REVIEW
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The *Legionella pneumophila* Dot/Icm type IV secretion system and its effectors

Daniel C. Lockwood[1], Himani Amin[2], Tiago R. D. Costa[2,*] and Gunnar N. Schroeder[1,*]

Abstract

To prevail in the interaction with eukaryotic hosts, many bacterial pathogens use protein secretion systems to release virulence factors at the host–pathogen interface and/or deliver them directly into host cells. An outstanding example of the complexity and sophistication of secretion systems and the diversity of their protein substrates, effectors, is the Defective in organelle trafficking/Intracellular multiplication (Dot/Icm) Type IVB secretion system (T4BSS) of *Legionella pneumophila* and related species. *Legionella* species are facultative intracellular pathogens of environmental protozoa and opportunistic human respiratory pathogens. The Dot/Icm T4BSS translocates an exceptionally large number of effectors, more than 300 per *L. pneumophila* strain, and is essential for evasion of phagolysosomal degradation and exploitation of protozoa and human macrophages as replicative niches. Recent technological advancements in the imaging of large protein complexes have provided new insight into the architecture of the T4BSS and allowed us to propose models for the transport mechanism. At the same time, significant progress has been made in assigning functions to about a third of *L. pneumophila* effectors, discovering unprecedented new enzymatic activities and concepts of host subversion. In this review, we describe the current knowledge of the workings of the Dot/Icm T4BSS machinery and provide an overview of the activities and functions of the to-date characterized effectors in the interaction of *L. pneumophila* with host cells.

INTRODUCTION

Legionella pneumophila was discovered in 1976 as the causative agent of a fatal outbreak of pneumonia at an American Legion convention in Philadelphia [1, 2]. Since then, this Gram-negative, rod-shaped gammaproteobacterium has been recognized as a significant human respiratory pathogen and more than 65 other *Legionella* species have been described [3]. Aside from *L. pneumophila*, the dominant species causing human disease globally, and *L. longbeachae*, which causes at least as many or even slightly more cases than *L. pneumophila* in Australia and New Zealand, several other species have been identified as notable, but mostly infrequent causes of opportunistic human infection [4–6]. Disease caused by *Legionella* species, termed collectively Legionellosis and in its most severe pneumonic manifestation, Legionnaires' disease, is typically not communicable and humans represent a terminal host [5, 7]. Only one event of human-to-human transmission has been described [8, 9]. The main route of infection is exposure and inhalation of the bacteria from environmental or contaminated artificial reservoirs, such as water systems [7, 10–12]. In these reservoirs, the bacteria are facultative intracellular parasites of protozoa, phagocytic predators of other microorganisms [13–15]. To overcome predation by diverse protozoa, *Legionella* species have evolved virulence factors that facilitate evasion of sequestration or, turning the table, exploitation of the protozoa as a replicative niche (Fig. 1) [16]. In

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Author affiliations: [1]Wellcome-Wolfson Institute for Experimental Medicine, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, BT9 7BL, Northern Ireland, UK; [2]MRC Centre for Molecular Bacteriology and Infection, Department of Life Sciences, Imperial College, London, SW7 2AZ, UK.
***Correspondence:** Gunnar N. Schroeder, G.Schroeder@qub.ac.uk; Tiago R. D. Costa, t.costa@imperial.ac.uk
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Abbreviations: cryo-EM, cryo-electron microscopy; cryo-ET, cryo-electron tomography; CTD, C-terminal domain; Dot/Icm, Defective in organelle trafficking/ Intracellular multiplication; DUB, deubiquitinase; ER, endoplasmic reticulum; FIC, Filamentation induced by cAMP; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; IM, inner membrane; LCV, *Legionella* containing vacuole; MAPK, mitogen-activated protein kinase; mART, mono-ADP-ribosyltransferase; NLS, nuclear localization signal; NTD, N-terminal domain; OM, outer membrane; OMC, outer membrane complex; PA, phosphatidic acid; PAMP, Pathogen associated molecular pattern; PDE, phosphodiesterase; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI3P, phosphatidylinositol-3-phosphate; PIP, phosphatidylinositol phosphate; PTM, post-translational modification; pUb, ribose-monophosphate ubiquitin; rDNA, ribosomal DNA; SNARE, Soluble NSF attachment protein receptor; T4BSS, type IVB secretion system; T4CP, type 4 coupling protein; T2SS, type II secretion system; T3SS, type III secretion system; T4SS, type IV secretion system; Ub, ubiquitin; UPR, unfolded protein response.

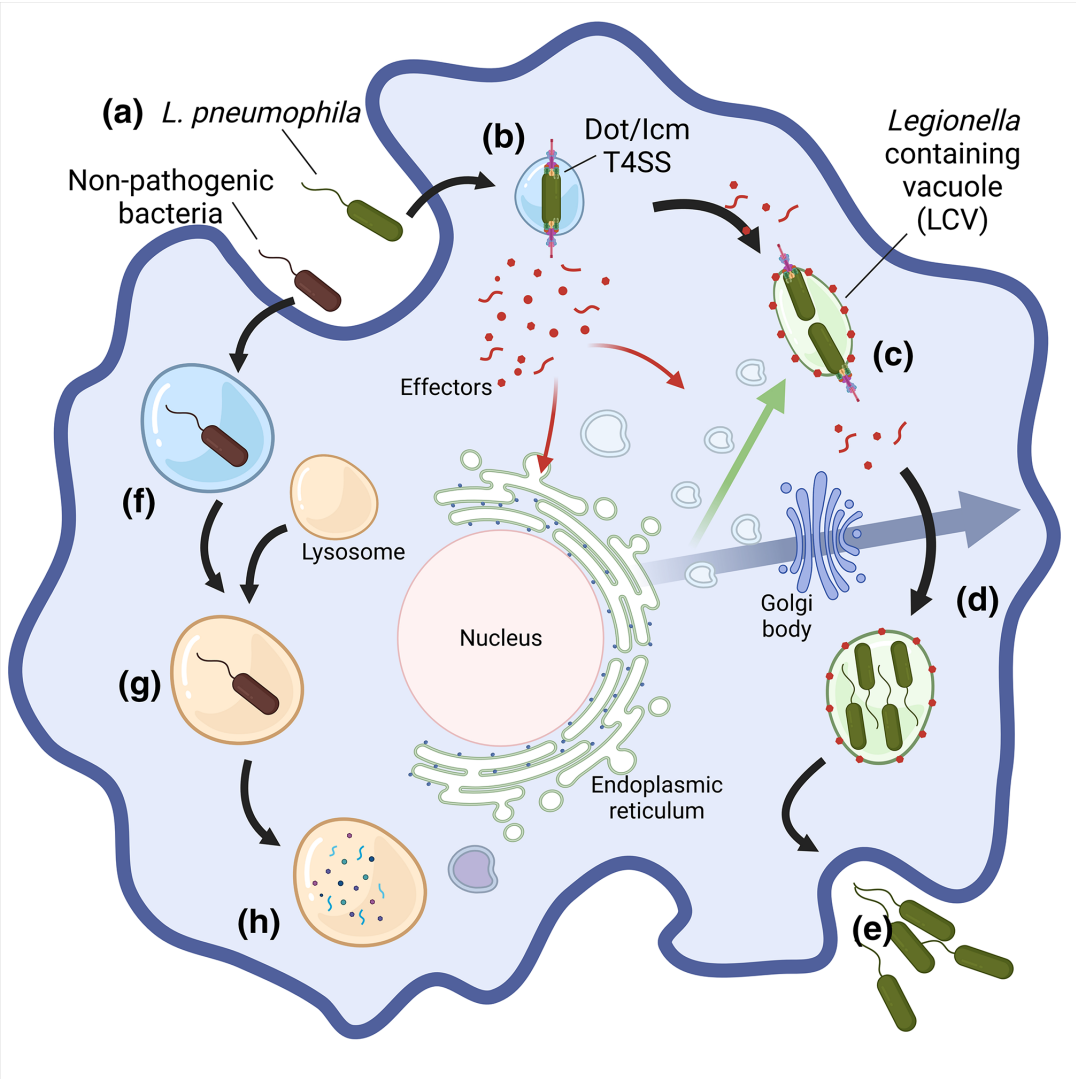

Fig. 1. The intracellular life cycle of *L. pneumophila*. (a) *L. pneumophila* is taken up into a host cell via phagocytosis. (b) Within the phagosome, *Legionella* rapidly employs its Dot/Icm T4BSS to deliver effectors into the host cell. With a large array of effectors, many processes in the host cell are targeted, in particular vesicle/endomembrane trafficking, enabling evasion of phagolysosomal degradation and instead creation of the endoplasmic reticulum (ER)-like *Legionella* containing vacuole (LCV). (c) Once the LCV is established, *L. pneumophila* switches to a non-motile and less virulent replicative form. (d) After successful replication, when nutrients in the host cell become limiting, the bacteria revert into an infectious, motile, transmissive form, preparing the bacteria to leave the cell. (e) The bacteria egress from the cell, ready to infect new host cells and repeat the life cycle. (f) Non-pathogenic bacteria cannot evade the phagolysosomal degradation pathway. (g) After phagocytosis, the phagosome undergoes a series of maturation steps ultimately fusing with lysosomes, resulting in an acidic microenvironment containing enzymes such as lipases and proteases. (h) The bacteria are completely degraded.

the absence of transmission between and selection in human hosts, the conservation of fundamental cellular processes between protozoa and human macrophages is believed to enable the bacteria to use these virulence factors to also thrive in human macrophages and cause disease [17].

The most important virulence factor for the exploitation of phagocytes by *Legionella* species is the Defective in organelle trafficking/Intracellular multiplication (Dot/Icm) Type IV secretion system (T4SS) [18–22]. This large multi-protein machine is assembled at the poles of the bacteria [23] and facilitates the translocation of effector proteins from the bacteria directly into host cells, where they manipulate cellular processes [24]. First and foremost, the T4SS and its effectors enable avoidance of phagolysosomal degradation and instead drive the formation of the *Legionella*-containing vacuole (LCV) [18, 20, 22, 25] (Fig. 1). The LCV is a unique compartment that shares characteristics of the endoplasmic reticulum (ER) studded with ribosomes but is also heavily decorated with ubiquitinated proteins [26–29]. It is a safe haven within the host, in which the bacteria replicate. Remarkably, work over the past two decades has revealed that each *L. pneumophila* strain employs the T4SS to translocate over

300 effectors, more than any other bacterial pathogen, allowing the bacteria to comprehensively modulate host cell physiology [30–36]. Here, we review the current understanding of the assembly and operation of the Dot/Icm T4SS machinery and the effectors, for which enzymatic activities, host targets and/or roles in infection have been determined to date.

Architecture and operation of the Dot/Icm T4BSS

The Dot/Icm T4SS machinery acts as the paradigm for type IVB secretion systems (T4BSSs) that represent complex machineries, both in number of proteins involved and also in the architecture. This prototype T4BSS comprises 27 proteins, mostly encoded in two large gene clusters [24]; however, at least three additional proteins associated with the machinery have recently been discovered [37]. All components, with exception of IcmR, which is sometimes replaced by non-homologous, but functionally similar proteins [38] are present across *L. pneumophila* isolates and *Legionella* species [3, 39]. The proteins show overall high conservation at the protein level, >50% identity, with some components that are probably host-exposed being less conserved than parts of the core bacterial membrane-spanning machinery [40]. Moreover, T4BSS-related secretion systems with differing degrees of conservation are found in *Coxciellaceae* and other *Legionellales* [24]. The T4BSSs are distinct from the type IVA secretion systems (T4ASSs), also referred to as the 'minimized' systems, which are typically composed of 12 components and represented by the VirB/D4 prototype machinery from the plant pathogen *Agrobacterium tumefaciens*. Despite the complexity of the Dot/Icm T4BSS machinery, remarkable progress has been made in dissecting its structure and function.

The Dot/Icm T4BSS core complex

Early studies investigating the subcellular location of T4BSS components suggested the existence of a five-protein core complex that spans across the bacterial inner and outer membranes [41]. The first structural insight into this core complex provided by transmission electron microscopy of intact *L. pneumophila* showed ring-shaped structures at the bacterial surface [42]. Isolation and MS confirmed that the complex is indeed made from the five Dot proteins.

In the complex, the inner membrane proteins DotF and DotG associate with the outer membrane complex in the presence of DotC, DotD and DotH [41]. The proteins DotC, DotD and DotH are crucial for complex formation, and the association of DotH with the outer membrane occurs through the action of the outer membrane lipoproteins DotD and DotC [41]. The importance of the membrane association of these two proteins is highlighted by the observation that mutations within their lipobox motif, a signal peptide that becomes acylated and is key for membrane retention of bacterial lipoproteins, causes partial defects in the intracellular growth of *L. pneumophila* and that these defects are additive [43]. DotD is associated with the membrane via cysteine 18, and the crystal structure showed that following a disordered N-terminal domain, DotD contains a globular region with high-order similarity to the N0 domain of secretins found in type II and type III secretion systems [44]. A similar structuring of the N terminus has been observed in *Xanthomonas citri* VirB7, which comprises a short linker followed by an N0 domain [45]. DotH and DotG are thought to be functional counterparts of T4ASS components VirB9 and VirB10, respectively. This highlights remarkable similarity and structural conservation amongst the secretion systems.

Surprisingly, unlike in T4ASSs, DotG is not an essential component and deletion of the gene does not entirely abrogate complex formation. A subcomplex lacking DotG is, however, inactive and unable to translocate effectors [22]. In the absence of DotG, the central channel is missing from the complex, indicating its role in shaping the outer membrane complex (OMC) secretion channel. This is reminiscent of the 'minimized' systems where the homologous protein, VirB10, is located adjacent to the central lumen of the channel.

DotF does also not seem to be an essential component since stable complexes were visualized in a Δ*dotF* background; however, DotF is important to stabilize and generate an effectively operating system, as effectors could only be translocated at a substantially reduced level by the *L. pneumophila* Δ*dotF* mutant [42, 46]. In the absence of DotF the DotG level was considerably reduced compared to the other three Dot proteins. This indicated that while functional complexes were formed consisting of DotCDHG at a low efficiency, for robust complex formation, DotF is required and is likely to facilitate the proper integration of DotG into the complex. Therefore, based on mutational studies coupled with visualization by negative staining, a model of the assembly of the active robust core complex was proposed, which involved an essential outer membrane portion composed of DotCDH, with which DotF associates, enhancing the docking of DotG to shape the secretion channel [42]. This general architecture is supported by a cryo-electron tomography (cryo-ET) structure that shows DotG density in the centre of the core complex with DotCDH located towards the C terminus of DotG at the outer membrane, and DotF situated below the DotCDH association [46]. Furthermore, this study indicated that the secretion channel between inner membrane (IM) and outer membrane (OM) is formed by an ~14 nm long funnel with an ~4 nm lumen that leads into a bell-shaped ~32 nm wide secretion chamber [46].

