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Ubiquitin and *Legionella*: From bench to bedside

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ABSTRACT

Legionella pneumophila, a Gram-negative intracellular bacterium, is one of the major causes of Legionnaires' disease, a specific type of atypical pneumonia. Despite intensive research efforts that elucidated many relevant structural, molecular and medical insights into *Legionella*'s pathogenicity, Legionnaires' disease continues to present an ongoing public health concern. *Legionella*'s virulence is based on its ability to simultaneously hijack multiple molecular pathways of the host cell to ensure its fast replication and dissemination. *Legionella* usurps the host ubiquitin system through multiple effector proteins, using the advantage of both conventional and unconventional (phosphoribosyl-linked) ubiquitination, thus providing optimal conditions for its replication. In this review, we summarize the current understanding of *L. pneumophila* from medical, biochemical and molecular perspectives. We describe the clinical disease presentation, its diagnostics and treatment, as well as host-pathogen interactions, with the emphasis on the ability of *Legionella* to target the host ubiquitin system upon infection. Furthermore, the interdisciplinary use of innovative technologies enables better insights into the pathogenesis of Legionnaires' disease and provides new opportunities for its treatment and prevention.

1. Background – medical insight into the *Legionella* infection

1.1. A brief history of Legionnaires' disease and its causative pathogen L. pneumophila

L. pneumophila was identified in 1976, when an unknown pathogen caused an outbreak of pneumonia, affecting more than 200 participants at the Annual convention of the American Legion, resulting in a mortality rate of 15,9% [31]. Due to the high mortality rates, the Centers for Disease Control and Prevention (CDC) initiated investigation that identified a new Gram-negative intracellular rod-shaped bacterium as the causative agent of Legionnaires´disease (LD) [12,76]. Further studies not only established *L. pneumophila* as the causative agent of LD, but also of an influenza-like disease, subsequently named Pontiac fever [36].

Follow up studies established the optimal *in vitro* conditions for the growth of *Legionella* [29,98,127], which exhibit a unique characteristic of pleomorphism, changing their morphology depending on the life cycle. In the transmissive phase, they appear as thin 2–20 μm long pleomorphic bacilli, and contain fimbriae (pili) and one polar flagellum. The flagellum is responsible for their motility, which is lost during the replicative phase (https://www.who.int/publications/i/item/9241562978).

From the ultrascopic view, this facultative intracellular pathogen contains an inner and an outer membrane, a common feature of Gram-negative bacteria [115]. Once isolated from human materials or environment, a primary factor required for its growth in laboratory conditions is the amino acid L-cysteine, since most of the *Legionella* species lack specific enzymes required for its synthesis, such as cysteine synthase and serine acetyl-transferase. Conversely, the intracellular growth of *Legionella* (for example, in the macrophages, mammalian cell lines or natural amoebic hosts) does not depend on the same requirement, suggesting that the bacteria get this ingredient directly from the host cell. Cysteine auxotrophy is therefore one of the major *L. pneumophila* features that restricts this pathogen to the intracellular lifestyle [27].

Nowadays, more than 65 species belong to the *Legionella* genus, and are organized in more than 70 serogroups. The number of serogroups, subspecies and species is constantly increasing. *L. pneumophila*, the first described bacterium of the genus *Legionella,* is a "representative type" and contains 16 serogroups. Interestingly, *L. pneumophila* serogroup 1 is responsible for approximately 80% of all *Legionella*-related infections [93] (WHO BOOK, https://www.who.int/publications/i/item/9241562978). However, a common urine antigen test, which represents the first-line screening method for the detection of the *Legionella* cell wall, can only specifically recognize serogroup 1, potentially underestimating the contribution of other serotypes to the disease [77].

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The closest evolutionarily related intracellular pathogen to *Legionella* is *Coxiella burnetti*, a causative agent of a flu-like sickness Query (Q-) fever [74]. Even though these two bacteria are taxonomically similar, and both use the type IV secretion system (T4SS) for the delivery of effector proteins to the host cytosol [121], the two pathogens are showing different features, particularly in the context of their intracellular life styles. For detailed comparison of *Legionella* and *Coxiella*, we refer the readers to the recent review [103].

1.2. Legionella targets multiple hosts by the similar strategy

L. pneumophila is a ubiquitous pathogen commonly found in various types of aquatic and soil environments, including natural waters (reservoirs, rivers, lakes) and purpose-built systems (spa pools, air conditioning devices, cooling towers, evaporate condensers) (WHO book, https://www.who.int/publications/i/item/9241562978); [59]. It can be found either as a free-living bacterial biofilm or within its hosts. *Legionella* employs the similar strategy to hijack various hosts – a large number of effector proteins that are exported to the host cell *via* type II (T2SS) or type IV (T4SS) systems. As an opportunistic intracellular pathogen, *L. pneumophila* exploits amoebae and other protozoa, which are considered as "natural" *Legionella* hosts. At the same time, *Legionella* is able to infect and replicate within the human macrophages. However, there are only a few reports confirming human-to-human transmission of the bacteria [43,89]. Therefore, considering humans as "accidental" hosts remains a subject of debate. Most of the LD cases are sporadic, with only several reported LD outbreaks [96] that are closely monitored by CDC and European Centre for Disease Prevention and Control (ECDC). The last reported outbreak, with 12,5% mortality rate, took place in the North of Portugal in October 2020. (Communicable Disease Threats Report, Week 47, 15–21 November; ECDC) [14]. Aerosol inhalation and aspiration from the aquatic systems are commonly described transmission routes for *L. pneumophila*, whereas inhalation of the contaminated aerosol, derived from soil and potting mixes, is related to *Legionella longbeachae*, a major cause of LD in Australia and New Zealand [11,21]. Moreover, a genomic sequence analysis of *L. pneumophila* revealed that this bacterium contains a set of genes that code for the eukaryotic-like proteins, probably acquired from its protozoan hosts during evolution. It was also assumed that some of those proteins are actually effector proteins that are secreted into the cytosol of the host cell [15,37]. Moreover, 44 uncharacterized genes containing eukaryotic motifs were identified in a bioinformatic genome-wide screen, suggesting that some of *Legionella* effectors have been acquired *via* interdomain horizontal gene transfer (HGT). Their products (such as effectors LegL3, LegLC4, LegL5, LegL7, LegLC8, LegC2, LegC5, and LegG2) seem to be translocated into the host *via* Dot/Icm T4SS [22,139]. This special feature of *Legionella* molecular mimicry enables its own survival under various environmental conditions and ensures its dual host specificity [90].

1.3. Disease

Facultative intracellular pathogen *L. pneumophila* is the major cause of legionellosis (common term for all illnesses caused by *Legionella*), which can be commonly presented in two ways – as Legionnaires' disease and Pontiac fever. After the exposure to the aerosol containing *Legionella*, individuals may be asymptomatic or symptomatic, depending on their current health status, and may present with the clinical picture of respiratory disease, either as Pontiac fever (flu-like respiratory disease, usually determined as mild form of legionellosis) or Legionnaires' disease severe form of pneumonia, often complicated with extrapulmonary dissemination, leading to the 8–12% mortality rate [77]. Moreover, in rare cases, *Legionella* might be presented as an "isolated extrapulmonary *Legionella* infection", in the absence of pneumonia, affecting soft tissues, synovial fluid or heart [17]; (https://www.uptodate.com/contents/treatment-and-prevention-of-legionella-infection).

