Ubiquitination accomplished: E1 and E2 enzymes were not necessary

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Qui et al. (2016) show that a mono-ADP-ribosyltransferase, SdeA, from *Legionella pneumophila* catalyzes ADP-ribosylation of ubiquitin, allowing SdeA to modify substrate with ubiquitin in the absence of E1 and E2 enzymes.

Posttranslational attachment of ubiquitin (Ub), a small 76-residue protein, is one of the most abundant protein modifications in eukaryotic cells. The process of ubiquitination, covalently linking the C-terminus of Ub to a lysine ε-amine on a protein substrate, is carried out through a three-enzyme cascade (E1, E2, E3; Figure 1A). In the case of polyubiquitination, additional Ub molecules can be attached at eight positions (M1, K6, K11, K27, K29, K33, K48, or K63) on the previously linked Ub molecule, resulting in diverse polymeric Ub chains (Glickman and Ciechanover, 2002). This property enables polyUb chains to form distinct signals and hence the broad range of polyUb signaling pathways. As a mechanism to reverse ubiquitination, deubiquitinating enzymes (DUBs) act to depolymerize polyUb back to monomeric units and also remove the proximal Ub from substrates (Komander et al., 2009). Through tight regulation of ubiquitination cascades and DUBs, cells maintain a viable balance between free Ub and substrate attached Ub.

Given the important regulatory roles Ub signaling pathways play in eukaryotes, bacterial pathogens and viruses have evolved elaborate mechanisms to hijack host Ub signaling pathways and evade the immune response. This is highlighted by the numerous E3 Ub ligases and spectrum of DUBs encoded by bacterial and viral human pathogens (Maculins et al., 2016; Sheedlo et al., 2015). Since prokaryotic systems are essentially devoid of Ub, it is assumed that all bacterial effectors of Ub are present solely to manipulate the host. Therefore, in many instances bacteria simply employ their own E3 Ub ligases (generally a HECT-type), while relying on the native E1 and E2 enzymes of the host (Maculins et al., 2016). However, in a stunning turn, Qiu *et al*. (Qiu et al., 2016) characterize SdeA, a novel E3 Ub ligase of *Legionella pneumophila* capable of transferring Ub to Rab substrates independent of E1 and E2 enzymes.

Of the ~300 effector proteins *L. pneumophila* secretes within host cells, many are employed to override substrate selectivity of host SCF E3 complexes (Bruckert and Abu Kwaik, 2016; Quaile et al., 2015). Well-documented examples include LubX, a U-Box containing protein, and F-box proteins AnkB and LegU1. Interestingly, DUB activity in the N-terminal domain of SdeA has been previously characterized, as well as for two other SidE effector family members SdeB and SdeC (Sheedlo et al., 2015). Although K63-linked polyUb is the preferred linkage type for $SdeA^{1-193}$, the structure of the domain is not related to any of the five DUB families (Figure 1B), but rather the ULP1 fold, characteristic of SUMO specific proteases (Sheedlo et al., 2015). Thorough sequence analysis downstream of the DUB domain, Qui *et al*. discovered a canonical R-S-ExE

motif within SdeA, constituting a putative mono-ADP-ribosyltransferase (mART) domain (Figure 1B). As demonstrated on yeast and a protozoan host, the R-S-ExE motif is essential for virulence and manipulation of the endoplasmic reticulum (ER). Notably, two point mutations (R-S-AxA) abolished virulence and intracellular function of *L. pneumophila*. However, wide spread ADP-ribosylation activity was not detected using ^{32}P-NAD in human lysates. Yet the mART motif was required to induce a molecular weight shift in ER associated Rab proteins.