The high-resolution structure of the Dot/Icm T4BSS core complex was recently solved using single-particle cryo-electron microscopy (cryo-EM) [47]. Combined with additional cryo-EM data from a follow-up study, an atomic model was built [37] (Fig. 2a). The core complex can be divided into two distinct regions which have different symmetries: a 13-fold symmetric OMC and a periplasmic ring (PR) with 18-fold symmetry. Strikingly, the OMC contains a further feature which is the 16-fold symmetric dome (Fig. 2a). The presence of different symmetries within a core complex has also been observed in the *Helicobacter* Cag T4SS

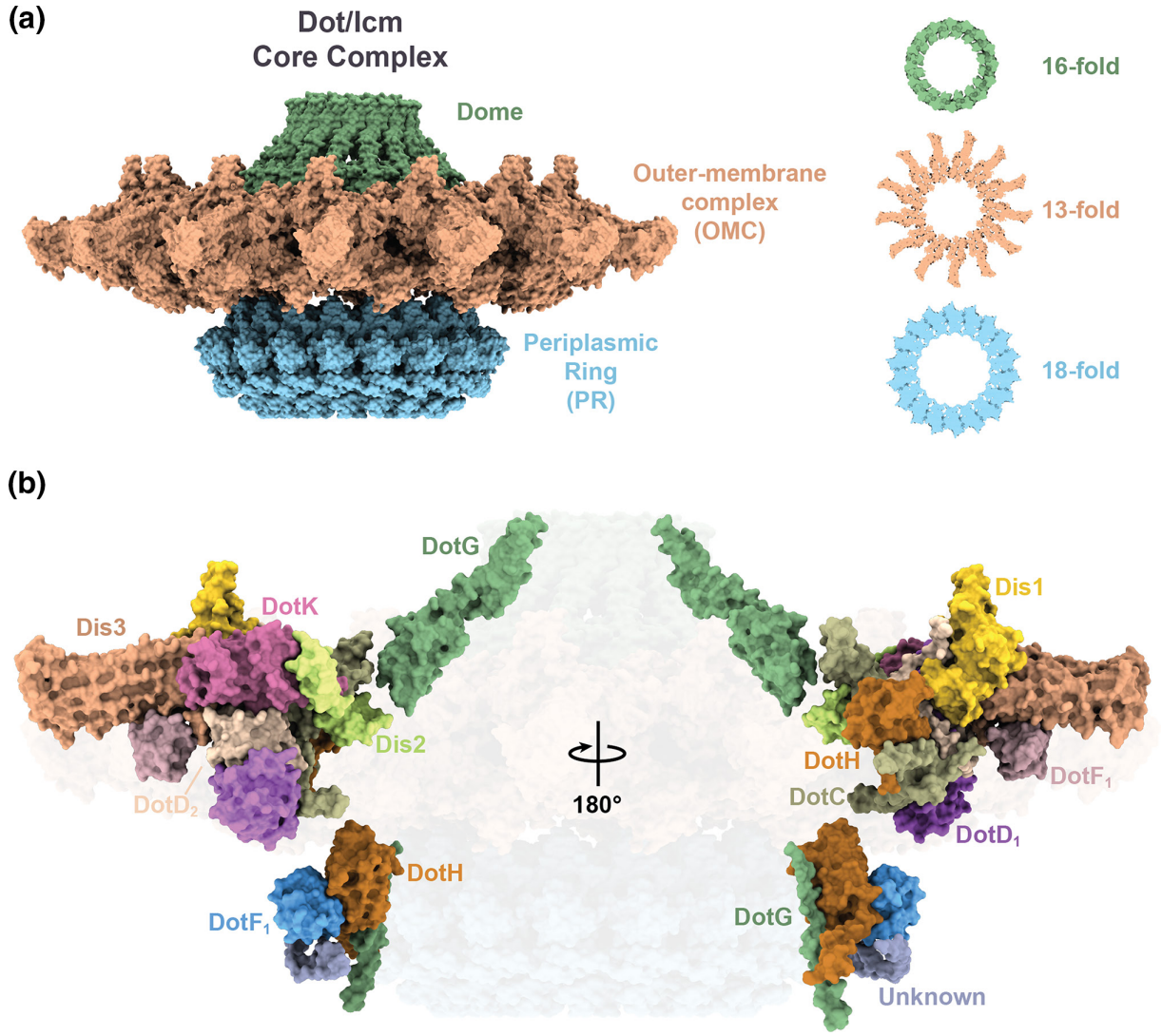

Fig. 2. The structure of the Dot/Icm T4BSS core complex. (a) Composite structure of the Dot/Icm T4BSS core complex with the Dome (PDB:7MUQ), outer membrane complex (OMC) (PDB:7MUD) and periplasmic ring (PR) (PDB:7MUE) coloured in green, orange and blue respectively. Cross-sections of the three regions show different symmetries for the dome (16-fold), OMC (13-fold) and PR (18-fold). (b) The side and 180° rotated view of the individual proteins that comprise the Dome, OMC and PR regions. All the protein structures are shown in surface representation.

and more recently in the *Escherichia coli* F T4SS [48, 49]. The core complex resembles overall the Cag core complex in terms of width (400 Å) but is more compact in height (165 Å) [50]. The dome appears to be formed from the C-terminal domain of DotG, which is in line with the observation that the core complex isolated from a DotG deletion strain lacks this particular region along with the PR [47] (Fig. 2b). This positioning of the C-terminal domain of DotG seems to be characteristic of VirB10 and homologous proteins since it has been seen in all VirB10 and homologous proteins resolved to date [37, 48, 50–52]. In addition to DotG, the PR consists of DotF and DotH (Fig. 2b). A short loop of DotG mediates the interaction with a globular section of DotH, similar to interactions previously reported between VirB9/VirB10 and CagX/CagY [52]. Furthermore, DotH was found to accommodate the symmetry mismatch by bridging the PR and OMC (Fig. 2b). This is made possible by a total of 18 DotH molecules existing in the entire structure with all 18 of their N-terminal domains (NTDs) forming part of the PR, of which 13 C-terminal domains (CTDs) then extend upwards to form part of the OMC. The remaining five CTDs only extend midway and into the intervening space, where the CTDs are reported to be flexible since the position of those five domains were not static in relation to the OMC or PR [37]. While this bridging of symmetries via DotH is reminiscent of the role of the Cag T4SS homologue CagX, similar densities in the surrounding space were not seen [53]. Instead, while the long helical expansion of CagX spans across the 14-fold OMC and 17-fold PR, an obvious connection for the remaining three domains of CagX was not observed [53].

It has been proposed that this difference and the flexibility conferred by it could be important for the Dot/Icm T4BSS secreting such a remarkably large repertoire of substrates.

The overall OMC consists of the core proteins DotC, DotD, DotH, DotF and DotG along with four additional components: DotK and three previously unrecognized T4BSS-associated proteins [Dis1 (Lpg0657), Dis2 (Lpg0823), Dis3 (Lpg2847)], which are not encoded by the *dot/icm* gene clusters. The final stoichiometry of the core complex was determined to be 32:26:18:18:13:13:13:1 3:13 (DotF: DotD: DotG: DotH: DotC: DotK: Dis1: Dis2: Dis3) (Fig. 2b) [37]. These latest findings highlight the remarkable size of the Dot/Icm T4BSS machinery as well as the complexity of the core complex architecture and suggest that additional proteins associated with the machinery might still be discovered.

The inner membrane complex and energy center

The inner membrane complex (IMC) has an important role in starting the T4BSS assembly at the bacterial poles, bridging the inner membrane and, as parts interface with the cytosol, providing contact sites for the Dot/Icm Type IV coupling complex and effector recognition. Moreover, three ATPases, DotO, DotB and DotL, that are homologous of T4ASS proteins VirB4, VirB11 and VirD4 respectively, are located here and provide energy for system assembly and threading of unfolded effectors through the system (Fig. 3a). While the membrane protein DotL was not required for recruitment of the other ATPases, the membrane-associated DotO associates with the IMC and recruits the mostly cytosolic DotB [41, 54, 55] (Fig. 3a). Interestingly, while resembling *A. tumefaciens* VirB11, DotB shares higher homology with PilT and EspE, ATPases belonging to the Type IV pilus system and the T2SS respectively [56]. This alludes to a potential evolutionary relationship and indicates a mosaic nature of the Dot/Icm T4BSS.

Some of the earliest steps in T4BSS assembly seem to depend on the two integral membrane proteins DotU and IcmF, which localize to the cell poles even in the absence of all other T4BSS components [46]. Moreover, their deletion results in reduced abundance of the core complex components DotH, DotG and DotF, and inability of these core components along with DotC and DotD to localize to the cell poles. This suggests that DotU and IcmF, which are counterparts of the T6SS proteins TssL and TssM respectively [57], are responsible for the polar targeting of the secretion machinery, acting as an anchor and stabilizing scaffold for recruitment of the core complex.

Experiments showing that DotU and DotI are required for the polar recruitment of DotO and that the inner membrane proteins DotA, DotU, IcmQ, IcmV, IcmT, DotJ, DotE and DotI are required for stable recruitment of the ATPase DotB, suggests assembly of an IM subcomplex to which the ATPases are subsequently recruited [46, 58]. *L. pneumophila* Δ*dotA* or Δ*icmT* are T4BSS-secretion deficient, highlighting the importance of these components for assembly of a functional system [25, 59, 60]. Given that DotI is homologous to VirB8, which is an integral component of the IMC in the VirB/D4 system, it is possible that DotI and its partial homologue DotJ are involved in the formation of a similar subcomplex.

Interestingly, time-lapse imaging demonstrated that native DotB is dynamic and moves between the two poles of the bacterial cell [54]; however, a $DotB^{E191K}$ mutant, which binds ATP but cannot hydrolyze it, localized exclusively at the cell poles. This suggests that the ATP-bound DotB stably attaches to the T4BSS apparatus.

Once recruited, DotO and DotB have critical roles in the structural organization of the T4BSS. Initially, based on cryo-ET densities protruding into the cytoplasm, it was proposed that DotO assembled as side-by-side hexamers as had been reported for VirB4 from the *E. coli* R388 conjugative plasmid [61, 62] (Fig. 3a). However, higher resolution structures suggest that DotO arranges as a hexamer of dimers at the base of the machinery [54] (Fig. 3a). Such an organization of the DotO homologue has also been observed in the F plasmid-encoded T4SS and in the Cag machinery, and more recently in the pKM101 encoded apparatus [49, 63]. Additional density was noted below the V-shaped structure of DotO. This was assigned to DotB, which arranges as a hexamer directly associated with DotO, and the overall dimensions correlate well with the X-ray structure of the DotB homohexamer. Together DotO and DotB form a cylindrical structure at the entrance of the T4BSS machinery. This docking of DotB to DotO is important in the translocation cascade causing a conformational change, resulting in opening of the channel at the IM [64] (Fig. 3a).

The Dot/Icm Type IV coupling complex (T4CP) and effector recruitment

While the membrane-spanning core of the T4BSS is a key element for the transport process, understanding how effectors are recruited to and threaded into the system, requiring interactions between machinery and effectors, has also been a subject of intense investigation. The third, cytoplasmic T4BSS ATPase, DotL, plays an important role in this process.

Initial work focusing on the identification of a recognition element or translocation signal sequence in effectors revealed that 20–30 aa residues at the C terminus of effectors were crucial for transport by the T4BSS [65]; however, even with an increasing number of identified effectors, definition of a universal T4BSS consensus sequence did not succeed. Only a reoccurring distribution pattern of amino acids with specific biophysical properties at the C terminus could be delineated [30] and a glutamate-rich E-block motif was identified in a large number of, but not all, effectors [33]. Aside from these, an internal translocation signal that interacts with the chaperone-like proteins IcmS and IcmW, which then act as T4BSS adaptors, was reported in some effectors such

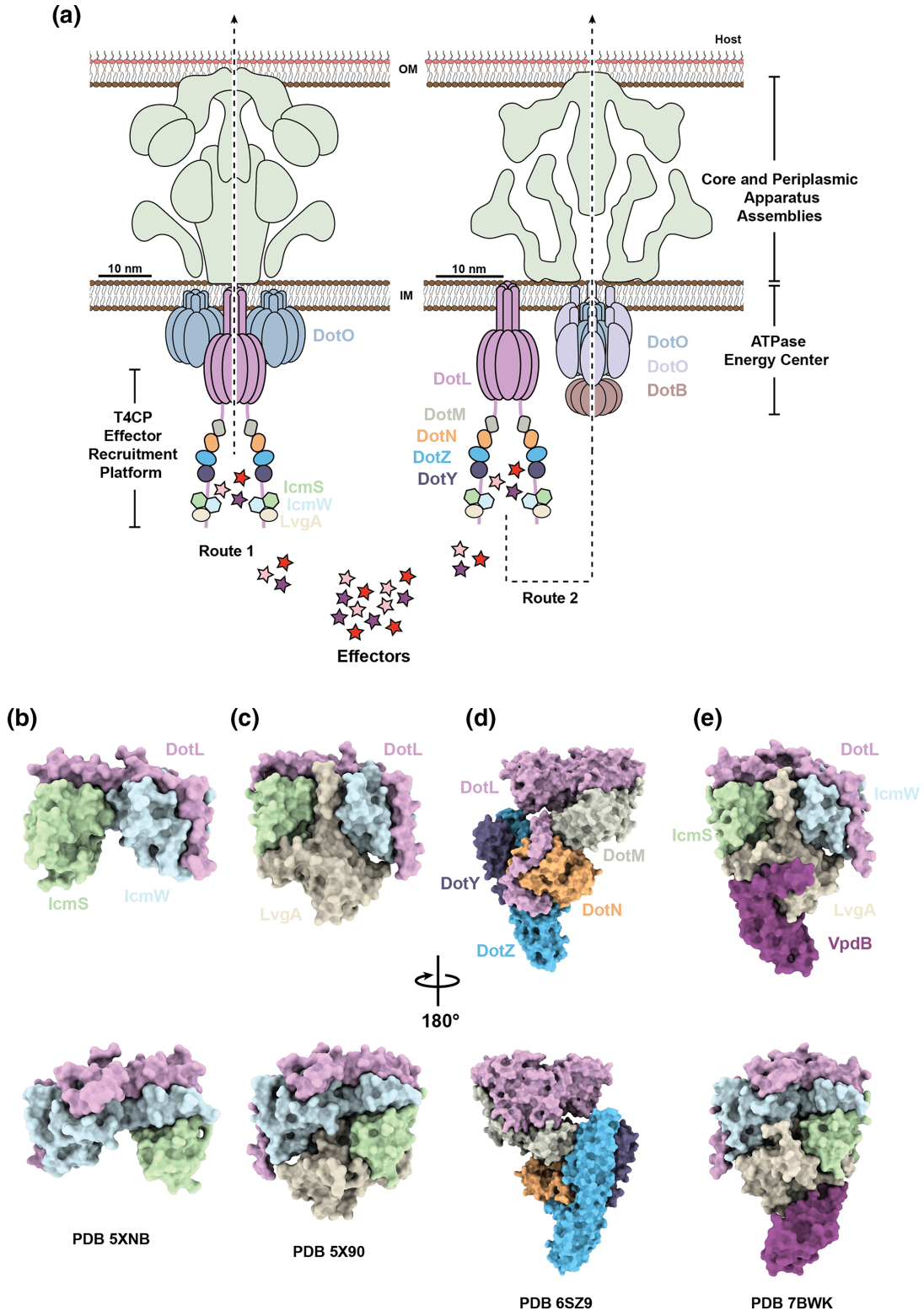

Fig. 3. Models for effector recruitment and transport through the Dot/Icm T4BSS. (a) Silhouettes of the Dot/Icm core and periplasmic apparatus assemblies obtained from two cryo-ET studies (EMD-0566 and EMD-7611) are shown in green. The type 4 coupling protein (T4CP) effector recruitment platform, shaped by the DotL C terminus together with the DotM, DotN, DotZ, DotY, IcmS, IcmW and LvgA proteins, locates either between two DotO hexameric rings (left) or beside the complex formed by the hexameric dimers of DotO, and DotB (right). Effector proteins are captured by the T4CP recruitment platform complex, and DotL energizes the secretion of the substrates across the bacterial cell envelope, via either its central channel (left) or through the DotO/DotB energy centre (right). (b–e) Different structural organizations of the T4CP recruitment platform. All the protein structures are shown in surface representation.

as SdeA and SidF [66–69]. The lack of a clear consensus signal and different dependencies for chaperone-like proteins indicates that different modes of recruitment and translocation might exist for different effectors.

Investigation of the complexes formed between effectors and the T4BSS has shed some light on this conundrum. At the heart of the machinery, DotL acts as the T4CP fulfilling a crucial role in the recognition and recruitment of effectors to the core transenvelope conduit complex [70–72]. The C terminus of DotL binds the heterodimer formed by the adaptor proteins IcmS and IcmW (IcmSW) [66, 68]. This C-terminal extension region, which extends approximately 200 aa, is common amongst T4BSS coupling proteins but not those found in the T4ASS. The crystal structures of the DotL C terminus in association with IcmSW and the additional proteins DotN and LvgA have been reported independently in different studies [73–76]. Using the established hexameric structure of the R388 TrwB (VirD4), a hexameric model of the DotL-DotN-IcmSW-LvgA complex was built [74, 76] (Fig. 3c). This T4CP holocomplex has an elongated bell-shaped architecture with a membrane-proximal ATPase hexamer followed by a membrane-distal complex, which consists of the DotL C-terminus and adaptor proteins and is designated as the substrate receptor. Based on this holocomplex structure, recognition and recruitment of most effectors to the DotL T4CP should proceed via contacts with adaptor proteins, IcmSW and/or LvgA (Fig. 3b, c).

Structural and mutational studies, combined with translocation assays, provided evidence to support and refine this model. Photo-crosslinking showed that SidF interacts via an elongated hydrophobic surface on the heterodimer with IcmSW complexed with the DotL C-terminus [75]. Studies of the interactions and adaptor-dependencies of VpdB, SidH, SetA and PieA revealed that these effectors interact with LvgA in the DotL C-terminus-IcmSW-LvgA complex and identified an LvgA binding motif, FxxxLxxxK, in a subset of effectors represented by VpdB and SidH [73] (Fig. 3e). However, only PieA, which does not contain the motif, was solely dependent on LvgA for delivery [73]. Overall, this suggests that, first, alternative LvgA binding motifs exist, and, second, some effectors can rely on additional adaptors or interactions with the T4CP complex for transport (Fig. 3a).