Pontiac fever is a mild, flu-like illness. Its symptoms can include fever and at least one of the following – headache, myalgia (muscle pain), arthralgia (joint pain), tiredness, non-productive cough and shortness of breath. The incubation period is between 30 and 90 h, and recovery starts after a week. As a diagnostic method, urine antigen test is commonly used. Reported cases are usually related to the legionellosis outbreaks (https://www.uptodate.com/contents/treatment-and-prevention-of-legionella-infection).

LD is a severe form of the legionellosis, which includes pneumonia with a wide spectrum of frequency and severity of disseminated extrapulmonary manifestations. In general, the symptoms include fever, headache, myalgia, accompanied by pneumonia-related signs (initially non-productive, followed by the productive cough), as well as extrapulmonary symptoms, such as diarrhea, nausea and confusion. The incubation period lasts between 2 and 14 days. LD is likely to be underdiagnosed, due to the lack of diagnostic approaches and overall surveillance system, as well as the variability of disease definitions. The higher risk groups are more likely to suffer severe symptoms, and this includes elderly population with comorbidities (such as COPD, diabetes, renal insufficiency), immunocompromised patients and potentially patients with a mutation in Toll-like receptors [81]. The healthy individuals can also develop LD, generally with milder symptoms. If not diagnosed promptly, LD can lead to the development of multiorgan failure and death. *Legionella* is responsible for both nosocomial/hospital-acquired pneumonia (HAP) and the community-acquired pneumonia (CAP), which appears outside the hospital. In HAP, the mortality rate is higher in comparison to the CAP, ranging between 15% and 34% [96]. The patients with microbiologically confirmed disease (*via* PCR test, urine antigen test or sputum culture) are commonly treated with the antibiotics, either levofloxacin or azithromycin, which are currently preferred due to the bactericidal features, good lung tissue penetration, high intracellular concentrations and efficiency to target multiple *L. pneumophila* serogroups. In the case of extrapulmonary *Legionella* infections, the patients are commonly treated with fluoroquinolone (such as levofloxacin), combined with various procedures (incision, drainage). (https://www.uptodate.com/contents/treatment-and-prevention-of-legionella-infection), https://www.cdc.gov/legionella/resources/guidelines.html [96].

Although the antimicrobial treatment for the *Legionella* infection is well known, the fatality rate in the case of LD remains quite high. Great efforts are being put into the development of a vaccine against *Legionella*, however, a sufficiently effective and non-toxic vaccine is still missing. Therefore, more microbiological, biochemical and molecular studies are required to improve the diagnostic approaches and treatment strategies to gain deeper insight into the pathophysiology of the LD. Biochemical studies, related to the effect of *Legionell*a on the molecular pathways, such as the ubiquitin machinery, will be further discussed in this review.

2. Host-pathogen interplay

2.1. Life cycle of L.pneumophila

The intracellular life cycle of *L. pneumophila* consists of two phases – the replicative/exponential and the transmissive/post-exponential phase, both of which are regulated by effector proteins affecting various intracellular pathways (Fig. 1). By secreting more than 330 effectors into the host cell using the Dot/Icm T4SS [25], *L. pneumophila* avoids its lysosomal-mediated degradation by escaping the endosomal-trafficking pathway and forming a unique single-membraned *Legionella*-containing vacuole (LCV). These specialized vesicles are rich in nutrients and lack lysosomal hydrolases, providing *Legionella* with an optimal environment for its replication. Moreover, vesicles derived from the host endoplasmic reticulum (ER) form the cluster around the nascent LCV, thus ensuring maturation of the LCV [109].

During the replicative phase, bacteria exploit the nutrient-rich LCV

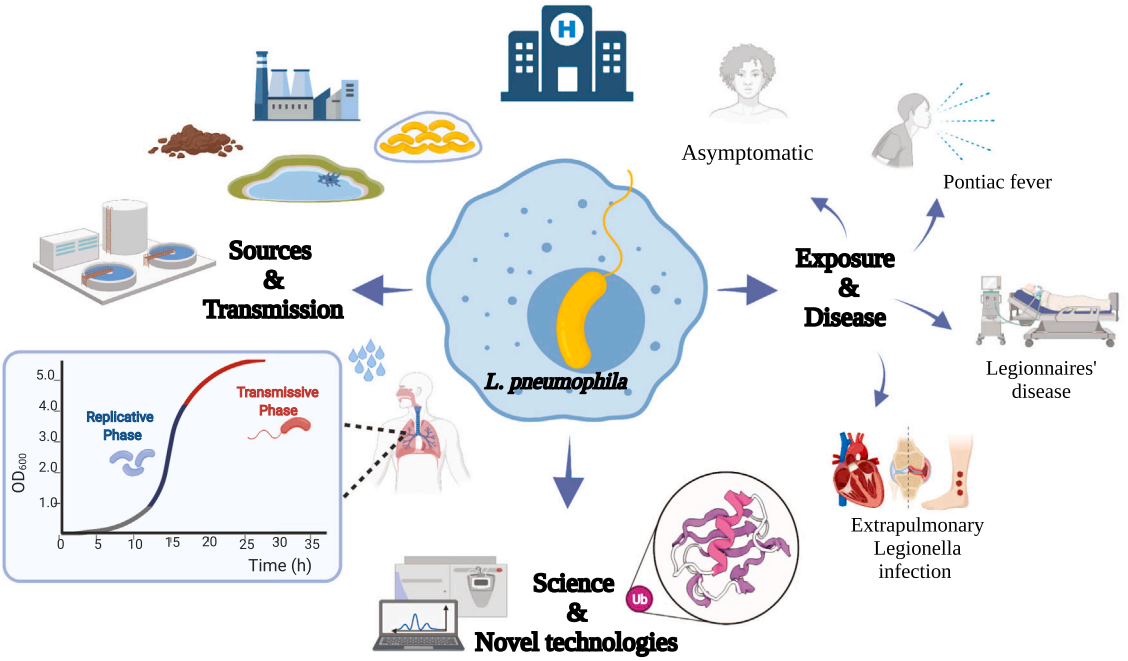

Fig. 1. *L. pneumophila* Overview. *L. pneumophila* is found as a free-living bacterial biofilm in artificial water systems or within its hosts (amoeba and human macrophages). People as "accidental hosts" are commonly infected *via* aerosol or potable water inhalation, thus developing various types of clinical pictures. Once when the host is attacked, *Legionella* enters a two-stage life cycle, consisting of the replicative/exponential and the transmissive/post-exponential phases differentially regulated by numerous effector proteins. New technologies ensure better understanding of the host-pathogen interplay and pathophysiology of the disease.

compartment and actively inhibit the phagosome-lysosome fusion [132]. As the bacterial density and the subsequent need for nutrients increase, *Legionella* effector proteins start to interfere with the host autophagy machinery. Among others, *L. pneumophila* hijacks the host sphingolipid metabolism to modulate autophagy [18,110]. A decrease in nutrient levels, caused by the high number of replicating bacteria in the LCV, triggers the transition of the bacteria to their infectious form. Such infectious transmissive bacteria change their shape, and obtain a thick cell wall, short rod form and a flagellum that ensures its fast motility. All of these features are required for the evasion of the lysosomal trafficking pathway and the exit from the host cell depleted of nutrients. Moreover, the transition is accompanied by the induction of many virulence factors, particularly Dot/Icm substrates (such as effector RalF that functions as an exchange factor for the ADP ribosylation factor (ARF) family of GTPases and plays an important role in LCV formation) [86]. Interestingly, effectors translocated through Dot/Icm system across the phagosomal membranes into the host cell can be transferred from one bacterial cell to the other, thus indicating a significant flexibility of the Dot/Icm system [70]. After successfully invading a new suitable host and establishing the intracellular niche, the transmissive bacterium converts back into the non-virulent replicative form [40,82].

