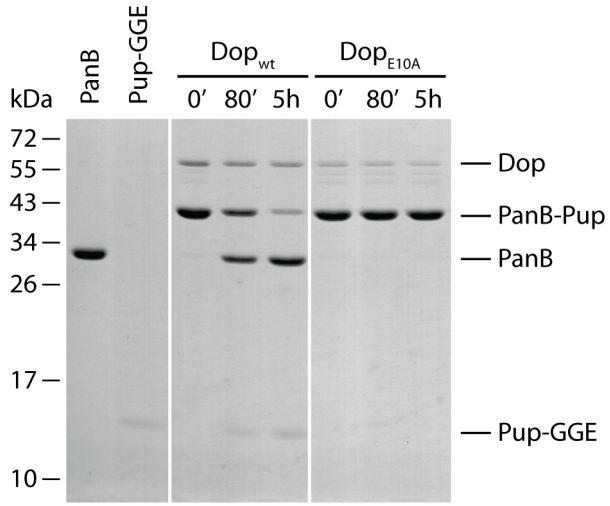
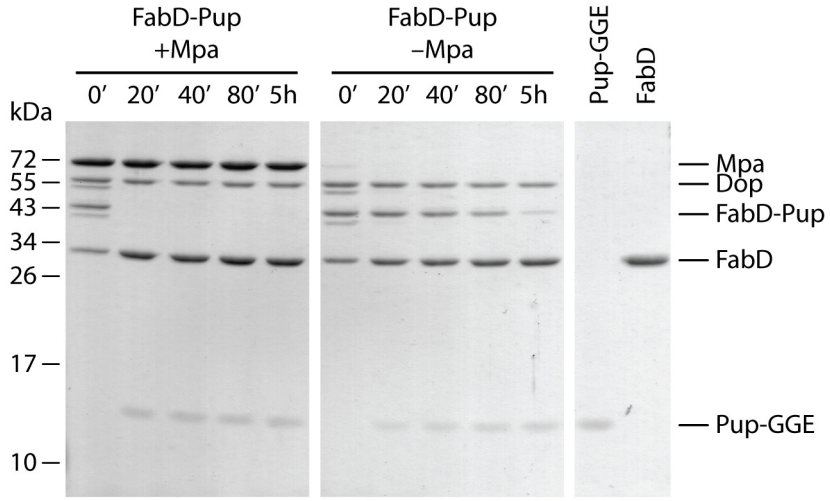
Imkamp *et al.* (2010) Dop functions as a depupylase in the prokaryotic ubiquitin-like modification pathway.