A different entry route into the T4BSS relying on DotM, which is associated with the IM via its N-terminus, was proposed for effectors that contain the Glu-rich/E-block motif [33, 68]. The crystal structure of the cytoplasmic domain of DotM revealed patches of positively charged residues, which could act as the binding site for the negatively charged E-block motif [77]. Mutations in this positively charged surface on DotM reduced the translocation of effectors, such as CegC3 or Lpg1663, and resulted in intracellular growth defects of *L. pneumophila* in *A. castellanii* and murine J774A.1 macrophage-like cells. Single-particle cryo-EM of an isolated T4CP complex consisting of DotL, DotM, DotN, IcmSW and two additional proteins, DotY and DotZ, allowed a hetero-pentameric complex to be defined, made up by the cytoplasmic domains of DotL, DotM, DotN, DotZ and the N-terminal region of DotY (DotLMNYZ) [78] (Fig. 3d).

A hexameric model was built of the hetero-pentameric complex with the overall structure resembling a starfish. Within the complex, a cavity is formed between DotM, DotZ and DotY. Thus, DotM-dependent effectors could enter the T4CP complex by interacting with DotM and passing through this cavity to reach the DotL channel. Additionally, DotZ and DotY, which depend on each other for association with the T4CP complex, were implicated in the polar localization of the T4CP complex and in constraining IcmSW in the motion trajectory, thereby guiding the IcmSW-bound effectors to the DotL channel and increasing effector delivery [79].

The use of distinct T4CP recruitment mechanisms is an effective way to flexibly accommodate diverse effectors and/or to orchestrate their delivery post-translation. Further work is needed to determine additional sequence and structural determinants that could direct effectors to a specific T4CP. Moreover, given that efficient translocation requires that effectors are unfolded [80], an intriguing question remains how effectors of vastly different size, ranging from for example the 21 kDa SnpL (Lpg2519, 186 aa residues) up to the 252 kDa SidH (Lpg2829, 2225 aa residues), are kept unfolded after translation. Similarly, while some evidence exists that, as for host membrane-inserting T3SS effectors, optimization of the hydrophobicity of transmembrane segments prevents premature insertion of many T4BSS transmembrane effectors in the bacterial IM, it was observed that this did not suffice for the effector LegC3 (Lpg1701) when expressed in *E. coli* [81]. If chaperones for effectors such as LegC3 ensure the efficient threading into the T4BSS in *L. pneumophila* or if some effectors could enter the T4BSS from the bacterial IM needs to be established.

These are important questions because, while a degree of transcriptional regulation for effectors subsets exists, high numbers of effectors are expressed at the same time, in particular in preparation for exit from an exhausted host cell, when bacteria switch from a replicative to a transmissive form [82]. The expression of the T4BSS machinery itself seems to underlie little growth phase variation and systems could be observed in the membrane during the stationary, elongation and division phases [83]. These characteristics indicate a 'loaded gun' model for the T4BSS allowing the bacteria to be ready to act if a host cell is encountered. In line with this model, membrane contact was demonstrated to be essential and sufficient to trigger translocation of effectors into host cells [84]. In the LCV the T4BSS tethers bacterial poles to the vacuole membrane, leading to a distinct indentation of the membrane contact site [85]; however, the molecular cues and mechanisms of host membrane permeation by the T4BSS remain unresolved. In the environment, this setup provides an effective way to swiftly fend off predators and/or maximize exploitation of potentially sporadic encounters with permissive hosts.

Fig. 4. Simplified scheme of the modular architecture of Dot/Icm T4BSS effectors. Effectors often, but not always, comprise the four following types of domains: a C-terminal translocation signal; a domain or motif that directs localization in the host cell during infection, for example a lipid-binding or transmembrane domain or prenylation or palmitoylation site; a target substrate-binding domain; and finally an enzymatic domain that exerts an effect on one or more target molecules. *Additional internal translocation signals can also be found.

DOT/ICM T4BSS EFFECTORS

General concepts and functional features

Discovery of the first T4BSS effector RalF [86] initiated a golden age of effector identification, establishing translocation for at least 330 effectors by *L. pneumophila* strain Philadelphia [30–36, 87–89]. About 65% of effectors are conserved between *L. pneumophila* isolates, revealing substantial plasticity, due to deletion, replacement or acquisition of effectors, even between the most frequently used laboratory strains *L. pneumophila* Philadelphia, Paris, Lens, Corby, 130b and Alcoy [3, 90]. Only nine effectors [Lpg0086 (LceA), Lpg0103 (VipF), Lpg0107 (RavC), Lpg0140 (CetLp1), Lpg1356 (LceB), Lpg2300 (LegA3/AnkH/AnkW), Lpg2815 (IroT/MavN), Lpg2832 and Lpg3000] are conserved in all analyzed *Legionella* species [3, 39, 91]. Interestingly, LegA3/AnkH has a companion protein LegA3C, which is not translocated, and only cognate LegA3–LegA3C orthologous pairs from different *Legionella* species function optimally together [91]. Overall, this low degree of conservation of the effector arsenals suggests that different networks of effectors, which cooperatively ensure intracellular survival, LCV formation and replication, exist. Caution has to be taken when extrapolating findings relating to individual effectors across strains.

The high number and diversity of effectors suggest that *Legionella* species have a high propensity to integrate new effectors into their arsenal. Strikingly, many effectors are eukaryotic-like, exhibiting overall or in parts higher homology to eukaryotic than bacterial proteins, suggesting direct repurposing of host genes [3, 31, 32, 88]. Several mechanisms of acquisition of host DNA or RNA and incorporation into the *Legionella* genome by homology or mobile-genetic element-facilitated recombination and/or alternative or non-homologous end-joining could mediate this process [92]; however, no transfer event has yet been observed experimentally and the mechanism(s) remain(s) unknown. While recombination hotspots and Dot/Icm T4SS effector-rich islands exist, the effectors are scattered across the whole genome [93, 94]. This is unlike T3SS effectors, which are often encoded in proximity of the secretion machinery and/or in mobilizable pathogenicity islands [95]. The flexibility of the T4BSS to accommodate different T4CP adaptors might simplify the evolution of translocation signals in newly acquired genes. Additional mechanisms probably drive further evolution and adaptation of eukaryotic-like effecter genes [92].

The presence of conserved *Legionella* effector domains in different combinations supports a model, in which new effectors evolve by gene recombination adding or swapping modules [3, 39, 92]. This generates a very diverse effector arsenal, containing small, single-domain and large effectors with a modular architecture containing a variety of domains, for example lipid- or protein-binding or catalytic domains mediating diverse enzymatic reactions, that dictate together the overall function of the effector (Fig. 4). Additionally, gene duplication, evidenced by families of effector paralogues, also contributes to the expansion of the arsenal [31, 35, 96].

This amassing of effectors seems perplexing, in particular as individual deletions of most effectors and even trimming down the arsenal by 31% demonstrated that many effectors are dispensable for survival and replication in macrophages [94]. Functional characterization and assessment of effector networks showed that this is at least in parts due to different forms of redundancy in the effector arsenal [97]: effectors may act on the same target (i) by identical or (ii) differing molecular mechanisms, or on different targets affecting (iii) the same signalling pathway, (iv) different signalling pathways modulating the same cellular process or (v) different cellular processes with overlapping global, system-wide consequences, for example sustained host cell survival despite redirection of resources to the bacteria. This multi-layered host manipulation might insure the bacteria against loss of individual effectors due to genetic mutation; however, more importantly, infection studies with different amoebae revealed an attenuation of the *L. pneumophila* strain lacking 31% of the effectors and a requirement of different subsets of effectors in different amoeba [83, 94]. Only seven effectors (MavN, SdhA, RavY, LegA3, Lpg1751, MavQ and MavO) were required for robust growth in all tested amoebae and, to varying extent, macrophages [83]. This demonstrates that the large effector arsenal predominantly evolved to enable a life as an environmental pathogen of a broad range of hosts, and functional redundancy in some hosts might be a side effect [83]. The recent discovery that the effector Lart1, an ADP-ribosyltransferase, only seems to modify fungal and protozoan NAD^+-dependent glutamate dehydrogenases (GDHs), but no mammalian targets, further highlights the role of environmental hosts as selective pressure to shape the effector arsenal [98].

The large numbers of non-redundant effectors also enable the bacteria to fine-tune the manipulation of host proteins, for example small GTPases. These are molecular switches and central regulators of eukaryotic cellular processes and in particular

Rab GTPases, the largest subfamily with about 60 members that control endomembrane trafficking [99, 100], are a prime target of effectors. Mimicry of antagonistic host proteins that control the activity cycle of small GTPases by effectors, for example, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), decouples a protein from the host's regulatory systems [101].

Moreover, as illustrated by the manipulation of the GTPase Rab1, *L. pneumophila* not only uses GEF and GAP effectors, SidM and LepB, but in addition relies on a second functional domain of SidM or the effector AnkX to carry out post-translational modifications (PTMs), AMPylation and phosphocholination (Table 1) [102–104], which protect the activation states of Rab1 against host reset. Both post-translational modifications can subsequently be reversed by the antagonistic effectors SidD and Lem3, respectively [105–107]. A growing list of similar antagonistic effector relationships highlights how the effector arsenal equips the bacteria to exert exact temporal and spatial control of host protein activity (reviewed in [108]). Moreover, the observation that co-expression but not individual expression of the effectors SidP and Lem14 inhibits yeast growth indicates that synergistic interactions also exist [109].

Fine-tuning also occurs at the effector level. 'Effectors of effectors', termed metaeffectors, directly modulate the activity of other effectors [110, 111] (Effector, Table 1). The first recognized metaeffector was LubX, an E3 ubiquitin (Ub) ligase, that ubiquitinates the effector SidH, inducing its degradation by the host's proteasomal system, and thus controls its concentration and half-life in the host [111]. A systematic screen identified several effector pairs and showed that the interactions occur by multiple mechanisms including steric complex formation, for example restricting access to a catalytic site, or by introduction or removal of PTMs [109]. Some metaeffectors control several cognate effectors; for example, Lpg2149 inhibits the paralogue effectors MavC and MvcA [112, 113] and SidJ deactivates SidE-family effectors (SidE, SdeA, SdbA, SdeB and SdeC) [114–117]. Surprisingly, the SidJ paralogue SdjA deactivates only the SidE-family members SdeB and SdeC and relieves the inhibition of SdeA by SidJ [118, 119]. This highlights that complex metaeffector-mediated regulatory circuits exist.

Some metaeffectors have additional host targets. SidP uses distinct domains to reduce cytotoxicity of the effector MavQ [109] and functions as a phosphatidylinositol-3-phosphate (PI3P) phosphatase [120]. The role of many metaeffectors during infection still requires further investigation; however, decreased progeny of *L. pneumophila* Δ*sidJ*, *L. pneumophila* Δ*lubX* and *L. pneumophila* lacking metaeffector MesI, which controls the cytotoxic effector SidI, in different infection models demonstrates the critical importance of balancing the activity of effectors to an optimal level [111, 121–123].

Taken together, host range expansion and fine-tuning of host subversion by effectors, probably to maximize host cell survival and therefore the time available for exploitation, emerge as key evolutionary drivers behind the accumulation of effectors by *Legionella* species.

The roles of effectors in the different phases of the interaction with host cells

Host cell invasion and manipulation of the actin cytoskeleton

Legionella species are facultative intracellular pathogens and, in the environment, protozoa provide an attractive, nutrient-rich niche for efficient replication. As protozoa are unicellular phagocytes, which hunt and actively ingest bacteria, there seems little selective pressure on *Legionella* species to evolve means to force host cell entry. However, while a T4BSS-deficient *L. pneumophila* strain is readily phagocytosed, uptake of wild-type bacteria into human and mouse macrophages is more efficient [124, 125]. Moreover, the T4BSS also enhances the invasion of *L. pneumophila* into non-phagocytic mammalian cells, for example lung epithelial cells [126]. Together, this suggests that effectors promote the uptake process; however, little is known about their identity.

LaiA, a SdeA homologue of *L. pneumophila* strain 80-045, was implicated in efficient uptake into human macrophages and epithelial cells [127], but translocation of LaiA by the T4BSS in this strain, the underlying mechanisms and relevance for other *L. pneumophila* isolates remain unclear.

The effector VipA enhances uptake of a filamentous form of *L. pneumophila* into lung epithelial cells [126]. VipA was previously demonstrated to drive polymerization of actin microfilaments [128] (Fig. 5); however, it was not associated with the LCV and rather localized to early endosomes in macrophages. VipA was therefore implicated in disruption of the endosomal system [128], but the effector could have alternative functions during the course of infection.

Notably, several effectors were found to directly target actin or the associated host proteins to inhibit actin polymerization (Table 1). *In vitro* studies showed that Ceg14/SidL, previously reported to block translation [129], interacts with and blocks actin polymerization by an unknown mechanism [130] and RavK is a cysteine protease effector that cleaves actin [131]; however, the role of these activities during infection have not yet been resolved. The effector LegK2 is a kinase that phosphorylates threonine residues on the ARPC1B and ARP3 subunits of the ARP2/3 complex inhibiting actin polymerization on the LCV and blocking association with late endosomes or lysosomes [132]. WipA is a phosphotyrosine phosphatase effector, which dephosphorylates, among other proteins, p-N-WASP, p-ARP3, p-ACK1 and p-NCK, disrupting phosphotyrosine signalling required for actin polymerization in eukaryotic cells [133–135].

Table 1. Dot/Icm T4BSS effectors with assigned biochemical activities/targets/functions during infection

Name(s)	Protein ID	Host target/substrates	Localization motif/domain	Activity and/or function	Selected references
RavA	Lpg0008	Rab11a			[357]
CegC1/PlcC	Lpg0012	Phosphatidylcholine		Zinc metallophospholipase C	[156]
Ceg3	Lpg0080	ANTs		ADP-ribosylates mitochondrial ADP/ATP translocases (ANTs) inhibiting their exchange activity	[260]
Larg1	lpg0081	ANTs		ADP-ribose glycohydrolase that counteracts Ceg3	[261]
Ceg4	Lpg0096	MAPK p38	TM	HAD protein-tyrosine phosphatase, attenuates MAPK signalling	[273]
VipF	Lpg0103			Acetyltransferase	[358]
Lart1	Lpg0181	GDH		ADP-ribosyltransferase inactivates fungal and protozoan glutamate dehydrogenase GDH	[98]
SidP	Lpg0130	PI3P/PI(3,5)P_2		PI-3-phosphatase controlling PIP-levels on the LCV and avoidance of the endosomal pathway	[120]
RavD	Lpg0160	Ub	PI(3/4)P	DUB cleaving linear Ub chains, dampens NF-kB signalling	[146, 282]
LegU1	Lpg0171	BAT3		U-box E3 ubiquitin ligase ubiquitinating BAT3; modulation of ER-stress response, apoptosis	[235]
LegK4, Pkn5	Lpg0208	Hsp70		Kinase, phosphorylates Hsp70 to block translation	[301, 311]
Ceg7	Lpg0227			DUB the cleaves K6-, K11-, K48- and K63-linked Ub	[208, 240]
SidE	Lpg0234	Numerous proteins including Rab33		Phosphoribosyl-ubiquitination of host targets allowing manipulation of tubular ER dynamics and generation of pseudovesicles, reduction of Golgi integrity, dampening of mTORC1 signalling	[188, 212, 213, 218, 219, 221]
Ceg9	Lpg0246	RTN4, Atlastin-1		Forms a complex with RTN4 and Atlastin-1, binding to both directly	[359]
SdhA	Lpg0376	OCRL		Maintenance of LCV integrity preventing cell death and type I IFN induction	[163, 164]
VipA	Lpg0390	Actin		Actin nucleator; modulation of vesicle trafficking	[128]
	Lpg0393	Rab5, Rab21, Rab22		GEF for Rab5, Rab21, Rab22	[174]
LegA9	Lpg0402			Enhancer of autophagy	[338]
SidL, Ceg14	Lpg0437	Actin		Inhibition of host protein synthesis leading to activation of the NF-kB pathway; interferes with actin polymerization	[129, 130]
Ceg17	Lpg0519	ATF6		Induces ATF6 cleavage	[322]
WipB	Lpg0642	vATPase, LAMTOR1		Ser/Thr phosphatase with homology to the eukaryotic phospho-protein phosphatase family, interacting with v-ATPase and LAMTOR1 on lysosomes *in vitro*	[325]
AnkX/AnkN/ LegA8	Lpg0695	Rab1, Rab35	PI(3/4)P	Phosphocholination of Rab1 and Rab35 to regulate their activity; modulation of endosomal trafficking	[103, 179, 360]
Lem3	Lpg0696	Rab1, Rab35		Dephosphorylcholinase relieving the AnkX-mediated modification on Rab1	[106]
LidA	Lpg0940	Rab1, Rab6a, Rab8	PI(3/4)P	Promotion of Rab1 recruitment and tethering of ER-derived vesicles to the LCV; stabilization of Rab-guanosine nucleotide complex	[225–230]
SidK	Lpg0968	VatA (Vma1)		Inhibition of LCV acidification	[181]
RavK	Lpg0969	Actin		Metalloprotease	[131]
Lem4/SmdA	Lpg1101		PI4P	Membrane-associated protein tyrosine phosphatase	[361, 362]
RavN	Lpg1111			E3 Ub Ligase U-Box like	[363]

