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The Role of Ubiquitin in the Survival of Legionella pneumophila in Eukaryotic Host Cells

Review Article

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Abstract

Background: Eukaryotic cells use essential ubiquitin-mediated pathways in their defense against pathogenic bacteria, such as Legionella pneumophila, the intracellular pathogen of Legionnaire's disease. Despite the protective role of these pathways, L. pneumophila virulence has evolved to secrete numerous effector proteins involved in co-opting host ubiquitin-mediated processes to facilitate their survival. Many of these effector proteins are of great research interest in the quest to demystify the molecular mechanisms underlying L. pneumophila pathogenesis as the bacterium has a vast repertoire of effector proteins.

Methods: Articles were obtained from scientific literature databases such as PubMed and the McGill library. Selected articles provided an overview of the ubiquitination pathway, eukaryotic autophagy, L. pneumophila pathogenesis, and structural and functional analysis of L. pneumophila and other bacterial effectors involved in subverting host ubiquitin systems.

Summary: This review discusses the current structural and functional characterization of L. pneumophila protein effectors involved in exploiting host ubiquitin machinery to facilitate intracellular bacterial survival. These protein effectors include those with E3 ubiquitin ligase activity, LubX, AnkB, and SidC, which respectively mediate bacterial nutrient acquisition, temporal regulation of other effectors, and remodelling of the L. pneumophila replicative niche; the SidE family of effectors, which mediates the first novel, single-enzyme ubiquitination pathway and deubiquitination; and ravZ, a protease promoting evasion of host autophagy. However, the exact molecular functions and biological consequences of these effectors as well as the full repertoire of L. pneumophila effectors facilitating ubiquitin-mediated survival still require further investigation.

Introduction

Legionella pneumophila

Legionella pneumophila is a gram-negative bacterium typically found in aquatic environments that is a facultative intracellular pathogen. (1) Its natural hosts are protozoans, such as the amoeba Acanthamoeba castellanii, but *L. pneumophila* also infects mammalian alveolar macrophages, causing an atypical form of pneumonia known as Legionnaires' disease. Legionnaires' disease has a fatality rate of 8-12% in healthy individuals and up to 34% in nosocomial cases. (2, 3) While person-to-person transmission of *L. pneumophila* infections has not been reported, humans often are known to contract *L. pneumophila* infections by inhaling water droplets contaminated with the bacteria spread through aerosolized systems such as cooling towers or air condition systems. (4) The increased presence of man-made systems is thought to have facilitated the evolution of *L. pneumophila* infection in humans, hence *L. pneumophila* is often referred to as an accidental human pathogen. (4)

Following uptake into host cells by phagocytosis, *L. pneumophila* uses a Dot/Icm type IV secretion system which translocates over 300 bacterial proteins, known as termed effectors, into the host cytosol. (5) Some of these effectors are known to modulate eukaryotic pathways to establish a replicative niche, the *Legionella*-containing vacuole (LCV), which evades the endosomal-lysosomal degradation pathway activated by the host cell's immune response. (1) Other effectors are likely involved in triggering apoptosis of macrophages and alveolar epithelial cells during early infection, bacterial replication and growth, and finally, a pore-formation mechanism that induces lysis of the host cell during late infection. (6) Eukaryotic cells modulate numerous host processes to support *L. pneumophila* proliferation, which makes these effectors potential targets for drug development. (7) While antibiotic resistance is not a current issue for *L. pneumophila*, many effectors interfere with host cell immune signalling pathways and characterizing these effectors may also facilitate the future adaption of bacterial effectors to treat human diseases such as autoimmune disorders. (8) However, functional elucidation of *L. pneumophila*'s effectors has proven challenging given their highly redundant nature and lack of homology to currently characterized proteins. (7)