In summary, using the strategy of the reversible two-phase expansion, *L. pneumophila* controls the energy costs needed for its replication. During the replicative phase, *L. pneumophila* is metabolically active and exhibits non-virulent features in order to spare energy levels, whereas during the transmissive form, *L. pneumophila* transforms into the virulent variant (Fig. 1) [13,108,91].

2.2. *Lpneumophila effector proteins: bacterial tools to modulate host intracellular pathways*

In order to ensure optimal conditions for replication and effective transmission, *L. pneumophila* employs various strategies to subvert multiple host cell signaling pathways, including the modulation of bacterial uptake, evasion from the endocytic pathway, interaction with the ER and formation of the LCV, modulation of the autophagy, kinase signaling, cell death and hijacking of the host ubiquitin machinery. In this review, we will discuss the impact of selected effector proteins on the LCV formation and autophagy (summarized in Table 1), with special emphasis on how *Legionella* targets ubiquitin system.

2.2.1. *Mechanism of LCV formation*

After bacterial ingestion, a primitive nascent phagosome needs to be transformed into the mature LCV that contains the ER and mitochondrial fragments. In order to ensure an extended stay in the host, *Legionella* also has to inhibit LCV fusion with the endo-lysosome to prevent its degradation.

Phagosome maturation seems to be tightly regulated by the *Legionella* T4SS system, since it re-directs vacuoles containing *L. pneumophila* away from the canonical endocytic pathway at an early infection stage. Several proteomics analyses of the purified LCVs have identified small GTPases, including Rab1, attached to the pathogen vesicle [44,123]. It has been shown that small GTPases, which regulate the formation of COPI- and COPII-coated vesicles, play an important role in the biogenesis of LCVs [52], indicating that *Legionella* hijacks vesicular trafficking from the ER exit sites in order to create ER-derived organelle to ensure an adequate replication environment.

The host protein Rab1 regulates vesicular and membrane trafficking processes and is a target of at least five different effector proteins during the formation of the LCV [72] (Table 1). The *L. pneumophila* multifunctional effector SidM (DrrA) acts as a highly specific Rab1 guanine nucleotide-exchange factor (GEF) that disrupts Rab1-mediated secretory transport to the Golgi by direct interaction, thus promoting Rab1 recruitment to the LCV, a process that is enhanced by the effector LidA [85]. The *Legionella* effector SidD catalyzes AMP release from Rab1, enabling its inactivation by LepB [87]. Furthermore, GTPase-activating protein (GAP) LepB localized on the early LCVs, directly binds to the GTP-bound Rab1, disrupting the early secretory transport [49]. Effector AnkX covalently attaches a phosphocholine moiety to Rab1 and Rab35, thus modulating membrane transport of both endocytic and exocytic pathways of the host cell [83]. AnkX-mediated modification of Rab1 is reversed by Lpg0696 (Lem3) that functions as dephosphorylcholinase

Table 1
Overview of the selected *Legionella* effector proteins promoting host ER recruitment and modulating autophagic response.

Effector protein	Direct host target	Affected host pathway	Benefit for *Legionella*	References
	Interaction with ER and LCV formation			
DrrA (SidM)	recruits Rab1 to LCV, acts as Rab1 GEF, AMPylates Rab1, Rab6 and Rab35	ER recruitment	LCV formation	Murata et al. [85], Machner and Isberg [73], Muller et al. [84,72]
SidD	Rab1 deAMPylation	ER recruitment	LCV formation	Neunuebel et al. [87]
LidA	stabilizes the Rab1-guanosine nucleotide complex, protecting it from inactivation by GAPs	ER recruitment	LCV formation	Machner and Isberg [72], Neunuebel et al. [88], Murata et al. [85]
LepB	Rab1 GAP	ER recruitment	LCV formation	Ingmundson et al. [49]
RaIF	activates and recruits the small GTPase Arf1 to the bacterial vacuole	ER recruitment	LCV formation	Nagai et al. [86]
AnkX	acts as a phosphocholine transferase for Rab1 and Rab35	ER recruitment	LCV formation	Mukherjee et al. [83]
Lem3	acts as a phosphocholine hydrolase for Rab1	ER recruitment	LCV formation	Tan et al. [120]
SetA	glucosylation of Rab1which leads to decreased interaction of Rab1 and GDI1	ER recruitment	LCV formation	Wang et al. [126]
VipD	Rab5-activated phospholipase A1, protects *L. pneumophila* from endosomal fusion by modulating early endosome lipoprotein composition	Protects from endosomal fusion	LCV formation	Lucas et al. [68], Gaspar and Machner [34]
RidL	inhibits retromer activity by directly binding to the VPS29 subunit of retromer	Blocks retrograde trafficking	LCV formation, promotes intracellular replication	Finsel et al. [30] Yao et al. [135]
	Modulation of autophagic response			
RavZ	LC3 deconjugation	Autophagy inhibition	LCV maturation	Choy et al. [18] Rolando et al. [111]
Lpg 1137	STX17 cleavage	Autophagy inhibition	LCV maturation	Arasaki et al. [4]
LpSPL	prevents autophagosome formation	Autophagy inhibition	LCV maturation	Rolando et al. [111]

GEF (guanine nucleotide-exchange factor), GAP (GTPase-activating protein)
GDI1 (GDP dissociation inhibitor 1)
VPS29 (vacuolar protein sorting-associated protein 29)

[120]. Although abovementioned effector proteins play an important role in LCV formation, the deletion of individual effectors does not detectably impact the biogenesis of the LCV in laboratory infection model, supported by the fact that knockout of single gene does not lead to the growth defects *in vitro*. The effector LidA is an exception, as it is essential for the integrity of the Dot/Icm apparatus [22,70,20].

2.2.2. *Inhibition of autophagy upon L.pneumophila infection*

One of the preferential pathways targeted by *L. pneumophila* is autophagy. Autophagy is an evolutionary conserved process in eukaryotes used to maintain cellular homeostasis by degrading non-functional organelles, as well as damaged, misfolded proteins, aggregates and pathogens [39,78]. The key event in autophagy is the formation of a double membrane called the phagophore. In mammals, phagophore biogenesis is triggered by a complex interplay of protein and lipid kinases, starting from inhibition of mTOR kinase, which in turn activates ULK1 kinase. ULK1 kinase is part of a major kinase complex in autophagy, comprising ULK1, ATG13, FIP200 and ATG101. ULK1 positively regulates class III VPS34 phosphatidylinositol 3-kinase (PI3K) complex I, which consists of a lipid kinase VPS34, Beclin1, ATG14L and p150 [78]. The phagophore elongates, sequestering its targeted cargo, and, upon its closure, forms a vesicle termed an autophagosome, formation of which is dependent on the production of lipidated ATG8 proteins. While in yeast there is only one Atg8 protein, in mammals ATG8 represents a family of six LC3 and GABARAP proteins. Eventually, a fully mature autophagosome fuses with a lysosome in order to degrade the autophagosomal cargo. Various bacteria have evolved different mechanisms to avoid autophagy or to hijack the autophagy machinery in order to survive in the host cell [134].

