Ubiquitination independent of E1 and E2 enzymes by bacterial effectors

Jiazhang Qiu1, Michael J. Sheedlo2, Kaiwen Yu3, Yunhao Tan†, Ernesto S. Nakayasu4, Chittaranjan Das2, Xiaoyun Liu* & Zhao-Qing Luo†

Signalling by ubiquitination regulates virtually every cellular process in eukaryotes. Covalent attachment of ubiquitin to a substrate is catalysed by the E1, E2 and E3 three-enzyme cascade, which links the carboxy terminus of ubiquitin to the ε-amino group of, in most cases, a lysine of the substrate via an isopeptide bond. Given the essential roles of ubiquitination in the regulation of the immune system, it is not surprising that the ubiquitination network is a common target for diverse infectious agents. For example, many bacterial pathogens exploit ubiquitin signalling using virulence factors that function as E3 ligases, deubiquitinases or as enzymes that directly attack ubiquitin. The bacterial pathogen Legionella pneumophila utilizes approximately 300 effectors that modulate diverse host processes to create a permissive niche for its replication in phagocytes. Here we demonstrate that members of the SidE effector family of L. pneumophila ubiquitinate multiple Rab small GTPases associated with the endoplasmic reticulum. Moreover, we show that these proteins are capable of catalysing ubiquitination without the need for the E1 and E2 enzymes. A putative mono-ADP-ribosyltransferase motif critical for the ubiquitination activity is also essential for the role of the SidE family in intracellular bacterial replication in a protozoan host. The E1/E2-independent ubiquitination catalysed by these enzymes is energized by nicotinamide adenine dinucleotide, which activates ubiquitin by the formation of ADP-ribosylated ubiquitin. These results establish that ubiquitination can be catalysed by a single enzyme, the activity of which does not require ATP.

The ability of the bacterial pathogen L. pneumophila to replicate within a phagocyte depends completely upon the Dot/Icm type IV secretion system that translocates hundreds of substrates (effectors) into host cells. The activity of these effectors supports the biogenesis of the Legionella-containing vacuole (LCV), an area that is made permissive for bacterial replication by manipulating such diverse host processes as vesicle trafficking, protein translation, autophagy, cell migration, gene expression and the biosynthesis of signalling lipids, often with sophisticated mechanisms. With a few exceptions the roles of Dot/Icm effectors in L. pneumophila infection of its host are not fully understood because deletion of these genes individually often does not affect intracellular bacterial replication. A biochemical function has been assigned to less than 10% of these effectors.

The SidE effector family contains four large proteins that are required for proficient intracellular bacterial replication. PSI-BLAST analysis identified a putative mono-ADP-ribosyltransferase (mART) motif (R-S-ExE) in the central region of each of these proteins that is also present in such bacterial toxins as LTA16, C3 exoenzyme17 and ExoS18 (Fig. 1a). Among these, the putative mART element in SdeA is R766–S767–E768–S769–E770–S771, a catalytic motif found in enzymes that transfer the ADP-ribosyl group from nicotinamide adenine dinucleotide (NAD) to arginine residues. To examine its role in SdeA-mediated yeast toxicity, we created the SdeAΔ51 mutant, in which Glu860 and Glu862 were mutated to alanine. This mutant has completely lost its toxicity to yeast and was also defective in inhibiting the secretion of the secreted form of the embryonic alkaline phosphatase (SEAP)22 by mammalian cells (Fig. 1b, c). SidE, SdeB and SdeC also significantly inhibited SEAP secretion in a manner dependent upon the predicted mART motif (Extended Data Fig. 1a). These results suggest that the putative mART motif is essential for the activity of the SidE family effectors.

A mutant missing the SidE family (ΔsidE) shows attenuated virulence against the protozoan host Dictyostelium discoideum15 (Fig. 2a). Expression of wild-type SdeA but not the SdeAΔ51 mutant in a ΔsidE strain almost completely restored its ability to grow within the host (Fig. 2a, b). In D. discoideum, LCVs containing wild-type bacteria efficiently recruit endoplasmic reticulum (ER) markers such as the GFP–HDEL fusion to their surface, which is a hallmark of L. pneumophila infection23,24. Similar to its defects in intracellular growth, the ΔsidE mutant no longer recruited GFP–HDEL to its vacuoles, even at 10 h post infection (Fig. 2c, d and Extended Data Fig. 1b, c). Again, SdeA but not SdeAΔ51 complemented such defects (Fig. 2c, d). Thus, the putative mART motif is important for the function of the SidEs during bacterial infection.

Next we attempted to determine the potential ADP-ribosyltransferase activity of SdeA. Despite extensive efforts, we were unable to detect SdeA-mediated ADP-ribosylation of eukaryotic proteins (Extended Data Fig. 2a), suggesting that this protein possesses a different biochemical activity. During L. pneumophila infection, members of the SidE family are transiently associated with the LCV15, an organelle resembling the ER23. Because Rab small GTPases are a common target of L. pneumophila effectors27, we examined whether SdeA attacks any of the ER-associated Rab proteins28 by co-expressing fluorescent Rab proteins with SdeA (Fig. 3a, b). In L. pneumophila-infected Dictyostelium cells, Rab5 but not Rab3b was efficiently recruited to the LCV (Fig. 3c, d). The molecular mass shift in Rab33b also was observed when it was co-expressed with other members of the SidE family (Extended Data Fig. 2b). To determine whether the potential post-translational modification occurs during bacterial infection, we infected mammalian cells expressing 4×Flag-tagged Rab1, Rab6A, Rab30 or Rab33b with this effector in mammalian cells. A clear shift in molecular mass was observed for all four Rab proteins purified from cells co-transfected with SdeA but not SdeAΔ51 (Fig. 3a, left and middle panels). Such a molecular mass shift did not occur for the endosomal Rab5 or the cytoskeletal small GTPase Rac1 (Fig. 3a, right panel), indicating potential substrate specificity. Among the proteins potentially modified by SdeA, the modification of Rab33b was the most extensive, suggesting that this protein is a preferred substrate. The molecular mass shift in Rab33b also was observed when it was co-expressed with other members of the SidE family (Extended Data Fig. 2b). To determine whether the potential post-translational modification occurs during bacterial infection, we infected mammalian cells expressing 4×Flag-Rab33b with L. pneumophila. Rab33b of higher molecular mass was detected in samples infected with the wild-type strain but not with strain lacking the Dot/Icm transporter or the SidE family (Fig. 3b). The defect in Rab33b modification exhibited by the ΔsidE strain can be complemented by expressing SdeA but not SdeAΔ51 (Fig. 3b). A similar SidE-dependent molecular mass

© 2016 Macmillan Publishers Limited. All rights reserved
The defect exhibited by the SdeA E/A mutant restored the strong SEAP inhibitor AnkX²² was used as a control. Error bars represent s.e.m. (n = 3). The expression of the proteins (the lower panel in b for yeast and the right panel in c for mammalian cells) was probed with indicated antibodies. The PGK (3-phosphoglyceric phosphokinase) and tubulin were probed as a loading control, respectively. SdeA E/A, SdeA with Glu860 and Glu862 mutated to Ala. IB, immunoblotting. The yeast toxicity results in b and protein levels in b and c are from one representative of three independent experiments. The SEAP results in c are one representative done in triplicate from three independent experiments. Uncropped blots are shown in Supplementary Fig. 1.