Surprisingly, mass spectrometry analysis of "modified" Rab33b revealed the presence of Ub. This finding directed Qui *et al*. to reconstitute an assay to identify the responsible E2 enzyme, but Rab33b was ubiquitinated under many unexpected conditions. Notably, ubiquitination still proceeded without ATP or Mg^{+2} and also following heat deactivation of lysate, which contains E1 and E2 enzymes. Even addition of the SdeA DUB domain and maleimide treatment did not alter the ubiquitinating activity of SdeA, highlighting a unique mechanism. Inline with the ADP-ribosylating function of the mART motif, ubiquitination was found to be mainly dependent on the presence of nicotinamide adenine dinucleotide (NAD). This left the gapping question as to how Ub transfer by SdeA was related to NAD.

In a most unconventional fashion, SdeA was still able to perform ubiquitination using Ub lacking the two C-terminal glycine residues and all possible Ub lysine mutants. However, modification of Rab33b appeared dependent on R42 of Ub, while also producing free nicotinamide. This suggested that SdeA was still functioning as a canonical mono-ADP-ribosyltransferase. Indeed, careful mass spectrometry analysis confirmed ADP-ribose was transferred to R42 of Ub. This finding was the missing link as to how Ub transfer was dependent on NAD. In a two-step process, ADP-ribose is added to R42 of Ub, which is then transferred to Rab substrates (Figure 1C). Qui *et al*. demonstrate significant autoubiquitination of $SdeA^{178\text{-}1,100}$, but can direct Ub transfer only to substrates using a truncated form, $SdeA^{519\text{-}1,100}$. From a mechanistic view, this suggests that transfer of mono-ADP-ribosylated-Ub proceeds directly to the substrate. Future studies will be required to determine the mechanism of SdeA-catalyzed Ub transfer and how Ub is attached to the substrates.

The findings of Qui *et al*. represent the first instance of a Ub specific mono-ADP-ribosyltransferase, as well as the first documentation of E1/E2 independent ubiquitination. Post-translational modification of Ub certainly adds layers of complexity to our understanding of the Ub signal and should be taken into account for future investigations. Just as PINK1 phosphorylation of S65 in Ub dramatically alters Ub's signaling properties, mono-ADP-ribosylation of R42 could also and should be further investigated. The cross-talk between Ub signaling pathways and ADP-ribosylation has just begun to be explored and in one example, assembly of 26S proteasome was enhanced by poly-ADP-ribosylated PI31 (Cho-Park and Steller, 2013). Tankyrase, the human poly-ADP-ribose polymerase that modifies PI31 also functions in Wnt/β-catenin signaling and maintenance of telomeres, but it is not clear how this directly affects Ub signaling (Smith and de Lange, 2000; Yang et al., 2016). In a more direct link, the E3 Ub ligase RNF146 uses a WWE domain to select poly-ADP-ribose modified proteins (BLZF1 and CASC3), which are subsequently ubiquitinated and degraded by the proteasome (Zhang et al., 2011).

Strictly in the context of mono-ADP-ribosylation and proteins containing the mART motif, many remain to be investigated both in pathogenic bacteria and humans. The presence of the mART motif in other members of the SidE effector family suggests the function of SdeA is conserved. Furthermore, the importance of SdeA in invasion does make SdeA a therapeutic candidate and Qui *et al*. have laid the groundwork. With additional structural and functional characterization this may be a possibility and provide a new means of intervention against deadly human pathogens. Clearly *L. pneumophila* has a diverse arsenal of biochemical weapons capable of affecting numerous host pathways, often without a clear link to Ub.

References

Bruckert, W.M., and Abu Kwaik, Y. (2016). Lysine11-Linked Polyubiquitination of the AnkB F-Box Effector of Legionella pneumophila. Infection and immunity *84*, 99-107.

Cho-Park, P.F., and Steller, H. (2013). Proteasome regulation by ADP-ribosylation. Cell *153*, 614-627.

Glickman, M.H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiological reviews *82*, 373-428.

Komander, D., Clague, M.J., and Urbe, S. (2009). Breaking the chains: structure and function of the deubiquitinases. Nature reviews. Molecular cell biology *10*, 550-563.

