

## Invited Mini Review

Ubiquitin-regulating effector proteins from *Legionella*Minwoo Jeong<sup>#</sup>, Hayoung Jeon<sup>#</sup> & Donghyuk Shin<sup>\*</sup>

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Ubiquitin is relatively modest in size but involves almost entire cellular signaling pathways. The primary role of ubiquitin is maintaining cellular protein homeostasis. Ubiquitination regulates the fate of target proteins using the proteasome- or autophagy-mediated degradation of ubiquitinated substrates, which can be either intracellular or foreign proteins from invading pathogens. *Legionella*, a gram-negative intracellular pathogen, hinders the host-ubiquitin system by translocating hundreds of effector proteins into the host cell's cytoplasm. In this review, we describe the current understanding of ubiquitin machinery from *Legionella*. We summarize structural and biochemical differences between the host-ubiquitin system and ubiquitin-related effectors of *Legionella*. Some of these effectors act much like canonical host-ubiquitin machinery, whereas others have distinctive structures and accomplish non-canonical ubiquitination via novel biochemical mechanisms. [BMB Reports 2022; 55(7): 316-322]

## INTRODUCTION

Regulation of cellular activities relies on various post-translational modifications (PTM), such as phosphorylation, glycosylation, or acetylation (1-3). Unlike the aforementioned PTM that transfers chemical moieties to the targets, ubiquitination ligates ubiquitin protein to the substrates (4, 5). The covalent attachment of the C-terminal carboxyl group of ubiquitin to the amine group of lysine residues on a target protein is called monoubiquitination. Ubiquitin also forms different types of chains, called polyubiquitin, through the ubiquitination on an amino group of 7 lysine residues (K6, K11, K27, K29, K33, K48, K63) or the first methionine (M1) residue of ubiquitin. The diverse conformational complexity of polyubiquitin chains gives cells abilities to differentiate and regulate various cellular processes. M1-linked linear polyubiquitin chain participates in inflammation and im-

mune response. In the NF-kappaB (NF-κB) pathway, linear ubiquitin chain assembly complex (LUBAC) modifies NEMO, a subunit of inhibitor of κB (IκB) kinase (IKK) with M1 linkage that triggers phosphorylation of IκB (6-10). K6-linked polyubiquitin participates in DNA repair events and mitochondria stability (11-13), while K11- and K48-linked polyubiquitin is related to proteasomal degradation (14-17). K27-linked polyubiquitin controls DNA damage response and innate immune response. For instance, K27-linked polyubiquitination on histone 2A caused by RNF168 is related to DNA damage response (18). K29-linked polyubiquitination is associated with the Wnt signaling pathway, which regulates embryogenesis and tumorigenesis. K29-linked polyubiquitination on Axin, a scaffold protein in the Wnt signaling, disturbs interaction with LPR5/6 and inhibits the Wnt signaling (19, 20). It is known that K33 linkage participates in the regulation of T-cell antigen receptor and AMP-activated protein kinase-related protein kinase (21, 22). Together with K11-linked chain, K48 linkage plays a crucial role in the proteasome-dependent degradation pathway and ERAD pathway. Ubiquitin receptor on proteasome recognizes homotypic K48 linkage chain on the substrates and guides the substrate to enter the proteasome (15, 23). K63 linkage participates in proteasome DNA damage repair and endosomal-lysosomal system (24, 25). Also, K63 linkage plays an essential role in immune signaling. K63 polyubiquitin chain acts as docking sites in immune pathway proteins. K63-linked polyubiquitin chain on MALT1 is related to IκBα degradation and NF-κB activation (26). Moreover, K63 linkage is associated with immunomodulatory function in T cells and NLR-mediated signaling, and STING signaling in viral infections (27, 28).

The canonical ubiquitination system consists of three stages of the catalytic cascade (1, 29). The ubiquitin-activating enzyme (E1) hydrolyzes ATP and catalyzes acyl adenylation of the ubiquitin. The AMP-Ub intermediate forms a thioester bond with the catalytic cysteine of the E1 (1, 30). Next, Ub on the E1 is transferred to the catalytic cysteine on the ubiquitin-conjugating enzyme (E2) (31, 32). Finally, E2 works with the ubiquitin ligation enzyme (E3) to attach Ub to the substrate. E3 ligases are divided into three major classes (HECT, RING, and U-box, RBR). Each of them has a unique mechanism to transfer ubiquitin to a substrate (33, 34). Most E3 ligases make an isopeptide bond between the carboxyl terminus of the ubiquitin and the amino group of Lys on the target protein or preceding Ub (1). All E3 ligases bind to E2-Ub thioesters and catalyze the transfer of ubiquitin from E2 to the substrate lysine. In particular,