Ubiquitination requires enzymes E1, E2 and E3 which activates, conjugates and transfers the ubiquitin molecule to the substrate,
were ubiquitinated by SdeA (Extended Data Fig. 4b). Similarly, SidE, that this compound is the only co-factor required for the activity. against a buffer containing EDTA (Extended Data Fig. 4a), suggesting NAD is sufficient for the activity of SdeA that had been dialysed treated NAD is active, which is consistent with the fact that boiled containing NAD but not ATP or Mg²⁺

In line with this observation, ubiquitination occurred in reactions NAD was withdrawn, no ubiquitination was detected (Fig. 4b, lane 3). Yet, when compound in our reactions. In reactions containing NAD, Mg²⁺

consistent with the fact that boiled containing NAD but not ATP or Mg²⁺

Consistently, SdeA does not detectably ADP-ribosylate Rab33b or Rab1 (Extended Data Fig. 5a).

Since ubiquitin ligases often self-modify¹, we incubated SdeA with glutathione S-transferase (GST)-tagged ubiquitin to probe such self-ubiquitination. Proteins of higher molecular mass were detected in reactions containing SdeA but not SdeAɛA, again in a NAD-dependent manner (Fig. 4c). The central domain of SdeA remains toxic to yeast²⁰, suggesting that it is still biochemically active. Indeed, SdeA78-1000 robustly ubiquitimates itself and Rab33b in a manner that requires both NAD and the mART motif (Fig. 4d). These results demonstrate that the N-terminal deubiquitinase (DUB) domain²⁸ of SdeA does not interfere with its ubiquitin conjugation activity. Indeed, the SdeAC118A mutant defective in the DUB activity²⁸ robustly ubiquitimates and Rab33b in a manner that requires both NAD and the mART motif (Fig. 4d). These results demonstrate that the N-terminal deubiquitinase (DUB) domain²⁸ of SdeA does not interfere with its ubiquitin conjugation activity. Indeed, the SdeAC118A mutant defective in the DUB activity²⁸ catalyses ubiquitination indistinguishably to that of the wild-type protein (Extended Data Fig. 5b, c).

Mass spectrometric and mutational analyses revealed that Arg42 of ubiquitin is important for SdeA-mediated, but not for canonical ubiquitination catalysed by the E1–E2–E3 cascade (Extended Data Fig. 6a, b). Consistent with these results, SdeA ubiquitinites Rab33b with all lysine variants of ubiquitin, as well as the ubiquitin derivative containing an alanine substitution in the last two glycine residues or with six histidine residues attached to its carboxy terminus (Extended Data Fig. 6c–e). Further, ubiquitination catalysed by SdeA is insensitive to the cysteine alkylation agent maleimide, suggesting that a cysteine conjugation of ubiquitin does not form during the reaction (Extended Data Fig. 7). Finally, ubiquitination by SdeA affected the GTP loading and hydrolysis activity of Rab33b but did not detectably affect its stability (Fig. 3a and Extended Data Fig. 8). The nucleotide binding status of Rab33b did not affect its suitability as the substrate of SdeA (Extended Data Fig. 8e).

We detected AMP, nicotinamide, ubiquitin and NAD in SdeA-catalysed reactions (Extended Data Fig. 9). The release of AMP suggests the formation of an ubiquitin-AMP adduct during the reaction. Yet, the ubiquitin-AMP adduct could not be detected by 32P-α-NAD or by TCA precipitation followed by high-performance liquid chromatography–mass spectrometry (HPLC–MS) (Extended Data Fig. 10a). The release of nicotinamide and the requirement of Arg42 of ubiquitin respectively¹. We thus used in vitro reactions to determine whether SdeA directly participates in the ubiquitination of Rab33b. In a series of reactions each containing E1 and one of several E2 enzymes, no ubiquitination of Rab33b was detected (Extended Data Fig. 3c). We thus tested the hypothesis that an unknown E2 is required for the activity of SdeA by adding cell lysates to the reactions, which led to ubiquitination of Rab33b in an mART-dependent manner (Fig. 4a). Unexpectedly, ubiquitination still occurred in reactions receiving heat-treated cell lysates (Fig. 4a, lane 3), suggesting that both E1 and the putative SdeA-specific E2 are heat-stable or that SdeA is able to catalyse ubiquitination by itself but only in the presence of heat-stable molecule(s) from cells. To distinguish between these two possibilities, we added E. coli lysates to the reaction. Notably, ubiquitination of Rab33b did occur (Fig. 4a, lane 4). These results demonstrate that SdeA catalyses E1/E2-independent ubiquitination in a process that requires one or more heat-stable molecules present in cells.

Classic ubiquitination requires the conserved E1 that activates ubiquitin in a process powered by hydrolysis of ATP, which binds the enzyme in a Mg²⁺-dependent manner.¹ We thus determined the requirement of these molecules in SdeA-mediated ubiquitination. Because of the importance of the mART motif in the cleavage of NAD by canonical ADP-ribosyltransferases,¹⁹, we included this compound in our reactions. In reactions containing NAD, Mg²⁺ and ATP, ubiquitination of Rab33b occurred (Fig. 4b, lane 2). Yet, when NAD was withdrawn, no ubiquitination was detected (Fig. 4b, lane 3). In line with this observation, ubiquitination occurred in reactions containing NAD but not ATP or Mg²⁺ (Fig. 4b, lanes 4 and 5). Heat-treated NAD is active, which is consistent with the fact that boiled cell lysates allowed SdeA to function (Fig. 4b, lane 8). Exogenous NAD is sufficient for the activity of SdeA that had been dialysed against a buffer containing EDTA (Extended Data Fig. 4a), suggesting that this compound is the only co-factor required for the activity. SdeAɛA is unable to catalyse the modification even in the presence of NAD (Fig. 4b, lane 9). Under this condition, both Rab1 and Rab6A were ubiquitinated by SdeA (Extended Data Fig. 4b). Similarly, SidE, SdeB and SdeC ubiquitinated Rab33b (Extended Data Fig. 4c).