Maculins, T., Fiskin, E., Bhogaraju, S., and Dikic, I. (2016). Bacteria-host relationship: ubiquitin ligases as weapons of invasion. Cell research *26*, 499-510.

Qiu, J., Sheedlo, M.J., Yu, K., Tan, Y., Nakayasu, E.S., Das, C., Liu, X., and Luo, Z.Q. (2016). Ubiquitination independent of E1 and E2 enzymes by bacterial effectors. Nature *533*, 120-124.

Quaile, A.T., Urbanus, M.L., Stogios, P.J., Nocek, B., Skarina, T., Ensminger, A.W., and Savchenko, A. (2015). Molecular Characterization of LubX: Functional Divergence of the U-Box Fold by Legionella pneumophila. Structure *23*, 1459-1469.

Sheedlo, M.J., Qiu, J., Tan, Y., Paul, L.N., Luo, Z.Q., and Das, C. (2015). Structural basis of substrate recognition by a bacterial deubiquitinase important for dynamics of phagosome ubiquitination. Proceedings of the National Academy of Sciences of the United States of America *112*, 15090-15095.

Smith, S., and de Lange, T. (2000). Tankyrase promotes telomere elongation in human cells. Current biology : CB *10*, 1299-1302.

Yang, E., Tacchelly-Benites, O., Wang, Z., Randall, M.P., Tian, A., Benchabane, H., Freemantle, S., Pikielny, C., Tolwinski, N.S., Lee, E., *et al*. (2016). Wnt pathway activation by ADP-ribosylation. Nature communications *7*, 11430.

Zhang, Y., Liu, S., Mickanin, C., Feng, Y., Charlat, O., Michaud, G.A., Schirle, M., Shi, X., Hild, M., Bauer, A., *et al*. (2011). RNF146 is a poly(ADP-ribose)-directed E3 ligase that regulates axin degradation and Wnt signalling. Nature cell biology *13*, 623-629.

Figure 1. Canonical and SdeA mediated ubiquitination. (A) The conventional ubiquitination cascade used across all eukaryotes. E1 binds Mg^{+2} cofactor and forms a thioester intermediate between its catalytic cysteine and G76 of Ub's C-terminus in an ATP dependent process (E1~Ub; ~ indicates thioester bond). E2 is recruited to "loaded"

E1~Ub and Ub is transferred to the active site cysteine in E2, again forming a thioester intermediate with Ub's C-terminus (E2~Ub). In the final step, E3 simultaneously binds E2~Ub and a substrate, catalyzing the formation of an isopeptide linkage between Ub's C-terminus and a lysine sidechain of the substrate. (B) Domain architecture of SdeA from *L. pneumophila* includes an N-terminal ULP1 fold (purple) with DUB activity (Sheedlo et al., 2015), and the newly characterized mART motif (blue). Bottom left panel, the crystal structure of SdeA DUB domain (purple) bound to Ub (green) is shown (PDB: 5CRA). Bottom right panel, the active site of a representative mART domain from *Clostridium botulinum* C3 exoenzyme (PDB: 2C8C) bound to NAD (green), highlights conserved residues Q212 and E214 (red sticks) essential for catalysis. (C) SdeA mediated Ub transfer. First, SdeA catalyzes ADP-ribosylation to R42 of Ub. Subsequently, Ub is ligated to the Rab substrate with the release of AMP via an unknown mechanism. How Ub is conjugated to Rab substrate and what amino acid residue(s) on Rab substrate is modified require future investigation.

