Oesterlin LK, Goody RS, Itzen A. Posttranslational modifications of Rab proteins cause effective displacement of GDP dissociation inhibitor. *Proc Natl Acad Sci U S A*. 2012; 109:5621–5626. [PubMed: 22411835]
33. O'Connor TJ, Adepoju Y, Boyd D, Isberg RR. Minimization of the *Legionella pneumophila* genome reveals chromosomal regions involved in host range expansion. *Proc Natl Acad Sci U S A*. 2011; 108:14733–14740. [PubMed: 21873199]
34. Nogueira CV, Lindsten T, Jamieson AM, Case CL, Shin S, Thompson CB, Roy CR. Rapid pathogen-induced apoptosis: a mechanism used by dendritic cells to limit intracellular replication of *Legionella pneumophila*. *PLoS Pathog*. 2009; 5:e1000478. [PubMed: 19521510]
35. Niebuhr K, Giuriato S, Pedron T, Philpott DJ, Gaits F, Sable J, Sheetz MP, Parsot C, Sansonetti PJ, Payrastre B. Conversion of PtdIns(4,5)P(2) into PtdIns(5)P by the *S. flexneri* effector IpgD reorganizes host cell morphology. *EMBO J*. 2002; 21:5069–5078. [PubMed: 12356723]
36. Newton HJ, Sansom FM, Dao J, McAlister AD, Sloan J, Cianciotto NP, Hartland EL. Sell repeat protein LpnE is a *Legionella pneumophila* virulence determinant that influences vacuolar trafficking. *Infect Immun*. 2007; 75:5575–5585. [PubMed: 17893138]
37. Newton HJ, Sansom FM, Bennett-Wood V, Hartland EL. Identification of *Legionella pneumophila*-specific genes by genomic subtractive hybridization with *Legionella micdadei* and identification of lpnE, a gene required for efficient host cell entry. *Infect Immun*. 2006; 74:1683–1691. [PubMed: 16495539]
38. Neunuebel MR, Mohammadi S, Jarnik M, Machner MP. *Legionella pneumophila* LidA affects nucleotide binding and activity of the host GTPase Rab1. *J Bacteriol*. 2012; 194:1389–1400. [PubMed: 22228731]
39. Neunuebel MR, Chen Y, Gaspar AH, Backlund PS Jr, Yergey A, Machner MP. De-AMPylation of the small GTPase Rab1 by the pathogen *Legionella pneumophila*. *Science*. 2011; 333:453–456. [PubMed: 21680813]
40. Nagai H, Kagan JC, Zhu X, Kahn RA, Roy CR. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. *Science*. 2002; 295:679–682. [PubMed: 11809974]
41. Murata T, Delprato A, Ingmundson A, Toomre DK, Lambright DG, Roy CR. The *Legionella pneumophila* effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. *Nat Cell Biol*. 2006; 8:971–977. [PubMed: 16906144]
42. Muller MP, Peters H, Blumer J, Blankenfeldt W, Goody RS, Itzen A. The *Legionella* effector protein DrrA AMPylates the membrane traffic regulator Rab1b. *Science*. 2010; 329:946–949. [PubMed: 20651120]
43. Mukherjee S, Liu X, Arasaki K, McDonough J, Galan JE, Roy CR. Modulation of Rab GTPase function by a protein phosphocholine transferase. *Nature*. 2011; 477:103–106. [PubMed: 21822290]
44. Monroe KM, McWhirter SM, Vance RE. Identification of host cytosolic sensors and bacterial factors regulating the type I interferon response to *Legionella pneumophila*. *PLoS Pathog*. 2009; 5:e1000665. [PubMed: 19936053]
45. Molmeret M, Zink SD, Han L, Abu-Zant A, Asari R, Bitar DM, Abu Kwaik Y. Activation of caspase-3 by the Dot/Icm virulence system is essential for arrested biogenesis of the *Legionella*-containing phagosome. *Cell Microbiol*. 2004; 6:33–48. [PubMed: 14678329]
46. Molendijk AJ, Ruperti B, Palme K. Small GTPases in vesicle trafficking. *Curr Opin Plant Biol*. 2004; 7:694–700. [PubMed: 15491918]
47. Mellman I, Warren G. The road taken: past and future foundations of membrane traffic. *Cell*. 2000; 100:99–112. [PubMed: 10647935]
48. Machner MP, Isberg RR. A bifunctional bacterial protein links GDI displacement to Rab1 activation. *Science*. 2007; 318:974–977. [PubMed: 17947549]
49. Machner MP, Isberg RR. Targeting of host Rab GTPase function by the intravacuolar pathogen *Legionella pneumophila*. *Dev Cell*. 2006; 11:47–56. [PubMed: 16824952]
50. Luo ZQ, Isberg RR. Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. *Proc Natl Acad Sci U S A*. 2004; 101:841–846. [PubMed: 14715899]