Rab33b ubiquitination. Flag–Rab33b purified from cells co-expressing SdeA (c) or infected with wild-type L. pneumophila (e) was subjected to mass spectrometric analysis and tryptic ubiquitin fragments were identified in proteins of the shifted bands (d, f, g). Overexpression of Rab33b restricts intracellular bacterial growth. COS1 cells transfected with Rab33b and the indicated mutants were infected with L. pneumophila and the formation of replicative vacuoles was determined. IB, immunoblotting. Data shown are one representative experiment of three independent experiments (a–f); results in g are one representative done in triplicate from three independent experiments. Error bars represent s.e.m. (n = 3). a–c, e, f, SdeA induces respectively¹. We thus used in vitro reactions to determine whether SdeA directly participates in the ubiquitination of Rab33b. In a series of reactions each containing E1 and one of several E2 enzymes, no ubiquitination of Rab33b was detected (Extended Data Fig. 3c). We thus tested the hypothesis that an unknown E2 is required for the activity of SdeA by adding cell lysates to the reactions, which led to ubiquitination of Rab33b in an mART-dependent manner (Fig. 4a). Unexpectedly, ubiquitination still occurred in reactions receiving heat-treated cell lysates (Fig. 4a, lane 3), suggesting that both E1 and the putative SdeA-specific E2 are heat-stable or that SdeA is able to catalyse ubiquitination by itself but only in the presence of heat-stable molecule(s) from cells. To distinguish between these two possibilities, we added E. coli lysates to the reaction. Notably, ubiquitination of Rab33b did occur (Fig. 4a, lane 4). These results demonstrate that SdeA catalyses E1/E2-independent ubiquitination in a process that requires one or more heat-stable molecules present in cells.

Classic ubiquitination requires the conserved E1 that activates ubiquitin in a process powered by hydrolysis of ATP, which binds the enzyme in a Mg²⁺-dependent manner¹. We thus determined the requirement of these molecules in SdeA-mediated ubiquitination. Because of the importance of the mART motif in the cleavage of NAD by canonical ADP-ribosyltransferases¹⁹, we included this compound in our reactions. In reactions containing NAD, Mg²⁺ and ATP, ubiquitination of Rab33b occurred (Fig. 4b, lane 2). Yet, when NAD was withdrawn, no ubiquitination was detected (Fig. 4b, lane 3). In line with this observation, ubiquitination occurred in reactions containing NAD but not ATP or Mg²⁺ (Fig. 4b, lanes 4 and 5). Heat-treated NAD is active, which is consistent with the fact that boiled cell lysates allowed SdeA to function (Fig. 4b, lane 8). Exogenous NAD is sufficient for the activity of SdeA that had been dialysed against a buffer containing EDTA (Extended Data Fig. 4a), suggesting that this compound is the only co-factor required for the activity. SdeAɛA is unable to catalyse the modification even in the presence of NAD (Fig. 4b, lane 9). Under this condition, both Rab1 and Rab6A were ubiquitinated by SdeA (Extended Data Fig. 4b). Similarly, SidE, SdeB and SdeC ubiquitinated Rab33b (Extended Data Fig. 4c).
implied ADP-ribosylation of this side chain as a possible step before ubiquitin conjugation, which is consistent with the requirement of the R-S-ExE motif found in members of the SdeE protein family. Thus, we probed the reaction intermediate by obtaining SdeA178-1000, a fragment that retained the ability to modify Rab33b but had lost the self-ubiquitination activity (Extended Data Fig. 10b, c). Incubation of SdeA178-1000 with NAD and ubiquitin led to the release of nicotinamide (Extended Data Fig. 10d), suggesting the formation of ADP-ribosylated ubiquitin. Furthermore, inclusion of 32P-γ-NAD in the reaction produced 32P-labelled ubiquitin in an Arg42-dependent manner and the ADP-ribosyl moiety linked to Arg42 of ubiquitin can be detected by mass spectrometric analysis (Extended Data Fig. 10e–g). Thus, ADP-ribosylated ubiquitin is the reaction intermediate. The production of AMP in reactions with full-length SdeA could be a subsequent step in the attack of an acceptor nucleophile (from the Rab proteins or SdeA itself in the self-conjugation reaction) on the ADP-ribosylated ubiquitin leading to the modification of the target protein.

In a canonical ubiquitination reaction, ubiquitin activated by E1 is delivered to E2 to form the E2–Ub thioester. For the E3 ligases of theRING family, ubiquitin is directly transferred from the E2 to a substrate facilitated by the ligases, whereas members of the HECT and RBR E3 families transfer ubiquitin to a catalytic cysteine in the E3 before delivering it to the substrate1. Clearly, SdeA defines an all-in-one ubiquitin conjugation enzyme that directly activates ubiquitin; the fact that SdeA178-1000 defective in auto-ubiquitination can still modify Rab33b suggests that the activated ubiquitin is directly transferred to the substrate.

The discovery that ubiquitin can be modified by ADP-ribosylation expands the post-translational modification on this prevalent signalling molecule, which has been shown to be modified by acetylation and phosphorylation25. Whether ADPR–Ub itself is directly used to modify proteins is unknown, but it is clear that such modifications can potentially lead to significant expansion of the ubiquitin code and its functions in cellular processes and disease development25. The mART motif is present in a family of mammalian proteins, some of which are unable to catalyse ADP-ribosylation25. In light of the mART-dependent ubiquitination activity of SdeA, it will be interesting to determine whether any of these mART-containing proteins is capable of catalysing ubiquitination, and if so, whether the reaction requires E1 and E2. The identification of eukaryotic mART proteins with such a capability will surely expand the spectrum of cellular processes regulated by ubiquitination.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 2 October 2015; accepted 14 March 2016.

Published online 6 April; corrected online 4 May 2016 (see full-text HTML version for details).

1. Komander, D. & Rape, M. The ubiquitin code. *Annu. Rev. Biochem.* **81**, 203–229 (2012).

2. Zimnegre, J., Montinario, A., Peltzer, N. & Walczak, H. Ubiquitin in the immune system. *EMBO Rep.* **15**, 28–45 (2014).

3. Zhou, Y. & Zhu, Y. Diversity of bacterial manipulation of the host ubiquitin pathways. *Cell. Microbiol.* **17**, 26–34 (2015).

4. Cui, J. et al. Glutamyl deamidation and dysfunction of ubiquitin/NEDD8 induced by a bacterial effector family. *Science* **329**, 1215–1218 (2010).

5. Xu, L. & Luo, Z. Q. Cell biology of infection by *Legionella pneumophila*. *Microbes Infect.* **15**, 157–167 (2013).
18. Ganesan, A. K., Frank, D. W., Misra, R. P., Schmidt, G. & Barbieri, J. T. 
Pseudomonas aeruginosa exoenzyme S ADP-ribosylates Ras at multiple sites. 
J. Biol. Chem. 273, 7332–7337 (1998).

