Cell Host & Microbe

A Single Legionella Effector Catalyzes a Multistep Ubiquitination Pathway to Rearrange Tubular Endoplasmic Reticulum for Replication

Graphical Abstract



Highlights

- Legionella Sde proteins remodel tubular ER to initiate intracellular replication
- The tubular ER protein reticulon 4 is targeted by Sde proteins
- ER remodeling requires ubiquitin transfer by Sde proteins
- Ubiquitin transfer involves ADP-ribosyltransferase and nucleotidase activities

Authors

Kristin M. Kotewicz, Vinay Ramabhadran, Nicole Sjoblom, ..., Jessica Behringer, Rebecca A. Scheck, Ralph R. Isberg

Correspondence

ralph.isberg@tufts.edu

In Brief

Intracellular pathogens, including Legionella, target host organelles for replication. Kotewicz et al. show that Legionella generates an ERencompassed replication compartment via Sde protein-mediated ubiquitination of host reticulon 4. Ubiquitination is mediated by sequential action of the ADP-ribosyltransferase and nucleotidase activities of Sde and independent of the host ubiquitination system.





A Single *Legionella* Effector Catalyzes a Multistep Ubiquitination Pathway to Rearrange Tubular Endoplasmic Reticulum for Replication

Kristin M. Kotewicz,^{1,6} Vinay Ramabhadran,^{1,3,6} Nicole Sjoblom,² Joseph P. Vogel,⁴ Eva Haenssler,^{1,7} Mengyun Zhang,¹ Jessica Behringer,⁵ Rebecca A. Scheck,² and Ralph R. Isberg^{1,3,8,*}

¹Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 150 Harrison Ave., Boston, MA 02111, USA ²Department of Chemistry, Tufts University, 62 Talbot Ave., Medford, MA 02155, USA

³Howard Hughes Medical Institute, Tufts University School of Medicine, 150 Harrison Ave., Boston, MA 02111, USA

⁴Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO 63110, USA

⁵Program in Genetics, Tufts University School of Medicine, 150 Harrison Ave., Boston, MA 02111, USA

6Co-first author

⁷Present address: Qiagen GmbH, 40724 Hilden, Germany

⁸Lead Contact

*Correspondence: ralph.isberg@tufts.edu

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SUMMARY

Intracellular pathogens manipulate host organelles to support replication within cells. For Legionella pneumophila, the bacterium translocates proteins that establish an endoplasmic reticulum (ER)-associated replication compartment. We show here that the bacterial Sde proteins target host reticulon 4 (Rtn4) to control tubular ER dynamics, resulting in tubule rearrangements as well as alterations in Rtn4 associated with the replication compartment. These rearrangements are triggered via Sde-promoted ubiquitin transfer to Rtn4, occurring almost immediately after bacterial uptake. Ubiquitin transfer requires two sequential enzymatic activities from a single Sde polypeptide: an ADP-ribosyltransferase and a nucleotidase/phosphohydrolase. The ADP-ribosylated moiety of ubiquitin is a substrate for the nucleotidase/phosphohydrolase, resulting in either transfer of ubiquitin to Rtn4 or phosphoribosylation of ubiquitin in the absence of a ubiquitination target. Therefore, a single bacterial protein drives a multistep biochemical pathway to control ubiquitination and tubular ER function independently of the host ubiquitin machinery.

INTRODUCTION

Legionella pneumophila is an intravacuolar pathogen of both humans and amoebae (Rowbotham, 1980). As the agent of Legionnaires' disease, infection is initiated by inhalation of contaminated water sources, followed by bacterial growth within alveolar macrophages (Copenhaver et al., 2014). *L. pneumophila* utilizes its main virulence factor, a type IVB secretion system (T4SS) known as Icm/Dot, to translocate \geq 300 proteins into the host cytosol, establishing an endoplasmic reticulum (ER)-associated *Legionella*-containing vacuole (LCV) (Swanson and Isberg, 1995). Bacteria lacking the T4SS are unable to form an LCV (Wiater et al., 1998). The formation of this intracellular *Legionella* ER compartment is evolutionarily conserved from amoebae to mammals (Abu Kwaik, 1996; Berger and Isberg, 1993).

The ER is formed from membrane tubules and flattened sacs that can be classified into the perinuclear, ribosome-associated ER sheets and tubular ER (Voeltz et al., 2006). Recent high-resolution studies of the ER reveal that these classifications are an oversimplification of the breadth of ER structures, as architecture formerly described as peripheral sheets are instead composed of cross-linked ER tubules, termed ER matrices (Nixon-Abell et al., 2016). The tubular ER is a vast network of elongated cylinders, enriched in structural ER membrane proteins, such as Dp1/Yop1p and the reticulon family (English et al., 2009). Reticulons (Rtns) are evolutionarily conserved from yeast to humans (Yang and Strittmatter, 2007), with four subfamilies in mammalian cells (Yan et al., 2006). Reticulon 4 (Rtn4), also known as neurite outgrowth inhibitor (NOGO), is a highly abundant ER protein with three isoforms (Rtn4a/Nogo-A, Rtn4b/Nogo-B1, and Rtn4d/Nogo-B2), at least one of which is expressed in most mammalian cells (Yang and Strittmatter, 2007). Rtns generate ER curvature through their two conserved hydrophobic hairpins inserted in the cytoplasmic leaflet of the lipid bilayer (Zurek et al., 2011). Homo- and hetero-Rtn oligomers are believed to establish arc-like scaffolds (Zurek et al., 2011).

Previous studies hypothesized that the LCV acquires ERassociated markers by hijacking ER vesicles destined for the Golgi (Tilney et al., 2001; Kagan and Roy, 2002), but a recent report indicates that this may be preceded by association with tubular ER (Haenssler et al., 2015). The demonstration that the LCV acquires phosphoinositide-4-phosphate prior to acquisition of vesicular ER markers (Weber et al., 2014) further argues for early association of peripheral ER. Therefore, interaction with peripheral ER tubules may represent the first step of ER association involved in LCV biogenesis. Many Icm/Dot translocated substrates (IDTSs) control membrane trafficking and immune



function by post-translational modifications (PTMs), which regulate protein stability, localization, and enzymatic activities (Ribet and Cossart, 2010; Zhou and Zhu, 2015). Enzymatic PTM by bacterial proteins is a common strategy used by a wide swath of bacterial pathogens, including ADP-ribosylation (ADPr) and ubiquitination (Price et al., 2009; Ribet and Cossart, 2010; Ravikumar et al., 2015; Michard and Doublet, 2015). Although there is deep insight into how PTMs can regulate the activity and stability of targets, little is known regarding how bacterial-induced modifications could induce changes in quaternary interactions in the targeted proteins (Pieters et al., 2016).