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HECT (Homologous to E6-associated protein C terminus) type E3s have catalytic cysteine that accepts ubiquitin from E2 via the transthioesterification reaction (35). In contrast, RING (Really Interesting New Gene) type E3 ligases have no active site residues and mediate direct ubiquitin transfer from E2 to the substrates. RING E3 ligase contains a RING (or RING-like) domain responsible for binding to E2 and stimulating the ubiquitin transfer (36). The RING domain generally adopts a cross-brace structure with two structural Zn<sup>2+</sup> ions. A related domain, known as a U-box, is similar in function and structural fold but has a hydrophobic core instead of the structural metal ion (37). The RBR (RING-between-RING) has two RING domains but combines roles of both RING and HECT E3 ligase (38, 39). One of the RING domains of RBR binds to E2 like the canonical RING domain, while the other RING domain accepts ubiquitin in a similar way to the HECT E3 ligases. Similar to other PTM, ubiquitination is also a reversible process, and the ubiquitin on the target proteins is recycled by deubiquitinase (DUB), which cleaves the isopeptide bond between ubiquitin and the substrate. About 100 types of DUBs have been identified in humans, and they are classified into seven superfamilies. Six of them (USP, OTU, MJD, UCH, MINDY, and ZUFSP) are cysteine proteases, whereas JAMM belongs to a zinc-containing metalloprotease (40, 41).

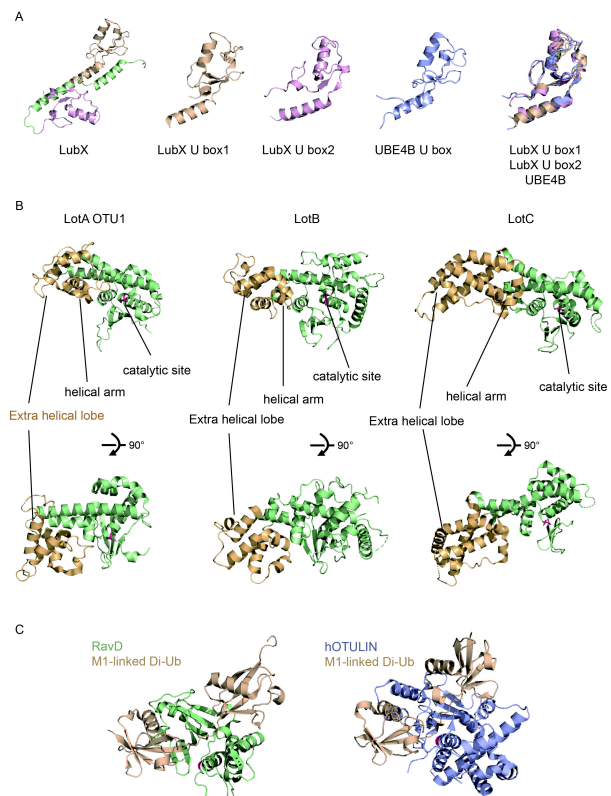
*Legionella pneumophila* is a gram-negative pathogenic bacteria causing Legionnaires' disease (42). *Legionella* uses more than 300 effector proteins during the infection. These effectors disrupt the host cellular processes and create an ideal environment for bacterial survival and replication inside the host cell (43). Many of these effectors target the host-ubiquitin system. For instance, RavZ (Region allowing vacuole colocalization Z) hydrolyzes carboxyl-terminal glycine of Atg8 and blocks the host autophagy (44). The SidE (Substrates of Icm/Dot transporter E) family effector mediates non-canonical phosphoribosyl ubiquitination, which is required for ER- or Golgi compartment disruption (45, 46). In addition, RavD (Region allowing vacuole colocalization D) effector cleaves the M1 polyubiquitin chain to block the host NF- $\kappa$ B signaling pathway (47). These results show that *Legionella* has developed various machinery that alters host ubiquitin signaling in canonical or non-canonical ways. Structural and biochemical studies on these effectors revealed that some effectors mimic the host proteins to participate in the host cellular processes, whereas others have completely different structures and mediate novel biochemical reactions. This review highlights structural and biochemical differences of *Legionella* proteins involved in the host ubiquitin system.