51. Luo ZQ. Legionella secreted effectors and innate immune responses. *Cell Microbiol.* 2012; 14:19–27. [PubMed: 21985602]
52. Luo ZQ. Striking a balance: modulation of host cell death pathways by legionella pneumophila. *Front Microbiol.* 2011; 2:36. [PubMed: 21687427]
53. Losick VP, Isberg RR. NF-kappaB translocation prevents host cell death after low-dose challenge by Legionella pneumophila. *J Exp Med.* 2006; 203:2177–2189. [PubMed: 16940169]
54. Losick VP, Haenssler E, Moy MY, Isberg RR. LnaB: a Legionella pneumophila activator of NF-kappaB. *Cell Microbiol.* 2010; 12:1083–1097. [PubMed: 20148897]
55. Lomma M, Dervins-Ravault D, Rolando M, Nora T, Newton HJ, Sansom FM, Sahr T, Gomez-Valero L, Jules M, Hartland EL, Buchrieser C. The Legionella pneumophila F-box protein Lpp2082 (AnkB) modulates ubiquitination of the host protein parvin B and promotes intracellular replication. *Cell Microbiol.* 2010; 12:1272–1291. [PubMed: 20345489]
56. Liu Y, Gao P, Banga S, Luo ZQ. An in vivo gene deletion system for determining temporal requirement of bacterial virulence factors. *Proc Natl Acad Sci U S A.* 2008; 105:9385–9390. [PubMed: 18599442]
57. Laguna RK, Creasey EA, Li Z, Valtz N, Isberg RR. A Legionella pneumophila-translocated substrate that is required for growth within macrophages and protection from host cell death. *Proc Natl Acad Sci U S A.* 2006; 103:18745–18750. [PubMed: 17124169]
58. Kubori T, Shinzawa N, Kanuka H, Nagai H. Legionella metaeffector exploits host proteasome to temporally regulate cognate effector. *PLoS Pathog.* 2010; 6:e1001216. [PubMed: 21151961]
59. Kubori T, Hyakutake A, Nagai H. Legionella translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions. *Mol Microbiol.* 2008; 67:1307–1319. [PubMed: 18284575]
60. Kinch LN, Yarbrough ML, Orth K, Grishin NV. Fido, a novel AMPylation domain common to fic, doc, and AvrB. *PLoS One.* 2009; 4:e5818. [PubMed: 19503829]
61. Kerscher O, Felberbaum R, Hochstrasser M. Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu Rev Cell Dev Biol.* 2006; 22:159–180. [PubMed: 16753028]
62. Kagan JC, Stein MP, Pypaert M, Roy CR. Legionella subvert the functions of Rab1 and Sec22b to create a replicative organelle. *J Exp Med.* 2004; 199:1201–1211. [PubMed: 15117975]
63. Kagan JC, Roy CR. Legionella phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nat Cell Biol.* 2002; 4:945–954. [PubMed: 12447391]
64. Jensen D, Schekman R. COPII-mediated vesicle formation at a glance. *J Cell Sci.* 2011; 124:1–4. [PubMed: 21172817]
65. Ivanov SS, Charron G, Hang HC, Roy CR. Lipidation by the host prenyltransferase machinery facilitates membrane localization of Legionella pneumophila effector proteins. *J Biol Chem.* 2010; 34686–34698. [PubMed: 20813839]
66. Isberg RR, O'Connor TJ, Heidtman M. The Legionella pneumophila replication vacuole: making a cosy niche inside host cells. *Nat Rev Microbiol.* 2009; 7:13–24. [PubMed: 19011659]
67. Ingmundson A, Delprato A, Lambright DG, Roy CR. Legionella pneumophila proteins that regulate Rab1 membrane cycling. *Nature.* 2007; 450:365–369. [PubMed: 17952054]
68. Huynh KK, Grinstein S. Regulation of vacuolar pH and its modulation by some microbial species. *Microbiol Mol Biol Rev.* 2007; 71:452–462. [PubMed: 17804666]
69. Huang L, Boyd D, Amyot WM, Hempstead AD, Luo ZQ, O'Connor TJ, Chen C, Machner M, Montminy T, Isberg RR. The E Block motif is associated with *Legionella pneumophila* translocated substrates. *Cell Microbiol.* 2010; 13:227–245. [PubMed: 20880356]
70. Hsu F, Zhu W, Brennan L, Tao L, Luo ZQ, Mao Y. Structural basis for substrate recognition by a unique Legionella phosphoinositide phosphatase. *Proc Natl Acad Sci U S A.* 2012; 109:13567–13572. [PubMed: 22872863]
71. Horwitz MA, Maxfield FR. Legionella pneumophila inhibits acidification of its phagosome in human monocytes. *J Cell Biol.* 1984; 99:1936–1943. [PubMed: 6501409]
72. Hilbi H, Weber S, Finsel I. Anchors for effectors: subversion of phosphoinositide lipids by legionella. *Front Microbiol.* 2011; 2:91. [PubMed: 21833330]
73. Hicks SW, Galan JE. Hijacking the host ubiquitin pathway: structural strategies of bacterial E3 ubiquitin ligases. *Curr Opin Microbiol.* 2009; 13:41–46. [PubMed: 20036613]