Continued

Table 1. Continued

Name(s)	Protein ID	Host target/substrates	Localization motif/domain	Activity and/or function	Selected references
RavO	Lpg1129			SH2 domain	[33, 276]
	Lpg1137	Syntaxin 17		Serine protease, inhibition of autophagy and staurosporine-induced apoptosis	[364]
LupA	Lpg1148	LegC3		DUB, CE clan protease	[109]
Lem8	Lpg1290	Phldb2, 14-3-3ζ		Cysteine protease activated by 14-3-3ζ binding, cleaving itself and Phldb2, interfering with cytoskeleton organization and host cell migration	[365]
Lgt1	Lpg1368	eEF1A	PI3P	Glucosyltransferase; inhibition of host protein synthesis	[129, 366, 367]
LicA	Lpg1408	Skp1		F-box containing protein, shown to interact with SKP1 during infection	[235]
LegK1	Lpg1483	IκBα	PI3P	Phosphorylation of inhibitory IκBα to induce its degradation activating the NF-kB pathway	[284, 285]
Lgt3/LegC5	Lpg1488	eEF1A	PI3P	Glucosyltransferase; inhibition of host protein synthesis	[129, 304]
RavX	Lpg1489			Blocks translation	[301]
LotB, Ceg23	Lpg1621	COPI, Sec22b	TM	OTU-type DUB, cleaves K63-linked Ub. Deubiquitinates Sec22b to facilitate non-canonical SNARE pairing	[234, 239]
LamA	Lpg1671	Glycogen		Hydrolyzes glycogen triggering nutritional immunity in macrophages and blocking cyst formation in amoeba	[368]
RavZ	Lpg1683	ATG8	PI3P	Protease; cleavage of ATG8 to block autophagy	[333]
LecA	Lpg1692			Activator of host phosphatidic acid (PA) phosphatase protein	[154]
LegC3/PpeA	Lpg1701	VAMP4	TM	Inhibitor of homotypic yeast vacuole fusion; modulation of vesicle trafficking	[237, 369, 370]
RomA/ LegAS4	Lpp1683 Lpg1718	Histone H3		Histone methyl transferase; methylates K4 and K14 histone H3 to modulate transcription	[289, 290]
Lem14	Lpg1851			Synergizes with SidP to inhibit yeast growth	[109]
LegC2/YlfB	Lpg1884	VAMP4		Modulation of endosomal trafficking	[237]
LpdA	Lpg1888	Phospholipids		Phospholipase D; modulation of cellular phosphatidic acid levels	[153, 154]
LegK7	Lpg1924	MOB1		Mimics host Hippo kinase (MST1) to modulate transcription	[287]
RalF	Lpg1950	ARF1		ARF1 GEF; ARF1 recruitment and promotion of ER-derived vesicle fusion to LCV	[86, 371]
LegC4	Lpg1953			Promotes inhibition of intracellular *Legionella* replication within TNF of IFN-γ activated mouse macrophages	[340]
PieE/ Lem18	Lpg1969	Rab1, 2a, 5c, 6a, 7, 10		Modulation of vesicle trafficking, tethering of membranes	[170]
LegG1/MitF	Lpg1976	RanBP10	CAAX	Activation of Ran leading to microtubule stabilization; controls LCV motility and cell migration. Prenylated by host farnesyltransferase and class I geranylgeranyltransferase	[202, 255, 256, 372]
SetA	Lpg1978	Rab1, SNX1	PI3P	PI3P-activated promiscuous *O*-glucosyltransferase; modulation of vesicular trafficking; promotes robust nuclear localization of TFEB	[245, 246, 324, 373]
LegA6	Lpg2131			Induces ER stress response element driven transcription	[322]
LegK2	Lpg2137			Protein kinase; promotion of ER-recruitment	[374]
AnkB/ LegAU13/Ceg27	Lpg2144/Lpp2082	Various, Parvin B, SKP1, Cullin 1	CAAX Box	Ubiquitination of proteins on the LCV to induce their degradation; modulation of Parvin B signalling by preventing ubiquitination. Prenylated by host farnesyltransferase and class I geranylgeranyltransferase	[193, 194, 198, 202]

Continued

Table 1. Continued

Name(s)	Protein ID	Host target/substrates	Localization motif/domain	Activity and/or function	Selected references
MavC	Lpg2147	UBE2N		Ubiquitin deamidase and transglutaminase, targeting UBE2N	[112, 113, 116]
MvcA	Lpg2148	UBE2N-Ub		UBE2N-specific deubiquitinase and ubiquitin deamidase counteracting MavC	[112, 113]
	Lpg2149	MavC, MvcA		Blocks Ub deamidation activity of MavC and MvcA	[112, 113]
SdeC	Lpg2153	Numerous host proteins including e.g. RTN4, Rab33b, Rag GTPases		Phosphoribosyl-ubiquitination of host proteins allowing manipulation of tubular ER dynamics and generation of pseudovesicles, reduction of Golgi integrity dampens mTORC1 signalling	[188, 212, 213, 218, 219, 313]
DupA	Lpg2154			PDE, Removes phosphoribosyl-Ub from substrates	[218, 219]
SidJ	Lpg2155	SidE-family effectors	Calmodulin	Promotion of ER-recruitment; exerts control over LCV localization and inhibits SidE-family members by glutamylation	[114, 117, 121]
SdeB	Lpg2156	Numerous host proteins including, e.g. RTN4, Rab33, RagGTPases		Phosphoribosyl-ubiquitination of target proteins allowing manipulation of tubular ER dynamics and generation of pseudovesicles, reduction of Golgi integrity, dampening of mTORC1 signalling	[188, 212, 213, 218, 219, 221, 313]
SdeA	Lpg2157	Numerous proteins including e.g. RTN4, Rab33, Rab1, Rab6a, Rag GTPases, Grasp55		Phosphoribosyl-ubiquitination of target proteins allowing manipulation of tubular ER dynamics and generation of pseudovesicles, reduction of Golgi integrity, dampening of mTORC1 signalling	[188, 212, 213, 218, 219, 221, 313]
Lpg2160	Lpg2160	BAT3		Associates with BAT3 and LegU1, with BAT3 association being LegU1-indepenent	[235]
LegS2/ LpSpl	Lpg2176 Lpp2128			Sphingosine-1-phosphate lyase (SPL); modulation of lipid metabolism and autophagy	[264, 265]
PpgA	Lpg2224	RANGAP1		Activates Ran	[256]
LotA, Lem21	Lpg2248		PI3P	Promiscuous DUB modulating LCV ubiquitination, cleaves K13-, K48- and K63-linked UB.	[241]
LegC7/YlfA	Lpg2298	VAMP4		Modulation of endosomal trafficking	[237, 375]
AnkH/ LegA3/AnkW	Lpg2300	Larp7		Transcriptional reprogramming by interfering with transcriptional elongation by RNA polymerase (Pol) II	[298]
RidL/ Ceg28	Lpg2311	Vps29	PI3P	Inhibition of retrograde trafficking	[175]
LegA5, AnkK	Lpg2322	PI		Phosphoinositide 3-kinase	[150]
MavE	Lpg2344			Evasion of phagolysosomal degradation	[184]
HipA	Lpg2370			UB ligase, containing F-Box domain	[363]
LegA14, AnkF, Ceg31	Lpg2452			UB ligase	[363]
SidM/DrrA	Lpg2464	Rab1; Plasma membrane Syntaxins	PI4P	Rab1 GEF/GDF; Rab1 recruitment to the LCV, AMPylation of Rab1; Rab1-facilitated induction of non-canonical SNARE-pairing	[102, 104, 232, 233, 376]
SidD	Lpg2465	Rab1		De-AMPylation of Rab1	[107, 225, 242]
LepB	Lpg2490	Rab1, PI3P	PI3P	Rab1 GAP; promotes non-lytic release from protozoa; PI4 kinase generating $PI(3,4)P_2$ contributing to PIP conversion on the LCV	[105, 148, 352, 377, 378]
MavJ	Lpg2498			UB ligase	[363]
SidI	Lpg2504	eEF1A and eEF1Bγ, GDP-mannose		Inhibition of host protein synthesis activating the NF-κB pathway; glycosyl hydrolase for GDP-mannose	[110, 129]
MesI	Lpg2505	SidI		Metaeffector, inhibits SidI	[122, 123]
SdjA	Lpg2508	SidE, SdeA, SdeB, SdeC	Calmodulin	Bi-functional enzyme inhibiting SidE, SdeB and SdeC by glutamylation; de-glutamylase for SdeA	[118, 119]

Continued

Table 1. Continued

Name(s)	Protein ID	Host target/substrates	Localization motif/domain	Activity and/or function	Selected references
DupB	Lpg2509			PDE, Removes phosphoribosyl-ubiquitin from substrates	[218, 219]
SdcA	Lpg2510	E2 Ubiquitin conjugating enzymes (UbcH5)	PI4P	Paralogue of SidC; recruitment of ER and poly-ubiquitin conjugates to the LCV; implicated in ubiquitination of Rab1	[142, 204, 205, 379]
SidC	Lpg2511	E2 Ubiquitin conjugating enzymes; (UbcH7), Rab1, Rab10	PI4P	Paralogue of SdcA; recruitment of ER and poly-ubiquitin conjugates to the LCV, implicated in ubiquitination of Rab1 and Rab10	[142, 204, 205, 379]
MavL	Lpg2526	UBE2Q1		Binds ADP-ribose via ADP-ribosyltransferase fold. Does not transfer ADP-ribose *in vitro*, proposed to act as a signalling molecule in infection	[380]
LnaB	Lpg2527			Activation of the NF-κB pathway	[285]
LotC	Lpg2529	Rab10		OTU type DUB removing K6-, K11, K48- and K63-linked UB from substrates, e.g. Rab10	[239]
LecE	Lpg2552		TM	Localizes to the LCV, induces toxicity in yeast by activating PA phosphatase Pah1, influences diacylglycerol levels	[154]
MavM	Lpg2577			UB ligase	[363]
SidF	Lpg2584	BNIP3 and Bcl-rambo, $PI(3,4)P_2$ and $PI(3,4,5)P_3$		Neutralizes BNIP3 and Bcl-rambo to inhibit host cell death; phosphatidylinositol polyphosphate 3-phosphatase modulating of PIP levels on the LCV	[149, 267]
LegS1	Lpg2588			Acid sphingomyelinase-like phosphodiesterase	[381]
Lem28	Lpg2603		PI4P; inositol hexakisphosphate	Predicted kinase	[362, 382]
WipA	Lpg2718	p-N-WASP, p-ARP3, p-ACK1, p-NCK1		Phosphotyrosine phosphatase, inhibits actin polymerization via dephosphorylation of p-N-WASP, p-ARP3, p-ACK1, and p-NCK1. Implicated in endocytosis	[133–135]
LepA	Lpg2793			Non-lytic release from protozoa	[352]
MavN/ DimB IroT	Lpg2815 Lpp2867	Iron	TM	Iron-acquisition by bacteria in the LCV	[91, 94, 347]
LppA	Lpg2819	*Myo*-inositol hexakisphosphate		*Myo*-inositol hexakisphosphate (phytate) phosphatase (phytase)	[350]
LegU2/ LubX	Lpg2830	SidH, Clk1		U-box E3 ubiquitin ligase; SidH degradation; cell cycle modulation via Clk1	[111]
VipD	Lpg2831	Rab5, Rab22 PI3P		Phospholipase A1, removes PI3P from early endosomes generating PI	[171–173]
Lgt2/LegC8	Lpg2862	eEF1A		Glucosyltransferase; inhibition of host protein synthesis	[129, 304]
LegCE	Lpg2907			Acetyltransferase	[383]
MavQ	Lpg2975	PI	SidP	Phosphoinositide 3-kinase	[151]
LncP	Lpw_31961	ATP	TM	Mitochondrial Carrier Family (MCF) protein; transports ATP across membranes	[258]
LtpD	Lpw_03701	IMPA1	PI3P	Modulation of phospholipid metabolism	[155]
LseA	LPC_2110	Qa-, Qb- and R-SNAREs		Homology to the Qc-SNARE subfamily; manipulation of vesicle trafficking	[238]
PieG	Lpp1959	Ran, RanGAP1	CAAX	Contains RCC1 repeats which activate the GTPase Ran. Associated with LCV motility/microtubule stability	[256]
LtpM	Lpp0356		PI3P	Glucosyltransferase	[183]

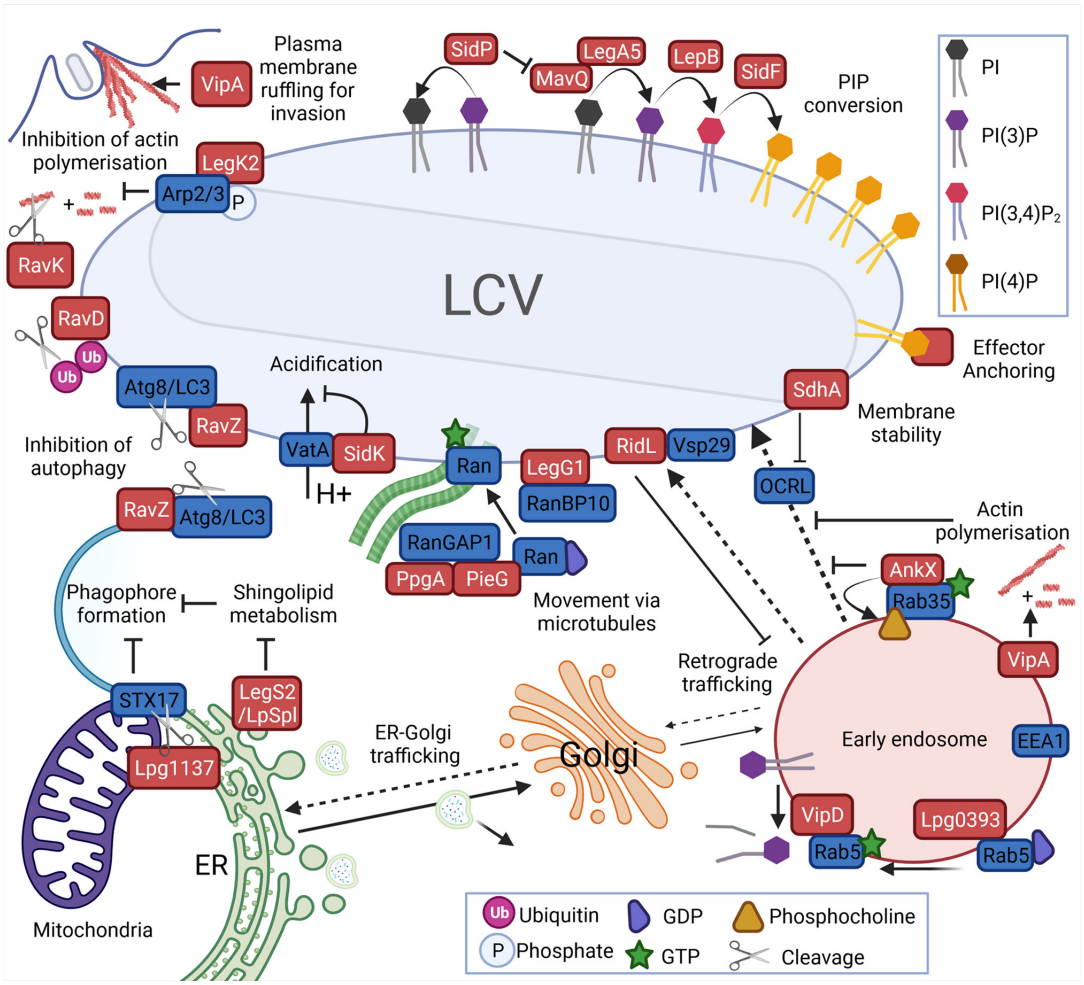

Fig. 5. Scheme highlighting Dot/Icm T4BSS effectors implicated in decoupling of the LCV from the phagolysosomal and autophagolysosomal pathway by manipulating the actin cytoskeleton, regulating the motility of the LCV, shaping the PIP composition of the LCV membrane and interfering with recruitment and function of endosomal proteins and drivers of autophagy on the LCV as well as on endosomal vesicles and ER–mitochondria contact sites, which are involved in autophagosome biogenesis. In addition, the effector SdhA reduces association of endosomal proteins with the LCV, guarding the integrity of the LCV membrane, which hides the bacteria from cytoplasmic host defences. Ub, ubiquitin; GDP, guanosine diphosphate; GTP, guanosine triphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate.