## CANONICAL UBIQUITIN SYSTEM-RELATED *LEGIONELLA* EFFECTORS

### LubX (*Legionella* U-box E3-ligase)

Some effectors have been demonstrated to interact directly with the host ubiquitination machinery using the U-box or F-box domain. Both LegU1 and LegAU13 are F-box proteins and inte-

grate into mammalian SCF complexes (Skp, Cullin, F-box containing complex), and the LegU1 SCF complexes ubiquitinate host chaperone protein BAT3 (48, 49). Another *Legionella* effector, LubX (Lpg2830), is a U-box E3 ligase (50). Like the eukaryotic U-box, LubX has E2 specificity and interacts with eight E2s (UBE2D1, UBE2D3, UBE2D2, UBE2D4, UBE2E2, UBE2E3, UBE2W1, and UBE2L6) (51, 52). Intriguingly, LubX has two U-box motifs (U-box-1 and U-box-2). However, the LubX U-box-2 domain alone shows no E3 activity, whereas LubX U-box-1 is sufficient for interacting with E2s and catalyzes polyubiquitination. Structural analysis of LubX U-box-1 in a complex with human UBE2D2 reveals that most of the LubX U-box-1:UBE2D2 binding interface residues are not conserved in LubX U-box-2 (53). The overall structure of LubX:UBE2D2 is similar to the human UBE4B U-box:UBE2D3 complex (Fig.



**Fig. 1.** Structures of canonical ubiquitin system-related *Legionella* effectors. Structures of effectors that participate in or regulate canonical ubiquitin systems are presented. (A) Crystal structures of LubX (PDB:4WZ3), LubX U-Box-1 (PDB:4WZ3), LubX U-Box-2 (PDB:4X11), and human UBE4B U box (PDB:3L1Z) are shown (from left to right, respectively). Superimposed structures of LubX U-box-1/2 and human UBE4M U-box are shown on the right. (B) Crystal structures of LotA OTU1 (PDB:7F9X), LotB (PDB:6KS5), and LotC (PDB:6YK8) are presented. The conserved OTU catalytic core is depicted as green, and the extra helical lobe (EHL) is yellow. (C) Complex crystal structures of RavD:di-ubiquitin (PDB:6NJD) and hOTULIN:di-ubiquitin (PDB:3ZNV).

1A) (54). However, residues at the binding interface differ from each other. Although both LubX U-box-1 and human UBE4B bind to the Ala-Pro-Ser hydrophobic patch on E2s, LubX U-box-1 makes additional hydrogen bond networks between Ile39-Arg5, Lys68-Ser91, and Arg75-Glu92. Importantly, these residues are not conserved in LubX U-Box-2. These structural analyses indicate that *Legionella* U-box distinguishes E2s through the sequence diversity on the U-box:E2 interface, just as the other U-box does. Functional roles of LubX E3 ligase is also reported. As a metaeffector, LubX target another substrate of the Dot/Icm system (52). LubX promotes human Cdc2-like kinase1 (Clk1) degradation. Clk1 plays an important role in the modulation of host cellular processes. As a result, Clk1 contributes to maximum *Legionella* growth in mouse macrophages. In addition, LubX mediates proteasomal degradation of SidH (another effector protein of Dot/Icm system) (51, 52).

#### **Legionella OTU-like deubiquitinases (Lot-DUBs)**

Not only E3 ligases but also deubiquitinases are found in *Legionella*. Among seven DUB superfamilies, the OTU (Ovarian Tumor deubiquitinase) family explicitly shows linkage specificity toward polyubiquitin chains (55). For example, human OTUB1 preferentially cleaves the Lys48-linked chain, and human OTULIN cleaves the M1-linked chain (56-58). Interestingly, *Legionella* has several OTU-like DUBs (*Legionella* OTU-like DUBs, LotA, LotB, and LotC), and they also show different linkage specificities (59). Intriguingly, LotA has two OTU domains (LotA OTU1, LotA OTU2), whereas LotB and LotC have a single OTU domain (60, 61). The LotA OTU1 domain preferentially cleaves K6-linked di-ubiquitin, but the LotA OTU2 domain cleaves longer K48- and K63-linked chains (60). LotB cleaves K63-linked di- and polyubiquitin chains, and LotC cleaves K6-, K11-, K33-, K48-, and K63-linked di-ubiquitin (Di-Ub) chains (62-64).