19. Simon, N. C., Aktories, K. & Barbieri, J. T. Novel bacterial ADP-ribosylating 
toxins: structure and function. Nature Rev. Microbiol. 12, 399–611 (2014).

20. Hayve, J. C. & Roy, C. R. Toxicity and SidJ-mediated suppression of toxicity 
require distinct regions in the SidE family of Legionella pneumophila effectors. 
Infect. Immun. 83, 3506–3514 (2015).

21. Jeong, K. C., Sexton, J. A. & Vogel, J. P. Spatiotemporal regulation of a Legionella 
pneumophila T4SS substrate by the metaeffect SidJ. PLoS Pathog. 11, 
e1004695 (2015).

22. Tan, Y., Arnold, R. J. & Luo, Z. Q. Legionella pneumophila regulates the small 
GTPase Rab1 activity by reversible phosphorylcholination. Proc. Natl Acad. Sci. 
USA 108, 21212–21217 (2011).

23. Swanson, M. S. & Isberg, R. R. Association of Legionella pneumophila with the 
macrophage endoplasmic reticulum. Infect. Immun. 63, 3609–3620 (1995).

24. Liu, Y. & Luo, Z. Q. The Legionella pneumophila effector SidJ is required for 
efficient recruitment of endoplasmic reticulum proteins to the bacterial 
phagosome. Infect. Immun. 75, 592–603 (2007).

25. Sherwood, R. K. & Roy, C. R. A. Rab-centric perspective of bacterial pathogen-
occupied vacuoles. Cell Host Microbe 14, 256–268 (2013).

26. Ortiz Sandoval, C. & Simmen, T. Rab proteins of the endoplasmic reticulum: 
functions and interactors. Biochem. Soc. Trans. 40, 1426–1432 (2012).

27. Itoh, T. et al. Golgi-resident small GTPase Rab33B interacts with Atg16L and 
modulates autophagosome formation. Mol. Biol. Cell 19, 2916–2925 (2008).

28. Sheedlo, M. J. et al. Structural basis of substrate recognition by a bacterial 
deubiquitinase important for dynamics of phagosome ubiquitination. 
Proc. Natl Acad. Sci. USA 112, 15090–15095 (2015).

29. Herhaus, L. & Dikic, I. Expanding the ubiquitin code through post-translational 
mapping. EMBO Rep. 16, 1071–1083 (2015).

30. Glowacki, G. et al. The family of toxin-related ecto-ADP-ribosyltrasfersas in 
humans and the mouse. Protein Sci. 11, 1657–1670 (2002).

Supplementary Information is available in the online version of the paper.

Acknowledgements We thank P. Hollenbeck (Purdue University) for critical 
reading of the manuscript. J. Barbieri (Medical College of Wisconsin) for 
plasmids. This work was supported by National Institutes of Health grants 
R56AI103168, K02AI085403 and R21AI105714 (Z.-Q.L.), 2R01GM103401 
(C.D.) and National Natural Science Foundation of China grants 21305006 and 
21475005 (X.L.).

Author Contributions J.Q. and Z.-Q.L. conceived the general ideas for this 
work. J.Q. and Z.-Q.L. planned, performed and interpreted experiments. 
Y.T. initiated the project, performed the bioinformatics analysis and 
determined the importance of the predicted mART motif in yeast toxicity. 
M.S., E.S.N., J.Q. and C.D. determined the reaction intermediates. K.Y., X.L. 
and E.S.N. performed mass spectrometric analyses. J.Q. and Z.-Q.L. wrote the 
manuscript and all authors provided editorial input.

Author Information Reprints and permissions information is available at 
www.nature.com/reprints. The authors declare no competing financial 
interests. Readers are welcome to comment on the online version of the 
paper. Correspondence and requests for materials should be addressed to 
Z.-Q.L. (luoz@purdue.edu).
METHODS

Bacterial, yeast strains and plasmid construction. L. pneumophila strains used in this study were derivatives of the Philadelphia 1 strain Lp02 (ref. 31) and were grown and maintained on CYE medium or in AYE broth as previously described31. When necessary antibiotics were included as described31. The ΔsidE strain was made by step-wise deletion of the 4 members using an established method31. For complementation experiments, the genes were inserted into pZL507 (ref. 32). All infections were performed with bacterial cultures grown to the post-exponential phase as judged by optical density of the cultures (OD600 = 3.0–3.8) as well as increase of bacterial motility. For expression in mammalian cells, genes were cloned into pEGFP-C1 (Clontech) or a 4 × Flag vector32. The integrity of all constructs was verified by sequencing analysis.

Cell culture, infection, transfection and co-immunoprecipitation. HEK293 or 293T cells (ATCC) were cultured in Dulbecco’s modified minimum Eagle's medium (DMEM) supplemented with 10% FBS. Cells grown to about 80% confluence were transfected with Lipofectamine 3000 (Life Technology) following a standard protocol used by the manufacturer’s instructions. U937 cells (ATCC) were differentiated into macrophages as described33. D. discoideum strains AX4 and AX4–HDEL–GFP were cultured in HL-5 medium as described earlier34. Strains of L. pneumophila used for infection were grown in AYE to post-exponential phase judged by optical density (OD600 = 3.2–4.0) and by increase in motility. 2 × 10^6 D. discoideum cells seeded in 24-well plates were infected with an MOI of 0.05 for growth experiments and of 5 for immunostaining. In all cases, one hour after adding bacteria to cultured cells, infections were synchronized by washing the infected cells three times with warm PBS buffer. Total bacterial counts at indicated time points were determined by plating serially diluted saponin lysates onto bacterial media. To determine the development of the LCV in COS1 cells (ATCC) expressing Rab33b and its mutants, cells transfected for 14 h were infected with wild-type L. pneumophila and samples were fixed 14 h after bacterial uptake. Intracellular and extracellular bacteria were differentially stained with a Legiorella-specific antibody and secondary antibodies conjugated to different fluorescence dyes. The category of LCVs was scored visually under a fluorescence microscope. All cell lines used were directly purchased from ATCC and were free of mycoplasma contamination by monthly testing using the PlasmoTest Kit (InvivoGen).

For infections to determine the modification of Rab33b, HEK293 cells were transfected to express 4 × Flag-Rab33b and FC-RII for 24 h with Lipofectamine 3000 (Life Technology). Bacteria of relevant L. pneumophila strains were opsonized with rabbit anti-Legionella antibodies32 at 1:500 for 30 min before infecting the cells at an MOI of 10 for 2 h. Lysates prepared from infected cells with RIPA buffer (Thermo Fisher Scientific) were subjected to immunoprecipitation with Flag beads (Sigma–Aldrich).