Legionella pneumophila Growth Requires Ubiquitin

Upon microbial infection, eukaryotic cells activate ubiquitin-mediated processes, such as proteasomal degradation of ubiquitinated pathogenic proteins, as part of their defense response. (9) Despite this protective role in host cells, studies delineate a paradoxical importance of ubiquitin in *L. pneumophila* infection. (10-12) LCVs are enriched in polyubiquitinated conjugates, and this vacuolar membrane remodelling is credited to the functions of effector proteins, although the exact mechanisms are not well established. (10) A proteomic study revealed that the majority of these ubiquitinated proteins are involved in host immune response, signaling, regulation, intracellular trafficking, and amino acid transport pathways. (11) Furthermore, inhibition of ubiquitin-mediated proteasome function using dsRNA-mediated knockdown of the proteasomal subunit Rpn11 or the proteasomal inhibitor Mg-132 resulted in a significantly decreased intracellular bacterial replication in *Drosophila* cells. (10) SiRNA depletion of the host cdc48/p57 complex, an AAA ATPase required for proteasomal degradation of polyubiquitinated proteins, also diminished *L. pneumophila* proliferation and produced an accumulation of ubiquitinated proteins on the LCV surface. (10) In accordance to this dependence on host ubiquitin systems, *L. pneumophila* has been shown to employ several effectors to co-opt these processes and facilitate its survival. (12) This review will explore the effectors involved and the current understanding of how they manipulate ubiquitin-mediated processes in *L. pneumophila* infections.

Overview of the Canonical Ubiquitin System

Ubiquitination is a highly conserved and regulated eukaryotic post-translation modification that targets proteins for degradation or modifies their function. (9, 13) Specifically, ubiquitination is the addition of the 8.5 kDa eukaryotic protein ubiquitin on amino groups of residues, frequently lysine, in protein substrates through covalent linkages. The seven lysine residues within ubiquitin can be used to conjugate subsequent ubiquitin

moieties, forming polyubiquitin chains. (14) This molecular modification regulates a myriad of intracellular processes such as endocytosis, signal transduction, and transmembrane protein trafficking. (15, 16) Substrates may be monoubiquitinated or multiubiquitinated, where multiple lysine residues are monoubiquinated. (17) In addition, Lys63-linked chains mark substrates involved in lysosomal degradation, DNA damage repair, cellular signaling, intracellular trafficking, and ribosomal biogenesis. (18, 19) Among these forms of ubiquitination, substrates with polyubiquitin chains linked through the Lys48 side chains of ubiquitin are destined for proteasomal degradation.

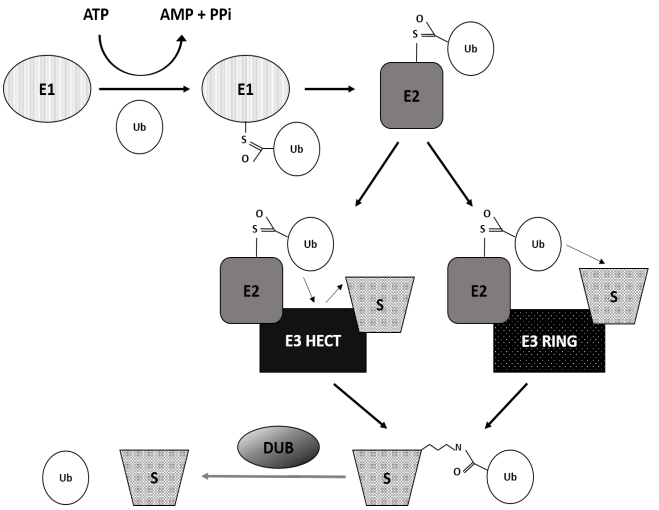

Figure 1: Eukaryotic Ubiquitination Pathway. E1 binds activates ubiquitin (Ub) using ATP before transferring it to E216. E3 binds the E2-Ub complex, catalyzing ubiquitination of the substrate (S) on a lysine residue through a HECT domain, which covalently bind ubiquitin before transferring it to substrates, or a RING domain, which bring ubiquitin and substrates close together16. DUBs remove ubiquitin16.