Clearly, control of the actin cytoskeleton is a part of the infection strategy; however, evidence only weakly supports a role in supporting invasion and rather indicates that the actin cytoskeleton is manipulated to set the *Legionella*-containing phagosome on the right path to become the LCV and ensure efficient intracellular replication. Interestingly, analysis of an *L. pneumophila* strain lacking *wipA* and *legK2* indicates that the inhibition of actin polymerization by the two effectors is not additive, suggesting interdependencies or compensatory host cell mechanisms [133]. Additional work is required to determine if and how the effectors, which target the actin cytoskeleton, collaborate in the infection process.

Manipulation of the endosomal membrane system and formation of the LCV

Arguably the most important role of effectors is subversion of the host cell endomembrane system and the formation and maintenance of the LCV, protecting *L. pneumophila* from the fate of non-pathogenic bacteria, which are taken up in a phagosome that matures along the endosomal pathway into a phagolysosome, creating an acidic, degrative microenvironment including proteases and lipases (Fig. 1). To achieve this, effectors deactivate, override or redirect the activities of key host proteins regulating this process and modulate the lipid composition of membranes, which has a critical role in defining organelle and vesicle identity and selective recruitment of proteins [136]. Events that enable the blockade of progression along the endosomal pathway and formation of the replication-permissive LCV are closely intertwined, but to enhance readability we will divide this broadly into different subsections here.

Exploitation and subversion of membrane lipids

Phosphatidylinositol phosphates (PIPs), glycerophospholipids, serve according to their phosphorylation state as important selective membrane receptors and anchors for proteins with PIP-binding domains on the cytosolic leaflet of membranes [137]. Indeed, a large number of *Legionella* effectors exploit predominantly PI3P- and PI4P-specific lipid-binding domains to associate with membranes and shape PIP metabolism and distribution in the cell (Fig. 5) [138, 139] (Effector, Table 1). These PIP-binding and PIP-converting effector domains do not show obvious sequence homology to eukaryotic proteins, suggesting they evolved by convergent evolution [140–142].

The conversion of PIPs is a rapid and integral part of membrane trafficking events and an early step preceding ER recruitment to the LCV [143]. While differences between host organisms and exact uptake pathways exist, the formation of a phagocytic cup is generally accompanied by the conversion of $PI(4,5)P_2$, predominant in the plasma membrane, to $PI(3,4,5)P_3$ and to a lesser extent PI4P by PIP phosphatases and kinases [144]. Upon closure of the phagosome, swift turnover to $PI(3,4)P_2$ and ultimately PI3P creates the nascent phagosomal membrane. A similar sequence was observed for the early *L. pneumophila*-containing phagosome in the soil amoeba *Dictyostelium discoideum* [143]. However, while PI3P on a common cargo-containing phagosome serves as a critical signalling molecule and anchor for proteins such as Early Endosomal Antigen 1 (EEA1), initiating a cascade of protein–protein and protein–lipid interactions leading to early endosome, late endosome and ultimately phagolysosome formation [145], it disappears from the LCV, decoupling it from the phagolysosomal pathway [143].

Instead, *L. pneumophila* effectors, in particular its own PIP kinases and phosphatases, act in concert to enrich the LCV membrane in PI4P [139, 146], the dominant lipid found in the Golgi-apparatus and secretory vesicles [147] (Fig. 5). At the heart of this are the effectors LepB, a PI4 kinase that transforms PI3P into $PI(3,4)P_2$ [148], and SidF, a PI3P phosphatase that hydrolyzes preferentially $PI(3,4)P_2$ to generate PI4P [149]. Surprisingly, *L. pneumophila* also translocates two PI3 kinase effectors, MavQ and LegA5, which both can generate PI3P from PI *in vitro,* but do not seem to be redundant in infection [150, 151]. While the role of LegA5 remains to be established, MavQ provides additional substrate for the LepB-SidF machinery [151], indicating that changing the membrane identity to avoid the phagolysosomal pathway is only one aspect and PI4P is of more general importance for the biogenesis of the LCV. Given that the ER is the main site of PI biosynthesis from phosphatidic acid (PA) [152], MavQ could maintain a constant PI3P supply once the LCV has matured and acquired ER membranes, providing anchors for PI3P-binding effectors and lipid precursors for the expansion of the LCV membrane.

Four additional effectors, (1) LpdA, a palmitoylated phospholipase D that produces PA from PI, PI3P and PI4P, and phosphatidylglycerol, (2) LecE, a PA phosphatase that produces diacylglycerol [153, 154], (3) LtpD, an effector that binds inositol (myo)−1 (or 4)-mono-phosphatase-1 (IMPA1) [155], as well as (4) the zinc metallophospholipase C effector, PlcC, that hydrolyzes a broad spectrum of lipids including PI [156] could contribute to the control of phospholipid biosynthesis on the LCV and beyond. While this is an attractive model, the interplay between effectors and definite links in this network still need to be experimentally established. For example, SidP is a PI3P phosphatase effector preferentially hydrolysing PI3P and $PI(3,5)P_2$ [120] and metaeffector suppressing toxicity of MavQ in yeast [109]. This indicates a mechanism by which generation and hydrolysis of PI3P in the same place could be avoided; however, while SidP and MavQ interact, this does not affect the PI3K activity of MavQ *in vitro*, implying a more complex relationship of these effectors [151].

The use of its own PIP-converting enzymes makes *L. pneumophila* independent from the host machinery; however, while no effectors have been identified that directly target the corresponding host enzymes, their role in *L. pneumophila* host interaction has been analyzed in great detail [139] and depletion studies implicate at least host PI4 kinase IIIβ in the acquisition of PI4P by the LCV [157]. If PI4 kinase IIIβ contributes predominantly on the LCV [157] or through the generation of PI4P-containing vesicles and membranes on the trans-Golgi, which associate with the maturing LCV, remains to be dissected [158]. OCRL, a PI5 phosphatase with the substrates $PI(4,5)P_2$ and $PI(3,4,5)P_3$ that is recruited to the LCV by the T4BSS-independent *Legionella* virulence factor LpnE, was implicated in PIP conversion [159, 160]. However, OCRL and its *D. discoideum* homologue Dd5P4 actually restrict intracellular growth [160, 161]; and *L. pneumophila* deploys the effector SdhA to shield the LCV from OCRL [161].

Subversion and exploitation of endosomal proteins

SdhA binds OCRL, reducing the interaction of the LCV with endosomal vesicles, as indicated by increased levels of endosomal marker proteins such as EEA1 on the LCV of *L. pneumophila ΔsdhA* strains [161, 162]. Endosomal Rab GTPases, in particular Rab5 on early endosomes and Rab7 on late endosomes, are equally important as PIPs in orchestrating the maturation of phagosomes and their fusion with lysosomes. In the absence of SdhA, endosomal proteins such as Rab5 promote instability of the LCV [161, 162], leading to leakage of bacterial molecules such as DNA in the host cytoplasm. This triggers inflammasome activation and cell death before the bacteria can efficiently replicate, restricting growth in murine macrophages and virulence in both *Galleria* and A/J mice models [163–166]. SdhA also seems to balance destabilizing effects of the type 2 secretion system (T2SS)-secreted phospholipase A PlaA, suggesting an interplay between T2SS- and T4BSS-delivered proteins in the host cell [163, 167].

Despite the action of SdhA, analysis of the LCV proteome showed that some endosomal proteins including Rab5a, Rab14, Rab21 are still detectable [168]. Moreover, Rab7 was reported on the LCV in HeLa cells, but could not facilitate phagosome maturation

[169]. This suggests that *L. pneumophila* uses additional effectors, which deactivate these RabGTPases on the LCV. The effector PieE has coiled-coil domains, which bind several RabGTPases including Rab5 during infection [170]; however, the consequence of this still needs to be revealed.

Alternatively, residual Rab5 might be highjacked by the bacteria for their own purpose. The effector VipD binds active but not inactive Rab5 and Rab22a, for membrane association and subcellular targeting, and the interaction activates its phospholipase A_1 domain, which hydrolyzes PI3P [171–173] (Fig. 5). This contributes to PIP conversion on the LCV, but also induces loss of PI3P-binding proteins such as EEA1 from endosomes, rendering them fusion-incompetent and reducing their association with the LCV. The action of VipD could be enhanced by the effector Lpg0393 that contains a Vps9-like domain homologous to the host Rab GEF Rabex-5 and activates Rab5, Rab21 and Rab22 *in vitro* [174].

To build the LCV, *L. pneumophila* not only blocks endosomal maturation, but also other endosomal trafficking pathways. The effector RidL binds PI3P on the LCV and Vps29, a subunit of the retromer complex which controls retrograde vesicle trafficking from endosomes to the trans-Golgi network and the plasma membrane [175]. RidL occupies the binding site and displaces the regulator TBC1D5, a Rab7 GAP, from the retromer and the LCV, disrupting retrograde transport [176, 177]. This disruption, aided by not fully defined activities of the released TBC1D5, promotes intracellular replication [175, 176].

The effector AnkX interferes with microtubule-dependent endocytic recycling of transferrin and MHC-I and contributes to decoupling of the LCV from the endosomal system, preventing accumulation of lysosomal proteins such as LAMP-1 [178, 179]. AnkX contains ankyrin repeats, typically eukaryotic protein–protein interaction motifs, a PI3P-/PI4P-binding and a Filamentation induced by cAMP (FIC) domain [103, 178, 179]. The FIC domain of AnkX catalyzes the modification of the GTPase Switch II regions of Rab35, which regulates recycling pathways between the plasma membrane and early endosomes, and Rab1, which controls traffic between the ER and Golgi, with a phosphocholine moiety [103], blocking interactions with endogenous host proteins. While the manipulation of Rab1 could contribute to the hijacking of the secretory pathway (discussed below), targeting of Rab35 is probably the dominant factor for the AnkX-mediated suppression of the association of endosomal proteins with the LCV. A detailed dissection of the kinetics of the modification of both Rab GTPases, but also of additional host targets such as PLEKHN1 [180], will be required to fully understand their contributions. The fact that *L. pneumophila* also translocates Lem3, which removes the PTM [106], suggests that exact temporal and/or spatial exploitation of the activity is advantageous for the bacteria.

In line with its multi-layered subversion strategy, *L. pneumophila* is also prepared to defuse proteins involved in key steps of phagolysosomal maturation apart from membrane trafficking. The effector SidK directly targets subunit VatA of the multi-protein v-ATPase complex, which is the dominant factor controlling organelle acidification [181]. Binding of SidK to VatA reduces its ATPase activity and transport of protons into the LCV, promoting survival of the bacteria [181, 182].

More effectors that facilitate the evasion of phagolysosomal degradation probably await discovery. For example, the effector LtpM is a new type of glucosyltransferase, which is activated by binding to PI3P and slows the movement of endosomes upon ectopic expression [183] and the effector MavE was recently found to be indispensable for preventing progression of the LCV through the endosomal system [184]; however, the exact functions that these effectors fulfil remain to be uncovered.

Transformation of the LCV in an ER-like compartment

In parallel to blocking interactions with the endosomal pathway, the bacteria rapidly convert the LCV in an ER-like compartment, including decoration with ribosomes, in which replication takes place [28, 29, 185, 186] (Figs. 1 and 6). The remodelling involves interception of smooth ER vesicles from the secretory pathway [28, 187], interactions with smooth ER tubules, tubule-derived pseudovesicles and acquisition of rough ER, which typically occurs 6 h post-infection [188]. Moreover, in recent years it has emerged that the manipulation of the host-ubiquitin system by effectors leading to ubiquitination of the LCV and shaping ubiquitin-dependent signalling processes works hand in hand with the hijacking of key regulators of vesicle recruitment and membrane fusion, such as Rab GTPases and soluble NSF attachment protein receptors (SNAREs), to mature the LCV (Fig. 6).

The Ub system is integral for maintaining host cell homeostasis, mediating spatial and temporal control of cell signalling, protein half-life and quality as well as host cell defences [189, 190]. It is governed by a myriad of ubiquitin ligases and deubiquitinases (DUBs). A large number of effectors exploit the Ub system, using molecular mimicry of eukaryotic proteins, but some also catalyze non-canonical ubiquitination of targets (Fig. 6) [191]. These effectors have been reviewed recently in great detail [101, 191, 192]. Here, we present them in the context of the different aspects of the targeted steps in the infection process.

AnkB was the first effector implicated in the acquisition of ubiquitinated proteins to the LCV. AnkB possesses an F-Box domain, typically found in eukaryotic E3 Ub ligases [193, 194]. These canonical E3 ligases are part of a three-step cascade, in which a Ub-activating enzyme (E1) transfers a Ub molecule onto a Ub-conjugating enzyme (E2) that then collaborates with single- or multi-protein RING or U-Box type E3 ligases to directly modify or with HECT-type E3 ligases to indirectly via an E3-Ub intermediate modify the lysine of a target protein [195]. In the last step, the E3 ligase typically recruits and connects the target

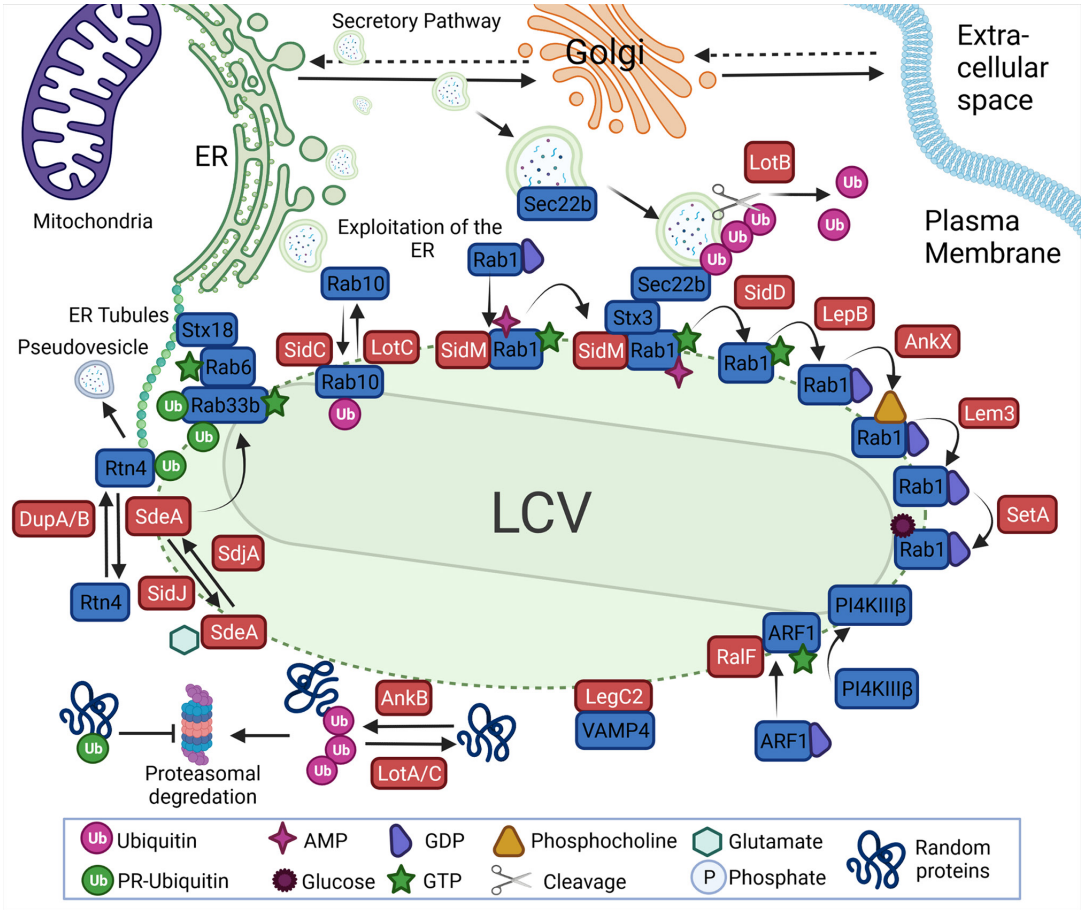

Fig. 6. Scheme highlighting Dot/Icm T4BSS effectors implicated in the maturation of the LCV by modulating canonical and non-canonical ubiquitination and highjacking ER vesicles and ER tubules to create the ER-like LCV. In particular, all steps of the GTPase cycle of the small GTPase Rab1, involved in recruitment of ER-derived vesicles, are tightly regulated by effectors. Moreover, polyubiquitination by effectors also targets host proteins for proteasomal degradation, liberating free amino acids as nutrients. Ub, ubiquitin; PR, phosphoribosyl; AMP, adenine monophosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate.

protein to the E2. F-Box proteins are commonly part of multi-subunit E3 ligase complexes, such as the SCF complex [196]. Indeed, AnkB interacts with the SCF subunit SKP1 and functions as part of the complex [197, 198].