The crystal structure of LotA OTU2, LotB, and LotC revealed that all three Lots consist of a catalytic domain (green) and an extended helical lobe (EHL) (yellow) (Fig. 1B). Because the EHL is not found in any other OTU family, EHL defines *Legionella* OTU DUBs as a unique OTU subfamily. Notably, the EHL domain provides a ubiquitin-binding site that interacts with ubiquitin through hydrophobic or electrostatic interactions in *Legionella* (60, 61, 63). LotA OTU2 binds to positively charged ubiquitin residues (R42, R72, R74) through the acidic patch (D407, D410, D412) on the EHL. Indeed, the D410R mutant decreases the catalytic activity against K48- and K63-linked polyubiquitin. In addition, LotA OTU2 EHL binds to the hydrophobic I44 patch of distal ubiquitin through its hydrophobic surface around V398. LotB EHL also plays an important role in ubiquitin-binding. F143 and M144 of LotB EHL bind to the hydrophobic patch around F45 and A46 on the ubiquitin (60, 63). The EHL on LotC also provides a binding site for ubiquitin, and mutations on EHL (Y119R or Y149R) decrease the K48-linked ubiquitin cleavage activity of LotC. Therefore, the EHL domain, which is not found in the eukaryotic OTU-DUB, seems to be a

critical determinant for providing the linkage specificity of *Legionella* OTUs (60). Though the exact functional role of all three Lots during infection is not clear, proteomics studies revealed their substrates, and further studies are awaited to reveal how Lots regulates these substrates (60).

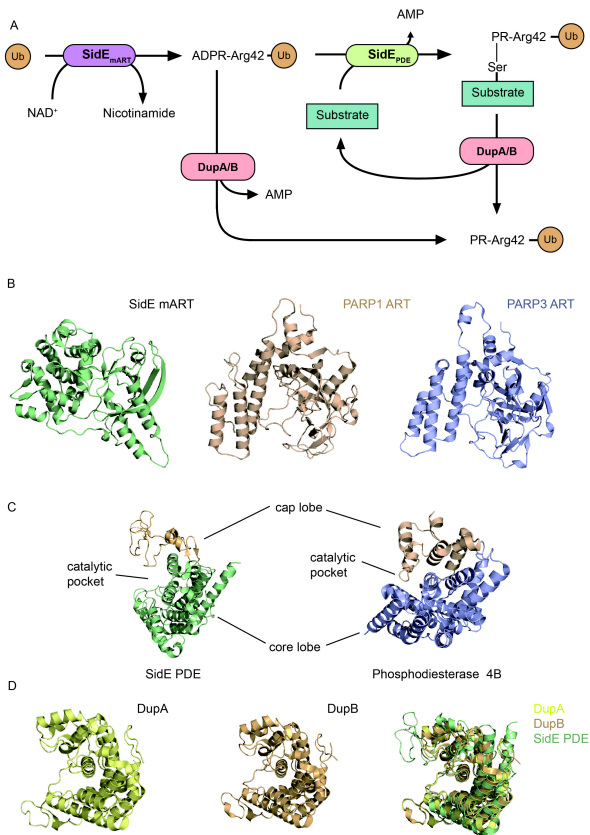
#### **RavD (region allowing vacuole colocalization D)**

RavD is another *Legionella* DUB that explicitly cleaves the M1-linked linear ubiquitin chain. The structure of RavD in complex with a linear di-ubiquitin chain revealed that di-ubiquitin uses the same binding interface as it binds to human M1-specific deubiquitinase OTULIN (Fig. 1C) (57). Given the crystal structure of RavD, it was suggested that RavD does not use a substrate-assisted catalytic activation mechanism as OTULIN does. However, recent studies indicate that both RavD and OTULIN use a common mechanism for recognizing the M1-ubiquitin chain (65, 66). Molecular dynamic simulations on the microsecond scale of RavD in complex with di-ubiquitin suggest that ubiquitin at the S1' site has flexibility and that the binding affinity is substantially weaker than at the S1 site. These results indicate that RavD also uses substrate-assisted catalysis as OTULIN does (47, 66).