The aforementioned forms of ubiquitination are catalyzed by the same sequential, three-step enzymatic cascade (Fig. 1). (17) This process begins with the E1 ubiquitin activating enzyme, which catalyzes the formation of a thioester bond between its catalytic cysteine residue and the C-terminal glycine of ubiquitin in an ATP and Mg2+-dependent manner. (17) Following the transfer of the activated ubiquitin to E2 ubiquitin-conjugating enzymes, E3 ubiquitin ligases coordinate the final transfer of ubiquitin onto substrates. (17) Finally, this process is reversible through the action of substrate-specific deubiquitinases (DUBs), which hydrolyses linkages between the substrate and ubiquitin or between ubiquitin moieties. (13)

E3 ligases mediate substrate selectivity, allowing cells accordingly to encode numerous E3 enzymes: human cells, for example, have two E1 enzymes, 37 E2 enzymes, and over 600 E3 ligases. (17) E3 ligases are classified according to four particular domains: HECT (homologous to the E6-AP C-terminus) domain, RING (really interesting new gene) finger domain, a U-box domain, or an RBR (Ring Between Ring) domain. (20, 21) HECT domains bind ubiquitinated E2 and catalyzes the formation of a thioester linkage between its cysteine residue and ubiquitin prior to transferring ubiquitin to the substrate. (20) In contrast, RING finger domains function as adaptors, forming protein binding motifs stabilized by the coordination of Zn2+ with their cysteine and histidine residues, which serve as scaffolds that bring E2 and the substrate close together to catalyze ubiquitin transfer. (20) Skp1-Cullin-F-box (SCF) complex is a major RING finger-containing E3 ubiquitin ligase family: the F-box binds target substrates, Cullin is a scaffold protein, and Skp1 acts as an adaptor protein. (22) U-box domains are classified as modified RING domains that function as adaptors. While they structurally resemble RING domains, U-box domains lack the key residues involved in Zn2+ chelation. (23) Lastly, the RBR E3 ligases are multi-domain proteins comprising of an IBR (InBetweenRING) domain and two domains whose sequences bear resemblances to the RING1 and RING2 domains. (21) While all three domains contain several cysteine residues that co-ordinate Zn2+, the IBR lacks the catalytic cysteine required for ubiquitination. (21) Furthermore, the RING2-like domain does not structurally conform to canonical RING2 domains, but it contains the essential catalytic cysteine which mediates ubiquitin transfer from an E2 enzyme to the substrate via a thioester linkage. (21) Given the diversity and function of E3 ubiquitin ligases, *L. pneumophila* have unsurprisingly developed several effectors mimicking E3 ubiquitin ligases. (24)

Discovery of Noncanonical Ubiquitination in Legionelle pneumophila

The SidE effectors represent the first examples of an all-in-one ubiquitination machineries. (25) Recently, Qiu et al. discovered that the SidE effector family of *L. pneumophila* mediates ubiquitination independent of E1 and E2 enzymes. (26) Previously, ubiquitination has been reported to occur with E2 enzymes directly ubiquitinating proteins containing a ubiquitin-binding domain. (27) SidE ubiquitination proceeds in the absence of not only E1 and E2 enzymes but also of cofactors ATP and Mg2+. (26) Furthermore, the C-terminal glycine and lysine residues of ubiquitin were non-essential. (26)

The SidE family consists of SdeA, SdeB, SdeC, and SidE which all reside on the cytosolic face of the LCV. (28) Through sequence analysis, all four proteins were found to contain a mono-ADP-ribosyltransferase (mART) motif, R-S-ExE, which catalyzes the transfer of ADP-ribose groups from nicotinamide adenine dinucleotide (NAD) to arginine residues of substrates. (28) This noncanonical ubiquitination (Fig. 2) begins with the transfer of ADP-ribose onto R42 of ubiquitin by the mART motif followed by the transfer of the activated ubiquitin to its substrate. (26, 28) Currently, known substrates of the SidE effectors are the eukaryotic Rab GTPases Rab1, Rab6A, and Rab33b. (25, 26) However, the biochemical consequences of the SidE effector family, substrate selectivity, the mechanisms of activated ubiquitin transfer to the substrate, and the nature of the linkage between ubiquitin and Rab remain to be investigated. (28)