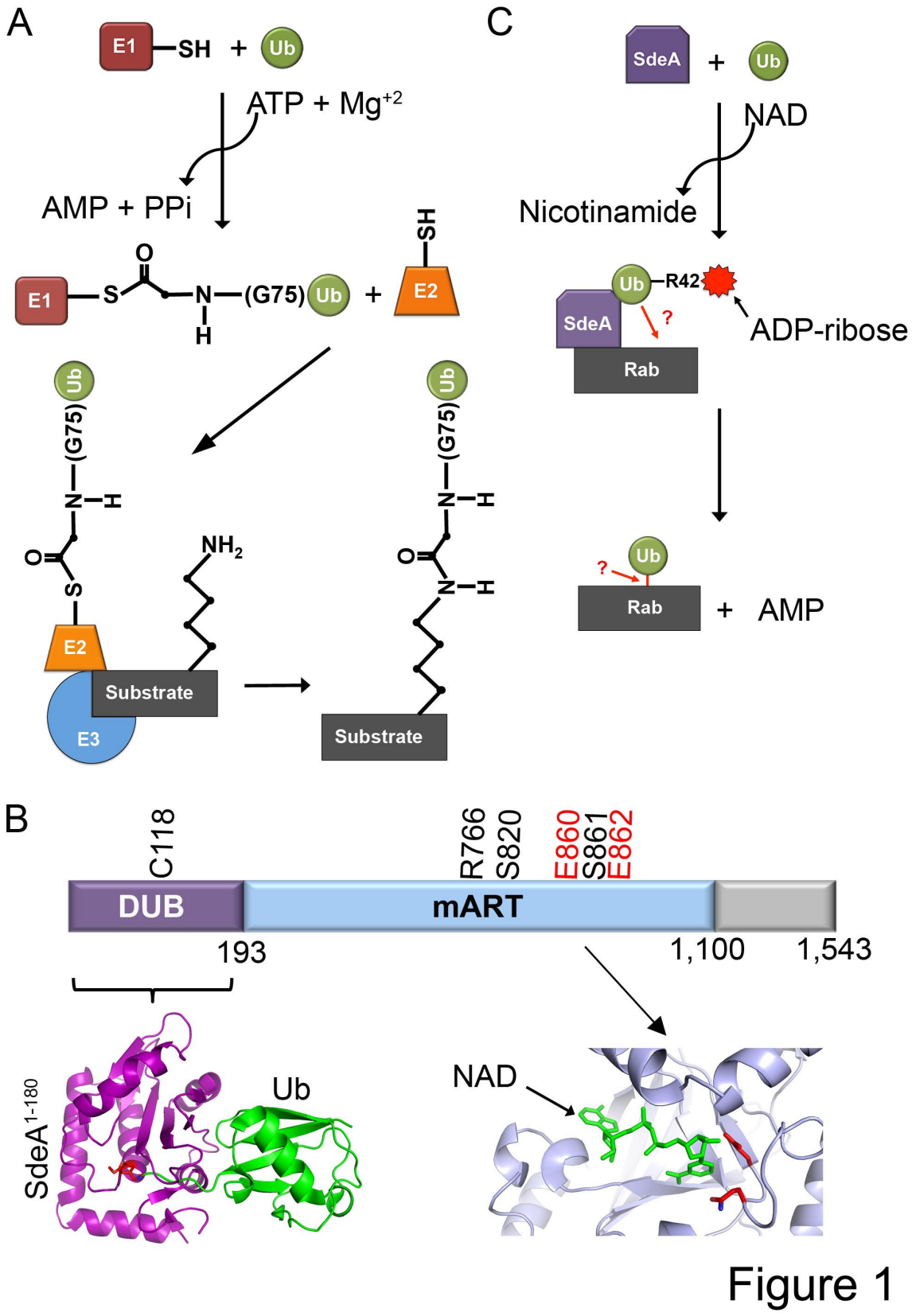
A
E1
SH
Ub
ATP + Mg+2
AMP + PPi
E1
S
O
N
(G75)
Ub
H
+
E2
SH
Ub
(G75)
N
H
O
S
E2
NH2
Substrate
E3
Substrate
C
SdeA
+
Ub
NAD
Nicotinamide
Ub
R42
SdeA
?
ADP-ribose
Rab
Ub
?
Rab
+
AMP
B
C118
R766
S820
E860
S861
E862
DUB
mART
193
1,100
1,543
SdeA1-180
Ub
NAD

Figure 1