The Sde family is a group of IDTSs that edit host proteins (Sheedlo et al., 2015; Qiu et al., 2016). L. pneumophila encodes multiple gene paralogs, with the Philadelphia-1 strain having four, three of which are organized in a single contiguous locus (sdeA, sdeB, and sdeC) (Bardill et al., 2005). Members of the protein family are all 170+ kDa proteins that contain an N-terminal deubiquitinase (DUB) and a central domain similar to mono-ADP-ribosyltransferases (ARTs) (Sheedlo et al., 2015; Qiu et al., 2016). Loss of the entire sde family results in defective L. pneumophila intracellular growth within amoebal hosts (Bardill et al., 2005; Jeong et al., 2015; Qiu et al., 2016). Sde proteins are able to ubiquitinate several ER-associated Rab proteins, dependent on their ART domain (Qiu et al., 2016), with ubiquitination occurring independently of host ubiquitin (Ub) machinery (Pickart and Eddins, 2004). Although there is a connection between the ART domain and ubiquitination, ADP-ribosylation of mammalian substrates by full-length Sde members is not observed. Mass spectrometry of reactions containing an SdeA ART domain fragment results in ADPr of Ub at residue 42, but the role of the ART domain in this particular ubiquitination mechanism remains cryptic, particularly because the full-length protein shows no evidence of this activity (Qiu et al., 2016).

In this study, we analyzed early events in the interaction between *L. pneumophila* and host cells. We show that Sde family members modulate tubular ER function by catalyzing a biochemical pathway in which ADPr of Ub provides a substrate for a nucleotidase/phosphohydrolase, promoting transfer of Ub to Rtn4, resulting in dramatic ER reorganization. An independent study has similarly identified this single peptide-catalyzed biochemical pathway (Bhogaraju et al., 2016). That study argues that the modified Ub is involved in disrupting the host ubiquitination system.

RESULTS

Sde Family Promotes Rtn4 Rearrangement

To probe association of tubular ER with the LCV, we challenged mammalian cells with *L. pneumophila* and analyzed them by immunofluorescence microscopy. As previously shown (Haenssler et al., 2015), detergent-resistant Rtn4 formed a reticular network proximal to the LCV by 40 min post-infection (MPI), which then condensed circumferentially by 8 hr post-infection (hpi) in Triton X-100-permeabilized samples (Figures 1A and 1B). Our inability to detect the tubular reticulon network throughout the cell was investigated by changing permeabilization conditions. Consistent with previous reports (Haenssler et al., 2015), Rtn4 colocalization with the LCV required a func-

tional T4SS (Figure 1C). Strikingly, staining of the entire Rtn4 network could only be detected in the absence of detergent extraction, while colocalization of Rtn4 with the LCV persisted even in the presence of 5% SDS. Therefore, the Rtn4 associated with the LCV resulted from structural changes that distinguish it from the cellular pool of Rtn4 (Figure 1C). To determine whether these structural changes could be detected on SDS gels, we challenged cells with the wild-type (WT) or *dotA3* strains and analyzed SDS extracts. A high molecular weight (HMW) Rtn4 species was identified in extracts from infections with the WT strain, while another ER membrane protein, calnexin, showed no altered migration (Figure 1D).

To decipher which L. pneumophila T4SS substrate was responsible for altering Rtn4, we challenged cells for 2 hr with L. pneumophila lacking Ceg9, a T4SS substrate that interacts with Rtn4 (Haenssler et al., 2015), or a L. pneumophila strain lacking 12.7% of the genome (*Apent*; O'Connor et al., 2011). Both mutant strains generated the Rtn4 HMW species (Figure 1E). Based on this result, we performed a transfection screen to assay the effect of individual L. pneumophila proteins on Rtn4 by selectively screening gene candidates encoded by these mutants. Plasmids encoding individual GFP-tagged L. pneumophila T4SS substrates were transfected into mammalian cells, and then cell extracts were fractionated by SDS-PAGE (Figure 1F). Of the >60 L. pneumophila substrates examined (Table S2), three members of the Sde family-SdeC, SdeB, and SdeA-induced a HMW Rtn4 species. In addition, a modified form of Rtn4 that migrated just above the abundant Rtn4b/d monomer (~50 kDa) was observed (Figure 1F, modified). These three large L. pneumophila T4SS substrates are organized in a contiguous locus with lpg2154 and sidJ, a known regulator of Side family function (Figure 1G).

To determine whether *L. pneumophila* lacking the *sde* family was capable of inducing colocalization of detergent-resistant Rtn4, we challenged cells for 1 hr with *L. pneumophila* and analyzed them by immunofluorescence microscopy (Figure 1H). More than 70% of WT LCVs were associated with Rtn4, while no colocalization was observed with *dotA3*. Both a complete *sde* family deletion (KK099, *AsdeC AsdeB-A AsidE*; Table S1) (Jeong et al., 2015) and an *sde* locus deletion (KK034, *AsdeC-A*; Table S1) were unable to induce Rtn4 association (Figure 1H; Figure S1A). Expression of plasmid-encoded SdeC or SdeB was able to completely restore Rtn4-LCV association to WT levels in both *sde* deletion backgrounds, while there was partial restoration with SdeA (Figure 1I).

To survey the dynamics of Rtn4-LCV association in real time, we transfected Cos7 cells with Rtn4b-GFP, challenged them with WT *L. pneumophila* or Δsde strains expressing mCherry, and monitored them (Movies S1 and S2). The Rtn4b-GFP signal illustrates a high-resolution outline of the ER network, which strongly contrasts with the poor resolution of endogenous Rtn4 in micrographs after concentrated detergent extraction (Figures 1J and 1K; Movies S1 and S2). In response to the WT infection, an Rtn4 signal intensified around the vacuole membrane and then dramatically nucleated outward from the LCV in Rtn4-rich tubular protrusions, some of which formed junctions with other protrusions (Figure 1J; Movie S1). In a Δsde challenge, there was no observable change in Rtn4 localization (Figure 1K; Movie S2).



Figure 1. Sde Family Members Promote Rtn4 Rearrangements in Response to L. pneumophila Challenge

(A and B) Bone-marrow-derived macrophages (BMDMs) from A/J mice were challenged for 40 min (A) or 8 hr (B), followed by fixation, permeabilization with 0.1% Triton X-100, and probing with α -Rtn4 (green), α -L. pneumophila (red), and Hoechst (blue). Scale bar, 5 μ m.