To determine protein translocation by L. pneumophila, cells infected with the indicated bacterial strains were lysed with 0.2% saponin, which lysed membranes of mammalian but not of bacterial cells. The lysates were directly probed for GST-tagged protein, while all other proteins were purified as His₆–tagged proteins. pQE30–4 × Flag–Rab33b was sub-cloned from the mammalian expression vector p4 × Flag–Rab33b to produce His₆–4 × Flag–Rab33b. For protein production, 30 ml of overnight culture of the E. coli strain harbouring the appropriate plasmid was transferred to 750 ml LB medium (ampicillin 100 μg ml⁻¹) and grown until OD₆₀₀ of 0.6–0.8 was reached. After adding IPTG (isopropyl thio-d-galactopyranoside) to a final concentration of 0.2 mM, the cultures were further incubated in a shaker at 18 °C for 16–18 h. Bacterial cells were harvested by spinning at 12,000 g and lysed by sonication in the presence of protease inhibitors. The soluble fractions were collected by centrifugation at 12,000 g twice at 4 °C. His-tagged proteins were purified with Ni²⁺–NTA beads (Qiagen), and eluted with PBS containing 300 mM imidazole; GST–Rab1 were purified with Glutathione Sepharose 4 Fast Flow beads (GE healthcare), and proteins bound to beads were eluted with 25 mM reduced glutathione in 20 mM Tris–HCl, pH 8.0, 100 mM NaCl. Purified proteins were dialysed in a buffer containing 25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM DTT. To determine the potential involvement of the ions and other co-factors in the activity of SdeA, the protein was dialysed against the same buffer containing 10 mM EDTA for 14 h at 4 °C. Protein concentrations were determined by Bradford assay. For proteins used in in vitro biochemical assays, extensive dialysis was performed with at least two buffer changes. The purity of proteins was larger than 95% as assessed by Coomassie brilliant blue staining.

In vitro ubiquitination assays. E1, E2 and ubiquitin were obtained from Boston Biochem and were used at 100 nM for each 50-μl reaction. Ubiquitination assays were performed at 37 °C for 2 h in a reaction buffer containing 50 mM Tris–HCl (pH 7.5), 0.4 mM 3-mercaptopropionic acid adenine dinucleotide (NAD) (Sigma–Aldrich) and 1 mM DTT. Each 50-μl reaction contains 10 μg ubiquitin, 5 μg SdeA, SdeB, SdeC, SdeD or their mutant proteins and 5 μg substrates. When necessary, ATP and Mg²⁺ were added to a final concentration of 2 mM and 5 mM, respectively. When needed, 50 μg of mammalian or E. coli lysates were added. Heat treatment of cell lysates or NAD was performed at 100 °C for 5 min. When necessary maleimide (MEM) was added to in vitro reactions at a final concentration of 50 μM.

Antibodies, immunostaining and immumobloting. Antibodies against Legionella and GFP were described elsewhere35. Antibodies specific for SdeA were prepared by injecting rabbits with purified protein (Pocono Rabbit Farm and Laboratory, Canadensis, PA) following a standard procedure used by the service provider. When necessary, antibodies were affinity-purified against the same proteins covalently coupled to an Affigel matrix (Bio-Rad) using standard protocols36. Cell fixation, permeabilization and immunostaining were performed as described37. For immunostaining, anti-Legionella antisera were used at 1:1,000 (ref. 32). Intracellular bacteria were distinguished from extracellular bacteria by differential immunostaining with secondary antibodies of distinct fluorescence dyes. Processed samples were inspected and scored using an Olympus IX-81 fluorescence microscope.

For immunoblotting, samples resolved by SDS–PAGE were transferred onto nitrocellulose membranes. After blocking with 5% milk, membranes were incubated with the appropriate primary antibody; anti-GFP (Sigma, cat. no. G7781), 1:10,000; anti-GST (Sigma, cat. no. G6539), 1:10,000; anti-Flag (Sigma, F8180), 1:20,000; anti-ICDH1, 1:10,000; anti-PGK (Life Technology, cat. no: 459250), 1:3,000; anti-SdeA, 1:1,000; anti-SdeC, 1:1,000; anti-UB (Santa Cruz, cat. no: sc-8017), 1:1,000; anti-His (Sigma, cat. no. H1029), 1:10,000. Tubulin (DSHB, E7), 1:10,000. Membranes were incubated with an appropriate IRDye infrared secondary antibody (Li-Cor’s Biosciences Lincoln, Nebraska, USA) and the signals were obtained by using the Odyssey infrared imaging system.

GTP loading assay. For S²–GTP incorporation assays, 20 μg of 4 × Flag–Rab33b was loaded with unlabelled GDP (5 mM) before ubiquitination as described32. GDP loaded 4 × Flag–Rab33b was used for ubiquitination assays in the presence of either SdeA (10 μg) or SdeEDF (10 μg) for 2 h at 37 °C. 20% of the samples were analyzed in a standard assay of 4 × Flag–Rab33b and Coomassie staining. Ubiquitinated or non-ubiquitinated 4 × Flag–Rab33b was incubated in 50 μl nucleotide exchange buffer containing 25 mM Tris–HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl₂, and 0.1 mM EDTA with 5 μl of S²–GTP (Perkin–Elmer). GTP-loading reactions were performed at 22 °C. Aliquots of reactions were withdrawn at indicated time points, passed through nitrocellulose membrane filters (Hawp02500; Millipore) and placed onto a vacuum platform attached to a waste liquid container. Membranes were washed three times using the exchange buffer to remove the free nucleotides, and were then transferred into scintillation vials containing 8 ml scintillation fluid (Beckman). Incorporated S²–GTP was detected as a radioactivity signal on a scintillation counter at 1 min per sample.

GTPase assay. 20 μg of 4 × Flag–Rab33b was used for ubiquitination assays in the presence of either SdeA (10 μg) or SdeEDF (10 μg) for 2 h before S²–GTP (Perkin–Elmer) was added to the reactions. Nucleotide loading was performed at 22 °C for 30 min. Aliquots of the reactions were withdrawn and passed through
Detection of reaction intermediates by 32P-labelled ATP and NAD. To detect the ubiquitin intermediate, 5 μg of SdeA or SdeA19-1100 was incubated with 10 μg GST-ubiquitin, GST-ubiquitin∆56, GST in the presence of 32P-α-NAD (5 μCi) in a reaction buffer containing 50 mM Tris-HCl (pH 7.5). The reaction was performed at 37 °C for 6 h and stopped by adding 5 x SDS loading buffer. A reaction containing the E1 activating enzyme (1 μg), GST-ubiquitin or GST (10 μg), 32P-α-ADP (5 μCi) in the presence of 50 mM Tris-HCl (pH 7.5) and 2 mM MgCl₂ was used as a positive control. The 32P-labelled intermediates were detected by autoradiography.