74. Hernandez LD, Hueffer K, Wenk MR, Galan JE. Salmonella modulates vesicular traffic by altering phosphoinositide metabolism. *Science*. 2004; 304:1805–1807. [PubMed: 15205533]
75. Heidtman M, Chen EJ, Moy MY, Isberg RR. Large-scale identification of *Legionella pneumophila* Dot/Icm substrates that modulate host cell vesicle trafficking pathways. *Cell Microbiol*. 2009; 11:230–248. [PubMed: 19016775]
76. Gomez-Valero L, Rusniok C, Cazalet C, Buchrieser C. Comparative and functional genomics of legionella identified eukaryotic like proteins as key players in host-pathogen interactions. *Front Microbiol*. 2011; 2:208. [PubMed: 22059087]
77. Ge J, Xu H, Li T, Zhou Y, Zhang Z, Li S, Liu L, Shao F. A *Legionella* type IV effector activates the NF-kappaB pathway by phosphorylating the IkappaB family of inhibitors. *Proc Natl Acad Sci U S A*. 2009; 106:13725–13730. [PubMed: 19666608]
78. Garduno RA, Chong A, Nasrallah GK, Allan DS. The *Legionella pneumophila* Chaperonin - An Unusual Multifunctional Protein in Unusual Locations. *Front Microbiol*. 2011; 2:122. [PubMed: 21713066]
79. Gao LY, Abu Kwaik Y. Activation of caspase 3 during *Legionella pneumophila*-induced apoptosis. *Infect Immun*. 1999; 67:4886–4894. [PubMed: 10456945]
80. Gal-Mor O, Zusman T, Segal G. Analysis of DNA regulatory elements required for expression of the *Legionella pneumophila* icm and dot virulence genes. *J Bacteriol*. 2002; 184:3823–3833. [PubMed: 12081952]
81. Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, Sharrar RG, Harris J, Mallison GF, Martin SM, McDade JE, Shepard CC, Brachman PS. Legionnaires' disease: description of an epidemic of pneumonia. *N Engl J Med*. 1977; 297:1189–1197. [PubMed: 335244]
82. Franco IS, Shohdy N, Shuman HA. The *Legionella pneumophila* effector VipA is an actin nucleator that alters host cell organelle trafficking. *PLoS Pathog*. 2012; 8:e1002546. [PubMed: 22383880]
83. Forgac M. Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nat Rev Mol Cell Biol*. 2007; 8:917–929. [PubMed: 17912264]
84. Fontana MF, Banga S, Barry KC, Shen X, Tan Y, Luo ZQ, Vance RE. Secreted bacterial effectors that inhibit host protein synthesis are critical for induction of the innate immune response to virulent *Legionella pneumophila*. *PLoS Pathog*. 2011; 7:e1001289. [PubMed: 21390206]
85. Flannagan RS, Cosio G, Grinstein S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol*. 2009; 7:355–366. [PubMed: 19369951]
86. Fields BS, Benson RF, Besser RE. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev*. 2002; 15:506–526. [PubMed: 12097254]
87. Fernandez-Moreira E, Helbig JH, Swanson MS. Membrane vesicles shed by *Legionella pneumophila* inhibit fusion of phagosomes with lysosomes. *Infect Immun*. 2006; 74:3285–3295. [PubMed: 16714556]
88. Feng F, Yang F, Rong W, Wu X, Zhang J, Chen S, He C, Zhou JM. A *Xanthomonas* uridine 5'-monophosphate transferase inhibits plant immune kinases. *Nature*. 2012; 485:114–118. [PubMed: 22504181]
89. Ensminger AW, Isberg RR. E3 ubiquitin ligase activity and targeting of BAT3 by multiple *Legionella pneumophila* translocated substrates. *Infect Immun*. 2010; 78:3905–3919. [PubMed: 20547746]
90. Dorer MS, Kirton D, Bader JS, Isberg RR. RNA interference analysis of *Legionella* in *Drosophila* cells: exploitation of early secretory apparatus dynamics. *PLoS Pathog*. 2006; 2:e34. [PubMed: 16652170]
91. Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. *Nature*. 2006; 443:651–657. [PubMed: 17035995]
92. Derre I, Isberg RR. *Legionella pneumophila* replication vacuole formation involves rapid recruitment of proteins of the early secretory system. *Infect Immun*. 2004; 72:3048–3053. [PubMed: 15102819]
93. Deretic V, Singh S, Master S, Kyei G, Davis A, Naylor J, de Haro S, Harris J, Delgado M, Roberts E, Vergne I. Phosphoinositides in phagolysosome and autophagosome biogenesis. *Biochem Soc Symp*. 2007:141–148. [PubMed: 17233587]