(C) BMDMs challenged for 1 hr with LP02 (WT) or an *icm/dot*⁻ (*dotA3*) mutant were fixed, permeabilized as noted, and probed. Scale bar, 5 µm. Arrows indicate location of bacterium within infected cells.

(D and E) Altered electrophoretic migration of Rtn4, relative to unaltered Calnexin, after WT (D) or a $\Delta ceg9$ or $\Delta pent$ (E) *L. pneumophila* challenge. HeLa cells were challenged for 2 hr, solubilized in SDS at room temperature, fractionated by SDS-PAGE, and probed. Lanes: Un, uninfected.

(F) Sde family members result in Rtn4 electrophoretic variants. 40–46 hr after transfection into Cos1 cells of noted plasmids, cells were extracted and gel fractionated, and blots were probed with α-Rtn4.

(G) The chromosomal arrangement of the sde genes.

(H) Rtn4 rearrangements in BMDMs dependent on presence of *sd*e family members at 1 hpi (see Figure S1A). Scale bar, 5 μm.

(I) Deletion of sde family (KK099) prevents Rtn4 rearrangements about the LCV. BMDMs were challenged for 1 hr prior to probing as in (A). Mean ± SEM.

(J and K) Sde family members promote immediate Rtn4 rearrangements after host cell contact. Cos7 cells harboring Rtn4b-GFP were challenged with *L. pneumophila* that either produces (J) or lacks (K) Sde proteins, and then images from live cells were captured over a 10 min period (see Movies S1 and S2). Scale bar, 5 µm. Images displayed at 1.15× the captured sizes. See Figure S1 and Tables S1 and S2.

Sde-Dependent ER Rearrangements Result in Pseudovesicles

To perform high-resolution probing for Sde-mediated ER changes, we generated an Rtn4b-APEX2-GFP fusion protein (Figure S1B). This fusion allows Rtn4 localization to be determined by coupling the protein to an engineered peroxidase reporter, which can be detected by transmission electron microscopy (TEM) after addition of the substrate diaminobenzidine (DAB). Cells were transiently transfected with an Rtn4b-

APEX2-GFP fusion (Figure S1B), challenged with the WT or *Asde* strains expressing mCherry for 1 hr, and analyzed for deposition of DAB by microscopy. Bright-field microscopy revealed strong DAB depositions associated with WT LCVs, mimicking the Rtn4b structures seen previously by fluorescence microscopy (Figure S1B).

1 hr after infection, TEM images of WT revealed vesiclemimicking structures (termed pseudovesicles) with dense DAB deposition about their surface, as well as projections extending

Cos7(Rtn4-GFP-APEX2)



Bone marrow macrophages



out from pseudovesicles (Figures 2A and 2B). LCV membranes adjacent to the WT were darkly stained by DAB depositions, indicating high levels of Rtn4 contiguous with the vacuole membrane (Figure 2A). In contrast, vacuole membranes encompassing the ⊿sde mutant had little evidence of pseudovesicles or DAB staining (Figures 2C and 2D). To determine whether pseudovesicular structures were generated by the fusion protein, we performed TEM of bone-marrow-derived macrophages (BMDMs) challenged with WT L. pneumophila. Analogous pseudovesicular structures, as well as linear projections from these structures, were observed surrounding the LCV (Figure 2E; Figure S2). There is strong precedence for this observation, as pseudovesicular structures occurring immediately after infection have been observed numerous times (Table S3). There were no pseudovesicular structures in BMDMs challenged for 1 hr with L. pneumophila mutants lacking sde. Instead, the LCV was associated with long membranous structures that resembled irregularly stacked rough ER sheets, indicating premature association of rough ER with the LCV (Figure 2F; Figure S3). Rough ER asso-

Figure 2. Sde-Dependent ER Rearrangements Generate Rtn4-Stained Pseudovesicles or Linear Stacks

(A–D) Cos7 cells harboring Rtn4b-GFP-APEX2 (see Figure S1B) challenged for 1 hr with either LP02 WT (A and B) or the $\varDelta sde$ strain (KK099) (C and D) subjected to DAB staining, followed by TEM. (A) and (B) are TEM images of different sections from the same cell. (B) is a high-magnification image of Rtn4-rich region abutting bacterium that can be seen in (A). Arrows point to membranes in direct apposition to the LCV (A) or projections of Rtn4-associated membranes (B).

(E and F) BMDMs challenged for 1 hr with either LP02 (WT) (E) or $\triangle sde$ strain (KK034) (F). Boxed area at higher magnification (E, inset). Arrowhead points to projections from round structure. Stacks of ER surrounding $\triangle sde$ strain (KK034) (F). See Figures S1, S2, and S3 and Table S3.

ciation with the LCV during the earliest phase of infection has rarely, if ever, been observed in the literature (Table S3) and was only occasionally observed in micrographs of cells challenged with WT (Figure S2D).

Sde Family Members Induce Rtn4 Ubiquitination

We next investigated the nature of the Rtn4 modification in response to Sde proteins (Figure 1F, modified). GFP-SdeC was transiently transfected into cells to produce the modified Rtn4 species, and the modified Rtn4 species was excised from an SDS gel for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Figure 3A). The modified Rtn4 sample showed almost complete coverage of Ub, although the

classic Gly-Lys isopeptide diagnostic of ubiquitination could not be detected (Table S4). Several peptides present in the control Rtn4 monomer samples were absent from the modified Rtn4 species, consistent with those peptides containing residues targeted by the modification (Table S4). Furthermore, the migration of the modified Rtn4 was consistent with mono-ubiquitination (8.5 kDa). To confirm Ub modification of Rtn4, we transiently co-expressed hemagglutinin (HA)-tagged Ub with GFP-SdeC, and then transfected cell extracts were subject to Rtn4 immunoprecipitation (IP). Eluates of immunoprecipitates from SdeCtransfected cells revealed two prominent HA-positive bands above 50 kDa (Figure 3B, compare E lanes). The migrations of the two species were consistent with single and double Ub modification of Rtn4, although the higher molecular weight (MW) species could not be detected by silver stain analysis. No ubiquitination of Rtn4 was observed in eluates from GFP control transfections (Figure 3B, E lanes).