Legionella employs various direct or indirect strategies to modulate autophagy. Directly, *via* the effector RavZ and modulation of autophagosomal SNARE protein Syntaxin 17 (STX17), and indirectly by affecting sphingolipid metabolism. *L. pneumophila* inhibits autophagy through its effector protein RavZ (Table 1), which irreversibly inactivates LC3 and GABARAP proteins during the bacterial infection. The amide bond between the carboxyl-terminal Gly residue and an aromatic residue in ATG8 proteins is hydrolyzed by RavZ, thus producing an ATG8 protein that cannot be re-conjugated by ATG7 and ATG3 to phosphatidylethanolamine [18].

Moreover, phosphorylation of STX17 regulates the initiation of autophagy [61]. In nutrient-rich conditions, STX17 modulates the activity of Drp1, a mitochondrial fission factor. However, during starvation, STX17 dissociates from Drp1 and binds ATG14L, promoting the recruitment of VPS34 PI3K complex I to the mitochondria-associated membranes (MAMs), inducing the formation of PI3P-rich, cradle-like domains called omegasomes, which will define the spots for phagophore biogenesis. *Legionella* effector protein Lpg1137, a serine protease localized in mitochondria and MAMs, cleaves the host STX17, leading to STX17 degradation, disassembly of STX17-ATG14L and inhibition of STX17-Drp1 complex, thereby inhibiting an early key step of autophagy [4]. Furthermore, cleavage of STX17 also enables *L. pneumophila* to inhibit staurosporine-induced apoptosis (SIS), thus ensuring the continuous bacterial replication [6]. More studies revealed that the effector protein LpSpl exhibits sphingosine-1-phosphate lyase activity during infection, thus disrupting the sphingolipid metabolism of the host cell and decreasing starvation-induced autophagy [111].

3. Targeting ubiquitin system upon *L.pneumophila* infection

3.1. *Canonical vs non-canonical PR ubiquitination*

Ubiquitination is an evolutionary conserved posttranslational modification (PTM) critical for the regulation of numerous cellular processes, including cell cycle progression, DNA damage repair, immune

response, vesicular trafficking and protein homeostasis [42]. Multiple studies have shown that *Legionella* uses a sophisticated strategy to hijack the host ubiquitin system in eukaryotic cells, targeting both canonical and non-canonical ubiquitination [56,104,101]. Recent genome-wide genetic screen established the critical role of the host ubiquitination pathway components for the efficient Dot/Icm effector translocation and *L. pneumophila* intracellular replication [92].

Canonical ubiquitination *per se* can be described as the formation of a covalent bond between the α-carboxyl group of the terminal glycine of ubiquitin and, more commonly, the ε-amino group of the substrate's lysine residue. To a lesser extent, other residues can also be ubiquitinated, such as cysteine, serine, threonine, as well as the free N-terminus of proteins, which form thioester, hydroxyester and peptide bonds, respectively [75].

In the canonical/conventional ubiquitination, conjugation occurs through the well-conserved 3-enzyme cascade process (Fig. 2). Firstly, a free ubiquitin is activated by the E1 (ubiquitin-activating enzyme) in an ATP-dependent manner, thus forming a ubiquitin-AMP-intermediate that is important for the E1 modification by a thiol-ester bond between the cysteine residue on E1 and carboxyl-terminus of ubiquitin. Secondly, E1-linked ubiquitin is transferred to a cysteine residue on an E2 (ubiquitin-conjugating enzyme) through a trans-thiolation reaction. Lastly, an E3 (ubiquitin protein ligase) catalyzes the covalent isopeptide bond between the carboxyl-terminus of ubiquitin and ε-amino group of the lysine residue on the substrate [42]. In the mammalian system, there are two genes encoding the E1 enzymes, approximately 30 genes encoding the E2 and almost 1000 genes for the E3 ligases, which determine substrate specificity [112,136]. E3 ligases are classified mechanistically into three major subgroups: HECT (homologous to the E6AP carboxyl terminus) domain family, RING (really interesting new gene) family E3 ligases and the RBR (RING-between-RING) family, and are able to generate ubiquitin linkages of various architectures using different catalytic motifs and mechanisms of catalytic reactions [79,80,130]. Moreover, there are more than 100 deubiquitinating enzymes (DUBs) that hydrolyze peptide/isopeptide bond between ubiquitin and its substrate, thus reversing the activity of ubiquitin machinery [19]. DUBs are classified into 7 families: UCHs (ubiquitin-carboxyl-terminal hydrolases), USPs (ubiquitin-specific proteases), MJD (Machado-Joseph domain), OTU (ovarian-tumor), JAMM (JAB1/MPN/MOV34 metalloenzymes), MINDY (motif interacting with Ub-containing novel DUB family) and ZUFSP/ZUP1 [1,131,62,41]. Development of various tools and methods in the field of ubiquitination ensured better understanding of its role in the other signaling pathways, innate immunity and cancer discovery. Multiple assays are commonly used to study how ubiquitin receptors/readers recognize ubiquitin code to induce specific cellular responses [48], as well as to identify substrates modified by ubiquitin and various types of ubiquitin chains, develop antibodies specifically recognizing ubiquitin linkages, and to quantify ubiquitin modifications