Compared to wild-type *L. pneumophila*, strains lacking all SidE genes were observed to have reduced virulence in the natural host D. discoideum. (29) However, this effect was not observed in the infection of alveolar macrophages and the exact downstream effects of the SidE effectors in eukaryotic hosts are still unclear. (29) Since SidE proteins are expressed early in host cell infection and interact with Rab proteins, which are involved in membrane trafficking and phagosome formation, they are hypothesized to play a role in the evasion of the endocytic pathway and/or LCV maturation. (26, 30)

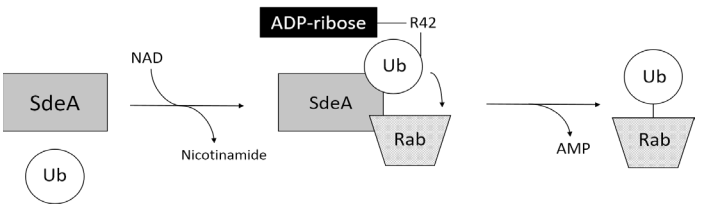

Figure 2: Schematic of Noncanonical Ubiquitination Mediated by SdeA. SdeA catalyzes ADP-ribosylation of R42 on ubiquitin (Ub) using NAD. SdeA then ubiquitinates its substrate, Rab proteins24. Currently, the mechanism of ubiquitin transfer and the nature of the substrate-ubiquitin linkage remain to be elucidated24.

Deubiquitinating Activity in *L. pneumophila*

Numerous bacterial DUBs have been characterized, such as ChlaDub1, YoP, and YopJ from Chlamydia trachomatis, Yersinia enterocolitica, and Yersinia pseudotuberculosis respectively, which all function to inhib-

it host cell NF-κB activation. (31-33) DUBs are postulated to exist in *L. pneumophila*, although few have been discovered or fully characterized. (24, 34) Currently, effectors of the SidE family are known to possess an N-terminal DUB domain. (35) Through structural studies, this particular DUB domain was found to contain a canonical ubiquitin-like protease domain, which cleaves ubiquitin from substrates. (35, 36) Mechanistically, the DUBs of the SidE family mediate deubiquitination of Lys11, Lys48, and Lys63-linked polyubiquitinated proteins on the LCV using its conserved Cys-His-Asp catalytic triad. (35) Infection of mouse bone marrow macrophages with *L. pneumophila* lacking all members of the SidE family exhibited decreased proliferation and a 90% increase in ubiquitinated species surrounding the LCV. (35) However, while the addition of an inactivated catalytic cysteine to alanine mutant of SdeA with a DUB domain mutation rescued the growth defect, the accumulation of ubiquitin species was not restored to wild-type levels. (35) This DUB domain was shown to be non-essential to the novel ubiquitination mechanism, but is believed to play a role in polyubiquitination of the LCV. (35)

E3 Ubiquitin Ligases in *Legionella pneumophila*

Although bacteria lack the proteins involved in a canonical eukaryotic ubiquitin system, numerous studies indicate that bacteria have developed an array of compatible ubiquitin ligase-like effectors. (37) These effectors enable bacteria to hijack host ubiquitin systems and modulate a variety of signalling cascades to secure their survival. (37) For example, *Salmonella typhimurium* contains SspH2 and SlrP, E3 ubiquitin ligase-like effectors involved in inducing IL8 secretion and host cell death respectively. (38, 39) In *L. pneumophila*, several secreted effectors were found to mimic eukaryotic E3 ubiquitin ligases through their possession of F-box or U-box domains, which facilitate *L. pneumophila* co-option of its host ubiquitin system. (40) Bioinformatic analyses hypothesize that acquisition of these eukaryotic-like effectors occurred through an inter-domain horizontal gene transfer, the process by which pathogens acquire and incorporate foreign eukaryotic genetic material into their genome. (41) This review will focus on the currently identified and characterized E3 effectors in *L. pneumophila*, summarized in Table 1, with their observed functions.