To analyze whether Rtn4 ubiquitination occurs during *L. pneumophila* infection, we transiently transfected cells with



HA-Ub, challenged them with L. pneumophila, and then subjected them to Rtn4 IP. Immunoprecipitates from WT challenge predominantly resulted in ubiquitination of the smaller Rtn4b isoform within 10 min of infection (Figure 3C, WT). By 3 hr, both Rtn4b and Rtn4d isoforms were robustly mono-ubiquitinated, with evidence of HMW ubiquitinated forms (Figure 3C, WT, 180 MPI). The absence of the sde family resulted in the complete loss of Rtn4 ubiquitination, similar to mock-infected cells (Figure 3C, vector, mock). The *Asde* strain harboring SdeC showed complementation, albeit inefficiently, with evidence of ubiquitinated HMW forms, whereas complementation with either SdeB or SdeA was robust, producing substantial Rtn4 mono- and multi-ubiquitination (Figure 3C). The pattern of Rtn4 ubiquitination in these strains was broadly reminiscent of a WT infection, with an abundance of detectable mono- and di-Ub-modified Rtn4.

Rtn4 Reorganization Requires ART Activity

Sde family proteins have been reported to contain a conserved arginine-mono-ADP-ribosyltransferase domain necessary for Rab ubiquitination, although the ART activity could not be detected in full-length WT protein (Qiu et al., 2016). To probe the connection between the ART activity and ER reorganization, we challenged cells with a panel of Sde ART domain point mutants and analyzed them for Rtn4-LCV association (Barth et al., 1998). The predicted enzymatic residues were mutated to alanine in each Sde member (Figure 3D: for SdeB/C, E859A or R763A; for SdeA, E862A). Each Sde ART mutant was completely unable to restore Rtn4 association with the LCV, consistent with the ART being essential for Rtn4 reorganization (Figures 4A and 4B; Figure S4A).

We next evaluated the role of the ART in ubiquitination of Rtn4 after *L. pneumophila* challenge. WT *L. pneumophila* promoted Rtn4 ubiquitination after infection whereas the Δsde mutant

Figure 3. Sde Family Members Promote Rtn4 Ubiquitination

(A) GFP-SdeC or GFP (vector) was transiently expressed in HeLa cells for 24 hr, followed by Rtn4 IP. Eluates were fractionated by SDS-PAGE and stained.

(B) HA-Ub was transiently co-expressed with either GFP or GFP-SdeC in HeLa cells for 24 hr, then subjected to IP with α -Rtn4 IP, fractionated by SDS-PAGE, and probed for Ub-modified Rtn4 with α -HA (E, eluate; FT, flow-through; T, total).

(C) HEK293T cells were transiently transfected with HA-Ub for 24 hr, the cell culture medium was replaced with 10 μ M MG132 (Millipore) medium 30–60 min prior to challenge with *L. pneumophila*, and the infection was allowed to proceed (MPI, minutes post-infection) prior to IP with α -Rtn4.

(D) Domain structure of Sde family proteins, with endpoints noted for SdeC (Qiu et al., 2016). See Table S4.

was clearly defective (Figures 3C and 4C). Expression of WT SdeB in a $\triangle sde$ background was able to complement Rtn4 ubiquitination, whereas the ART

mutant, SdeB R763A, was indistinguishable from a Δsde mutant infection (Figure 4C, compare SdeB R763A to SdeB WT).

We hypothesized that any ADPr modifications were either inefficient or unstable. To address this problem, we devised an ADPr assay that exploited an analog of β -NAD, ethenoNAD (ϵ NAD) (Klebl et al., 1997), in which ADPr of substrates could be monitored by western blotting with α -ethenoadenosine (α - ϵ Ado) (Krebs et al., 2003). To assay for SdeC ART activity directed against mammalian proteins while simultaneously monitoring cellular ubiquitination changes in response to Sde proteins, we incubated recombinant full-length SdeC with cell extracts and recombinant HA-Ub in the presence of *ɛ*NAD. A 5 min reaction with either WT SdeC or a DUB-defective derivative resulted in robust laddering of ADPr substrates, in a pattern reminiscent of polyubiquitin chain laddering (Figure 4D, 5 min, WT and C118S). By 60 min, evidence for the ε Ado signal was greatly reduced, with the only remaining signal being above 250 kDa (Figure 4D, 60 min, WT and C118S). Probing with α-HA revealed that both SdeC_{WT} and SdeC_{C118S} induced robust HA-Ub polymerization, but the Ub polymerization was unchanged over time (Figure 4D, lower panel). In contrast, there was no ADPr or HA-Ub polymerization by the SdeC_{E859A} ART mutant (Figure 4D). In the absence of HA-Ub, a HMW species above 250 kDa was recognized by α - ε Ado and disappeared over time (Figure 4D, upper panel, WT No HA-Ub). These results indicate that SdeC promotes ubiquitination of host proteins dependent on the ART domain.

To simplify the ε ADPr assay, we excluded cell extracts, using recombinant polyubiquitin as a substrate. Recombinant K63-linked Ub tetramers and ε NAD were incubated at 37°C with SdeC_{WT} for various times before termination. Immediate introduction of SDS buffer resulted in the appearance of a prominent ADP-ribosylated Ub (ADPr-Ub) tetramer (Figure 4E, 0 min). This signal was dramatically reduced, however, if incubations were



Figure 4. Sde Family Mono-ADP-Ribosyltransferase Activity Is Required for Rtn4 Restructuring and Ubiquitination

(A) A/J BMDMs were challenged for 1 hr, fixed, permeabilized with 1% Triton X-100, and probed with α-Rtn4 (green), α-L. pneumophila (red), and Hoescht (blue). Data are mean ± SEM. 50 L. pneumophila vacuoles were assessed for Rtn4 colocalization per coverslip.

(B) Representative micrographs of Rtn4 association with the LCV at 1 hpi (hour post-infection). Scale bar, 5 µm.

(C) HEK293T cells transiently transfected with HA-Ub were challenged with *L. pneumophila*, and extracts were subjected to IP with α-Rtn4. Eluates were analyzed for Ub by probing with α-HA.

(D) HEK293T extracts were incubated at 37°C with 10 nM recombinant SdeC and 20 μ M recombinant human HA-Ub monomer. Reactions were separated by SDS-PAGE and probed for α - ϵ Ado (ADPr) and α -HA (Ub).