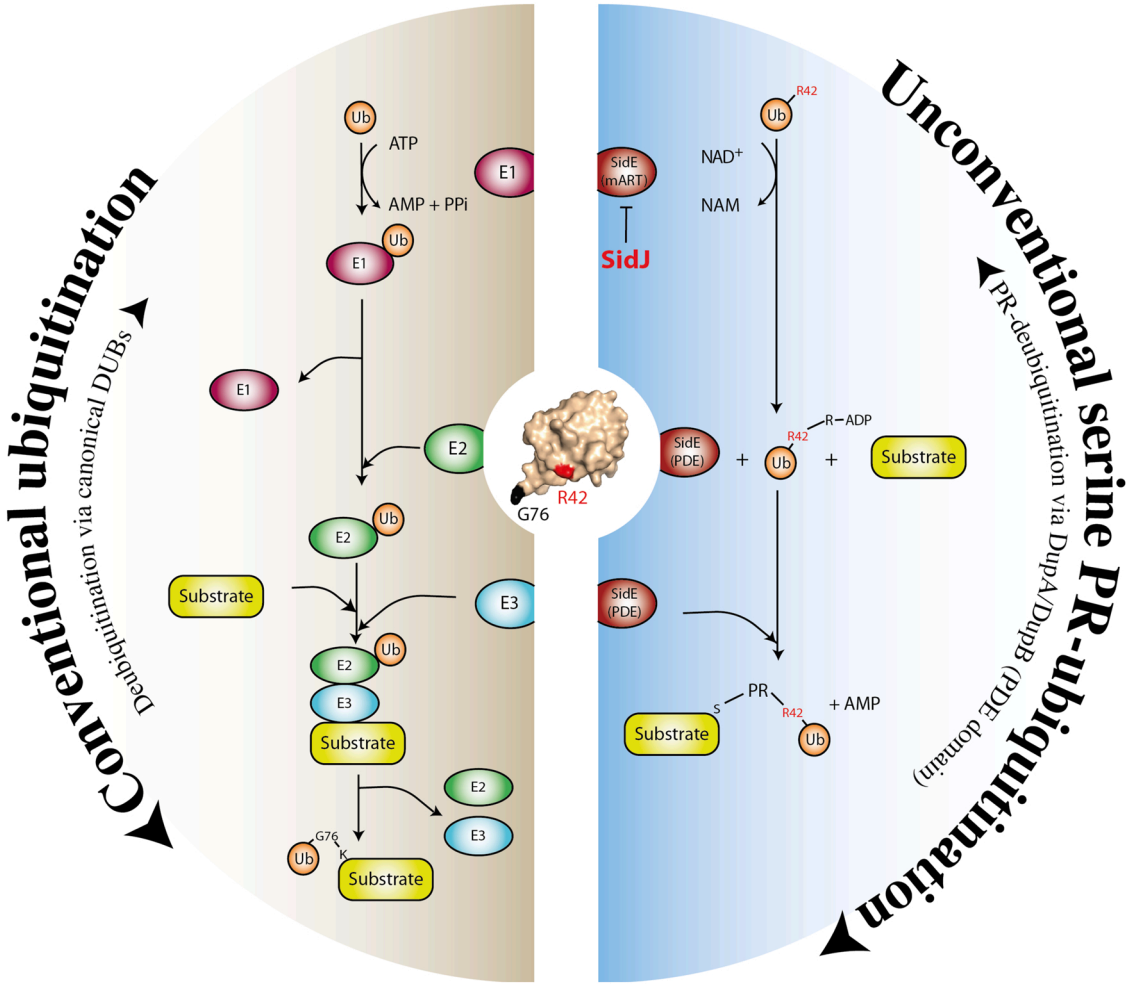

Fig. 2. Schematic overview of the conventional and unconventional serine PR ubiquitination. Left: A three-enzyme cascade (E1-E2-E3 enzymes) catalyzes canonical ubiquitination of the substrate, in an ATP-dependent manner, linking Gly76 of ubiquitin and the Lys residue of the substrate with an isopeptide bond, Right: SidE effectors harboring "three-in-one" ligase activity catalyze PR ubiquitination in a two-step reaction, utilizing NAD^+. In the first step, the mART domain of the SiDE effectors, ADP-ribosylates ubiquitin at Arg42 (Arg42 ADPR-Ub). Next, ADPR-Ub is further processed by the PDE domain, resulting either in the transfer of PR ubiquitin to a serine residue of a substrate, or the hydrolysis to AMP and generation of free PR ubiquitin.

[57]. In parallel, the ubiquitin system is extensively studied as a potential drug target, both in terms of developing novel DUB inhibitors [28] and proteolysis targeting chimeras (PROTAC) technology [54].

Recently discovered non-canonical ubiquitination triggered by *L. pneumophila* effectors is important for its pathogenicity [9,104]. Members of the SidE effector protein family are the first known E1/E2-independent ubiquitin ligases, which bypass the classical E1-E2-E3 enzymatic cascade. The SidE family contains four paralogues – SidE, SdeA, SdeB, and SdeC – all of which modulate bacterial infection *via* the unconventional serine phosphoribosyl (PR) ubiquitination of the host substrates. The SidE family is required for a full bacterial virulence in a protozoan host, and appear functionally redundant in bone marrow-derived macrophages in mammals [7,70]. These enzymes contain three domains: (1) an N-terminal DUB domain preferentially catalyzes the hydrolysis of lysine 63-linked polyubiquitin chains [114]; (2) Phosphodiesterase (PDE) and (3) mono-ADP-ribosyltransferase (ART) domains participate in the catalysis of ubiquitin phosphoribosylation and serine ubiquitination through phosphoribosyl linkages [9,104]. In the first step of the reaction the ART domain utilizes β-nicotinamide adenine dinucleotide (NAD^+) to ADP-ribosylate ubiquitin at arginine 42 (Arg42 ADPR-Ub). Next, ADPR-Ub is further processed by the PDE domain, resulting either in the transfer of PR-ubiquitin to a serine residue of a substrate, or the hydrolysis to AMP and generation of free PR ubiquitin. Interestingly, PR ubiquitination blocks conventional ubiquitination cascade, thus impairing a plethora of cellular processes that depend on canonical ubiquitination [9]. SidE family can also target tyrosine residues for PR ubiquitination, expanding the range of sites that can be modified [138]. PR ubiquitination is a reversible process, in which *L. pneumophila* effectors DupA and DupB play key roles [117,125]. Structurally, these DUBs for PR ubiquitination (DUPs) resemble the PDE domain of SdeA, but have much higher binding affinities towards PR ubiquitin in comparison to SidE PDEs [117], which allows them to cleave PR ubiquitin from serine residues of the substrates. DUPs can also convert ADPR-Ub into PR-Ub, in a similar manner to SidE PDEs, but they do not transfer PR ubiquitin to the substrate. Using a unique strategy of hijacking the host cell ubiquitin system *via* conventional and unconventional PR ubiquitination, *L. pneumophila* creates an environment for its optimal replication, thereby increasing the complexity of ubiquitin biology in eukaryotic system. Differences between the canonical and non-canonical serine PR ubiquitination are summarized in the Fig. 2.

3.2. *L.pneumophila effector proteins targeting the ubiquitin system*

Since prokaryotes do not have any genes encoding for ubiquitin, the canonical ubiquitination machinery and related pathways are missing in bacteria. However, the ubiquitination machinery and related processes are highly conserved among eukaryotes, pointing out the importance of this network for the cellular integrity. Therefore, successful modulation of the host ubiquitin system represents an important target for intracellular pathogens, including *Chlamydia, Salmonella* and *Legionella* [102]. Depending on the species, exploitation of the host ubiquitin system can be achieved by diverse strategies. Effector proteins aimed at manipulating host ubiquitination might either mimic "classical" E3 ligases or function in an E1/E2-independent manner as "three-in-one" ubiquitin E3 ligase enzymes. Moreover, *L. pneumophila* releases effector proteins with a range of functions into the host cell, including – (1) the "classical" DUBs, (2) DUBs that exhibit specificity for PR ubiquitination (DUPs) or (3) meta-effector SidJ, which possesses glutamylation activity that counteracts the function of the SidE family [8,10,33,119]. Table 2 summarizes the key *Legionell*a effector proteins that target host ubiquitin system.

3.2.1. *L. pneumophila effector proteins with the E3ligase function*

Effector proteins mimicking the "classical" E3 ligases include F-box and U-box proteins, which are relevant for the recruitment of the polyubiquitinated species to the LCV [99,100,46]. Additional effector proteins related to the modulation of the ubiquitin system include SidC and its paralog SdcA, which have been described as the new type of E3 ligases, also known as SidC N-terminal E3 ligases (SNL E3). They are important for the recruitment of the ER-derived vesicles and ubiquitinated proteins to the LCV membrane [46,69]. It was also shown that the N-terminal domain of SidC is sufficient for this recruitment, by anchoring to the phosphatidylinositol-4-phosphate (PI4P)-binding domain both in amoeba and macrophages [106]. Interestingly, the crystal structure of the N-terminal domain of SidC does not resemble any hitherto characterized protein [35,45,46]. Hsu et al. reported that SdcA and SidC exhibit E3 ligase activity that requires the Cys46-His444-Asp446 catalytic triad [46]. In addition, L. *pneumophila* also contains a third group of the effector proteins, described as "three-in-one" E3 ubiquitin ligases of the SidE family, including SidE, SdeA, SdeB and SdeC. As mentioned before, they catalyze the transfer of the PR ubiquitin to the serine residues of the host proteins [9].