LubX

LubX (*Legionella* U-box protein) is a 215 amino acid long effector that contains two U-box domains. (42) In vitro reactions indicate that LubX mediates auto-ubiquitination and polyubiquitination. (43) This process is E1 and E2 dependent in which LubX interacts with the currently defined subset of E2 enzymes: UBE2D1, UBE2D3, UBE2D2, UBE2D4, UBE2E2, UBE2E3, and UBE2W1. (42, 43) Of note, U-box 1, the motif with the most N-terminals, retains canonical E3 ubiquitin ligase activity while U-box 2, located after U-box 1, binds target substrates, a non-canonical function

Effector	Lpg No.	E3-like Domain	Function(s)	Substrate(s)	References
LubX	2830	U-box	Promote bacterial growth in macrophages; regulate function/activity of other effectors	CLK1, SidH	31, 32, 33
AnkB	2144	F-box (RING domain)	Increase protein turnover, generating amino acids to support proliferation	ParvB, TRIM21	34, 35, 36
SidC	2511	Noncanonical 1	Recruit ER proteins and polyubiquitinated conjugates to the LCV	Unknown	37, 38, 39

Table 1: Summary of E3 Ubiquitin Ligases Secreted by L. pneumophila and Their Functions

previously not observed in eukaryotic U-box domains. (43) This functional difference is explained in part by the conservation of hydrophobic residues critical for E2 binding in U-boxes which have been lost in U-box 2. (42) Furthermore, sequence alignment indicates that an invariant proline residue found in active U-box domains was missing from loop 2 of U-box 2. (42, 44) While both U-box domains are highly similar to eukaryotic versions, these amino acid differences are postulated to account for their divergent functions. (42)

LuxB substrates have been found to be similar to Cdc2 kinase 1 (Clk1) through yeast two-hybrid and co-immunoprecipitation assay and SidH through bioinformatics assessment. (43, 45) Clk1, a eukaryotic protein whose expression is essential to *L. pneumophila* virulence, is involved in regulating alternative mRNA splicing by phosphorylating members of a family of serine and arginine-rich splicing factors expression. (43, 46) While LubX mediates Clk1 polyubiquitination, Clk1 is not degraded and the biological consequences which are likely linked to modulation of gene expression are unclear. (43) However, splicing regulation has been observed to be involved in disarming host-induced antimicrobial responses. A recent study showed that *L. pneumophila* secretes effectors lgt1 and lgt2 which inhibit splicing of the XBP1 mRNA, suppressing the host unfolded protein response that arises following *L. pneumophila* infection. (46) In contrast, LubX, whose expression is elevated in late phases of infection, regulates the function of SidH, an effector expressed early during infection. (45) This activity in *L. pneumophila* makes LubX the first identified metaeffector, an effector that regulates the function of other effectors. (45) Unlike Clk1, LubX-mediated polyubiquitination of SidH leads to proteasome degradation in late stages of host-cell infection. (45) This temporal downregulation is necessary for *L. pneumophila* proliferation. Infection of *Drosophila melanogaster* with LubX mutants led to hyper-lethality in the flies and also decreased intracellular viable bacterial counts relative to wild-type *L. pneumophila*. (45) These phenotypes were rescued through introducing SidH mutants with LubX mutants. (45) Prolonged SidH expression is toxic to both host and bacteria, necessitating the ubiquitination activity of LubX. (45, 47)

AnkB

AnkB is essential for *L. pneumophila* virulence as AnkB mutants exhibit severe defects in proliferation in both human and amoeba hosts. While the LCV of these mutants retained proper vacuolar remodelling, and evaded lysosomal fusion, the loss of AnkB activity was associated with a decreased level of ubiquitinated proteins at the LCV. (48, 49) However, supplementing AnkB null mutants with amino acids, especially cysteine, serine, and pyruvate, rescued the growth defect and indicated that AnkB activity caters to the nutritional needs of *L. pneumophila*. (48)