Detection of reaction intermediates by 32P-labelled ATP and NAD. To detect AMP generated in reactions catalysed by SdeA, reactions were set up with 50 μg SdeA178-1000, 10 mM NAD and 100 μg of SdeA or SdeA19-1100 incubated with 10 μg GST-ubiquitin, GST-ubiquitin∆56, GST in the presence of 32P-α-NAD (5 μCi) in a reaction buffer containing 50 mM Tris-HCl (pH 7.5). The reaction was performed at 37 °C for 2 h. Controls used were 1 mM solutions containing only NAD, nicotinamide or AMP. Samples for mass spectrometric analysis were obtained by using His₆-ubiquitin in reactions containing SdeA19-1100 and NAD for 2 h, SdeA19-1100 and other components were removed by Ni²⁺ beads chromatography. Eluted proteins were segregated in SDS–PAGE and the band corresponding ubiquitin was excised and digested with trypsin. Resulting peptides were analysed in a NanoAcquity nano-HPLC system (Waters) by loading peptides into a trap column (5 cm × 150 μm i.d. column packed in-lab with 5 μm Jupiter C18 stationary phase) and separated in a 40 cm × 75 μm i.d. column packed in-lab with 3 μm Jupiter C18 stationary phase. The elution was carried out at 300 nl per min with the following gradient: 0–8% B solvent in 2 min, 8–20% B in 18 min, 12–30% B in 55 min, 30–45% B in 22 and 97–100% B in 3 min, before holding for 10 min at 100% B. Eluting peptides were introduced to the mass spectrometer (Q-Exactive HR, Thermo Fisher Scientific) using electrospray ionization and mass spectra were collected from 400–2,000 m/z with 100,000 resolution at m/z 400. HCD tandem-mass spectra were collected by data-dependent acquisition of the 12 most intense ions using normalized collision energy of 30%. A dynamic exclusion time of 45 s was used to discriminate against previously analysed ions. Spectra were analysed manually by de novo sequencing.