Furthermore, several studies have shown a link between the ubiquitination and trans-glutamination upon *Legionella* infection, thus indicating the diversity of *L. pnemuophila* effectors hijacking host ubiquitin machinery using various strategies. Bacterial effector MavC (Lpg2147) acts as a transglutaminase, which is able to ubiquitinate host E2 enzyme UBE2N by catalyzing the formation of an isopeptide bond between glutamine residue 40 (Gln40) of ubiquitin and lysine residues of UBE2N. Consequently, UBE2N E2 activity is blocked, thus affecting Lys63-linked ubiquitination and blocking NFκB signaling [32,124,133] (Fig. 3).

3.2.2. *Lpneumophila effector proteins with the DUB function*

As expected, recent studies revealed that some of the *L. pneumophila* effector proteins contain DUB activity and are important for the bacterial phagosome remodeling. Proteins of the SidE family contain a DUB domain at their N terminus, which contains Cys118–His64–Asp80 catalytic triad and can preferentially remove Lys63-linked polyubiquitin chains [114]. Effector LupA (Lpg1148) belongs to the ubiquitin-like protease (UBP) family and contains a canonical Cys–His–Asp protease triad. Once it is mutated within the triad, ubiquitinated LegC3 can be detected in the cell, suggesting that LupA might act as a DUB for LegC3 [122].

It has recently been shown that prolonged inhibition of UBE2N activity by MavC is reversed by MavC ortholog MvcA (Lpg2148). MvcA possesses ubiquitin deamidase activity and can remove MavC-conjugated ubiquitin from UBE2N by using the same catalytic triad required for its deamidase activity (Fig. 3) [32,133].

Moreover, three *Legionella* OTU-like DUBs (Lot) are related to the eukaryotic OTU family. LotA has two catalytic cysteine residues (Cys13 and Cys303), which are able to cleave polyubiquitin chains, with Cys13 having preference for Lys6-linked ubiquitin chains [60]. It has also been demonstrated that LotA is recruited to the LCV in order to cleave polyubiquitin chains decorating the LCV [60]. Recently identified effector Lem27 acts as a DUB, and counteract the activity of SidC upon *Legionella* infection [65]. Interestingly, deletion of LotA did not show a growth defect compared to the wild-type *L. pneumophila* strain, most probably due to the functional redundancy with LotB and LotC. LotB exhibits high specificity toward Lys63-linked ubiquitin chains and a unique catalytic motif, which differentiates it from other OTU-DUBs in eukaryotic cells [71,113,116]. LotB contains a unique extended helical region between the variable and a cysteine loop. Moreover, its preference for Lys63-linked ubiquitin chains is explained by an additional ubiquitin-binding site. Crystal structure of LotC revealed similar catalytic characteristics when compared to LotB [65,116]. However, the extra ubiquitin-binding site observed in LotB was not present in LotC that consequently does not exhibiti any particular cleavage specificity for any ubiquitin chain. When ectopically expressed in cells, LotB and LotC localize in the ER and cytosol, respectively. Both proteins are recruited to the LCV upon the infection, suggesting a role for LotB and LotC in the replicative cycle of *L. pneumophila* [116]. Furthermore, RYK,

Table 2
Summary of key *Legionella* effector proteins that target host ubiquitin system.

Effector	Protein group	Direct host target	Specific features	(De)ubiquitination	References
	Effectors with E3 ligase function	Modulation of host ubiquitin machinery			
LubX	RING-type U-box	Clk1 kinase	U-box 1 critical for the ubiquitin ligation, U-box 2 mediates interaction with Clk1; catalyzes degradative ubiquitination of SidH effector	Canonical ubiquitination with non-canonical substrate binding	Quaile et al. [105], Kubori et al. [60]
GobX	U-box	Not known	Exploits host cell S-palmitoylation to localize to Golgi membranes	Canonical ubiquitination	Lin et al. [63]
RavN	U-box	Not known	Function not known	Canonical ubiquitination	Lin et al. [64]
SidC/SdcA	SNL E3 ubiquitin ligase	Rab1 and Rab10	Recruitment of the ER proteins and ubiquitin signals to the LCV; *in vitro* preferably catalyzes Lys11- and Lys33-linked polyubiquitin chains	Canonical ubiquitination	Hsu et al. [46, 69], Ragaz et al. [106]
MavC	Transglutaminase	UBE2N E2	Ubiquitination of UBE2N E2 *via* transglutamination, affecting Lys63-linked ubiquitination, thus blocking NFκB signaling	Non-canonical ubiquitination *via* transglutamination	Valleau et al. [124,33,133]
SidE family: SidE SdeA SdeB SdeC	"Three–in–one" ligase	> 180 host proteins, including: Rab GTPases, RTN4 & GRASP55,65	Recruitment of the ER vesicles *via* ER-fragmentation to the LCV; Golgi fragmentation	Non-canonical serine PR ubiquitination	Qiu et al. [104], Bhogaraju et al. [9] Kotewicz et al. [58] Shin et al. [117]
	Modulators of host ubiquitination machinery	Modulation of Host Ubiquitin Machinery			
AnkB	Contains F-box	ParvB	F-box interacts with the Skp1 component of the E3 ligase complex SCF, modulates Lys48-polyubiquitination of host proteins	Modulates canonical ubiquitination	Price et al. [99, 100]
LegU1	Contains F-box	BAT3	F-box interacts with the Skp1 component of the E3 ligase complex SCF, modulates polyubiquitination of BAT3	Modulates canonical ubiquitination	Ensminger and Isberg [26]
LicA	Contains F-box	Not known	F-box interacts with the Skp1 component of the E3 ligase complex SCF, function not known	Modulates canonical ubiquitination	Ensminger and Isberg [26]
	Effectors with DUB function	Modulation of Host Ubiquitin Machinery			
LupA	ubiquitin-specific protease A domain	Counteracts effector LegC3	Meta-effector, modulates host organelle trafficking, putative DUB, contains catalytic triad	Canonical deubiquitination (?)	Urbanus et al. [122]
RavD	papain-like fold, unconventional Cys–His–Ser catalytic triad	Linear polyubiquitin chains on LCV	Specifically cleaves Met1-linked linear polyubiquitin chains from the LCV, but not branched isopeptide-bond linked chains in mammalian system	Canonical deubiquitination	Pike et al. [97], Wan et al. [125]
Lot A/B/C	OTU domain	Polyubiquitin chains on LCV	Recruited to the LCV in order to cleave polyubiquitin chains on LCVs	Canonical deubiquitination	Liu et al. [65, 116]
Dup A/B	PDE domain	Multiple PR ubiquitin substrates	Specifically cleaves PR ubiquitin from SidE targets	Non-canonical serine PR deubiquitination	Shin et al. [117]
	Meta-effectors with inhibiting function	Modulation of Host Ubiquitin Machinery			
SidJ	Glutamylase	Controls SidEs activity; targets host proteins: CKB, HMGB2, PDIA3 and PDIA4	Inhibits PR ubiquitination *via* SidJ/calmodulin-mediated glutamylation	Impairs PR ubiquitination	Bhogaraju et al. [8] Song et al. [118]
SdjA	Glutamylase/deglutamylase	Controls the activity of SdeB, SdeC	Inhibits ADP-ribosyltransferase activity *via* CaM-dependent glutamylation	Impairs PR ubiquitination	Song et al. [118]
MvcA	Ubiquitin–deamidase	Counteracts MavC activity	Removes MavC-conjugated ubiquitin from UBE2N	Impairs UBE2N ubiquitination	Gan et al. [33]

Rab13 and PCYT1A were identified as putative LotB substrates, and VAT1, HMOX1 and PPP2R1A as possible LotC substrates (reference). Further characterization on how ubiquitination of these proteins is regulated by LotB or LotC will unveil biological roles of these enzymes during infection.