Through bioinformatics and structural analysis, AnkB was confirmed to possess an N-terminal F-box domain which interacts with the host SCF E3 ubiquitin ligase complex. (50, 51) Furthermore, in vitro ubiquitination assays confirmed that AnkB mediates robust ubiquitination in the presence of E2 enzymes UBCH4A and UBCH5C. (52) AnkB is anchored to the LCV membrane through host-mediated farnesylation at the C-terminal CaaX farnesylation motif. (11) Here, the effector recruits Lys48-linked polyubiquitinated proteins to the LCV, which are subsequently degraded by the proteasome to release free amino acids. (11, 48) These amino acids represent a major carbon-rich source *L. pneumophila* use to produce energy via the tricarboxylic acid cycle to power bacterial growth and replication. (48)

AnkB substrates are not well established, but two interaction partners have been identified: eukaryotic parvinB (ParvB) and Trim21 proteins. (49) ParvB is involved in focal adhesion, cellular motility, and pro-apoptotic pathways. (53) Its deficiency decreases *L. pneumophila* proliferation but does not affect normal host viability. (49) Interestingly, AnkB null strains results in increased ParvB ubiquitination. (49) Furthermore, wild-type *L. pneumophila* was associated with increased caspase-3 activation and DNA fragmentation during infection compared to AnkB null strains. (49) This observation suggests that AnkB protect ParvB from ubiquitination by competing with endogenous ubiquitin ligases for ParvB binding, leading to apoptotic processes stimulation. (49) However, the biological importance of these effects to *L. pneumophila* survival is not clearly established. (49) In addition, Trim21 is a host E3 ubiquitin ligase which was recently

found to mediate Lys11-linked polyubiquitination on AnkB. (54) Host proteins ubiquitinated by Trim21 are typically degraded by the proteasome, but this does not occur for AnkB. (54) Although this phenomenon is the first example of an interaction between Trim21 and a bacterial effector protein, the biological significance remains to be elucidated. (54)

SidC

Unlike AnkB and LubX, SidC exhibits E3 ubiquitin ligase activity mediating polyubiquitination through several lysine linkages while having no structural homology to canonical E3 domains. (55) SidC contains a catalytic triad in the N-terminal domain, termed the SNL domain, that is typical of cysteine-based proteases and DUBs. The triad comprises of amino acids cysteine (C46), histidine (H444), and aspartic acid (D446). (55) While the crystallized SNL domain differs structurally from HECT domains, the cysteine residue of the triad is postulated to function similar to a nucleophile and SidC is thought to define a unique family of ubiquitin ligases. (55)

SidC binds to phosphoinositide lipid PI(4)P, which is abundant on mature LCV surfaces, through its C-terminal PI(4)P binding domain, P4C. (56) Binding of PI(4)P was associated with an increase in the ubiquitin ligase activity of SidC, presumably due to a conformational change in SidC which increased accessibility of the catalytic site. (56) Crystal structures of near full length SidC indicated that hydrophobic interactions mediate interactions between P4C and SNL domains. (56) Accordingly, mutation of a leucine residue in the P4C domain involved in this interaction to an arginine residue (L629R) resulted in a mutant SidC that preferably adopted an open conformation of the catalytic site and exhibited increased E3 ubiquitin ligase activity. (56) This result suggests a model of SidC regulation where SidC is inactive upon secretion and active when it is attached to the LCV and interacts with the host ubiquitin system. (56)

SidC functions as a tethering factor that recruits host ER vesicles, polyubiquitin conjugates, and arf-1 to the LCV via its SNL domain. (56, 57) *L. pneumophila* defective for SidC and its paralog SdcA showed delayed establishment of replicative vacuoles due to decreased recruitment of host ER proteins and polyubiquitin conjugates, necessitating E3 ubiquitin ligase activity in proper tethering. (55, 57) Interestingly, SidC is required in the monoubiquitination of Rab1, but this modification is not a result of direct ubiquitination by SidC. (55) Rather, SidC is thought to tether Rab1 and bring it proximal to its ubiquitin ligase. (55) Based on SidC's tethering functions, SidC ubiquitinated substrates ubiquitinated are currently unknown but are hypothesized to be host proteins involved in trafficking between the ER and membranes. (55)