### **NON-CANONICAL UBIQUITINATION-RELATED LEGIONELLA EFFECTORS**

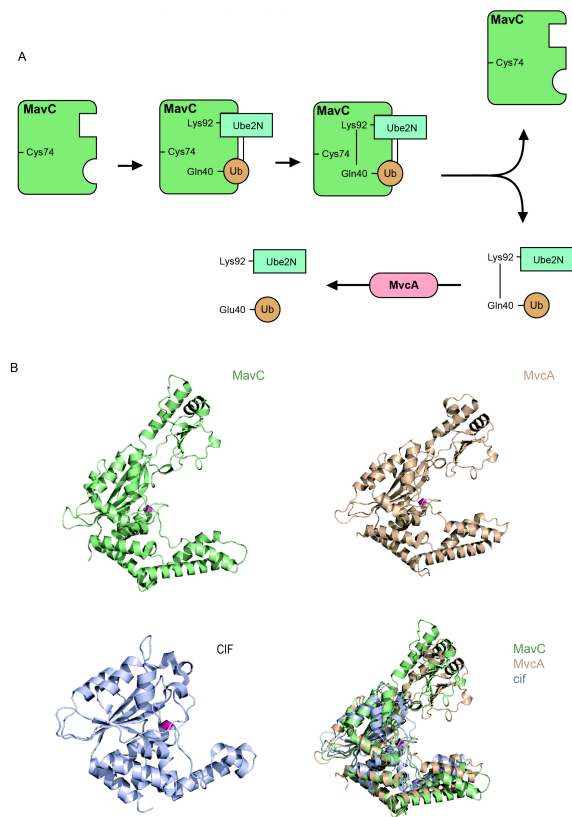
#### **Phosphoribosyl ubiquitination**

SidE family effectors (Substrates of Icm/Dot transporter E, SidEs) are multi-domain proteins. They consist of a deubiquitinase domain, PDE (phosphodiesterase) domain, mART (mono ADP ribosyl transferase) domain, and a coiled-coil domain. When SidEs are translocated in the host cell, they catalyze a unique ubiquitination process on host proteins, called phosphoribosyl ubiquitination (Fig. 2A) (67). The mART domain on SidEs transfers the ADP-ribose moiety from NAD<sup>+</sup> (nicotinamide adenine dinucleotide) to Arg42 of ubiquitin and generates ADP-ribosylated ubiquitin (ADPR-Ub). The ADPR-Ub is then processed by the PDE domain (68). PDE releases the AMP moiety from ADPR-Ub and produces phosphoribosyl ubiquitin (PR-Ub) in the absence of a substrate. When there is a substrate during the ADPR-Ub processing, PR-Ub is transferred to a serine residue on substrate proteins. Like canonical ubiquitination, deubiquitinases specific to PR-Ub are also present (46, 69). DupA and DupB (Deubiquitinase for PR-ubiquitination A and B) are also *Legionella* effectors, and each has a phosphodiesterase domain. DupA/B cleaves and releases PR-Ub from serine residues in the substrate. *Legionella* also regulates SidEs by directly inhibiting the mART domain using the glutamylation by SidJ or SdjA (70-75). Most of the PR-ubiquitination machinery's structures are determined (46, 67, 68, 70, 71, 73, 76-79), and structures of both mART and PDE domains of SidEs differ from functionally similar enzymes found in other organisms. In humans, ADP-ribosylation is catalyzed by the poly-ADP-ribose polymerase (PARP) family (80). A comparison of SidE mART with



**Fig. 2.** The overall scheme of phosphoribosyl ubiquitination and structures of key effectors. (A) Phospho-ribosyl ubiquitination pathway. SidE family effectors (SidEs) consist of a PDE (phosphodiesterase) domain and a mART (mono ADP ribosyl transferase) domain, which can cause phospho-ribosyl ubiquitination. The mART domain on SidEs transfers the ADP-ribose moiety from NAD<sup>+</sup> (nicotinamide adenine dinucleotide) to Arg42 of ubiquitin and generates ADPR-ubiquitin (ADPR-Ub). The PDE domain then processes the ADPR-Ub. PDE releases the AMP moiety from ADPR-Ub and produces phosphoribosyl ubiquitin (PR-Ub). PR-Ub is transferred to a serine residue on substrate proteins. DupA and DupB are phosphodiesterases that cleave and release PR-Ub from serine residues in the substrate. (B) Structural comparison between the SidE mART domain (PDB ID: 5ZQ5), PARP1 ART (PDB ID: 4DQY), and PARP3 ART domain (PDB ID:4GV4). (C) The structure of the SidE PDE domain (PDB ID:5ZQ5) and human Phosphodiesterase 4B (PDB ID:5OHJ) are compared. (D) Structural comparison of DupA (PDB:6RYB), DupB (PDB:6B7M), and SidE PDE (PDB ID 5ZQ5).