Supplementary information

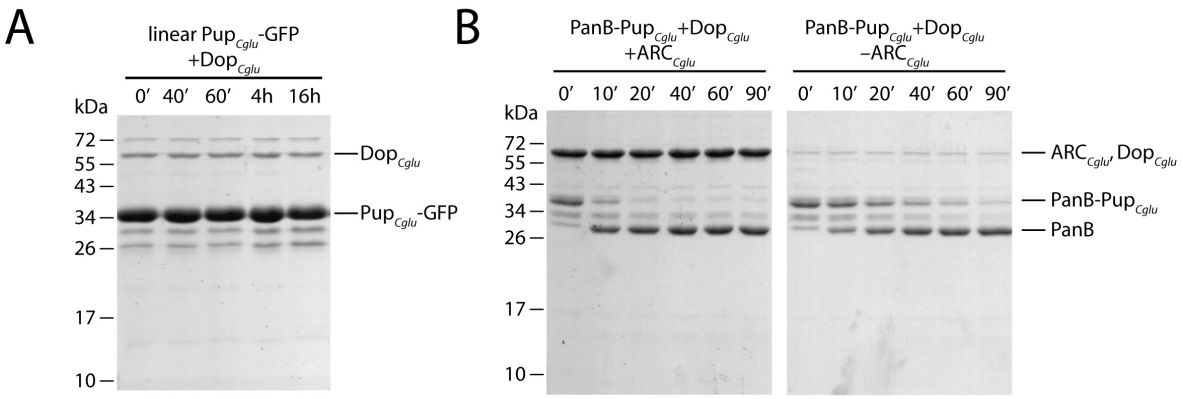
Supplementary Figures



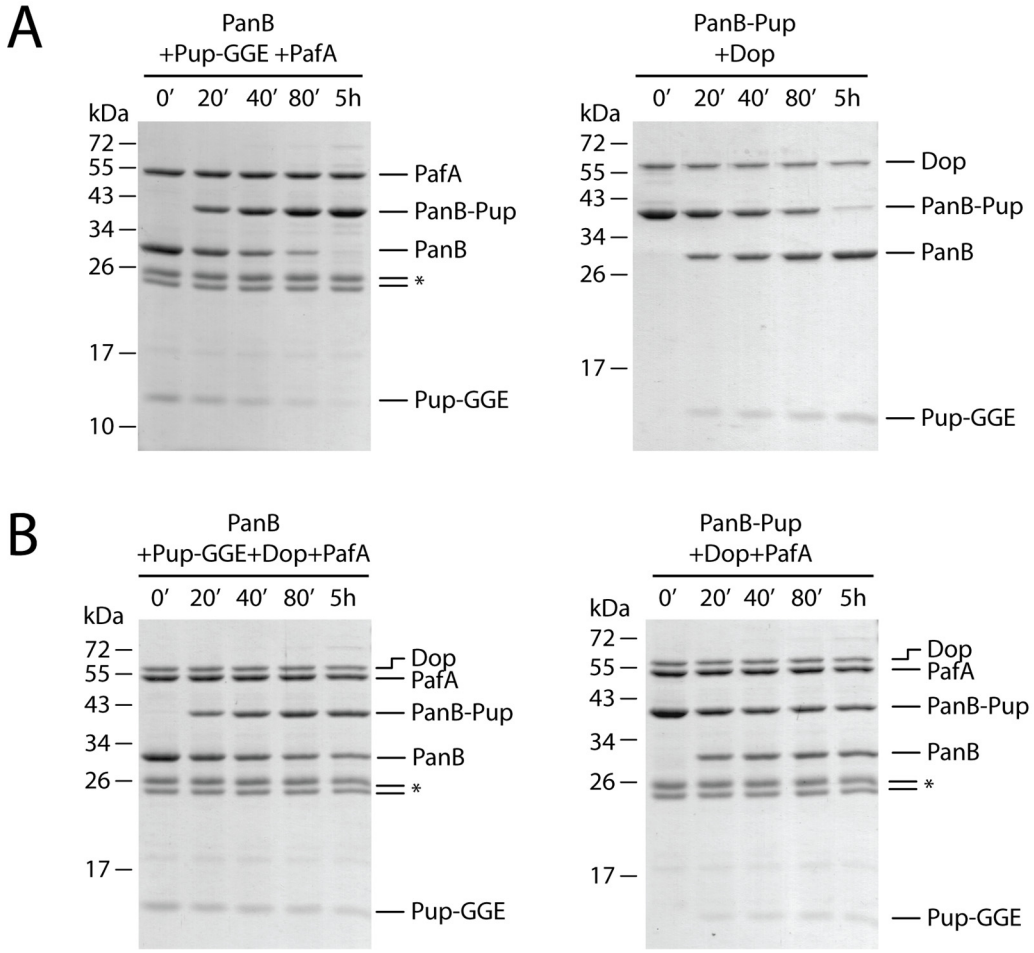
Supplementary Figure S1: An ATP binding site mutant of Dop is unable to depupylate PanB-Pup. PanB-Pup (3 μM), a substrate modified with Pup using the ligase PafA, is depupylated by Dop (0.5 μM) (middle panel) but not by DopE10A (right panel) as analysed by SDS–PAGE and Coomassie staining. For comparison, PanB and Pup-GGE are shown (left panel).



Supplementary Figure S2: Mpa enhances the depupylation of the proteasomal substrate FabD-Pup *in vitro*. FabD-Pup (3 μM), a substrate modified with Pup using the ligase PafA, is depupylated by Dop (0.5 μM) in the presence of ATP (5 mM) as analysed by SDS–PAGE and Coomassie staining (middle panel). For comparison, Pup and FabD are shown (right panel). The presence of Mpa (0.2 μM) increases the rate of depupylation of FabD-Pup by Dop (left panel).



Supplementary Figure S3: Corynebacterial Dop is specific for the isopeptide bond and the depupylation reaction is accelerated by ARC. (**A**) Pup$_{Cglu}$-GFP (3 µM), in which Pup$_{Cglu}$ is N-terminally fused to GFP, is not depupylated by Dop$_{Cglu}$ (0.5 µM). (**B**) Depupylation of PanB-Pup$_{Cglu}$ (3 µM) by Dop$_{Cglu}$ (0.25 µM) in absence (right panel) or presence (left panel) of ARC$_{Cglu}$ (0.8 µM). Reactions were analysed by SDS–PAGE and Coomassie staining. Proteins from *Corynebacterium glutamicum* are labeled with the subscript *Cglu*.



Supplementary Figure S4: Pupylation and depupylation occur on similar time scales and influence the pupylation state of a substrate population *in vitro*. (**A**). To observe a time course for the pupylation reaction, the ligase PafA (0.5 μM), Pup-GGE (4 μM) and PanB (3 μM) were mixed and samples were drawn after different incubation times and visualized by SDS-PAGE and Coomassie staining (left panel). Similarly, to observe a time course for the depupylation reaction, the depupylase Dop (0.5 μM) was mixed with PanB-Pup (3 μM) (right panel) (**B**) To observe the above time courses in presence of the respective opposite activities, PafA (0.5 μM) was incubated with Pup-GGE (4 μM) and PanB (3 μM) in presence of Dop (0.5 μM) (left panel) or Dop (0.5 μM) was incubated with PanB-Pup (3 μM) in presence of PafA (0.5 μM) (right panel). All reactions contained ATP (5 mM). The asterisk denotes protein impurities present in the PafA preparation.