3.2.3. Inhibition of PR ubiquitination by SidJ/calmodulin-mediated glutamylation

SidJ has been characterized as a meta-effector that is required for an efficient growth of *L. pneumophila* in the host cell. Moreover, initial observations described that SidJ counteracts SidE effects on intracellular growth of *L. pneumophila* [51]. Having revealed the enzymatic activity of SidE family members, several research groups conducted studies to elucidate how SidJ inhibits PR ubiquitination and why such activity is important for replicative cycle of *L. pneumophila*. Four independent studies identified SidJ as a glutamylase that modifies the catalytic glutamate (Glu860) in the mART domain of SdeA, thus blocking its activity. Moreover, SidJ was shown to abolish the activity of all SidE family members [8,10,33,119]. The activity of SidJ requires ATP/Mg^{2+} and the interaction with the host protein calmodulin (CaM). Structural and biochemical studies revealed that CaM interacts with the IQ motif of SidJ, which also implies the role of Ca^{2+} in the regulation of SidJ activity upon the infection. Importantly, the LCV is glutamylated 3 h upon infection with the wild-type *L. pneumophila* strain, unlike ΔsidJ strains [8]. Interestingly, Bhogaraju et al. also proposed that SidJ targets host proteins, such as CKB, HMGB2, PDIA3 and PDIA4. Moreover, mutation in *SidJ* leads to significant growth defects in both ameba and macrophages, whereas mutated *SdjA* causes growth defects only in protozoa. Those findings provide an explanation why SidJ has a very important role in *L. pneumophila* proliferation in host cells [66]. A recent study reported that SdjA, a paralogue of SidJ, also possess a CaM-dependent glutamylation activity towards SdeB and SdeC, thus inhibiting their ADP-ribosyltransferase activity [118]. Unlike SidJ, deletion of SdjA does not affect the replication of *L. pneumophila* inside the cell. The same study provided evidence that SdjA also contains deglutamylase activity specifically against SdeA previously modified by SidJ. Further structural

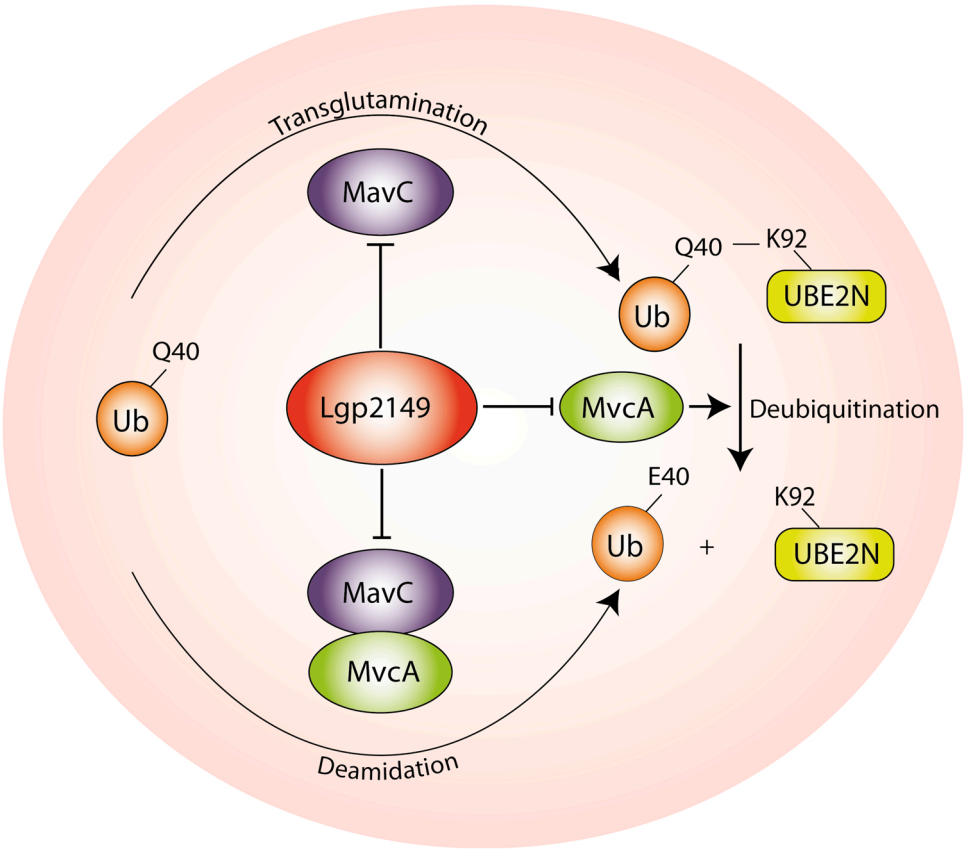

Fig. 3. Effector proteins catalyzing ubiquitin transglutamination. Effector proteins MavC and MvcA show ubiquitin deamidase activity. The transglutaminase MavC catalyzes ubiquitination of UBE2N, which is reversed by MvcA, specifically hydrolyzing the Gln40–Lys92 isopeptide bond between ubiquitin and UBE2N. Effector Lpg2149 inhibits both MavC and MvcA [124,33,133].

and biochemical studies should provide mechanistic insights into the deglutamylation activity of SdjA.

4. The effects of *Legionella* SidE effector protein family on various organelles and ubiquitin-related pathways

After discovering the key features of the non-canonical PR ubiquitination, one of the challenges was to determine the host proteins targeted by the SidE family. Shin et al. took advantage of the catalytically inactive DupA and its high affinity for PR ubiquitin to develop a PR ubiquitin "trapping system". They captured nearly 200 host proteins targeted by SidE effectors 2 h after *Legionella* infection [117]. The identified targets included a large number of ER-, Golgi-, autophagy-, endo-lysosome- and mitochondria-associated proteins (Fig. 4), prompting further functional studies to understand the role of PR ubiquitination in the infection process at the organellar level.