(E) SdeC was incubated with K63-linked Ub tetramers at 37°C. Reactions were fractionated by SDS-PAGE and assayed for altered migration and ADPr of Ub by silver staining and immunoblotting. Lanes: WT, WT SdeC; C118S, DUB mutant; E859A, ART mutant; –, no SdeC; WT (No HA-Ub), WT SdeC (No HA-Ub added) (see Figures S4 and S5).

allowed to continue (Figure 4E, α - ε Ado; Figure S5). This indicates that ADPr modification by SdeC is rapid and transient, explaining why previous studies were unable to detect the modification with full-length protein (Qiu et al., 2016). The ADPr signal required an intact ART domain as no *eADPr* signal was observed in SdeC_{E859A} reactions (Figure 4E, α-εAdo E859A; Figure S5). On further analysis of the SdeC-modified poly-Ub on silver-stained gels, it was clear that loss of the eAdo signal was not due to total reversal of the modification. After initial appearance of an ADPr signal, a slower migrating Ub tetramer relative to an unmodified Ub tetramer predominated (Figure 4E, WT 0), and this form persisted without detectable change in migration, even as the ADPr signal disappeared (Figure 4E, WT 120). This is consistent with Ub chains being ADPr modified followed by additional processing retaining an unknown modification (Figure 4E, WT silver stain). Therefore, the Sde family generated ADPr-Ub as an intermediate reaction species prior to loss of the ε Ado epitope.

Another striking aspect of the ART activity was observed in these assays: although SdeC has an amino terminal DUB domain, there was minimal cleavage of poly-Ub when SdeC was incubated with ϵNAD (Figure 4E, WT, silver stain). When the assay was repeated using the SdeC ART mutant, the DUB activity was restored, with 120 min incubation resulting in an accumulation of mono-Ub (Figure 4E, E859A, silver stain; Figure S5). Therefore, the ART activity strongly interfered with the DUB activity, and this inhibition continued after the ADPr was processed.

Sde Family Nucleotidase/Phosphohydrolase Domain Is Required for Ubiquitin Conjugation and Biological Function

The Sde proteins contain a region between the DUB and ART domains (Figure 3D) with sequence homology to the *Legionella* IDTS Lem10, which has been crystalized, revealing structural similarities to nucleotidases and other phosphohydrolases (Wong et al., 2015; Morar et al., 2015). We hypothesized that this nucleotidase/phosphohydrolase (NP) domain could be responsible for processing of ADPr-Ub, resulting in the loss of



Figure 5. Sde Family NP Domain Is Required for Rtn4 Rearrangements, Intracellular Growth, and Functions Cooperatively with ART Domain to Conjugate Ub

(A and B) A/J BMDM were challenged for 1 hr, followed by fixation and permeabilization with 1% Triton X-100, and probed as in Figures 4A and 4B. *L. pneumophila* vacuoles were assessed for Rtn4 colocalization (see Figure S4A). Representative micrographs (B) of Rtn4 association with the LCV from (A). Scale bar, 5 µm.

(C) Dictyostelium discoideum was challenged with WT (Lp02) or mutant L. pneumophila expressing luciferase (PahpC::/lux). L. pneumophila intracellular growth (luminescence) was monitored hourly. Mean \pm SEM for every 5 hr increment; results representative of \geq 3 replicate experiments (see Figures S4B–S4D).

(D) HEK293T extract was incubated at 37°C for the indicated time with recombinant SdeC, ε NAD, and recombinant human HA-Ub. Rtn4 Ub and ε ADPr were assessed by immunoblot with indicated antibodies.

(E) Recombinant Ub or poly-His-Ub monomers were incubated with eNAD and recombinant SdeC at 37°C for the indicated time. Ub ADPr was assessed as in (D). (F) A four-component system is sufficient to ubiquitinate Rtn4. Purified GST-Rtn4 was incubated with noted components and SdeC derivatives for 1 hr. Proteins were fractionated by SDS-PAGE and visualized by silver stain (see Figure S6). Lanes: WT, WT SdeC; E859A, ART mutant; H416A, NP mutant (see Figures S4 and S6).

 ε Ado, while leaving a modification that retarded Ub migration. To explore this possibility, we selected several potential NP catalytic residues in SdeC for site-directed alanine mutagenesis based on sequence similarity to the nucleotide-binding pocket in Lem10 and introduced them on plasmids into a Δsde background. The SdeC NP mutant SdeC_{H416A} was completely incapable of generating Rtn4 structures associated with the LCV after 1 hr challenge, in contrast to the behavior of the SdeC_{WT} derivative, which fully restored Rtn4-LCV association (Figures 5A and 5B).

To determine whether the Sde NP domain, similar to the ART domain (Figures S4B–S4D), was important for promoting intracellular replication in natural hosts, we used WT or mutant *L. pneumophila* strains expressing luciferase to challenge the amoebal species *Dictyostelium discoideum*, and replication was monitored over 4–5 days. A plasmid harboring $sdeC_{H416A}$ in a Δsde strain could not restore *L. pneumophila* intracellular

growth to levels observed with either the WT or the deletion strain harboring $sdeC_{WT}$. Instead, expression of the SdeC NP mutant mimicked the poor intracellular growth observed with Δsde infection (Figure 5C). Therefore, the Sde NP domain is required for promoting bacterial replication during amoebal challenge.

To probe the biochemical role of the NP domain, we simultaneously monitored ADP-ribosylation and Rtn4 ubiquitination in cell extracts in the presence of recombinant SdeC derivatives. Reactions with SdeC_{WT} produced several altered Rtn4 migration forms consisting of ~9 kDa shifts, consistent with the addition of one or more Ub moieties (Figure 5D, α -Rtn4). These modified Rtn4 species were not observed in the absence of SdeC or with addition of the ART mutant SdeC_{E859A} or NP mutant SdeC_{H416A} (Figure 5D, α -Rtn4), indicating that the ART and NP domains collaborate for ubiquitination. When probed with α -εAdo, a HMW ADP-ribosylated species was apparent that dissipated over 1 hr in SdeC_{WT} reactions. At 5 min post-SdeC addition,



weak laddering of ADPr proteins was also observed, presumably due to modification of endogenous poly-Ub (Figure 5D, α - ϵ Ado). The SdeC_{E859A} mutant phenotypically mimicked reactions lacking SdeC, with the residual ADPr signal dependent on endogenous enzymes from the extract (Figure 5D, α - ϵ Ado). Strikingly, the SdeC_{H416A} construct, which showed no evidence of Rtn4 ubiquitination (Figure 5D, α -Rtn4), was able to produce robust ADPr of numerous cell extract proteins, including a protein that migrated at the size predicted for Ub (Figure 5D, α - ϵ Ado). Therefore, the presence of persistent ADPr modification in the NP mutant negatively correlated with Rtn4 laddering.