human PARP1 and PARP3 clearly shows the overall structural differences (Fig. 2B). SidE mART is also different from the mART of other organisms (HopU1 from *Pseudomonas syringae*, XopA1 from *Xanthomonas axonopodis*) and shows unique conformations (77). The PDE domain of SidEs also shows unique structural features. Most of the phosphodiesterases found in humans have a cap lobe sitting on the top of the catalytic core. In contrast, the SidE PDE cap lobe is not located next to the catalytic core (Fig. 2C). Interestingly, both DupA and DupB



**Fig. 3.** The overall scheme of transglutaminase-mediated ubiquitination of Ube2N and structural comparison of MavC and MvcA. (A) Transglutaminase-induced Ube2N ubiquitination mechanism. MavC (Lpg2147) binds to activated Ube2N-Ub. Intramolecular transglutamination generates an isopeptide bond between the Gln40 of ubiquitin and the Lys92 of Ube2N. MvcA (Lpg2148) specifically cleaves Ub from the Ube2N-Ub produced by MavC. (B) Structural comparison of MavC (PDB ID:5TSC), MvcA (PDB ID:6K11), and cif (PDB ID:4F8C). Catalytic cysteines are color-coded in red.

share a structure similar to that of the SidE PDE domain and use identical catalytic sites while they mediate opposite reactions (PR-ubiquitin transfer and PR-ubiquitin cleavage) (Fig. 2D). It is shown that DupA/B have a strong affinity to Ub, ADPR-UB, and PR-ubiquitinated peptides. In contrast, the SdeA PDE domain has a weak affinity and doesn't integrate with PR-ubiquitinated peptides. High affinity to the PR-ubiquitinated substrate results in PR-deubiquitinase activity of DupA/B (46).

### Transglutaminase induced ubiquitination

MavC (More regions allowing vacuole colocalization C, Lpg2147) and MvcA (MavC paralog A, Lpg2148) were initially discovered as ubiquitin Gln40 deamidase (81). Structural and biochemical studies reveal that MavC binds to an activated Ube2N-Ub conjugate and catalyzes an intramolecular transglutaminase reaction to produce a covalent bond between Gln40 of Ub and



Lys92 of Ube2N (82, 83). In contrast, MvcA cleaves ubiquitin from Ube2N and reverses the MavC-mediated Ube2N ubiquitination (Fig. 3A) (84). Another *Legionella* effector, Lpg2149, binds to MavC and MvcA and blocks their function (81, 85). Structural comparison of MavC and MvcA to the Cif effector revealed that both MavC and MvcA have a large insertion domain on the top of the catalytic pocket (Fig. 3C) (81). An interesting point is that MavC and MvcA share a 50% sequence identity, use the same catalytic triad, and have similar structural folds while performing opposite reactions. Structural studies reveal that the substrate-recognition region on the MvcA defines its role as a deubiquitinase (84).

## CONCLUSION

This mini-review summarized current understandings of the *Legionella* effector proteins that regulate host ubiquitin signaling. *Legionella* hijacks host ubiquitination systems by using effectors similar to canonical ubiquitination machinery or alters ubiquitination systems by introducing non-canonical ubiquitination systems, such as phosphoribosyl ubiquitination and transglutaminase-induced ubiquitination. Structural analysis of both canonical and non-canonical ubiquitin effectors revealed that these effectors have structures that distinguish them from host ubiquitination systems. *Legionella* OTU-like deubiquitinases (LotA/B/C), mART, PDE, and MavC/MvcA, have additional structural motifs or insertion regions that participate in the catalytic reaction or ubiquitin recognition. More importantly, it seems to be a general phenomenon in *Legionella* that a pair of similar effectors performs opposite reactions. SidE PDE transfers PR-ubiquitin, while DupA/B PDE removes PR-ubiquitin. MavC induces Ube2N ubiquitination, whereas MvcA reverses this reaction. They use identical catalytic residues but have different binding affinity or specificity for the substrates to define their roles.

Interestingly, the non-canonical ubiquitination mechanisms—phosphoribosyl ubiquitination and transglutaminase-induced ubiquitination—are found only in *Legionella*. Because *Legionella* is not the only pathogen that translocates effectors to the host cells, it might be possible to discover similar ubiquitin-regulating machinery from other pathogens. Further research is awaited to see whether the novel non-canonical ubiquitination system exists in another organism and to understand how these unique effectors are developed in *Legionella*. Yet, other human pathogens do not have such systems.

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## CONFLICTS OF INTEREST

The authors have no conflicting interests.

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