The ER network is continuously rearranged and fragmented, depending on the specific cellular demands, including *L. pneumophila* infection [117]. *Legionella* is able to utilize PR ubiquitination to modify ER-remodeling proteins, such as FAM134A, FAM134B, FAM134C, RTN3 and TEX264, resulting in ER membrane fragmentation and defects in ER membrane dynamics [117]. Importantly, these proteins are characterized as ER-phagy receptors that link ER fragments with the autophagic machinery for their subsequent degradation within the lysosome [38,107,16,3]. Moreover, SidE proteins target host Rtn4 (Reticulon 4) during early infection, in order to rearrange and control tubular ER dynamics [58]. At the same time, *L. pneumophila* utilizes its effector RavZ to block autophagy and prevent the delivery of cytosolic components to the lysosome [18]. Thus, the proposed model suggests that ER fragments generated upon *Legionella* infection cannot be delivered to the lysosome

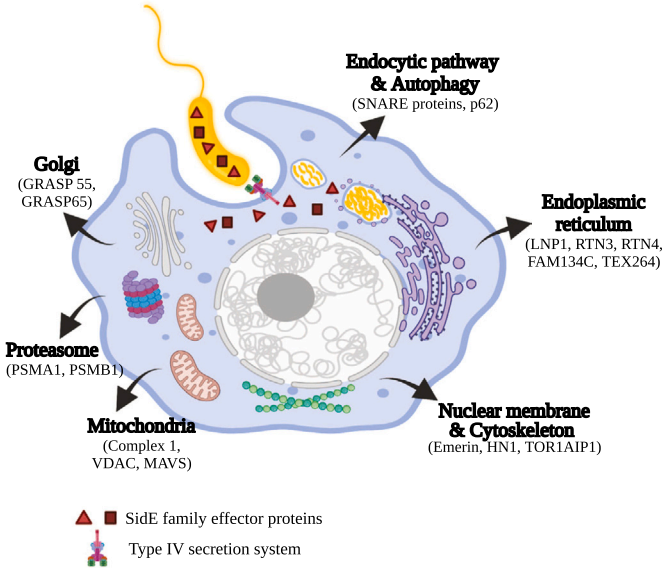

Fig. 4. Multiple host proteins targeted by SidE family effectors are PR ubiquitinated. SidE family targets more than 180 host proteins, which are modified with PR ubiquitin, thus affecting ER fragmentation, disrupting Golgi structure, affecting endo-lysosomal system, mitochondria as well as proteasomal subunits, cytoskeleton and nuclear membrane proteins [117].

for degradation, but are rather used as the main source of membranes for the formation of the LCV. Yet, precise molecular details of the role of PR-linked ubiquitination, as well as ER vesicle sorting towards the LCVs,

are lacking. Moreover, SdeA was shown to PR ubiquitinate several ER-associated Rab GTPases, resulting in the downregulation of their activity, which in turn favors the delivery of membrane to the LCV [9, 104].

The Golgi complex functions as the posttranslational modification factory for glycan maturation and is a trafficking hub in the secretory pathway for proteins and lipids in the cells. It is composed of a complex network of cisternae with three separate modules: the *cis*-, which is close to the ER and receives the ER output, *medial* and *trans*-Golgi network (TGN) that contain glycosylation enzymes and process cargo proteins and lipids in order to sort them into different destinations [47]. In this regard, PI4P was identified as a key phosphoinositide in the control of membrane trafficking at the Golgi complex [128]. Interestingly, the LCV is able to capture PI4P vesicles derived from the Golgi complex in order to establish a replication-permissive compartment [129]. Following this line, it has been shown that SdeA localizes predominantly to the ER, but also in the Golgi complex, disrupting its ribbon structure in a mechanism that involves PR ubiquitination of several Golgi-associated proteins [67, 125]. Among them, GRASP55 and GRASP65 were identified as the major substrates of the SidE family during infection [117]. These two proteins form trans-oligomers to maintain the Golgi stacks [2], which is required for the accurate protein trafficking, glycosylation and sorting. GRASP55 was also shown to facilitate autophagosome-lysosome fusion, expanding its roles in cellular trafficking [137]. Liu et al. proposed that Golgi complex disruption upon *L. pneumophila* infection is dependent on GRASP55/GRASP65 PR ubiquitination, thus leading to defects in host secretory pathway [67].

The endo-lysosomal system and mitochondria are also targeted by SidE family members of *L. pneumophila* [117]. However, how PR ubiquitination regulates the activity of these organelles is still not clear. Lysosomes are the main degradative compartment in living cells. They are involved in the turnover of receptors from the cell surface through the endo-lysosomal pathway and removal of defective cytoplasmic components through autophagy [5]. Lysosomes can also degrade intracellular pathogens *via* selective autophagy pathway called xenophagy [24]. PR ubiquitination inhibits the activity of Rag small GTPases, which in turn abolish mTORC1 activity in the lysosomal membrane [23]. By inhibiting mTORC1, SidE family can therefore increase availability of free amino acids in the cell to favor replication of the bacteria. Even though several proteins related to the autolysosome fusion machinery are PR ubiquitinated (*e.g.* SNAP29 and STX17), the biological relevance during bacterial infection of this modification has not been studied in greater detail [117]. Finally, it has been shown that mitochondrial proteins undergo PR ubiquitination during infection (*e.g.*, proteins of the electron transport chain) [117]. However, how PR ubiquitination regulates mitochondrial structure and function remains unknown.

5. Future directions and concluding remarks

The development of the new technologies with the increased focus on the study of Legionnaires' disease has significantly contributed to our understanding of the molecular mechanisms underlying the disease, including complex host-cell interactions. New findings also enabled the development of advanced strategies to target specific infection-related processes, such as the chemical synthesis of novel probes in order to target *Legionella* effector proteins from the lysates [55] and omics-analyses of pathogen and host interactions combined with next-generation sequencing (NGS) [94].

Intriguingly, *Legionella* can promote a novel type of PR ubiquitination in the host cell, which has until now not been detected in the mammalian system. This raised many new questions that logically focus on the possible identification of putative orthologues of the SidE family in mammalian system. Based on the fact that the intermediate step of ADPR ubiquitination is critical for further PR ubiquitination, Kim et al. succeeded in synthesizing novel chemical Ub-ADPR analogs, providing the first step towards developing new tools for identifying and targeting PR ubiquitination in the mammalian system [55]. Whether PR ubiquitination indeed takes place in the mammalian system (independent of *Legionella* infection), remains unknown.

Moreover, development of advanced omics and NGS technologies enabled analysis of both transcriptomic and genomic data sets from various patient materials, enabling deeper insight into human physiology, pathophysiology and infection [95]. Genetic CRISPR-Cas9-based screens have also proven to be crucial for the determination of the specific genetic functions and gene interactions in the pathophysiology of various diseases with the potential to uncover mechanisms underlying the diseases and broaden therapeutic options [53]. In the field of *Legionella*, one of the crucial questions arising from the host side was – how does the host cell respond to *L. pneumophila* infection? Jeng et al. performed a genome-wide CRISPR-Cas9 screen in a macrophage-like cell line to identify host factors that regulate elimination of the pathogen upon infection. Besides identifying known host factors hijacked by *L. pheumophila*, they also discovered novel proteins, including C1orf043 and KIAA1109 as regulators of phagocytosis [50]. Further CRISPR-Cas9 screen will clearly provide more information about the intricate network of genetic interactions between *Legionella* and host cell.

As discussed in this review, *L. pneumophila* contains a large number of effector proteins hijacking various intracellular pathways, including the ubiquitin system of a host, which is modulated *via* canonical ubiquitination and non-canonical serine PR ubiquitination. The importance of the ubiquitin network in the virulence of *Legionella* is emphasized by its ability to employ two types of ubiquitination. Combined with novel technologies, studying these targets will ensure better understanding of the *L. pneumophila* pathogenesis.

Author contributions

IT designed the structure for the review. IT, AG and ID wrote the manuscript. IT prepared the tables and figures.

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Competing interests

Authors declare no competing interests.

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