A conserved loop sequence of the proteasome system depupylase Dop regulates substrate selectivity in Mycobacterium tuberculosis

Received for publication, August 2, 2022, and in revised form, September 1, 2022  Published, Papers in Press, September 10, 2022, https://doi.org/10.1016/j.jbc.2022.102478

Jin Hee Yoo, Shoshanna C. Kahne, and K. Heran Darwin*  From the Department of Microbiology, New York University School of Medicine, New York, New York, USA  Edited by George DeMartino

Mycobacteria use a proteasome system that is similar to a eukaryotic proteasome but do not use ubiquitin to target proteins for degradation. Instead, mycobacteria encode a prokaryotic ubiquitin-like protein (Pup) that posttranslationally modifies proteins to mark them for proteolysis. Pupylation occurs on lysines of targeted proteins and is catalyzed by the ligase PaFA. Like ubiquitylation, pupylation can be reversed by the depupylase Dop, which shares high structural similarity with PaFA. Unique to Dop near its active site is a disordered loop of approximately 40 amino acids that is highly conserved among diverse dop-containing bacterial genera. To understand the function of this domain, we deleted discrete sequences from the Dop loop and assessed pupylation in mutant strains of Mycobacterium tuberculosis. We determined that various Dop loop mutations resulted in altered pupylome profiles, in particular when mutant dop alleles were overexpressed. Taken together, our data suggest these conserved amino acids play a role in substrate selectivity for Dop.

Mycobacterium tuberculosis is a human exclusive pathogen that is transmitted by aerosols and causes the disease tuberculosis (TB). Although TB can be effectively treated with several antibiotics, treatment is prolonged, which often results in poor compliance and the emergence of drug-resistant strains. In an effort to find new targets for TB treatment, a screen for mutants sensitive to the host effector nitric oxide (NO) identified mutations in components of the bacterial proteasome system (1). In eukaryotes, proteins targeted for proteasomal degradation are posttranslationally modified by the small protein ubiquitin (reviewed in (2)), whereas bacteria have a different modification called Pup. In M. tuberculosis, Pup is translated as a 64 amino acid protein ending in glutaminine (Gln) that must be deamidated to glutamate (Glu) by deamidase of Pup (Dop) prior to attachment by the only known Pup ligase, proteasome accessory factor A (PaFA), to substrate lysines (3–5). The pupylation status of any protein is likely dynamic given that Dop can also remove Pup from substrates (depupylation), rescuing them from degradation (6, 7), and PaFA can potentially move Pup from one substrate to another (8). Given that over 60 proteins are targets of pupylation that comprise the “pupylome” (9–11), it is perhaps unsurprising that components of the Pup-proteasome system (PPS) are essential for the robust virulence of M. tuberculosis in animal models (1, 12–14). In fact, the accumulation of a single proteasome substrate, Log, results in a buildup of aldehydes that synergize with NO to kill bacteria and attenuate growth in mice, demonstrating the essential robustness of the PPS for resistance to host defenses and potentially other stressors (15).

A major gap in understanding the PPS is how proteins are selected for pupylation and depupylation. The expression of M. tuberculosis dop, pup, and pafa in Escherichia coli, which lacks a PPS, results in the pupylation of numerous proteins (16), suggesting that there is no mycobacteria-specific sequence motif that PaFA must recognize to pupylate a protein. PaFA and Dop are members of the glutamine synthetase superfamily and share numerous conserved residues in their active sites (13, 17, 18). While PaFA catalyzes a reaction similar to glutamine synthetases, Dop does not. Dop has an amidase activity that appears unique to it and its close homologs (17, 19, 20). Furthermore, Dop has a disordered loop sequence that is absent in PaFA and is a highly conserved region among Dops from diverse actinobacterial species (17). Deletion of the “Dop loop” does not diminish its activity nor does it convert Dop into a ligase. However, deletion of the loop and addition of an alpha helix from PaFA confers ligase activity to Mycobacterium smegmatis Dop (21).