Data quantitation and statistical analyses. Student’s t-test (two-sided) was used to compare the mean levels between two groups each with at least three independent samples. No statistical methods were used to predetermine sample size.
Extended Data Figure 1 | Inhibition of the secretion of SEAP by SidE, SdeB and SdeC and the recruitment of an ER marker by the *L. pneumophila* mutant lacking the SidE family. **a**, GFP fusions of the indicated proteins were co-expressed with SEAP in 293T cells for 24 h. The SEAP index was determined by measuring alkaline phosphatase activity in culture supernatant or in cells. Similar results were obtained in three independent experiments, and data shown are from one representative experiment done in triplicate. Note that mutations in the putative mART motif abolished the inhibitory effects. Error bars represent s.e.m. (*n* = 3). **b**, Quantitation of the vacuoles positive for GFP–HDEL. The indicated bacterial strains were used to infect a line of *D. discoideum* stably expressing GFP fusion to the ER retention signal HDEL and the recruitment of the GFP–HDEL signal to the phagosome was evaluated 10 h after infection. At least 150 phagosomes were scored in each sample done in triplicate. Results shown are from one representative experiment done in triplicate and similar results were obtained from three independent experiments. Error bars represent s.e.m. (*n* = 3). **c**, Representative images of *L. pneumophila* phagosomes associated with GFP–HDEL. Images are from one representative of three independent experiments with similar results. Scale bar, 5 μm.
Extended Data Figure 2 | SdeA does not ADP-ribosylate mammalian proteins, the modification of Rab33b by other members of the SidE family and SdeA-mediated post-translational modification of Rab1 during bacterial infection. a, SdeA, SdeA<sub>E/A</sub> or ExoS and 5 μCi <sup>32</sup>P-NAD were added to 100 μg total protein of 293T cells. After incubation at 22 °C for 1 h, samples were separated by SDS–PAGE. Gels were stained with Coomassie brilliant blue (left panel) and then by autoradiography for the indicated time duration (middle and right panels). In samples receiving SdeA, no ADP-ribosylation signal was detected in many experiments performed in various reaction conditions. Lane 1: <sup>32</sup>P-α-NAD + SdeA + 293T lysates; lane 2: <sup>32</sup>P-α-NAD + SdeA<sub>E/A</sub> + 293T lysates; lane 3: no sample; lane 4: <sup>32</sup>P-α-NAD + ExoS<sub>78-453</sub> + FAS + 293T lysates. b, Flag-tagged Rab33b was co-expressed with GFP-tagged testing proteins in 293T cells for 24 h. Cell lysates were subjected to immunoprecipitation with Flag beads and the precipitated products were probed with the Flag antibody (right panel). 5% of each lysate was probed for the expression of Rab33b (left panel) or for GFP fusions (middle panel). Proteins used: 1, GFP; 2, GFP–SdeB<sub>11-1751</sub>; 3, GFP–SidE; 4, GFP–SidE<sub>E</sub>. 293T cells transfected to express Flag–Rab1 were infected with the indicated L. pneumophila strains for 2 h and the Rab1 enriched by immunoprecipitation was probed by immunoblotting. For all panels, similar results were obtained from three experiments. a–c, Uncropped blots and autoradiograph images are shown in Supplementary Fig. 1.
Extended Data Figure 3 | The extracted ion chromatograms of ubiquitin tryptic fragments detected by mass spectrometry, expression of Rab33b and its mutants in COS1 cells, and in vitro ubiquitination of Rab33b by SdeA with E1 and a series of E2 proteins. a, Proteins in bands corresponding to normal (upper panel) or shifted (lower panel) Rab33b were digested with trypsin and the resulting protein fragments were identified by mass spectrometry. Note that the ubiquitin tryptic fragments are present only in the shifted band of higher molecular mass. b, COS1 cells were transfected with GFP or GFP fusion of Rab33b or its mutants for 14 h. Total cell lysates resolved by SDS–PAGE were probed with a GFP-specific antibody. Tubulin was detected as a loading control. c, Reactions containing E1 and the indicated E2 proteins were allowed to proceed at 37 °C for 2 h. Proteins in the reactions were resolved by SDS–PAGE followed by immunoblotting to detect ubiquitinated proteins with higher molecular mass (left panel). SdeA in the reaction was detected with specific antibodies by using 10% of the reactions (lower panel). Control reactions with wild-type Legionella E3 ligase SidC1-542 and its enzymatically inactive mutant SidC1-542C46A with E1 and the E2 UbcH7 were established to monitor the activity of E1 (right panel). Note the robust self-ubiquitination of SidC1-542 (second lane right panel). Results in a are representative of three experiments with similar results; b and c are a representative of two and five independent experiments, respectively. b, c, Uncropped blots are shown in Supplementary Fig. 1.
Extended Data Figure 4 | The activity of EDTA-dialysed SdeA and other members of the SidE family. a, SdeA or SdeA\textsubscript{EA} dialysed against a buffer containing 10 mM EDTA was used for \textit{in vitro} ubiquitination of Rab33b. Reactions were allowed to proceed for 2 h at 37 °C. Samples resolved by SDS–PAGE were detected by Coomassie staining (upper panel), by immunoblotting with antibodies specific for ubiquitin (middle panel) or for the Flag tag (lower panel). Note that the addition of exogenous NAD is sufficient to allow SdeA-mediated ubiquitination of Rab33b (lane 2). b, \textit{In vitro} ubiquitination of Rabs by SdeA. Reactions containing indicated proteins and NAD were allowed to proceed for 2 h at 37 °C. After SDS–PAGE, ubiquitinated proteins were detected by staining 50% of the reactions resolved by SDS–PAGE with Coomassie (upper panel) or by immunoblotting with antibodies specific for ubiquitin (lower panel). Similar results were obtained from two experiments. c, \textit{In vitro} ubiquitination of Rab33b by SidE, SdeB\textsubscript{1-1751} and SdeC. Indicated testing proteins were incubated with NAD, ubiquitin and Flag–Rab33b for 2 h at 37 °C. Proteins resolved by SDS–PAGE were detected by antibodies specific for Flag (upper panel) or for ubiquitin (middle panel). His\textsubscript{6}-tagged SdeA, SdeB\textsubscript{1-1751} and SdeC and SdeA\textsubscript{EA} used in the reactions were probed 10% of the proteins with an antibody against His (lower panel). Similar results were obtained from two independent experiments. a–c, Uncropped blots are shown in Supplementary Fig. 1.
Extended Data Figure 5 | SdeA does not detectably ADP-ribosylate Rab33b or Rab1 and the deubiquitinase (DUB) activity of SdeA does not interfere with its ubiquitin-conjugation activity. a, 5 μg of SdeA or SdeA\textsubscript{E/A} were incubated with 5 μg of GST–Rab1, 4 × Flag–Rab33b and 5 μCi of \textsuperscript{32}P–α-NAD. A reaction containing 200 ng of ExoS\textsubscript{78–453}, 2 μg of FAS and 5 μg Rab5 was established as a positive control. All reactions were allowed to proceed for 1 h at 22 °C before being terminated by adding 5 × SDS loading buffer. Samples resolved by SDS–PAGE were detected by Coomassie staining (upper panel) and then by autoradiography (middle and lower panels). Lane 1: \textsuperscript{32}P–α-NAD + SdeA + GST–Rab1; lane 2: \textsuperscript{32}P–α-NAD + SdeA\textsubscript{E/A} + GST–Rab1; lane 3: \textsuperscript{32}P–α-NAD + SdeA + 4 × Flag–Rab33b; lane 4: \textsuperscript{32}P–α-NAD + SdeA\textsubscript{E/A} + 4 × Flag–Rab33b; lane 5: no sample; lane 6: \textsuperscript{32}P–α-NAD + ExoS\textsubscript{78–453} + FAS + Rab5. Note the strong ADP-ribosylation signals in the reaction with ExoS\textsubscript{78–453} (lane 6). b, SdeA, its mutants SdeA\textsubscript{C118A} or SdeA\textsubscript{C118AE/A} was used for in vitro NAD-dependent ubiquitination of Rab33b. Reactions containing the indicated components were allowed to proceed for 2 h at 37 °C before being terminated with SDS sample buffer. Samples resolved by SDS–PAGE were probed by Coomassie staining (upper panel) or by immunoblotting with antibody specific for ubiquitin (middle panel) or for the Flag tag (lower panel). c, Reactions containing GST–ubiquitin were similarly established to detect self-ubiquitination by SdeA. Note that SdeA and SdeA\textsubscript{C118A} exhibited similar activity in these reactions. Data in all panels are one representative of two independent experiments with similar results. a–c, Uncropped blots and autoradiograph images are shown in Supplementary Fig. 1.
Extended Data Figure 6 | The reactivity of ubiquitin mutants in SdeA-mediated ubiquitination. a, Arg42 in ubiquitin is important for SdeA-mediated ubiquitination. Ubiquitin or ubiquitinR42A was included in reactions catalysed by SdeA or the bacterial E3 ubiquitin ligase SidC (E1 and the E2 UbcH7 were added in the latter category of reactions). After allowing the reaction to proceed for 2 h at 37 °C. Samples separated by SDS–PAGE were probed with antibody against the Flag tag (on Rab33b) (middle panel) or ubiquitin (right panel). Note that ubiquitinR42A can be used by ubiquitination catalysed by SidC but not SdeA. b, GST–ubiquitinR42A cannot be used for self-ubiquitination by SdeA. GST-ubiquitin or GST–ubiquitinR42A was used in reactions with SdeA or SdeAΔE. Self-modification was detected by the shift of SdeA detected by Coomassie staining (left panel) or by immunoblotting with a GST-specific antibody (right panel). c, The lysine residues or the carboxyl terminus of ubiquitin is not important for SdeA-catalysed Rab33b ubiquitination. Reactions containing SdeA or SdeAΔE, NAD, Flag-Rab33b and the indicated ubiquitin mutants were allowed to proceed for 2 h at 37 °C. d, Utilization of the ubiquitin di-glycine mutant by different ligases. Reactions with indicated components were allowed to proceed for 2 h at 37 °C. Proteins resolved by SDS–PAGE were detected by staining (upper panel) or by immunoblotting with antibodies specific to ubiquitin (lower panel). Note that the wild type but not the di-glycine ubiquitin mutant (AA) can be conjugated to proteins in a reaction containing E1 and E2 and the bacterial E3 ligase SidC (Lanes 6 and 7). This di-glycine mutant (AA) can still be attached to Rab33b by SdeA (Lane 4). e, Addition of 6 histidine residues to the carboxyl end of ubiquitin did not affect SdeA-mediated ubiquitination. Reactions containing the indicated components were established and allowed to proceed for 2 h at 37 °C. SDS–PAGE resolved samples were probed by Coomassie staining (left panel) or by immunoblotting with a GST-specific antibody (right panel). The data in all panels are one representative of three independent experiments with similar results. a–e, Uncropped blots are shown in Supplementary Fig. 1.
Extended Data Figure 7 | Ubiquitination catalysed by SdeA is insensitive to the cysteine modifying agent maleimide. a, Ubiquitination reactions by SdeA or SidC together with E1 and E2 were established; maleimide was added to 50 μM to a subset of these reactions. After incubation at 37 °C for 2 h, ubiquitination was detected by Coomassie staining (left panel) or by immunoblotting with the Flag- (middle panel) or ubiquitin-specific (right) antibody. Note that maleimide completely inhibits ubiquitination in the reaction catalysed by SidC, E1 and its cognate and E2 (lane 6) but does not affect the activity of SdeA (lane 4). b, Maleimide does not affect self-ubiquitination of SdeA. Reactions containing the indicated components were established and the modification of SdeA was probed by Coomassie staining (left panel) or by immunoblotting with the GST-specific antibody (right panel). For all panels, similar results were obtained from four independent experiments. a, b, Uncropped blots are shown in Supplementary Fig. 1.
Extended Data Figure 8 | SdeA-mediated ubiquitination affects the activity but not stability of Rab33b and SdeA ubiquitinates Rab33b independently of its nucleotide binding status. a, Evaluation of the ubiquitinated Rab33b. 4×Flag–Rab33b was loaded with unlabelled GDP (5 mM) before ubiquitination reaction. GDP-loaded Rab33b was subjected to ubiquitination by SdeA or SdeA E/A for 2 h at 37 °C; 20% of the samples were withdrawn to determine the extent of ubiquitination by Coomassie staining. b, Ubiquitination affected the GTP loading activity of Rab33b. Ubiquitinated or non-ubiquitinated 4×Flag–Rab33b was incubated in 50 μl nucleotide exchange buffer containing 5 μCi 35S-GTP at 22 °C. Aliquots of reactions were withdrawn at indicated time points and passed through nitrocellulose membrane filters. Membranes were washed for three times using exchange buffer before being transferred into scintillation vials containing scintillation fluid to detect incorporated 35S-GTP with a scintillation counter. c, Ubiquitination affected the GTPase activity of Rab33b. Samples withdrawn from Ub~Rab33b or Rab33b loaded with 32P-GTP were measured for the associated radioactivity to set as the starting point. Equal volumes of samples were withdrawn at the indicated time points to monitor intrinsic GTP hydrolysis. The GTP hydrolysis index was calculated by dividing the readings obtained in later time points by the values of the starting point. d, SdeA-mediated ubiquitination does not lead to degradation of Rab33b. GFP-fusion of SdeA or SdeA E/A was co-transfected with Rab33b for 14 h. The proteasome inhibitor MG132 (10 μM) was added to one of the SdeA samples. The levels of Rab33b were detected by immunoblotting following immunoprecipitation with the Flag-specific antibody. Note that the addition of MG132 does not affect the level of modified Rab33b in samples co-transfected with SdeA. Similar results were obtained from two independent experiments. e, The nucleotide binding status of Rab33b does not affect its suitability as substrate in SdeA-mediated ubiquitination. Equal amounts of Rab33b, its dominant negative mutant Rab33b(T47N), or the dominant positive mutant Rab33b(Q92L) was incubated with SdeA. Samples withdrawn at the indicated time points were detected for ubiquitination by Coomassie staining (upper panel); 293T cells transfected to express these mutants were infected the indicated L. pneumophila strains and ubiquitinated Rab33b or its mutants were probed by molecular mass shift in Rab33b obtained by immunoprecipitation (lower panel). Data in this panel are one representative of two independent experiments with similar results. a, d, e, Uncropped blots and gel images are shown in Supplementary Fig. 1.
Extended Data Figure 9 | Detection of the reaction intermediates in SdeA-catalysed ubiquitination. a, Controls were analysed by HPLC of NAD alone and in the presence of SdeA, Ub, and SdeA and Ub. In these reactions, AMP and NAD were identified with retention times of 3.6 and 6.8 min, respectively. b, Both AMP (left) and NAD (right) were additionally identified by ESI mass spectrometry. Both NAD and a product in which the nicotinamide group has been lost were observed in these experiments. c, To determine whether other fragments are generated in this reaction, retention time for nicotinamide mononucleotide (NMN, left) and nicotinamide (Nic, right) was determined by HPLC to be 5.6 and 2.6 min respectively. d, To identify additional components, a reaction was set up and the individual components were identified by HPLC. In the reaction mixture, AMP (3.5 min), nicotinamide (Nic 5.5 min), and NAD (6.5 min) were observed. An additional component to the reaction mixture (labelled X) was observed (6.1 min), but could not be further identified by mass spectrometry. Data in all panels are one representative from three independent experiments with similar results.
Extended Data Figure 10 | Detection of the ubiquitination intermediate by using SdeA<sub>519-1100</sub>. a, Full-length SdeA cannot produce 32P-labelled product in reactions using 32P-α-NAD. Reaction samples resolved by SDS–PAGE were detected by Coomassie staining (left panel) and then by autoradiography (right panel). Note the 32P-α-AMP-GST-ubiquitin complex can be detected in the reaction containing E1 but not SdeA.