As the SdeC_{H416A} NP domain mutant appeared to cause accumulation of ADPr-Ub, the effect of the NP domain on the modification of Ub was analyzed in an in vitro system free of cell extract. SdeC_{H416A} was able to robustly ADP-ribosylate both polyhis-tagged and untagged Ub over a 60 min reaction with no loss of the ε Ado signal (Figure 5E). In contrast, SdeC_{WT} showed a weak ADPr-Ub signal after only 1 min, and by 2 hr, the ADPr-Ub was undetectable, indicating that the NP activity efficiently removed ε Ado (Figure 5E, α - ε Ado). These results indicate that, even in the absence of a target to ubiquitinate, both the ART and NP domains collaborate to post-translationally modify ubiquitin, transitioning from ADPr-Ub to a second modification that lacks the ε Ado epitopes.

To demonstrate that Ub modification of Rtn4 by SdeC occurs catalytically on a natural substrate in the absence of any host components, we incubated SdeC derivatives with a 20× molar excess of glutathione S-transferase (GST)-Rtn4 in the presence or absence of ENAD. Impressively, within 1 hr, nearly the entire Rtn4 population was mono- or multi-ubiguitinated, with resulting species migrating \sim 8–24 kDa larger than GST-Rtn4, and no detectable modification of unfused GST (Figure 5F; Figure S6, α-Ub). Ubiquitination of Rtn4 required both the NP and ART domains, as neither SdeC_{H416A} nor SdeC_{E859A} could promote Rtn4 ubiquitination. The loss of either NP or ART activity, however, could be overcome by mixing the two mutant proteins together in the presence of GST-Rtn4, with extremely efficient ubiquitination after 1 hr (Figure 5F). These results are consistent with ADPr-Ub being a substrate of the SdeC NP domain, in which trimming of ADPr and transfer of Ub to Rtn4 requires the NP activity.

A SdeC-Promoted Biochemical Pathway Leads to Ribose-Monophosphate-Modified Ubiquitin

Our results argue that Ub conjugation to Rtn4 by the Sde family is a consequence of covalent modification of Ub followed by enzymatic processing and transfer to targets. To understand the nature of the transient Ub-modified intermediate and its apparent trimming, we incubated monomeric Ub with SdeC derivatives in the presence of ε NAD and analyzed it by liquid chromatography-mass spectrometry (LC-MS). In the absence of SdeC, the molecular mass of monomeric Ub was 8,564.57 amu, but after 1 hr incubation with the SdeC_{H416A} mutant, \geq 90% of Ub population increased by a mass of 565.05 to 9,129.62 amu (Figures 5E and 6A), consistent with a single ε ADP-ribose moiety added. Incubation of SdeC_{WT} with Ub, on the other hand, resulted in \geq 90% of the Ub population converted to 8,776.5 amu (Figures 5E and 6A). This 212 amu mass increase is consistent with ribose-monophosphate modification of Ub, as a consequence of cleavage at the diphosphate bridge between adenosine and ribose (Figures 6A and 6B). Therefore, in the absence of a Ub recipient, the ART domain recognizes and modifies Ub, followed by diphosphohydrolase processing to ribose-monophosphate by the NP domain (Figure 6B). The proposed reaction is similar to a subset of nucleotidases that show diphosphohydrolase activity toward ADPr-modified proteins (Daniels et al., 2015; Palazzo et al., 2015).

To determine whether the final reaction product is found on Ub residue R42 (Qiu et al., 2016), as predicted by diphosphohydrolase action on R42- ϵ ADP, we gel extracted the modified Ub species, subjected them to trypsin/AspN double digestions, and analyzed them by LC-MS/MS. The double digestion generated a 10-amino-acid fragment with an expected m/z(+2) = 694.334 for the ribose-monophosphate-modified form and m/z(+3) = 580.91 for the ϵ ADPr-modified form (Figures 6C and 6D). When extracted ion chromatograms (XIC) were analyzed for the m/ z(+2) expected for the ribose-monophosphate-modified form, only Ub incubated with SdeC_{WT} could generate significant amounts (Figure 6C). Similarly, XIC from the m/z(+3) predicted for the ϵ ADPr-modified Ub fragment showed that only the diphosphohydrolase mutant SdeC_{H416A} could generate significant levels of this product (Figure 6D).

To gain further evidence for a diphosphohydrolase activity, we incubated Ub with $SdeC_{WT}$ for 2 hr and then treated it with alkaline phosphatase (AP) to remove the predicted phosphate group. The +212 Da modification by SdeC was reduced to a +132 Da modification (Figure 6E, Ub+SdeC+AP), predicted for phosphatase processing to simple ribose (Figure 6F). Therefore, in the absence of a ubiquination substrate, the ART and diphosphohydrolase collaborate to promote phosphoribosylation of Ub.

Figure 6. ART and Diphosphohydrolase-Dependent Ribose-Monophosphate Modification of Ub

(B) Proposed pathway to generated modification of 212 amu.

(F) Likely products that lead to the generation of 132 amu modification.

⁽A) SdeC in presence of ε NAD results in a modification of 212 amu (atomic mass unit). Ub (black), Ub incubated with the NP mutant SdeC (H416A, gray), or SdeC (WT, red) were subjected to LC-MS analysis, and the deconvoluted masses of the peaks for each sample were displayed.

⁽C and D) Trypsin/AspN treatment of modified Ub species followed by extracted ion chromatography (XIC) analysis reveals predicted modifications displayed in (B). Shown are XIC chromatograms of species having displayed m/z values for ribose-monophosphate (C) or ε ADP-ribose (D) Ub modifications after incubation without SdeC (top) or with SdeC H416A (middle) or SdeC WT (bottom).

⁽E) Treatment of species 3 with alkaline phosphatase results in a product predicted for ribosylated Ub. Ub was treated with noted enzymes, followed by LC-MS, and the deconvoluted masses of the peaks for each sample are displayed.

⁽G) Electrospray ionization MS/MS spectrum of trypsin/AspN Ub fragment having +212 amu modification resulting from SdeC treatment. The b-type ion fragments are displayed above the trypsin/AspN peptide that has an increase of 212.01 amu over the predicted size of the unmodified Ub peptide. Each predicted b-type ion was identified and displayed along with identified y-type ion fragments. Ions marked #212 denote fragment sizes that correspond to the predicted b-type ions having an added 212 amu.