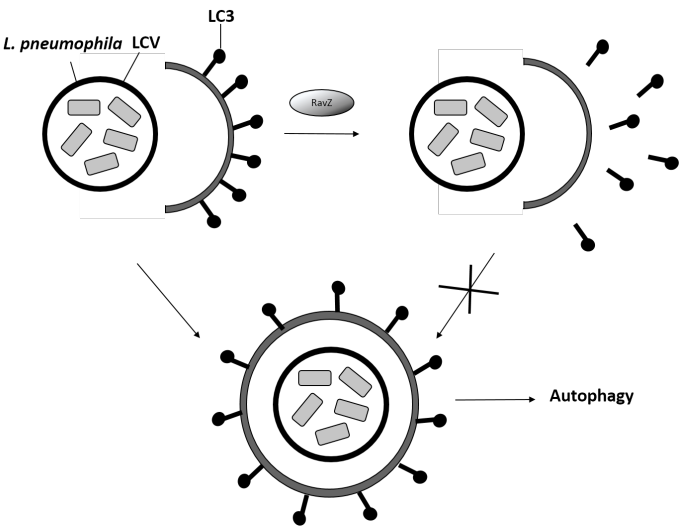

Figure 3: Evasion of Autophagy of L. pneumophila Mediated by RavZ. L. pneumophila secretes RavZ which decouples LC3 conjugated to PE on the membranes of autophagosomes. This blocks LC3-mediated extension of the autophagosome membrane, which prevents degradation of L. pneumophila by host autophagy pathways49.

Evasion of Autophagy by *Legionella pneumophila*

Eukaryotic cells engage in autophagy to selectively remove protein aggregates and damaged/surplus organelles as well as to non-specifically degrade proteins and organelles during cellular starvation to generate amino acids used to preserve essential processes such as protein synthesis. (58, 59) However, in response to bacterial infections, host cells can activate a form of autophagy termed xenophagy: the elimination of invading microorganisms by engulfing them in autophagosomes followed by fusion with lysosomes for degradation. (58) Selective autophagy of pathogens involves their ubiquitination, and in this case, ubiquitination of the phagosomal surface of the LCV. (60) Adaptor proteins, such as p62, bind to the LCV via the ubiquitin and also bind autophagosome-associated LC3-II which targets the ubiquitinated LCV to the autophagosome for degradation. (60) LC3 is the mammalian homolog of yeast autophagy related (ATG) 8 protein and is a ubiquitin-like protein that is cleaved by ATG4 to form LC3-II, which is conjugated through its C-terminal glycine to phosphatidylethanolamine (PE). (61) Functionally, LC3-II has been observed to facilitate autophagosomal membrane expansion. (61)

Despite facing elimination by the host cell's autophagy pathway, *L. pneumophila* has evolved mechanisms to circumvent ubiquitin-dependent xenophagy as evinced by the fact that *L. pneumophila* replicates in ubiquitinated LCVs evade the autophagy pathway. (62) Furthermore, *L. pneumophila* replication in its natural host, Dictyostelium discoideum, is increased when ATG9 is knocked out. (62) Following these observations, *L. pneumophila* was formally confirmed to interfere with xenophagy at the stage of autophagosome maturation via the secreted effector ravZ, a cysteine protease which functions similarly to ATG4 (Fig. 3). (62) However, unlike ATG4, ravZ is a deconjugating enzyme that specifically cleaves the amide bond between tyrosine and the PE-conjugated glycine of lipidated LC3, producing an LC3 product that cannot be re-conjugated to PE due to loss of the C-terminal glycine. (62) Hence, the loss of membrane-bound LC3 prevents p62 from delivering the ubiquitinated LCVs to the autophagy pathway. (61, 62) Intriguingly, macrophages infected with *L. pneumophila* lacking the ravZ gene were observed to retain the ability to prevent LC3 recruitment to LCVs, suggesting that multiple effectors are involved in disrupting the autophagy pathway. (62)