b, c, SdeA<sub>519-1100</sub> is defective in auto-ubiquitination. Reactions containing the indicated components were allowed to proceed for the indicated time duration and the production of ubiquitinated Rab33b (b) or SdeA<sub>519-1100</sub> was detected by immunoblotting. d, SdeA<sub>519-1100</sub> induces the production of nicotinamide from NAD and ubiquitin. Retention time for nicotinamide and NAD was first determined by HPLC and nicotinamide can only be detected in the reaction containing SdeA<sub>519-1100</sub>, NAD and ubiquitin.

e, SdeA<sub>519-1100</sub> induces the production of 32P-ADPR-labelled ubiquitin. GST-ubiquitin or GST–ubiquitin R42A was incubated with 32P-α-NAD and SdeA<sub>519-1100</sub> for 0.036 h. Classical E1 incubated with GST–ubiquitin was included as a control. Samples resolved by SDS–PAGE before autoradiography (20 min) (right panel). Note that GST–ubiquitin R42A cannot be labelled by 32P. Data in panels a–e are one representative from two independent experiments with similar results.

f, The detection of a peptide with m/z 737.33 corresponding to the tryptic peptide E<sub>34</sub>GIPPDQPDQRLIFAGK<sub>48</sub> containing one ADP-ribosylation site was detected only after ubiquitin was incubated with SdeA<sub>519-1100</sub>. As a loading control, another unmodified ubiquitin peptide T<sub>55</sub>LSYDIQK<sub>63</sub> was detected in both control and treated samples. g, Tandem mass analysis revealed that ADP-ribosylation occurred on Arg42 evidenced by the extensive fragmentation of the ADP-ribosylation into adenine, adenosine, AMP and ADP ions. Although not as extensive, the fragmentation of the peptide backbone helps confirm the peptide sequence. Data shown in all panels are one representative from two independent experiments with similar results. a–c, e, Uncropped blots and autoradiograph images are shown in Supplementary Fig. 1.

© 2016 Macmillan Publishers Limited. All rights reserved