In a study by the Gur lab, in vitro analysis found that deletions in the M. smegmatis Dop loop result in enzymes that more rapidly depupylate model substrates. Steady state pupylomes in M. smegmatis expressing mutant dop are reduced compared to the pupylome from a strain expressing wild-type (wt) dop, suggesting these Dop loop mutant alleles also hyperdepupylate in vivo (22). The authors of this work also showed that Dop binding to one substrate, Pup~IdeR, is unaffected by the Dop loop deletion, concluding the Dop loop regulates catalysis and not substrate binding. In contrast, the Weber-Ban lab found that replacement of loop residues with different amino acids made Corynebacterium glutamicum Dop more slowly depupylate a model substrate. Moreover, the authors proposed that the Dop loop promotes the dephosphorylation of an active site nucleotide (ATP), releasing a phosphate needed for amidase activity (20). It is possible that differences in Dop loop function described in these studies...
Dop loop affects substrate selection

were in part due to the use of Dop from different species (M. smegmatis Dop is 50% identical/75% similar to C. glutamicum Dop).

We sought to understand how this highly conserved and unstructured region of Dop affects the proteome of M. tuberculosis. We complemented a dop transposon mutation with either integrative or overexpression plasmids encoding various dop alleles, including a large deletion encompassing most of the conserved amino acids or several smaller deletions within the loop, and assessed the pupylomes of these strains. Deletion of the Dop loop resulted in an overall reduced pupylome and the accumulation of several established proteasome substrates, supporting observations in M. smegmatis (22). Smaller deletions of the Dop loop had variable effects, affecting only a handful of established PPS substrates. Most interestingly, the overexpression of mutant dop loop alleles resulted in dramatically different pupylomes. In particular, the expression of a specific dop loop deletion allele resulted in the accumulation of a single pupylated protein, suggesting the deleted amino acids are important for depupylating this substrate. Collectively, we propose that residues in the Dop loop help regulate depupylation, possibly by affecting access to substrates.

Results

Deletion of amino acids in the conserved Dop loop reduced pupylome abundance

In M. smegmatis, Dop lacking the loop depupylates faster than wt Dop in vitro and in vivo, suggesting that the Dop loop inhibits depupylation (22). To test if deletion of the loop would have a similar effect in M. tuberculosis, we complemented an M. tuberculosis dop transposon mutation with an integrative plasmid encoding various deletions from the dop loop sequence; dop alleles were expressed from the native dop promoter (see Table 1). We deleted the coding sequence for the 24 most conserved amino acids (“Δloop”) as well as made

Table 1

| Bacterial strains, plasmids, and primers used in this work | Relevant genotype: | Source or reference: |
|-----------------------------------------------------------|--------------------|---------------------|
| **E. coli:** | | Gibco, BRL. |
| DH5α | F-, B80ΔlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (rK− mK−) supE44 lacU169 thy−1 gyrA96 relA1 | | |
| **M. tuberculosis:** | | W. Bishai collection |
| CDC1551 | wild type | |
| MHD58 (MT2172) | CDC1551 dop::MycoMarT7; Kan’ | (13) |
| MHD375 | MHD58 pMV306; Hyg’, Kan’ | (13) |
| MHD376 | MHD58 pMV-dop; Hyg’, Kan’ | (13) |
| MHD1628 | MHD58 pMV-dopΔloop; Hyg’, Kan’ | This work. |
| MHD1631 | MHD58 pMV-dopΔAWDYEV; Hyg’, Kan’ | This work. |
| MHD1632 | MHD58 pMV-dopAESPHEL; Hyg’, Kan’ | This work. |
| MHD1630 | MHD58 pMV-dopARGF; Hyg’, Kan’ | This work. |
| MHD1633 | MHD58 pMV-dopΔLG; Hyg’, Kan’ | This work. |
| MHD1629 | MHD58 pMV-dopΔARGGPE; Hyg’, Kan’ | This work. |
| MHD671 | MHD58 pOLYG; Hyg’ | |
| MHD1097 | MHD58 pOLYG-dop; TAP-tagged; Hyg’, Kan’ | This work. |
| MHD1663 | MHD58 pOLYG-dopΔloop; TAP-tagged; Hyg’, Kan’ | This work. |
| MHD1664 | MHD58 pOLYG-dopΔAWDYEV; TAP-tagged; Hyg’, Kan’ | This work. |
| MHD1661 | MHD58 pOLYG-dopAESPHEL; TAP-tagged; Hyg’, Kan’ | This work. |
| MHD1660 | MHD58 pOLYG-dopARGF; TAP-tagged; Hyg’, Kan’ | This work. |
| MHD1669 | MHD58 pOLYG-dopΔLG; TAP-tagged; Hyg’, Kan’ | This work. |
| MHD1668 | MHD58 pOLYG-dopΔARGGPE; TAP-tagged; Hyg’, Kan’ | This work. |
| ΔnuoAN | CDC1551 with a deletion of nuoA through nuoN | (23) |
| MHD1701 | ΔnuoAN pOLYG-dop; Hyg’ | This work. |
| MHD1702 | ΔnuoAN pOLYG-dopΔloop; TAP-tagged; Hyg’ | This work. |
| MHD1703 | ΔnuoAN pOLYG-dopΔAWDYEV; TAP-tagged; Hyg’ | This work. |