94. de Felipe KS, Glover RT, Charpentier X, Anderson OR, Reyes M, Pericone CD, Shuman HA. Legionella eukaryotic-like type IV substrates interfere with organelle trafficking. *PLoS Pathog.* 2008; 4:e1000117. [PubMed: 18670632]
95. D'Souza-Schorey C, Chavrier P. ARF proteins: roles in membrane traffic and beyond. *Nat Rev Mol Cell Biol.* 2006; 7:347–358. [PubMed: 16633337]
96. Creasey EA, Isberg RR. The protein SdhA maintains the integrity of the Legionella- containing vacuole. *Proc Natl Acad Sci U S A.* 2012; 109:3481–3486. [PubMed: 22308473]
97. Collins CA, Brown EJ. Cytosol as battleground: ubiquitin as a weapon for both host and pathogen. *Trends Cell Biol.* 2010; 20:205–213. [PubMed: 20129784]
98. Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowski S, Cascales E. Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annu Rev Microbiol.* 2005; 59:451–485. [PubMed: 16153176]
99. Chong A, Lima CA, Allan DS, Nasrallah GK, Garduno RA. The purified and recombinant Legionella pneumophila chaperonin alters mitochondrial trafficking and microfilament organization. *Infect Immun.* 2009; 77:4724–4739. [PubMed: 19687203]
100. Chandra H, Reddy PJ, Srivastava S. Protein microarrays and novel detection platforms. *Expert Rev Proteomics.* 2011; 8:61–79. [PubMed: 21329428]
101. Berger KH, Isberg RR. Two distinct defects in intracellular growth complemented by a single genetic locus in Legionella pneumophila. *Mol Microbiol.* 1993; 7:7–19. [PubMed: 8382332]
102. Bartfeld S, Engels C, Bauer B, Aurass P, Flieger A, Bruggemann H, Meyer TF. Temporal resolution of two-tracked NF-kappaB activation by Legionella pneumophila. *Cell Microbiol.* 2009; 11:1638–1651. [PubMed: 19573161]
103. Banga S, Gao P, Shen X, Fiscus V, Zong WX, Chen L, Luo ZQ. Legionella pneumophila inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family. *Proc Natl Acad Sci U S A.* 2007; 104:5121–5126. [PubMed: 17360363]
104. Ashida H, Mimuro H, Ogawa M, Kobayashi T, Sanada T, Kim M, Sasakawa C. Cell death and infection: a double-edged sword for host and pathogen survival. *J Cell Biol.* 2011; 195:931–942. [PubMed: 22123830]
105. Arasaki K, Toomre DK, Roy CR. The Legionella pneumophila effector DrrA is sufficient to stimulate SNARE-dependent membrane fusion. *Cell Host Microbe.* 2012; 11:46–57. [PubMed: 22264512]
106. Arasaki K, Roy CR. Legionella pneumophila promotes functional interactions between plasma membrane syntaxins and Sec22b. *Traffic.* 2010; 11:587–600. [PubMed: 20163564]
107. Amor JC, Swails J, Zhu X, Roy CR, Nagai H, Ingmundson A, Cheng X, Kahn RA. The structure of RalF, an ADP-ribosylation factor guanine nucleotide exchange factor from Legionella pneumophila, reveals the presence of a cap over the active site. *J Biol Chem.* 2005; 280:1392–1400. [PubMed: 15520000]
108. Altman E, Segal G. The response regulator CpxR directly regulates expression of several Legionella pneumophila icm/dot components as well as new translocated substrates. *J Bacteriol.* 2008; 190:1985–1996. [PubMed: 18192394]
109. Al-Khodori S, Price CT, Habyarimana F, Kalia A, Abu Kwaik Y. A Dot/Icm-translocated ankyrin protein of Legionella pneumophila is required for intracellular proliferation within human macrophages and protozoa. *Mol Microbiol.* 2008; 70:908–923. [PubMed: 18811729]
110. Abu-Zant A, Santic M, Molmeret M, Jones S, Helbig J, Abu Kwaik Y. Incomplete activation of macrophage apoptosis during intracellular replication of Legionella pneumophila. *Infect Immun.* 2005; 73:5339–5349. [PubMed: 16113249]
111. Abu-Zant A, Jones S, Asare R, Suttles J, Price C, Graham J, Kwaik YA. Anti-apoptotic signalling by the Dot/Icm secretion system of L. pneumophila. *Cell Microbiol.* 2007; 9:246–264. [PubMed: 16911566]

