A further case of Dop-ing in bacterial pupylation

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Protein homeostasis is fundamental to the function of all cellular systems. In eukaryotes, the ubiquitin–proteasome pathway mediates regulated protein degradation. Intensive studies of the eukaryotic proteasome over the past decades have unravelled the complexity of this multi-subunit, ATP-dependent protease, and proteasome inhibitors are now established anticancer drugs (Finley, 2009). Prokaryotes use ATP-dependent proteases—such as Lon, ClpP and FtsH—for protein degradation. In addition, some bacteria in the class of Actinomycetes have acquired a proteasome which shares sequence and structural homology with its eukaryotic counterpart (Darwin, 2009). The function of the prokaryotic proteasome and its implication in pathogenesis is the subject of ongoing research. In *Mycobacterium tuberculosis*, proteasome activity is essential for the pathogen to persist in macrophages of the lung epithelium and could therefore be a target for antimicrobial treatment (Darwin, 2009).

Labelling substrates for proteasomal degradation is well understood in eukaryotes, in which ubiquitin is attached to proteins that are subsequently recognized by proteasomal subunits and degraded (Finley, 2009). A similar tagging system has recently been identified in *M. tuberculosis*, in which the prokaryotic ubiquitin-like protein (Pup) serves as a ubiquitin analogue (Pearce *et al*, 2008). Subsequent proteome-wide studies have identified hundreds of Pup-tagged substrates in different mycobacteria, defining the ‘pupylome’ (Festa *et al*, 2010; Poulsen *et al*, 2010). Pupylated proteins are recognized by the proteasome-associated ATPase Mpa, that unfolds proteins before they are degraded in the proteolytic core (Darwin, 2009).

Ubiquitination is reversed by specific deubiquitinases, but whether pupylation is also reversible was previously unknown. Two studies by Darwin and colleagues and—in this issue of EMBO reports—by Weber-Ban

and colleagues have now demonstrated that Pup is removed from substrates when incubated with mycobacterial lysates (Burns *et al*, 2010; Imkamp *et al*, 2010). This suggests the presence of one or more ‘depupylases’, and indicates that pupylation is a complex and versatile process, much like ubiquitination.

Pup and ubiquitin conjugation are mechanistically unrelated; ubiquitin is ligated by its carboxy-terminal glycine residue to lysine residues of target proteins by an enzymatic cascade, comprising E1, E2 and E3 enzymes (Dye & Schulman, 2007). By contrast, the pupylation machinery seems to be simpler; a single ligating

Fig 1 | Emerging roles for Dop. (A) The pupylation system. (1) Dop functions as a deamidase, converting Pup^{Gln} to Pup^{Glu}. PafA ligates Pup^{Glu} to substrates, which are targeted to Mpa and the proteasome and are degraded. (2) Dop can reverse pupylation on substrates and might rescue substrates from degradation. (B) Dop might act to recycle Pup, either (3a) at the Mpa/proteasome level or (3b) by binding to pupylated substrates, where Mpa-mediated substrate unfolding activates Dop. (C) (4) The existence of Dop in proteasome deficient bacteria might indicate that Dop antagonizes non-degradational roles for Pup. Dop, deamidase of Pup; Mpa, *Mycobacterium* proteasome-associated ATPase; PafA, proteasome accessory factor A; Pup, prokaryotic ubiquitin-like protein.

enzyme, proteasome accessory factor A (PafA), mediates isopeptide bond formation between the C-terminal glutamic acid side-chain carboxyl group of Pup and a substrate lysine residue (Sutter *et al*, 2010).

Only about half of the Pup-containing bacteria encode a glutamic acid residue at the C-terminus (Striebel *et al*, 2009). In the remaining species, including *M. tuberculosis*, the *Pup* gene encodes a C-terminal glutamine, which requires deamidation to glutamic acid before conjugation to substrates can occur. This activating deamidation step is carried out by the deamidase of Pup (Dop; Striebel *et al*, 2009). Curiously, the *dop* gene is conserved in all Pup-containing bacterial species (with the exception of *Plesiocystis pacifica*), including those in which initial deamidation is unnecessary.

Imkamp *et al* and Burns *et al* now identify Dop as a depupylase in the Pup-modification pathway. Hydrolysis of Pup from model substrates *in vitro* is abolished in a *dop*-deficient bacterial lysate, or in lysate expressing a mutant form of *dop*, but can be restored by complementation with *dop*. Dop is able to depupylate many proteins when tested against the pupylome, suggesting a broad substrate spectrum. By contrast, without Dop the pupylome is unchanged over time, indicating that Dop might be the main depupylase in Mycobacteria. Purified Dop from *M. tuberculosis* shows depupylase activity against model substrates. Finally, Imkamp *et al* analyse a Dop homologue from *Corynebacterium glutamicum* that encodes Pup^{Glu} and hence does not depend on deamidation. This Dop homologue is expressed recombinantly and purified from *Escherichia coli*—which does not harbour the Pup-proteasome system—and shown to be an active depupylase *in vitro*.

Both groups then investigated the functional relationship between Pup/Dop and the proteasomal ATPase Mpa. Burns *et al* found that Mpa is required *in vivo* for

depupylation of a proteasome substrate. Imkamp *et al* found that Mpa significantly increases depupylation activity *in vitro*. The mechanism for this remains unclear, but full-length Pup seems to be essential for Mpa-mediated activation, as depupylation is not enhanced with an amino-terminally truncated Pup. Previous work has indicated that the N-terminus of Pup is required to initiate substrate unfolding (Striebel *et al*, 2010), and Imkamp *et al* speculated that unfolding makes the isopeptide bond more accessible for interaction with Dop. Evidence for this comes from the observation that Dop can cleave a peptide substrate with an accessible isopeptide bond at the same rate in the presence or absence of Mpa. It is intriguing that Dop co-purifies with the pupylome (Burns *et al*, 2010), this suggests that Dop has significant affinity but low activity for pupylated substrates. This might, however, prime the system for depupylation after Mpa interaction.

Corynebacteria do not have a proteasome, but maintain the pupylation machinery comprising Pup, PafA, Dop and the proteasomal ATPase ARC (a homologue of Mpa). Here, the fate of Pup-tagged proteins cannot be proteasomal degradation, although substrate unfolding by ARC could initiate degradation by other proteases. However, pupylation in proteasome-deficient bacteria might suggest additional non-degradative functions for pupylation.

Both studies demonstrate that Dop acts as a depupylase in Pup-containing bacteria, in addition to the previously reported deamidation role of Dop in mycobacteria. In fact, the chemical reactions underlying depupylation and deamidation are mechanistically similar. The key functional question that remains is whether Dop protects substrates from proteasomal degradation. Alternative explanations are that Dop acts in conjunction with Mpa or the proteasome to recycle Pup, or that it reverses non-degradative roles of pupylation (Fig 1).

So far, nothing is known about the regulation of Dop. It will be interesting to analyse expression profiles to determine whether Dop is regulated independently of other proteins in this system. Other open questions remain about the existence of co-factors and binding partners, and the organization of the Pup–Dop–Mpa network. Structural studies of the Dop enzyme will hopefully increase our understanding of its roles in depupylation.

In conclusion, Dop in the pupylation system has the potential to combine all known functions of deubiquitinases in the ubiquitin system: processing of precursors, rescuing substrates from degradation, recycling the modifier and reversing potential non-degradative roles of pupylation. The identification of the first depupylase opens an exciting new research field to unravel the functional consequences of depupylation.

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