To conclusively demonstrate the proposed biochemical pathway, we subjected the AspN/trypsin 10-amino-acid fragment spanning R42 to b- and y-ion analysis after LC-MS/MS. If the ribose-monophosphate modification occurs on R42, beginning with the b_4 ion, each of the successive ions should have an increase in mass of +212.01 (Figure 6G, noted as $R^{\#}$). We were able to identify ions with high resolution that matched the predicted b_4 through b_9 ions, each with the expected mass increase (Figure 6G). In addition, we were able to identify an ion predicted to be the intact peptide with neutral loss of the modification (Figure 6G, m/z = 588.330), which has been observed in ribose-phosphate-modified peptides previously (Palazzo et al., 2015). Therefore, SdeC ART activity, followed by diphosphohydrolase processing of ADPr, occurs on the R42 residue.

DISCUSSION

Previous work has shown that as early as 10 min post-bacterial challenge, vesicle-like structures approximately 200 nM in diameter associate with the LCV (Abu Kwaik, 1996; Kagan and Roy, 2002; Tilney et al., 2001). These structures have been called ER-derived vesicles, based on the fact that ER vesicle-associated proteins rapidly associate with the LCV (Kagan and Roy, 2002; Tilney et al., 2001). The work described here argues that these circular structures are derived from tubular ER as a consequence of a biochemical pathway catalyzed by Sde family members (Figure 2A).

We propose that Rtn4 ubiquitination by the Sde biochemical pathway promotes structural transformations of ER tubules, potentially through enhanced Rtn4 oligomerization or generation of a scaffold to form tubule matrix-like structures (Nixon-Abell et al., 2016). For this reason, we have called these structures pseudovesicles, which have Rtn4-rich appendages extending from their cytoplasmic face. These appendages have not been a focus of interest in the field but have been observed previously at early time points (Robinson and Roy, 2006) (Figure 2B). Surprisingly, in the absence of the Sde proteins, rough ER sheets prematurely associated with the LCV (Figure 2F; Figure S3). An examination of 54 manuscripts from the literature indicates that rough ER recruitment typically occurs 6 hr post-infection of cells and is never observed as an early event (Table S3). In fact, the overwhelming consensus among these studies is that the earliest event observed is the formation of round compartments with identical morphology to the pseudovesicles (Table S3; Abu Kwaik, 1996). The observed ER transitions indicate that the bacterium engineers these ER transformations in a temporal process.

Our work shows that a single bacterial protein catalyzes a unique multistep biochemical pathway in the absence of host proteins that leads to ubiquitination and rearrangements of Rtn4. Bacterial and viral pathogens are known to directly subvert the Ub system through mimicry of eukaryotic Ub editing proteins, such as the *Legionella* SidE family deubiquitinases or the E3 ligases LubX and SidC (Qiu et al., 2016; Hsu et al., 2014; Horenkamp et al., 2014). It was previously demonstrated that the unique feature of Sde ubiquitination is that it occurs independently of the host Ub conjugation system and is dependent on an ART domain (Qiu et al., 2016). We argue here that ADPr of

the Ub R42 residue is merely the first step in a pathway that leads to direct conjugation of Ub to recipient host proteins. The model we favor is that the Sde pathway is initiated by highly efficient ADPr modification of Ub, which occurs catalytically at 0°C using a ratio of 1:50 SdeC:Ub, arguing for rapid ADPr modification of Ub immediately after Sde translocation. The ADPr-Ub is then used as a substrate by the NP domain that can either trim the ADPr to ribose-monophosphate *or* promote transfer of Ub to Rtn4.

Consistent with this model, we have shown that in a purified system using only Rtn4, Ub, and small quantities of SdeC, there is rapid conjugation of Ub, arguing that ADPr-Ub is an intermediate that is acted on by the NP domain to promote ubiquitination of substrates. This model is supported by the fact that when an NP-deficient mutant protein is mixed with an ARTdeficient mutant, ubiquitination of Rtn4 is extraordinarily efficient (Figure 5G; Figure S6). We believe that during intracellular growth, the amino terminal DUB domain also plays a role (Sheedlo et al., 2015), perhaps by preventing accumulation of K63-linked Ub about the replication vacuole, thereby making available a local pool of mono-Ub for the action of the Sde family.

In contrast to the specific transfer reaction described here, phosphoribosylated Ub has the potential to serve as a nonspecific reactive intermediate able to undergo a non-enzymatic Maillard reaction that would conjugate Ub to recipient proteins through an irreversible sugar crosslink. In Maillard reactions, the electrophilic carbonyl of a reactive sugar, such as glucose or ribose-phosphate, reacts with a free amino group of recipient proteins, generating advanced end products (AGEs) (Chuyen, 2006). The kinetics of AGE formation is typically guite slow, so a Maillard mechanism would require some accessary factors that could allow a biologically relevant reaction. We favor a model in which the primary role of Sde proteins is to use the combined action of the ART and NP domains to ubiquitinate highspecificity targets. Therefore, it is more likely that the formation of phosphoribose-Ub downmodulates Sde-mediated Ub conjugation, reducing the concentration of ADPr-Ub that can act as a substrate for Sde-mediated transfer to host target proteins. Consistent with this role, Sde overexpression in either mammalian or yeast cells results in inhibition of the host Ub system (Bhogaraju et al., 2016). Our results argue, however, that an inhibitory role of Sde proteins during intracellular growth may be of secondary importance. Inhibition of the host Ub system by SdeA requires the ART activity and is independent of the NP domain (Bhogaraju et al., 2016). We have shown, however, that a L. pneumophila NP mutant that is competent for interfering with the Ub system (Bhogaraju et al., 2016) is as defective for intracellular amoebal growth as a total sde family deletion (Figure 5C).

The unusual nature of Sde-mediated cellular effects, combined with its mechanism of action, indicates that the functions of these proteins have a broad range of consequences. It has already been shown that a subset of Rab proteins can be ubiquitinated by SdeA (Qiu et al., 2016) in addition to the structural ER membrane protein demonstrated in this study (Figure 5F). These results argue for multiple pools of specific targets. The spectrum of cellular functions controlled by this protein family is likely to be quite large, with tubular ER rearrangement being the most rapid and visually spectacular response, controlling a morphological change that had previously been a mystery for much of the past two decades.

EXPERIMENTAL PROCEDURES

Bacterial/Eukaryotic Culture, Antibodies, and Media

L. pneumophila derivatives used in this study were derived from Legionella pneumophila Philadelphia-1 strain (Berger and Isberg, 1993). BMDMs were isolated from A/J mice (Swanson and Isberg, 1995). Bacterial strains, primers, plasmid construction, and challenge of mammalian cells are detailed in the Supplemental Experimental Procedures and Table S1. Animal protocols were approved by the Institutional Animal Care and Use Committee of Tufts University. Antibody sources/concentrations are detailed in the Supplemental Procedures.