AnkB of *L. pneumophila* strain 130b was proposed to be an important driver of the association of poly-ubiquitinated proteins with the LCV and proteasomal degradation of proteins, releasing free amino acids as a carbon source and essential micronutrient for *L. pneumophila* [199], which is auxotrophic in the production of several amino acids [200]. Interestingly, AnkB itself can become ubiquitinated with Lysine 11-linked poly-Ub chains by the host E3-Ub ligase Trim21, but while this does not trigger degradation the functional consequences remain unknown [201]. Notably, while many strains encode AnkB, functional differences exist. AnkB from *L. pneumophila* strain 130b ($AnkB^{130b}$) is one of several effectors that are recruited to membranes by post-translational prenylation [197, 202]. Its homologue from *L. pneumophila* Paris lacks this modification site and targets the host protein Parvin B to promote intracellular replication [198]. Moreover, $AnkB^{130b}$ is one of two effectors, which are hydroxylated on asparagine residues by the host protein Factor inhibiting HIF1 and this modification is important for its function [203]. Whether AnkB from other strains is also hydroxylated is unclear. Differences in structure and function as well as the composition of effector arsenals probably explain why AnkB seems to have a less prominent role in LCV ubiquitination in *L. pneumophila* Philadelphia and Paris [198, 204].

In *L. pneumophila* Philadelphia the effector SidC and its paralogue SdcA contribute significantly to the ubiquitination and acquisition of ER to the LCV [142, 204]. SidC anchors on the LCV via a PI4P-binding domain [140, 142] and uses an N-terminal E3 Ub ligase domain, which relies on an unusual protease-like Cys–His–Asp catalytic triad-dependent mechanism, to mono-ubiquinate Rab1 and mono-ubiquinate and poly-ubiquitinate Rab10 without inducing degradation [204–206]. Rab1 is a well-established target of effectors with roles in highjacking of the secretory pathway (discussed below) and Rab10 promotes ER acquisition by the

LCV and replication [168, 206]; however, the exact role of ubiquitination for their functions still needs to be elucidated. Notably, *L. pneumophila* produces the DUB effector Lem27/LotC, a eukaryotic-like cysteine protease related to the ovarian tumour (OTU) superfamily, which removes the modification, suggesting that the PTM is employed to temporarily manipulate the function of the Rabs [207–209].

Moreover, SidC and its paralogue SdcA also play an important but probably indirect role in the recruitment of the small GTPase ARF1 to the LCV [205]. On the LCV, ARF1 is activated by the effector RalF, which possesses a Sec7-homology domain typically found in ARF GEFs [86]. ARF1 is implicated in shaping the LCV in the first 2 h of infection and interference with ARF1 function impairs LCV biogenesis [210]. The mechanistic details of ARF1 exploitation still need to be uncovered; however, ARF1-GTP was proposed to engage PI4KIIIβ [211], aiding the PIP conversion of the LCV.

Effectors of the SidE family, consisting of SidE, SdeA, SdeB and SdeC, are translocated early during infection and have important roles in ER acquisition and generation of a replication-permissive LCV [66]. A *L. pneumophila* strain lacking all members shows impaired intracellular growth during infection in *A. castellanii* and *D. discoideum,* but not in murine bone marrow macrophages [66, 212]. Their role during infection depends on a unique, non-canonical Ub-ligase activity that is independent of E1 and E2 enzymes [188, 212, 213]. The effectors employ a mono-ADP-ribosyltransferase (mART) domain and NAD to ribosylate Ub on an arginine residue. The resulting ADP-ribosylated Ub (ADP-Ub) is processed by an adjacent phosphodiesterase (PDE) domain releasing AMP and transferring ribose-monophosphate-Ub (pUb) on serine or tyrosine residues of target proteins or, in the absence of a target, generating free ribose-monophosphate modified Ub [188, 212–214]. In addition to the mART and PDE domains, SidE-family members also contain a cysteine-protease DUB domain, which cleaves several different types of polyUb chains [215]. The DUB activity reduces the amount of polyUb-chains on the LCV and makes free Ub available locally for subsequent phosphoribosyl ubiquitination of proteins. While the exact consequences for tailoring of the polyUb coat by SidE-family members, for example evasion of autophagy, need to be defined, it suggests that at least temporarily, early during infection pUb generation is more important for *L. pneumophila*.

Nevertheless, the amount of pUB and phosphoribosyl ubiquitinated proteins is also tightly controlled on several levels. The activity of SidE-family members is regulated by the metaeffector SidJ, a calcium-regulated glutamylase, which after binding the co-factor calmodulin in the host becomes active and polyglutamylates the mART domain, rendering the protein inactive [114, 115, 117, 216]. As mentioned above, SidJ is supported by its paralogue SdjA, which has overlapping but not identical activity, and catalyzes the polyglutamylation of SdeB and SdeC; however, deglutamylates SdeA, liberating it from inhibition [118, 119, 121, 217]. Two PDE effectors, DupA and DupB, can remove pUb from modified proteins, enabling temporal control and fine-tuned exploitation of this modification [218, 219]. In addition, DupA and DupB also hydrolyze ADP-Ub [219, 220]. This could help to mitigate undesired effects of SdeA reaction intermediates in the absence of protein targets, as ADP-Ub and pUb are incompatible with the canonical Ub-machinery and their accumulation has downstream effects, interfering with, for example, mitophagy and proteasomal degradation [213].

Several phosphoribosyl-ubiquitinated proteins have been identified, including Rab GTPases (Rab1, Rab6a, Rab30, Rab33b), ER-associated proteins such as Reticulon-4 (Rtn4) and FAM134C and Golgi-associated proteins such as Grasp55 [188, 212, 218, 219, 221]. While the pUb-dependent effects on the individual proteins are mostly unknown, phosphoribosyl ubiquitination of Rtn4 and ER-associated proteins remodels smooth ER tubules, which coalesce on the LCV, and generates ER pseudovesicles or fragments, which can be recruited to the LCV in addition to vesicles originating from the secretory pathway [188, 218].

To associate with ER tubules as well as capture and fuse ER-derived vesicles with the LCV, the bacteria are required to take over the host cell machinery that ensures that vesicles only fuse with specific target membranes. In eukaryotic cells, tethering of vesicles to target membranes is mediated by vesicle (v)-SNAREs and target membrane (t)-SNAREs, membrane-anchored proteins that form coiled-coil bundles and generate the mechanical force that pulls membranes together [222]. Selectivity of the fusion event is ensured by intrinsic compatibility determinants of specific pairs of v- and t-SNAREs as well as SNARE-associated proteins, in particular Rab GTPases [222, 223]. *L. pneumophila* overrides endogenous control mechanisms, to allow fusion of ER-derived vesicles with the normally incompatible plasma membrane-derived nascent LCV (Fig. 6). Multiple effector-mediated mechanisms exist, which operate in parallel and synergistically.

Phosphoribosyl-ubiquitination of GDP-loaded Rab33b, which usually regulates Golgi-to-ER retrograde membrane traffic, promotes its association with the LCV, where it is activated in a T4SS-dependent manner and in turn recruits Rab6A, which establishes the interaction with ER-associated SNAREs, such as the syntaxin Stx18, to tether the ER membrane and facilitate LCV remodelling [224]. The absence of several host mediators, which typically act in conjunction with Stx18 and Rab6A, suggests that additional effectors might replace them in this cascade. Candidates are the effectors PieE and LidA, which bind several Rab GTPases including Rab6A, and at least LidA has been implicated in facilitating ER recruitment to the LCV previously [170, 225–230].

To intercept ER-derived vesicles from the secretory pathway, *L. pneumophila* co-opts Rab1 (overview in Fig. 6) [231]. Within minutes of infection, SidM associates with the cytoplasmic leaflet of the LCV via a PI4P-binding domain, recruits and activates Rab1-GDP using its GEF and modifies it with AMP using its adenylyltransferase domain [102, 104, 232]. AMPylation restricts the interaction with cognate host GAPs and Rab effectors, locking Rab1 in its GTP-loaded, active state [104]. Active Rab1 then nucleates a complex including selected subunits (Sec5, Sec15 and perhaps Sec6) of the exocyst complex, which usually tethers exocytic vesicles to the plasma membrane, and plasma-membrane SNAREs such as Stx3 [233]. This complex facilitates tethering of ER-derived vesicles and membrane fusion via non-canonical SNARE pairing between Stx2, 3, 4 or SNAP23 and the ER-localized Sec22b [233]. Interestingly, T4BSS-dependent K63-linked polyubiquitination of Sec22b stabilizes the non-canonical SNARE complex early during infection, and removal of the Ub approximately 4 h post-infection by another OTU-like DUB effector, LotB, leads to release of Stx3 from the complex and LCV [234]. Which Ub-ligase effector modifies Sec22b remains to be determined. Apart from SidC, AnkB and LubX at least nine other canonical Ub-ligase effectors have been identified [191, 235, 236]; however, knowledge about their host targets is limited. Intriguingly, the SNARE-like effector LegC3 produced by *L. pneumphila* also seems to be susceptible to ubiquitination and this is reversed by the metaeffector DUB LupA [109]. LegC3 and related SNARE-like effectors LegC2 and LegC7 were demonstrated to bind the SNARE VAMP4, usually involved in trans-Golgi network-to-endosome transport, in infected cells, and VAMP4 was found to be required for proficient intracellular replication [237]. *In vitro*, the LegC-VAMP4 complex could not be resolved by a canonical mechanism relying on the N-ethylmaleimide-sensitive factor NSF; however, the half-life of this complex in infection still needs to be established. It is tempting to speculate that ubiquitination regulates the activities of the three LegC SNARE effectors as well as potentially the additional SNARE mimic LseA [238] similar to non-canonical SNARE pairing by Sec22b.

A large number of additional targets have been identified for LotB and LotC [207, 209, 239]; and two additional OTU-like DUB effectors, LotA and Ceg7, exist [208, 240]. LotA contains two distinct catalytic sites, conferring the ability to cleave distinct ubiquitin chains, and upon its association with the LCV via a PI3P binding domain LotA trims the poly-Ub coat of the LCV (Fig. 6) [241]. While more work on target identification is required, the recent discoveries about the dynamic interplay of effector Ub-ligases and DUBs expose the Ub-system as a critical post-translational regulatory mechanism for the temporal exploitation of protein signalling complexes during infection.

While ubiquitination controls the release of non-canonical SNARE pairing, SidM and Rab1 also become decommissioned when they have fulfilled their roles [105]. The effector SidD is a deAMPylase, which removes the lock inflicted by the PTM [107, 242]; and, not relying on host cell factors, the PI4K effector LepB employs an additional Rab1 GAP domain to deactivate and expose Rab1 to membrane extraction by the GDP dissociation inhibitor (GDI) [105].

Additional layers of Rab1 regulation exist. Rab1 is also efficiently phosphocholinated by AnkX [103]. While AnkX displays some preference for inactive Rab1-GDP [243], conformational changes induced by tight binding of Rab1 through ankyrin repeat and FIC domains also allow modification of the active Rab1-GTP form [244]. Phosphocholination of Rab1 changes its interactome, for example preventing interactions with GEFs and the GDI, until the antagonistic effector Lem3 removes the PTM and restores conventional Rab1 interactions [106, 243].

Recently, it also emerged that Rab1 is a target of the glucosyltransferase effector SetA, a promiscuous *O*-glucosyltransferase, which irreversibly modifies serine and threonine residues of numerous proteins *in vitro* [245–248]. While the modification of many of the *in vitro* targets needs to be confirmed in infected cells, modification of Rab1 and Snx1 was validated [245, 249]. The modification of Rab1 occurs on Thr75 in its switch II region (Thr75) inhibiting its intrinsic GTPase activity and interaction with a GDI, but not affecting the interaction with SidM or AnkX [249]. In line with this, modification by these two effectors post-glucosylation was possible *in vitro*, but not the other way round. The exact timing and role of phosphocholination and glucosylation for the intricate regulation of Rab1 and the interplay with other PTMs still needs to be dissected.

Notably, most of the effectors that are characterized and discussed here are involved in early steps, the first 4 h, of LCV formation. Less is known about effectors that drive association with the rough ER and membrane expansion, once replication has started. *L. pneumophila* lacking the effector RavY was recently shown to form LCVs, which contain classical markers of the maturing LCV and sustain survival but not efficient replication [250], suggesting that RavY belongs to an effector subset that manipulates host processes, on or beyond the LCV, after evasion of the phagolysosomal pathway has succeeded.

Modulation of host cell metabolism and energetics

Mitochondria are the powerhouse of eukaryotic cells, generating ATP via oxidative respiration within eukaryotic cells [251]. *L. pneumophila* translocates effectors that modulate mitochondrial physiology. After the LCV is established, it transiently associates with the mitochondria [28, 168, 187, 252, 253]. While this association is T4SS-independent, subsequent T4BSS-dependent mitochondrial fragmentation was observed [254]. The effector MitF/LegG1 (Lpg1976) was implicated in this mitochondrial fragmentation in macrophages. MitF/LegG1 possesses an RCC1 domain, typically found in GEFs of the small GTPase Ran. MitF/LegG1 binds the endogenous Ran GEF RanBP10, recruits Ran to the LCV and activates it [255, 256]. Active Ran promotes the polymerization of microtubules, host cell migration and keeps the early LCV motile until it stabilizes in a perinuclear position

[255, 257]. In addition, activation of Ran triggers accumulation of DNM1L, a member of the dynamin GTPase superfamily at the mitochondria, which promotes mitochondrial fission [254]. This compromises oxidative phosphorylation and induces a Warburg-like metabolism, reflected by diminished oxidative phosphorylation and increased glycolysis, which benefits intracellular replication [254]. *L. pneumophila* encodes two other RCC1 effectors, PpgA and PieG, which evolved different subcellular localization signals and target different steps of the Ran GTPase cycle, RanGAP1 (PpgA/PieG) and Ran itself (PieG). Both effectors ultimately also activate Ran and promote LCV motility [256]; however, if this impacts the mitochondria in addition to MitF is not known.

The *Legionella* nucleotide carrier protein (LncP) is an effector that shares similarity with eukaryotic mitochondrial carrier proteins comprising six transmembrane segments that integrate into the inner mitochondrial membrane, depending on its membrane potential [258]. LncP is a nucleotide carrier protein displaying strong affinities for ATP as well as GTP, making it likely that it exports ATP from the mitochondria; however, a *L. pneumophila* Δ*lncP* deletion mutant is not attenuated and the exact role during infection needs to be validated [258, 259]. LncP might collaborate with the effector Ceg3, which inhibits host ADP/ATP translocases by ADP ribosylation, to manage ATP transport across mitochondrial membranes and thus cellular energy levels [260]. The inhibition by Ceg3 can be relieved by the effector Larg1, which is an ADP-ribose glycohydrolase that removes the PTM [261]. Additional effectors such as Lpg1625 and Lpg0898, which localize to the mitochondria in transfected cells [262], could enable *L. pneumophila* to further tighten control over the cellular power plant.

Given the central role of the mitochondria in powering the cell, any manipulation of their physiology is likely to generate stress that could culminate in their collapse and cell death. In addition, many other cell death pathways in response to extra- and intracellular signals become licensed at the mitochondria [263]. As cell death before the resources of the cell can be exploited would be inefficient for the bacteria, the bacteria mitigate stress and blunt apoptotic pathways at the mitochondria.