Fig. 1. Intracellular life cycle of *L. pneumophila*

The *L. pneumophila* containing vacuole (LCV) bypasses the default endocytic trafficking pathway, which delivers the contents to the lysosomal compartments (left side of the cartoon). Immediately after uptake, the LCV avoids phagolysosomal fusion by modifying its membrane property using materials carried in vesicles originating from the endoplasmic reticulum (ER). It also attracts organelles such as the mitochondrion (mito) and the ribosome (Rb) at different stage of the infection. The bacterial phagosome is transformed into an ER-like compartment, providing a permissive niche for *L. pneumophila* multiplication. Ubiquitinated proteins (Ub) and the vacuolar ATPases are shown to be present on the LCV membrane.

Fig. 2. Hijacking of the Rab1 activity cycle by *L. pneumophila* effectors

Rab1 is recruited to the cytoplasmic surface of the *L. pneumophila* containing vacuole (LCV) by binding to SidM/DrrA, where its activity is controlled by a number of *L. pneumophila* effectors. 1, SidM/DrrA activates Rab1 by promoting the dissociation of GDI and inducing the exchange of bound GDP to GTP; 2, SidM/DrrA concomitantly modifies Rab1 by AMPylation, which locks the GTPase into the active form by restricting the access by GAPs; 3, SidD reverses the AMPylation of Rab1, thus making it sensitive to GAP; 4, LepB, a Rab1 GAP, completes the cycle by inactivating deAMPylation Rab1; 5–7, AnkX and Lem3, mediating the phosphorylation of Rab1 and the reverse reaction, respectively, may affect the binding of GDI. The dash line indicates that Rab1 subjected to AnkX/Lem3-mediated modifications may not occur on the surface of the LCV.

Fig. 3. Close linkage of *Legionella* genes with functional relevance

Schematic diagram of three pairs of Dot/Icm substrates with functional relevance. Arrows indicate the transcriptional orientation of the genes. Genes in red color codes for proteins that directly target host processes, whereas genes in blues direct the synthesis of proteins that negatively influence their counterparts. Note the short intergenic regions in all cases.

Fig. 4. Manipulation of phosphoinositide lipids by *L. pneumophila*

Some *Legionella* effectors present on the LCV such as SidM/DrrA, LidA, SidC and SdcA, probably by binding to PI(4)P. The abundance of phosphoinositide lipids on the LCV is regulated by *L. pneumophila* effectors, such as SidF, which catalyze the production of PI(4)P by removing the D3 phosphate on PI(3,4)P2. PI(4)P can also be produced from PI(3,4,5)P2 by SidF and the LCV-associated host PI phosphatase OCRL1, which converts PI(4,5)P2 into PI(4)P.