Supplementary Materials and Methods

Cloning of mycobacterial constructs. For expression of a Pup-FabD fusion protein, *pup* was cloned via BamHI/SacI and *fabD* via SacI/HindIII into a His_6-Thioredoxin-TEV fusion vector (Striebel *et al.*, 2009), resulting in a His_6-Thioredoxin-TEV-Pup-FabD construct containing a LGSGSG-linker between Pup-GGE and FabD.

Purification of mycobacterial proteins. Dop-His_6, PafA-His_6, Pup, PanB-His_6 and Strep-FabD (Striebel *et al.*, 2009) as well as PanB-Strep, Pup-GFP and Mpa variants (all from *Mtb*) (Striebel *et al.*, 2010) were expressed and purified as described. Pup-FabD was purified in analogy to Pup-GFP as TEV-cleavable His_6-Thioredoxin-TEV-Pup-FabD fusion construct (Striebel *et al.*, 2010).

Purification of the pupylome. Msm Δ*dop* cells expressing His_6-tagged *Mtb* PupGGE (Imkamp *et al.*, 2010) were grown to an OD_{600} of 1.5. Cleared, soluble lysate was applied on a HiTrap NiNTA column in 50 mM Tris pH 7.4, 150 mM NaCl, 20 mM imidazole, washed with ten column volumes 40 mM imidazole and eluted with two column volumes 150 mM imidazole in the same buffer. The eluate was then applied on a 30 kDa MWCO filter and buffer exchanged to 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA while at the same time removing a large portion of excess His_6-Pup-GGE. Depupylation assays were performed as described above for the *in vitro* substrates.

Cloning of corynebacterial constructs. All corynebacterial genes were obtained by PCR from *Corynebacterium glutamicum ATCC 13032* genomic DNA. Dop_{Cglu} was cloned with a C-terminal TEV-EGFP-His_6 tag via NdeI/EcoRI into a modified pET-vector (Novagen), resulting in the expression of a Dop_{Cglu}-TEV-EGFP-His_6 fusion protein. $pafA_{Cglu}$ was cloned with a C-terminal linker of five amino acids (KSSVE) followed by a His_6-tag via NdeI/SacI into a modified pET-vector (Novagen), resulting in the expression of $PafA_{Cglu}$-His_6. pup_{Cglu} and pup_{Cglu}-*gfp* fusions were cloned, expressed and purified as described for the *Mtb* constructs (Striebel *et al.*, 2009; Striebel *et al.*, 2010).

Purification of corynebacterial proteins. Dop_{Cglu} was expressed in *E. coli* BL21(DE3) from IPTG-inducible plasmids at 25 °C. $PafA_{Cglu}$ was expressed in *E. coli* Rosetta at 23 °C.
Dop_{Cglu} was expressed as Dop_{Cglu}-TEV-EGFP-His_6 fusion protein and purified by Ni^{2+}-affinity chromatography (HiTrap IMAC HP, GE Healthcare). After cleavage of the fusion protein with TEV-protease (Invitrogen), EGFP-His_6 and TEV-protease were removed by Ni^{2+}-affinity chromatography. Dop_{Cglu} was further purified by size-exclusion-chromatography on a Superdex200 column (GE Healthcare) in buffer P (50 mM Tris, pH 7.5, 300 mM NaCl, 10 % glycerol (v/v), 1 mM DTT, 1 mM EDTA).
$PafA_{Cglu}$ was purified by Ni^{2+}-affinity chromatography and subsequent size-exclusion-chromatography on a Superdex200 column in buffer P.

Production of Pup-modified substrates. Pup-modified substrates were generated by incubating the substrate (10 - 40 µM PanB-Strep, PanB-His_6, Strep-FabD or 10 mM lysine) with 10 - 50 µM Pup-GGE (a Pup Q64E variant), ATP (5 mM) and PafA-His_6 (1 µM). Subsequently, for Strep-tagged substrates and Pup-Lys, PafA-His_6 was removed by Ni^{2+}-affinity chromatography. For PanB-His_6, PafA-His_6 was removed by size exclusion chromatography (Superose 6). Excess Pup was removed by ultrafiltration as described (Striebel *et al.*, 2010).

Supplementary References

Striebel, F., Hunkeler, M., Summer, H. and Weber-Ban, E. (2010) The mycobacterial Mpa-proteasome unfolds and degrades pupylated substrates by engaging Pup's N-terminus. *EMBO J* **29**: 1262-1271

Striebel, F., Imkamp, F., Sutter, M., Steiner, M., Mamedov, A. and Weber-Ban, E. (2009) Bacterial ubiquitin-like modifier Pup is deamidated and conjugated to substrates by distinct but homologous enzymes. *Nat Struct Mol Biol* **16**: 647-651