To maintain mitochondrial homeostasis *L. pneumophila* reverses the ATP-synthase activity of the mitochondrial F_OF_1-ATPase to hydrolyze ATP, preventing collapse of the mitochondrial membrane potential [259]. The effector LegS2/LpSpl, a sphingosine-1 phosphate lyase that localizes to the ER and mitochondria and modulates autophagy (discussed below), contributes to this; however, the mechanistic details and other involved effectors still need to be identified [259, 264, 265].

Two effectors, SidF and Lpg1137, block mitochondria-associated pro-apoptotic signalling [266, 267]. Apart from its role as a PI3P phosphatase, SidF binds BNIP3 and Bcl-rambo [267]. Upon stimulation BNIP3 and Bcl-rambo localize to the mitochondria where they activate the protein BAX, resulting in mitochondrial outer membrane permeabilization and apoptosis [263]. Sequestration of the two proteins by SidF was proposed to block apoptosis [267].

Lpg1137 is a serine protease that cleaves syntaxin Stx17, which controls mitochondrial dynamics and BAX-induced apoptosis, enabling Lpg1137 to block staurosporine-induced cell death [266]; however, as Stx17 is a multifunctional protein with additional roles, for example in autophagy, the main function of the effector in infection remains unclear. Detailed analysis of infection-induced cell death in murine macrophages did not find critical roles for SidF or the host proteins BNIP3, Bcl-rambo and BAX for intracellular replication and killing kinetics, suggesting that additional mechanisms promote survival of the host cell [268]. Differences in the effector repertoires of the used strains might result in different requirements for effectors or host proteins, showing that further analysis is needed.

Subversion of transcription and translation

Manipulation of the mitochondria and the associated shifts in cellular metabolism, but also the exploitation of various other resources, such as nutrients, and the remodelling of endomembranes probably cause an imbalance in cellular physiology and trigger stress responses. Moreover, in macrophages intracellular and extracellular pattern recognition receptors induce immune signalling, aiming to increase the resilience to infection. In recent years, an increasing number of effectors have been recognized that defuse stress signals and dictate host responses by targeting transcription and translation (Fig. 7) [269, 270]. A combination of Pathogen Associated Molecular Pattern (PAMP)-driven, direct effector-mediated and indirect effector action-triggered stimuli (feedback loops) leads to waves of transcriptional reprogramming over the infection course.

Upon infection of macrophages with *L. pneumophila*, the earliest trigger for an immune response is the detection of extracellular PAMPs or cytokines by cell surface receptors, such as Toll-like or TNF receptors, and signals from intracellular pattern recognition receptors such as Nod-like receptors follow suit (reviewed by [271]). While the initial signal steps differ, several signalling cascades converge on the activation of IκB kinase (IKK), which phosphorylates inhibitors of κB (IκB) inducing their Ub-dependent proteasomal degradation, liberating the NF-κB transcription factor subunits for transit into the nucleus [272]. In addition, receptor activation can be relayed through mitogen-activated protein kinases (MAPKs), which drive transcriptional reprogramming independently or together with NF-κB.

The effector Ceg4 was shown to be an atypical HAD-like phosphotyrosine phosphatase that blocks activation of MAPKs *in vitro*, suggesting that *L. pneumophila* can modulate MAPK signalling with effectors [273]. No effectors which directly bind and activate the MAPKs are known, but their activation at later stages of infection is promoted by the inhibition of translation by effectors (see

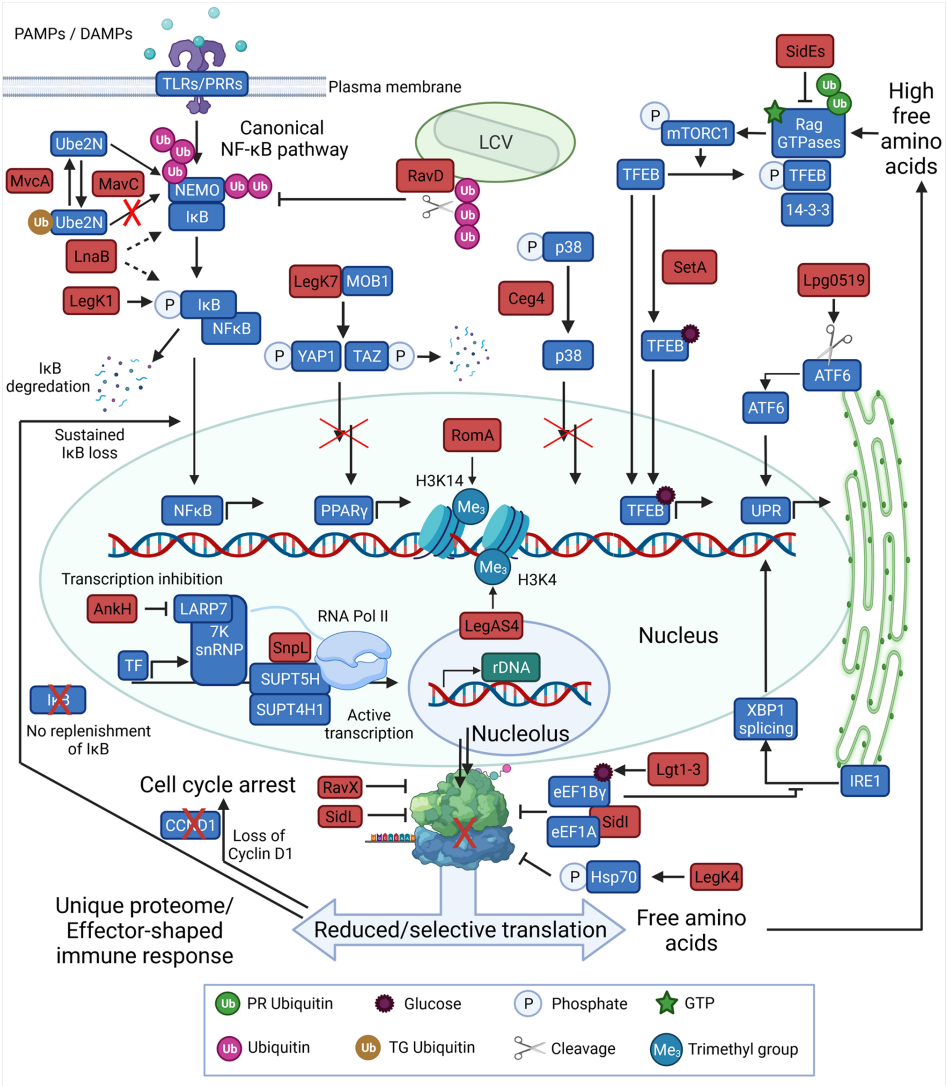

Fig. 7. Scheme summarizing the modulation of transcription and translation by Dot/Icm T4BSS effectors and the consequence for host cell physiology. Effectors manipulate cytoplasmic signalling cascades to block or trigger nuclear transit of transcription factors and modulate access to chromatin and activity of RNA polymerase II in the nucleus, shaping the global transcriptome. Congruent translation of the transcriptome into the proteome is thwarted by at least seven effectors, which inhibit translation. The translation block enables the bacteria to modulate ER stress responses, leads to a cell cycle arrest and increases the amount of free amino acids, which benefits intracellular replication. Decreased synthesis of signalling proteins such as IκB as well as increased amino acid levels result in feedback loops, which drive sustained NFκB signalling and activation of mTORC1 respectively. The activation of mTORC1 and subsequent cytosolic retention of the transcription factor TFEB is, however, dampened through the action of effectors of the SidE family and SetA. In macrophages, the sustained activation of NFκB promotes high transcription of selected immune genes, enabling escape from the block in translation and shaping a unique immune response. Ub, ubiquitin; PR, phosphoribosyl; AMP, adenine monophosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; TG, transglutamylated.

below) [274, 275]. *Legionella* species also encode effectors comprising eukaryotic Src homology 2 (SH2) domains (e.g. RavO), which are classical phospho-tyrosine binding domains involved in relaying signals, suggesting that these proteins tune into the host phosphorylation signalling networks [276].

Despite the absence of the NF-κB cascade in protozoa, *Legionella* translocates several effectors that manipulate signalling mediators (Fig. 7), suggesting that interactions with higher eukaryotic hosts occur in the environment or that protozoan targets have been integrated into new signalling pathways in mammals or evolved to serve as sensors for effector activities, similar to plant resistance proteins.

The effector MavC is another type of non-canonical Ub-ligase, which ligates Ub in a transglutamination reaction onto a lysine on the E2 Ub-conjugating enzyme UBE2N/Ubc13 [116, 277, 278], blocking its active site [279]. UBE2N usually catalyzes the

formation of K63-linked Ub chains and has vital functions in a large number of cellular processes, including ubiquitination of signalling components upstream of IKK such as NF-κB essential modifier (NEMO), a component of the IKK complex, leading to activation of IKK and thus induction of downstream NF-κB signalling [280]. Thus, by inactivating UBE2N, MavC dampens NF-κB signalling. However, this is only upheld within the first hour of infection because MvcA, a paralogue of MavC, is translocated shortly after MavC and reverses the ubiquitination of UBE2N [112, 113]. Interestingly, *L. pneumophila* can shut down both paralogues using the effector Lpg2149, which blocks access of Ub to the active site of the enzymes [113, 279]. This might allow precise temporal control or prevent undesired effects of the production of deamidated Ub, which MavC and MvcA generate in the absence of a protein target [113, 116].

The assembly of linear Ub chains on the N-terminal methionine of proteins plays also an important role in NF-κB signalling [281]. For example, in addition to K63-poly-ubiquitination, NEMO is also modified with a linear Ub chain, which enhances NF-κB signalling. The effector RavD is a DUB, which specifically cleaves linear Ub chains, reducing NF-κB signalling in concert with MavC [282].

While blocking NF-κB signalling at early stages of infection benefits the bacteria by preventing pro-inflammatory and antibacterial mechanisms, sustained activation of NF-κB later is believed to facilitate increased expression of pro-survival genes, allowing pro-longed exploitation of the host cell [283]. Two effectors drive NF-κB activation. LegK1 is a eukaryotic-like serine/threonine kinase and phosphorylates IκBα, leading to its ubiquitination and degradation [284]. In addition, LegK1 also promotes maturation of p100 into p52 (NF-κB2). Both events result in activation of NF-κB signalling and contribute to increased expression of NF-κB regulated genes. The effector LnaB also activates NF-κB signalling, but its mechanism of action has not yet been elucidated [285]. Notably, while both effectors are potent NF-κB activators upon ectopic expression, only an *L. pneumophila* Δ*lnaB* mutant showed a moderate reduction of NF-κB activity during infection [285], indicating that LegK1 might have alternative targets, or its activity is blunted by other effectors.

Additional phosphorylation-dependent signalling cascades are activated by *L. pneumophila*. The effector LegK7 highjacks the Hippo pathway, which is highly conserved across eukaryotes and regulates apoptosis, cell proliferation and developmental processes [286]. LegK7 mimics MST1/Hippo kinase, a core component of the Hippo kinase complex, and phosphorylates its target MOB1A [287]. Phosphorylation of MOB1A probably activates downstream kinases, such as LATS1/2, that relay the cascade resulting in phosphorylation of the co-transcriptional regulators YAP1 and TAZ, promoting their interaction with 14-3-3 proteins, retention in the cytoplasm and degradation [287]. Moreover, phosphorylation of MOB1A also promotes association with and enhances the kinase activity of LegK7 and the recruitment of downstream signalling components of the Hippo pathway, possibly YAP1, which in turn are directly phosphorylated by LegK7 [287]. The action of LegK7 leads to remodelling of the transcriptome, including upregulation of genes driven by PPARγ, a TAZ-modulated transcription factor, which was required for full virulence in infected macrophages; however, the consequences of LegK7 signalling at the proteome level have not yet been characterized [287].

Nucleomodulin effectors shape chromatin and transcriptional activity

Modulation of cytoplasm to nucleus signalling is not the only step targeted by *L. pneumophila* to control transcription. *L. pneumophila* produces nucleomodulins, a growing class of bacterial effectors that enter the nucleus and modulate DNA accessibility in nucleosomes and the transcriptional machinery (Fig. 7) (reviewed in [288]).

The effector RomA (*L. pneumophila* Paris)/LegAS4 (*L. pneumophila* Philadelphia) contains a canonical eukaryotic nuclear localization signal (NLS), allowing exploitation of the nuclear import machinery, and possesses a eukaryotic-like SET domain, that transfers a methyl group from *S*-adenosyl-L-methionine to the amino group of a lysine residue of histones, a key host mechanism to shape chromatin accessibility for transcription [289–291]. While RomA and LegAS4 share 96% similarity and both target histones, distinct modification sites are reported. RomA trimethylates K14 of histone H3, outcompeting acetylation of the residue leading to repression of transcription [290]. During infection of human macrophages, the modification affects genome-wide at least 4870 genes, including important immune genes such as TNFα and IL-6. Reduced intracellular replication of an *L. pneumophila* Δ*romA* indicates that this wide-reaching modulation is important for full virulence [290]. Additional, non-histone targets of RomA were identified, which might contribute, but their roles in infection remain to be established [292].

While *L. pneumophila* Philadelphia also induces robust H3K14 methylation during infection [270, 290], dependency on LegAS4 needs to be validated. Work so far has found that recombinant LegAS4 performs di- and trimethylation of H3K4 and H3K9 respectively [289]. In eukaryotic cells LegAS4 mainly localizes to the nucleolus, where an interaction with the heterochromatin proteins (HP) 1α and γ promotes ribosomal DNA (rDNA) association, leading to methyltransferase-dependent upregulation of rDNA transcription [289]. However, the absence of LegAS4 did not result in a notable change of rRNA gene transcription during infection with *L. pneumophila* Δ*legAS4*, suggesting the presence of functionally redundant effectors or other functions of LegAS4 during infection [289].

After a transcription factor occupies the promoter of a gene at an accessible chromatin site, RNA polymerase II (Pol II) is recruited; however, transcription initiation and elongation are further regulated by a multitude of protein and protein–RNA complexes [293, 294]. Pausing of Pol II 30–60 nt downstream of the transcription start site has emerged as a key, rate-limiting

step [295] and is facilitated by the negative elongation factor (NELF) and DRB-sensitivity-inducing factor (DSIF), which contains two subunits, SUPT4H and SUPT5H. Release from pausing is predominantly mediated by the positive transcription elongation factor-b, p-TEF-b, which phosphorylates multiple proteins including Pol II and NELF, leading to dissociation of NELF and a switch of DSIF from an elongation-inhibiting to an activating factor [296]. P-TEF-b itself underlies tight regulation and is initially recruited to promoter-proximal regions in its inactive form as part of the 7SK small nuclear ribonucleoprotein (snRNP) complex. Reshaping or disassembly of the 7SK snRNP by a number of host phosphatases and/or RNA helicase frees P-TEF-b and enables it to relieve pausing of Pol II [296].

Two effectors, SnpL and AnkH, enter the nucleus despite not containing a canonical NLS and interfere with the machinery regulating Pol II (Fig. 7). SnpL directly translocates to the nucleus, where it binds to SUPT5H [297]. Ectopic expression of SnpL leads to global upregulation of gene expression in macrophages, suggesting the SnpL–SUPT5H interaction suppresses pausing of Pol II, and is associated with increased macrophage death [297]. AnkH/LegA3, one of the conserved core effectors, sequesters the La-related protein 7 (LARP7), a component of the 7SK snRNP, disrupting the formation of the functional complex and probably releasing p-TEFb to drive transcription [298]. How SnpL and AnkH synergize during infection remains to be elucidated. SnpL is dispensable for efficient replication in murine macrophages and *A. castellanii* [297], whereas deletion of AnkH leads to the differential regulation of 405 genes in human macrophages and a replication defect [298–300]. This suggests that AnkH might have a dominant role, but alternative activities of AnkH might contribute to the replication defect. AnkH has a cysteine protease fold, and the residues of the putative catalytic triad are essential to complement an *L. pneumophila ΔankH* mutant [298]. However, actual protease activity, substrates and a link to the modulation of transcription still need to be established.

Inhibition of translation

While there are clear, global changes to the transcriptional landscape of *L. pneumophila*-infected cells, several studies revealed that these do not translate one-to-one into the proteome due to the inhibition of protein synthesis by at least seven effectors, Lgt1, Lgt2, Lgt3, SidI, LegK4, SidL and RavX (Fig. 7) (reviewed by [269]). The mechanisms of action of SidL and RavX [129, 301] have not yet been determined, but activities of the other effectors have been characterized.