The ATG8/LC3 protein also plays an important role in susceptibility to bacterial infections such as in Parkinson's disease, a neurodegenerative disorder caused by mutations in the PARK2 gene that can result in decreased parkin expression and impaired protein function. (63, 64) In *Mycobacterium tuberculosis* infections, the ubiquitin ligase parkin mediates K63-linked polyubiquitination of the bacteria-containing phagosomes and was found to be essential for macrophages to impede *M. tuberculosis* replication, supported by the fact that PARK2-/- mice are more sensitive to infection. (63) *D. melanogaster* flies deficient for parkin were also defective in ATG8 processing when infected with *L. pneumophila* monocytogenes, indicating a potential role for ubiquitin ligases in mediating proper autophagic immunity. (63) Furthermore, numerous studies have noted that genetic knockouts of specific ATG genes correlated to increased susceptibility to various bacterial strains. (65-69) Together, the role of ATG8/LC3 in parkin deficient cells and *L. pneumophila* infection along with ATG gene deletion assays suggests the existence of other bacterial mechanisms that interact with ATG proteins to dictate bacterial resistance and susceptibility. (70, 71)

Conclusion

Ubiquitin-mediated processes play important roles in defending host eukaryotic cells against bacterial invasion, yet these processes have been proven indispensable to *L. pneumophila* virulence. (9, 12) As described in this review, *L. pneumophila* secretes several effectors that modulate the host ubiquitin system to bolster their own survival, as summarized in Fig. 4. (12) These results include the SidE family of effectors, which mediate deubiquitination and the first and only E1 and E2 independent ubiquitina-

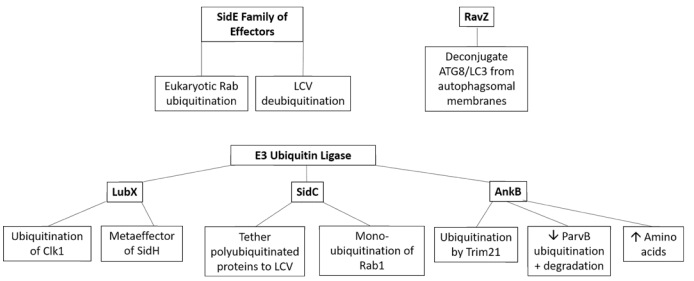

Fig. 4: Summary of L. pneumophila Effectors Involved in Ubiquitin-Mediated Survival Pathways in Eukaryotic Host Cells.

tion discovered to date; structural mimics of eukaryotic F-box and U-box type E3 ubiquitin ligases, which commandeer the canonical host ubiquitination machinery to mediate degradation and regulation of host proteins and bacterial effectors; and RavZ, which actively disrupts the ubiquitin-mediated autophagy pathway. (26, 35, 40, 62) Currently, these effectors are thought to affect various pathways such as LCV maturation, host gene expression, and bacterial nutrient acquisition. (6, 48) However, researchers still have much to elucidate regarding the largely unknown substrate spectrums and biological consequences of these effectors. As exemplified by parkin mediated bacterial resistance via ATG8/LC3, there may exist other undiscovered host protein-effector interactions causing differential susceptibility or fatality of *L. pneumophila* infections in infected individuals with other human diseases compared to healthy individuals. (3) Furthermore, research on numerous other pathogens indicate that bacteria secrete a wide variety of effectors that function as DUBS, post-translationally modify proteins in the host ubiquitin system, or interfere with pathogen ubiquitination in xenophagy. (24) Presently, these effectors are currently unobserved or not well characterized in *L. pneumophila*. (7) Part of the difficulty in identifying functions of effectors stem from the extensive redundancy of effectors in *L. pneumophila*. This challenge could potentially be circumvented by adopting new genetic screening methods, notably the insertional mutagenesis and depletion technique, continuing structural analysis, and developing novel robust assays, such as fluorescence resonance energy transfer, to elucidate novel effector-substrate relationships. (24, 37) Such efforts will facilitate the expansion of the current understanding and identified repertoire of effectors and substrates involved in the ubiquitin-mediated survival of *L. pneumophila*. In the future, this knowledge may contribute towards development of targeted antibacterial drugs and adoption of pathogenic molecules in treating human diseases. (7, 8, 24)

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