Plasmids

| Description | Reference |
|-------------|----------|
| pOLYG | Hyg’; shuttle plasmid for gene overexpression in mycobacteria |
| pMV306 | Hyg’; mycobacterial plasmid that integrates at attB site on mycobacterial chromosomes |

| Primers (sequences are 5’ to 3’): |
|----------------------------------|----------|
| pOLYGfor | CATGACCAACTTGCATAAAG |
| pOLYGrev | GACGACAGGTTTCCCGACTG |
| dopTAP_loop-WDYEV_R | GCGCAGCGCGCGGATCAGCCGGTTGCTGG |
| dopTAP_loop-WDYEV_F | GCCAACCGACCGCTGAATCGCCGCTGC |
| dopTAP_loop-ESPL_R | GAAGCCGGCGCGCGCTGACCCGTAGTCCC |
| dopTAP_loop-ESPL_F | TGGGACTACTGGTGACGAGGGGTTGTC |
| dopTAP_loop-DA_R | AAAATCGAGAGGCCCGAGACGGAGGATT |
| dopTAP_loop-DA_F | GAATCGAGAGGCCCGAGACGGAGGATT |
| dopTAP_loop-RG_F | CGACGGACTCAAATCGACGCGAGCCCGGATCCAC |
| dopTAP_loop-DLS_R | CCGGCTCGCCGACCGCGGGGCTTCCTGCGCCGCG |
| dopTAP_loop-DLS_F | GGGCCTCGCCGACCGCGGGGCTTCCTGCGCCGCG |
| dopTAP_loop-RSAGPP_R | GGCGTGGGACCACGCAGCAAGGACGGCAGGAGG |
| dopTAP_loop-RSAGPP_F | GGTTCGAGATTGAGATTTGAGTGAAGAGG |
| Dop_24cleandel_F | AGCAGTGACAAACCGACCGCAGCGAGGACGGAGG |
| Dop_24cleandel_R | TCCTGCGGATCAGCGAGCGAGGAGG |
| pMV306for | CCTTGCGTTCAGAGGCCTTA |
| pMV306rev | CGCGTGGTTCAGAGGCGAGGAGG |