Screen for Rtn4 Altered Electrophoretic Mobility

Cos1 or HeLa cells were transiently transfected with pDEST53 or pDEST53 harboring GFP fusions to *L. pneumophila* proteins (Losick et al., 2010) using Lipofectamine 2000 (Life Technologies) for 40–46 hr. Cells were collected in PBS and sample buffer (SB) lacking reducing agent at room temperature. A list of the transfected *Legionella* GFP fusions is in Table S2.

Rtn4 Colocalization

Rtn4 colocalization with the LCV was assayed by immunofluorescence microscopy (Haenssler et al., 2015). Infected BMDMs were PFA fixed and Triton X-100 permeabilized, stained with *α-L. pneumophila* rat serum and *α*-Rtn4, and detected with *α*-rat IgG Alexa 594 and goat *α*-rabbit Alexa 488. Hoescht 33342 was used to label DNA.

Immunoprecipitations

Prior to Rtn4 IP, HEK293T cells transiently transfected with pMT123 (HA-Ub, gift of D. Bohmann and S. Lippard; Treier et al., 1994), and 24 hr later, the transfection medium was replaced with DMEM + 10% fetal bovine serum (FBS) with 10 μ M proteasome inhibitor MG132 (Millipore) 30–60 min prior to *L. pneumophila* challenge at an MOI = 10. Then cells were collected and washed in PBS and stored at -80° C until IP.

See Supplemental Experimental Procedures for details of α -Rtn4 resin generation. For IPs, samples were lysed in 1% Triton X-100 for 20 min at 4°C. Cell debris was removed by centrifugation at 4°C, and cleared lysates were diluted with equal volume detergent-free buffer and incubated with resin. Resin binding was allowed to proceed at 4°C for \geq 4 hr, then washed \geq 5× in buffer containing 0.1% Triton X-100 at 4°C. For elution, Rtn4 resin was incubated with 0.1 M glycine (pH 2.8) with 0.2% Triton X-100 for 5 min in a spin column, centrifuged, then repeated, and neutralized with 0.5 M Tris (pH 10.55).

In Vitro DUB and ART/NP Assays

In DUB assays, 1 μ M recombinant Ub tetramers were incubated with 20 nM SdeC and 100 μ M nicotinamide 1,N6- ϵ -adenine dinucleotide (ϵ NAD) at 37°C in 1× ART buffer (see Protein Purification in the Supplemental Experimental Procedures) for the indicated time. Reactions were terminated by the addition of reducing SB and heated to 50°C–55°C for 20 min. ADPr assays in 1× ART buffer included 10 μ M HA-Ub, Ub, or polyHis-Ub, 20 nM SdeC, and 100 μ M ϵ NAD unless otherwise indicated. Reactions were terminated by addition of reducing SB and boiling.

For ART/NP assays with cell extracts, HEK293T cells were harvested in PBS and then lysed by Dounce homogenization, and nuclear material was removed by 3,000× relative centrifugal force (RCF) spins. Soluble extract was quantified, and 100 μ g was added to reactions, which were terminated by freezing on liquid N₂, lyophilization, resuspension in 8 M urea, and addition of reducing SB.

Rtn4 Ubiquitination In Vitro

In 1× ART buffer, 400 nM GST-HA-Rtn4 or GST, 10 μ M recombinant Ub, 20 nM recombinant SdeC, and 100 μ M ϵ NAD were combined and incubated for 1 hr at 37°C and then terminated by addition of reducing SB and boiling.

Intracellular Growth in Dictyostelium discoideum

D. discoideum strain Ax4 was cultured (Solomon and Isberg, 2000) and challenged with *L. pneumophila lux* derivatives at MOI = 0.5 for 2 hr, and then intracellular replication was monitored by luciferase production for \geq 100 hr in a microtiter lumimometer at 25.5°C; see Supplemental Experimental Procedures for details.

Electron Microscopy

For analysis of Rtn4b localization at the LCV (Lam et al., 2015), Cos7 cells were plated in 35 mm glass bottom No. 2 uncoated gridded dishes (MatTek). The following day, cells were transfected with Rtn4b-APEX2-GFP. Cells were then challenged with *L. pneumophila* mCherry or GFP for designated times, washed with PBS, fixed with 2% gluteraldehyde solution for ≥ 1 hr in cold PBS, and then washed in PBS before further processing for TEM, as detailed in the Supplemental Experimental Procedures.

Mass Spectrometry

Identification of Rtn4b/d-modification was performed after IP of Rtn4b/d from extracts of HeLa cells, fractionation on SDS-PAGE followed by silver staining, excision of Rtn4b/d electrophoretic variants that were trypsin digested and subjected to LC-MS/MS; see Supplemental Experimental Procedures. To determine molecular masses of Ub derivatives, we incubated 10 μ M Ub monomer with 20 nM SdeC_{WT} or SdeC_{H416A} and 100 μ M ϵ NAD for 2 hr and 1 hr, respectively, in ART buffer. Reactions were terminated in liquid N₂ and subjected to LC-MS analysis; see Supplemental Experimental Procedures for details.

Tandem Liquid Chromatography Mass Spectrometry

LC-MS/MS analysis was performed by the Taplin Biological Mass Spectrometry Facility at Harvard Medical School; method details in Supplemental Experimental Procedures.

Live Microscopy

Cos7 cells were seeded in 35 mm glass bottom No. 1.5 uncoated dishes (MatTek), and the next day cells were transiently transfected with Rtn4b-APEX2-GFP and then placed on the stage of a Zeiss AxioObserver fitted with environmental and temperature controls set at 37° C and 5% CO₂. After 15 min equilibration, mCherry expressing *L. pneumophila* derivatives were introduced at MOI = 20. Imaging was initiated immediately on a single cell and grabbed approximately every 15 s for 10 min; see Supplemental Experimental Procedures for details.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, four tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2016.12.007.

AUTHOR CONTRIBUTIONS

K.M.K. and R.R.I. conceived of and designed this study. K.M.K. wrote the manuscript with input from all authors. K.M.K. performed or guided experiments in all figures. V.R. designed and performed video microscopy and TEM experiments. N.S., E.H., M.Z., and J.B. performed experiments. J.P.V., R.A.S., and R.R.I. provided experimental guidance.

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