The three homologous effectors, Lgt1, Lgt2 and Lgt3, are metal ion cofactor-dependent type A glucosyltransferases and all mono-glucosylate the translation elongation factor eEF1A on S53, blocking its function in providing charged aminoacyl-tRNA for peptide chain extension [302–304]. While the modification of eEF1A efficiently blocks translation *in vitro* and these effectors are cytotoxic upon ectopic expression or microinjection, there is no strong evidence for a role in killing host cells during infection, suggesting that the modulation of the host proteome is the dominant role. Notably, while eEF1A plays an essential role in translation, it is a moonlighting protein involved in many cellular processes [305, 306]. Dissection of the impact of glucosylation on these functions as well as on additional targets such as Hsp70 subfamily B suppressor 1 (Hbs1), which is involved in salvaging stalled ribosomes [307], is an interesting area to be explored.

SidI also binds eEF1A and in addition eEF1Bγ, blocking translation [308]. SidI shares similarities with type B glycosyltransferases and hydrolyzes the sugar-donor GDP-mannose [123]. However, neither mannosyl transfer nor other SidI-dependent PTMs on eEF1A or other proteins have yet been detected [308]. SidI is inhibited by the metaeffector MesI [123, 309] and a *L. pneumophila* strain lacking MesI is attenuated in infection models [122], indicating that SidI is only beneficial at a certain activity level or time point during infection.

The effector LegK4 is a eukaryotic-like threonine kinase, which phosphorylates Hsp70 family chaperones at a single conserved residue [301, 310, 311]. Phosphorylation impairs the protein refolding capacity of the chaperone *in vitro*. In cells, phosphorylation of Hsp70 leads to its accumulation on translating ribosomes and causes a global block of translation, probably due to the compromised ability of Hsp70 to support folding of nascent proteins, stalling protein synthesis [311].

The enrichment of effectors targeting translation in *L. pneumophila*'s arsenal shows that this is an important mechanism of host subversion, which is covered by functional redundancy. Indeed, not all strains encode the full set of effectors. In particular, Lgt1–3 seem to overlap in their function; however, differential regulation of expression and different additional domains suggest that specific Lgt effectors might block translation only at different stages or cellular locations during infection and/or could have different additional targets beyond translation [304]. Notably, infection with *L. pneumophila* lacking all seven effectors still inhibits translation at a high level [312]. An *L. pneumophila* strain lacking Lgt1, Lgt2, Lgt3, SidI and SidL (Δ5) is attenuated in its growth in *D. discoideum*; however, neither the *L. pneumophila* Δ5 nor a strain lacking also RavX and LegK4 (Δ7) shows a replication defect in macrophages [129, 313]. The mTOR-dependent downregulation of translation initiation in response to pathogenic *L. pneumophila* probably contributes to this [314], but additional contributing effectors might exist [312, 313].

Consequences of translation inhibition and feedback signalling

Analysis of *L. pneumophila* Δ5 or Δ7 strains revealed that the overall block of translation has a profound impact on cellular signalling pathways and homeostasis, as protein pools that underlie continuous turnover cannot be replenished [129, 301]. This effect might be amplified by the increased ubiquitination and proteasomal degradation of proteins triggered by the bacteria. One

of the consequences of this is the rapid loss of the cyclin D1, a key regulator of cell cycle transition from G1 to S phase, which arrests the cell cycle prior to entry into S phase [315]. This arrest facilitates intracellular replication, as transition would destabilize the LCV and cells in S phase are less conducive to infection [315, 316]. This effect depends mainly on Lgt1 and Lgt3 and can be recapitulated by ectopic expression of the effectors.

Similarly, when translation is blocked, the failure to replenish the pool of IκB after the initial PAMP-driven spike in NF-κB activity early during infection leads to sustained activation of NF-κB signalling [129, 312]. This enhances the ability of host cells to respond to the translation blockade with the superinduction of some immune mediators such as IL1α and IL23α, overcoming the block in translation and producing a specific immune response to infection [129, 312, 317].

Biogenesis of the LCV is dependent on exploitation and remodelling of the ER, which has a key role in ensuring proper folding of newly synthesized proteins and maintaining proteostasis [318]. Three ER transmembrane proteins, PKR-like endoplasmic reticulum kinase (PERK), inositol-requiring kinase 1 (IRE1) and activating transcription factor 6 (ATF6), monitor deviations from ER homeostasis and trigger the unfolded protein response (UPR) [319]. The UPR signalling cascades slow translation and upregulate ER chaperones such as BiP or, in the case of catastrophic stress, pro-apoptotic factors such as CHOP [319]. In the absence of translation inhibiting effectors, *L. pneumophila* PAMPs and probably the exploitation and remodelling of the ER are potent triggers of the UPR, inducing XBP1 splicing, a hallmark of IRE1 signalling activation, and leading to upregulation of BiP and CHOP [320, 321]. Upon ectopic expression, Lgt1 and Lgt2 and to a lesser extent Lgt3, but not SidL or SidI, block chemically induced XBP1 splicing, and overexpression of Lgt2 or Lgt3 in *L. pneumophila* Δ5 restores the capacity to inhibit XBP1 splicing during infection, suggesting that these glucosyltransferase effectors are the main means of the bacteria to inhibit this pathway [320, 321].

Interestingly, investigation of the other two arms of the UPR revealed that, while no PERK activation occurred, infection with *L. pneumophila* Philadelphia but not Paris triggered a T4BSS-dependent non-canonical proteolytic activation of ATF6 and translocation of an ATF6 fragment into the nucleus where it enhanced transcription of UPR genes such as *BiP* [321, 322]. The effector Lpg0519 was identified as a likely candidate, which induces ATF6 cleavage upon ectopic expression; however, Lpg0519 does not share homology with known proteases and the mechanism therefore remains to be established [322]. Notably, while transcriptional upregulation of *BiP* is observed, production of the BiP protein is suppressed by LegK4 and other inhibitors of translation, leaving the question in which way activation of the ATF6 arm of the UPR benefits the bacteria unanswered [311].

Apart from modulating signalling, the global inhibition of translation by *L. pneumophila* has also been considered a means to increase the level of free amino acids, which are available as nutrients for replication. In eukaryotic cells, mTORC1 is the central master regulator, which integrates nutrient and energy states as well as exogenous signals to maintain homeostasis [323]. Activation of mTORC1 by high amino acid levels typically leads to phosphorylation and sequestration of the transcription factor TFEB in the cytosol, downregulating autophagy and lysosome biogenesis and upregulating translation initiation [323].

Translation inhibition by Lgt1-3 activates mTORC1, indicating that amino acids indeed become available; however, this activation is limited by SidE-family-mediated phosphoribosyl ubiquitination of Rag GTPases, which are essential amino acid-driven regulators of mTORC1 activity [313, 323]. This dampening of mTORC1 should preserve increased nuclear levels of TFEB. This might be further promoted by SetA [324]. Ectopically expressed SetA glucosylates several Ser/Thr residues of TFEB, of which S138 modification prevents export of TFEB from the nucleus and S211 modification perturbs binding to 14-3-3, which usually retains TFEB in the cytosol. In addition to these effectors, the effector WipB *in vitro* binds the two lysosomal v-ATPase subunits B and d1 and LAMTOR1, which are part of the lysosomal nutrient sensing (LYNUS) apparatus that modulates mTORC1 signalling, suggesting additional manipulations of this signalling hub, but the impact of WipB during infection remains unknown [325]. While TFEB might drive the expression of unknown genes, which are beneficial for the bacteria, it typically upregulates autophagy and lysosome biogenesis. These processes are commonly associated with adverse impacts on infections, but *L. pneumophila* has evolved additional effectors to mitigate these effects.

Modulation of autophagy and other cell autonomous defence mechanisms

Autophagy is a key process for recycling cellular components such as damaged organelles and liberation of nutrients during starvation, but also for the sequestration of intracellular pathogens, termed xenophagy [189, 326, 327]. The initiation and maturation of autophagosomes that ultimately fuse with lysosomes is highly regulated, involving a multitude of protein–protein and protein–lipid interactions [189]. Poly-Ub chains, branched and linear, on proteins, bacteria and their vacuoles can serve as a trigger and ligands for cargo-selecting adapters, e.g. p62/SQSTM1, NBR1, NDP52 or optineurin, which subsequently recruit the autophagic machinery [326], which itself is fine-tuned by dynamic ubiquitination [328].

L. pneumophila efficiently prevents recruitment of autophagy cargo adaptors, p62, NBR1 and optineurin on the LCV, whereas NDP52 accumulates in its proximity [329]. SidE-family proteins have been implicated in exclusion of p62 and redistribution of NDP52, suggesting that phosphoribosyl ubiquitination of proteins has different effects on recognition by these adaptors [329].

Removal of linear Ub chains from the LCV by RavD probably also contributes to the evasion of autophagy, in addition to its role in the blockade of NFκB signalling [282] (Fig. 5). While no direct evidence for this exists, increased association of the lysosomal marker LAMP-1 with LCVs containing the *L. pneumophila* Δ*ravD* mutant [146], could point to a reduced ability to prevent mechanisms driving autophagolysosome maturation. Other DUB effectors [192] that shape the poly-Ub landscape in the cell are likely to modulate this trigger as well as subsequent steps of autophagosome formation. However, to protect the LCV from autophagy despite acquisition of large amounts of poly Ub-chains during maturation of the LCV, *L. pneumophila* employs also additional effectors, which target different steps of autophagosome formation.

The effector LegS2 (Lpg2176)/LpSpl (Lpp2128) is highly homologous to and can functionally replace eukaryotic sphingosine-1 phosphate lyases, strongly supporting a trans-kingdom gene transfer event as the origin for the effector [264, 265]. Sphingolipids are increasingly recognized as regulators of autophagy, in particular in response to nutrient limitation [330]. *L. pneumophila* infection of macrophages reduces sphingolipid levels [265]. LpSpl contributes to the modulation of sphingolipid metabolism, in particular reducing sphingosine levels in infected human macrophages, and dampens autophagy at late stages of infection [265]. It might therefore contribute to the effort to balance the host's reaction to diminishing resources, which are consumed by replicating bacteria.

While interfering with the triggers of autophagy is efficient, *L. pneumophila* also blocks autophagy by targeting the proteins executing it. As discussed above, cleavage of Stx17 by Lpg1137 may contribute to suppression of apoptosis, but Stx17 is also integral to formation of pre-autophagosomal structures as well as the fusion of autophagosomes with lysosomes [331, 332] and its cleavage blocks autophagy *in vitro*.

The cysteine protease effector RavZ targets Atg8 (LC3) [333]. The ubiquitin-like protein Atg8, controlled by reversible lipidation with a phosphatidylethanolamine (PE) moiety on a C-terminal glycine residue, associates with membranes where it acts as a critical driver of cargo incorporation and autophagosomal membrane expansion [334]. It also binds autophagy adapters such as p62 to associate cargo with the early autophagosomal membrane. RavZ specifically recognizes the PE-conjugated protein on membranes and removes the C-terminal modified glycine, disrupting autophagosome formation and irreversibly deactivating the Atg8 [333, 335]. The mechanism of action of RavZ has been studied at the atomic level, involving membrane curvature recognition, protein–lipid and protein–protein interactions directing it specifically to its target on the early autophagosome membrane (reviewed recently [336]). During infection RavZ localizes to the LCV using its PI3P binding domain, protecting the LCV from autophagy, but in co-infection models the effector also disrupts xenophagy of *Listeria monocytogenes in trans*, suggesting that it operates throughout the cell [329]. Interestingly, while RavZ does not impact the ubiquitination of the LCV, it can also disrupt the ubiquitination of *Salmonella*-containing vacuoles even in the absence of Atg7, which mediates lipidation of LC3, suggesting that it might have additional targets or activities [337]. Notably, RavZ is not conserved in the majority of *L. pneumophila* strains, which are probably equipped with effectors that can replace it.

Despite the autophagy-inhibiting effector set, *L. pneumophila* also encodes effectors that appear to promote autophagy. A *L. pneumophila* Δ*legA9* strain showed reduced ubiquitin and p62/SQSTM1 association with LCVs in murine bone-derived macrophages [338]. Replication of the mutant was improved, strongly suggesting that LegA9 carries out an unknown host manipulation, which unintentionally promotes xenophagy. Alternatively, if its primary function is stimulation of autophagy, this might be beneficial in specific protozoan but not mammalian hosts. Only dissection of the exact mechanism will clarify which scenario applies.

Indeed, a growing number of effectors are linked to effector-triggered immunity due to different connectivity of their targets with host cell physiology in amoeba and in mammalian host cells (reviewed by [339]). The effector LegC4 promotes replication in amoeba but triggers increased cytokine release leading to growth restriction in murine macrophages [340]. LamA is an amylase that degrades glycogen, enabling the bacteria to interfere with encystation of amoeba; however, depletion of glycogen in human monocyte-derived macrophages induces pro-inflammatory signalling and nutritional immunity, through increased tryptophan depletion, limiting bacterial replication [341].

Exit from host cells

When nutrients become limited, due to consumption by replication or sequestration by the host, *L. pneumophila* undergoes a switch from replicative to its non-replicative, more infectious (transmissive) form [342, 343] (Fig. 1). While many triggers and sensors for this switch inside the macrophages still need to be discovered, limitation of iron was established as a critical factor. During the replicative phase, *L. pneumophila* employs the effector MavN/DimB (Lpg2815)/IroT (Lpp2867), one of the conserved 'core' effectors, to scavenge iron [3]. MavN is an iron-regulated, multipass transmembrane protein that integrates into the LCV membrane and is critical for the import of iron (Fe^{2+}) and to some extent also Mn^{2+}, Co^{2+} or Zn^{2+} into the LCV, and deletion of MavN leads to a severe replication defect [344–347]. Intriguingly, experiments with bacteria compromised in iron import show that iron limitation results in growth arrest as well as early exit from macrophages, highlighting the essentiality of iron as a nutrient source for intracellular replication and a cue for phenotypic adaptation [348].

Given the importance of metal ions for bacterial replication, restriction of their bioavailability is an antibacterial defence mechanism [349]. The small molecule phytate (*myo*-inositol hexakisphosphate), which is highly abundant in plants, but also synthesized by mammalian cells and protozoa, serves as phosphorus storage, but also chelates and withdraws metal ions from free circulation. Phytate impedes growth of *L. pneumophila*; however, the bacteria translocate the effector LppA, which degrades phytate, to overcome this inhibition [350].

Many aspects of the proteins that trigger and mediate host cell exit remain elusive. As discussed in the context of the functional description of SdhA, the release of bacteria from the LCV into the cytoplasm of macrophages can activate cell death pathways that trigger permeabilization of host cell membranes and liberation of the bacteria even without their active involvement. However, after successful exploitation of the host cell the bacteria can also actively promote their exit, and host type-specific, lytic or non-lytic, mechanisms seem to exist [351–354]. The effectors LepA and LepB localize to the LCV membrane and were implicated in facilitating non-lytic release, e.g. export of vesicles containing bacteria, from protozoa without host lysis [352, 355]. Mutants lacking the effectors also cause enhanced red blood cell lysis [352, 355]. However, the molecular mechanisms and host cell factors underlying these phenotypes and how they correlate with subsequently described biochemical activities of LepB remain unclear. Single cell studies of the last stages of infection of *A. castellanii*, *D. discoideum* and macrophages showed that within the LCV the bacterial population is heterogenous and LCV membrane-associated bacteria switch first from the replicative to transmissive form, including the characteristic production of flagella, and elaborate T2SS- and T4BSS-secreted phospholipases (PlaA, PlaB, PlaD, PlcC) that participate in the lysis of the LCV and host cell [356]. Individual contributions of the lipases and other bacterial factors involved in lytic exit and how non-lytic release could be orchestrated by effectors still need to be elucidated.

CONCLUSIONS

Since their discovery, the Dot/Icm T4BSS and its multitudinous effectors have fascinated and puzzled scientists. Why would a pathogen accumulate so many, seemingly redundant effectors and how could one secretion system deliver them? Seminal work, summarized in this review, provided extraordinary insight into the molecular architecture of the T4BSS, allowing formulation of models for effector loading and transport across the bacterial cell envelope, and has revealed that *L. pneumophila* does not simply flood the host with effectors, but imposes its own regulatory network, enabling fine-tuned spatial and temporal control of virtually all key host processes. This reminds of an occupant, who comes to stay rather than just an intruder who steals valuables and moves on. It is tempting to speculate that it indicates that long-term associations might be common between *Legionella* and environmental hosts. Furthering our understanding of the interaction with environmental hosts will in the future go hand in hand with the grand challenge to reveal the full complexity of the *L. pneumophila* effector network and how the T4BSS operates to deliver the right effector at the right time across the host membrane.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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