2 J. Biol. Chem. (2022) 298(10) 102478
Figure 1. Amino acid deletions in the Dop-loop affected pupylation levels in *M. tuberculosis*. A, amino acids deleted from the Dop loop region. In *M. tuberculosis*, these residues represent amino acids 48 to 71. B, an *M. tuberculosis* dop-null strain was complemented with integrative plasmids encoding *dop* with deletions in the Dop loop. Equivalent bacterial cell numbers were harvested and lysed for analysis on a 10% SDS-PAGE gel by immunoblotting (IB) for Pup. The pupylomes were quantified using Fiji and compared to the amount in the wt *dop* complemented strain. Arrowhead (<) indicates a unique species accumulating in the ΔWDYEV strain. Dop levels were checked by stripping the same membrane and incubating with antibodies to Dop. Molecular weight (MW) standards in kD are indicated on the left. Ponceau S-stained membrane before IB is shown at the bottom as a loading control. C, loop mutations did not affect NO sensitivity. The first four strains in (B) were incubated for 6 days in acidified media with or without 3 mM nitrite and then plated on agar to enumerate surviving colony forming units (CFU) 2 to 3 weeks later. Data are representative of three independent experiments, each performed in triplicate, with error bars signifying means ± standard deviation (SD). Statistical analysis was done by performing unpaired t tests comparing mutant strains to WT *dop*-complemented strain. *p < 0.05; ns = not statistically significant.
shorter deletions within the loop (Fig. 1A) and assessed pupylome levels at steady state by immunoblotting (Fig. 1B). As previously reported in M. tuberculosis, complementation of this dop mutant with WT dop restores a robust pupylome (Fig. 1B, lanes 1 versus 2) (13). The strain complemented with Δloop had a reduced pupylome (Fig. 1B, lane 3), similar to what was previously observed in M. smegmatis producing Dop lacking either 14 or 37 residues from its loop (22).

The smaller amino acid deletions in the Dop loop also resulted in decreased pupylome abundance. Deletions nearer to the amino terminus had greater decreases in pupylome levels; the strain producing Dop lacking the amino acids tryptophan, aspartate, tyrosine, glutamate, and valine (“ΔWDYEV”) had the most similar pupylome to the Δloop strain (Fig. 1B, lanes 3 versus 4). This decrease in pupylome abundance was specifically due to the deleted residues and not just the shortening of the Dop loop, given that deletion of six residues (arginine, serine, alanine, glycine, proline, proline; ΔRSAGPP) at the carboxyl terminus of the loop resulted in a pupylome like the wt-complemented strain (Fig. 1B, lanes 1 versus 8).

Deletion of the loop from M. smegmatis Dop does not affect deamidation activity (22). Thus, it seemed unlikely that the decreases in pupylome levels seen in Figure 1 were due to the reduced conversion of newly translated PupGln to PupGlu. Instead, we hypothesized that reduced pupylome levels were due to either slower or faster depupylation by the various Dop alleles. Hypodepupylation would result in more protein getting targeted to the proteasome, thus reducing the abundance of known proteasome substrates. In contrast, hyperdepupylation could rescue these substrates from degradation, thereby increasing the amount of a substrate relative to its abundance in wt bacteria. To determine which of these scenarios was more likely, we quantified and compared the proteome of the dop-null mutant to the proteomes of strains producing wt, Δloop, and ΔWDYEV Dop using tandem-mass tag mass spectrometry (TMT-MS). As expected, the dop-null mutant had the highest accumulation of several established proteasome substrates given that there is no pupylation in this strain (Tables 2, and S1). In the strains producing Δloop or ΔWDYEV alleles, several proteasome substrates accumulated to a lesser degree than what were observed in the dop-null strain (Tables 2 and S1). Nonetheless, this result suggested these mutant loop Dop alleles hyperdepupylated several known proteasome substrates, rescuing them from proteasomal degradation.

Defective protein degradation by proteasomes is associated with an increased susceptibility of M. tuberculosis to NO due to the failed degradation of the proteasome substrate Log (15). Log did not accumulate in any of the tested loop mutants (Table S1), but we nonetheless tested whether or not the small loop deletions affected NO susceptibility. Consistent with our observation that Log did not accumulate in any of the tested loop mutant strains, none of these strains was hypersensitive to NO (Fig. 1C).

The decrease in pupylome levels was unlikely due to changes in the abundance of the proteasome subunits (PrcA and PrcB) and mycobacterial proteasome activator Mpa because they were present at similar levels in the analyzed strains (Table S1). In contrast, there was less Pup in the mutant strains relative to the strain making wt Dop (Table S1). Pup is highly unstable when not conjugated to another protein in M. tuberculosis (13). Thus, the reduced Pup levels in the Dop loop mutants, along with the accumulation of known proteasome substrates, is consistent with a model in which hyperdepupylation occurs in these bacteria. However, we could not rule out an alternative explanation in which Dop loop mutations negatively influenced the ability of Dop to depupylate certain substrates, an activity that could also affect the overall Pup pool.

### Table 2

| Substrate      | MW (kD) | Dop null | Δloop | ΔWDYEV |
|----------------|---------|----------|-------|--------|
| FabD           | 31      | +        | +     | +      |
| KasA           | 43      | +        | +     | +      |
| Icl            | 47      | +        |       | +      |
| Log            | 20      | +        |       | +      |
| PanB           | 29      | +        |       | +      |
| Ino1           | 40      | +        |       | +      |
| FusA           | 77      | +        |       | +      |
| Bcp            | 17      |          |       | +      |
| LeuD           | 22      |          |       | +      |
| MtrA           | 25      |          |       | +      |
| NuoE           | 27      |          |       | +      |
| Rx20859c       | 32      |          |       | +      |
| Rv0073         | 36      |          |       | +      |
| FadA           | 42      |          |       | +      |
| MurA           | 44      |          |       | +      |
| PhoH2          | 47      |          |       | +      |
| PdaA           | 50      |          |       | +      |
| GlnU           | 52      |          |       | +      |
| SahH           | 54      |          |       | +      |
| Mpa            | 67      |          |       | +      |
| RecA           | 85      |          |       | +      |

See Table S1 for full list of quantified proteins. Abbreviation: MW, molecular weight.

Overexpression of loop mutant alleles revealed variable pupylomes

While the relative amounts of pupylated protein varied, the banding pattern of the pupylomes in our immunoblots did not appear different among the strains expressing the various loop alleles (Fig. 1B). However, an accumulated species of about 100 kD was apparent in the strain producing the ΔWDYEV allele (Fig. 1B, lane 4, arrowhead). Based on this observation, we hypothesized that specific residues in the Dop loop contributed to the depupylation of certain proteins. To begin to test this hypothesis, we overexpressed wt dop and mutant loop alleles in the dop-null M. tuberculosis strain, with the expectation that overexpression might magnify differences among the Dop alleles. We performed immunoblot analysis on total cell lysates of these strains and observed that several of the mutant dop allele-expressing strains had distinct pupylomes, with multiple pupylated proteins that were more prominent in several strains compared to each other or the WT dop-expressing strain (Fig. 2, lane 2 versus lanes 3–8). In most of the loop mutants, an approximately 100 kD species, herein called “protein X,” was present at greater levels.
than in the wt dop-expressing strain and most dramatically accumulated in the ΔWDYEV strain (Fig. 2, lane 4); it was likely that protein X was the same species seen in Figure 1B, lane 4. We hypothesized that the identity of protein X could give some insight into the significance of the WDYEV sequence in the Dop loop. To identify protein X, we performed immunoprecipitations using mAbs to Pup. After separating immunoprecipitated proteins by SDS-PAGE, we excised the region around 100 kD for MS analysis. After tryptic digestion and MS analysis, the top proteins with more than five peptide spectral matches included Pup and NuoG (Fig. 3A).

NuoG is an 85 kD protein and part of the 14-subunit type 1 NADH dehydrogenase complex that is encoded by the nuoA operon (23). To further test if NuoG was indeed protein X, we tested for protein X accumulation in a ΔnuoAN mutant lacking the entire operon and overexpressing wt or ΔWDYEV dop alleles. Robust pupylomes were seen in both the parental and ΔnuoAN strains when transformed with empty vector (Fig. 3B, lanes 1 and 4), whereas the overexpression of wt dop resulted in dramatically reduced pupylomes (Fig. 3B, lanes 2 and 5), most likely due to hyperdepupylation. Nonetheless, the overproduction of the ΔWDYEV mutant resulted in the appearance of protein X in the parental strain as seen in Figure 2 but not in the ΔnuoAN strain. Because none of the other proteins encoded in the nuoA operon was identified by our proteomics analysis and all of the Nuo proteins except NuoG are 66 kD or smaller, we concluded that protein X is Pup/C24 NuoG.

NuoG is a part of the peripheral arm of the type 1 NADH dehydrogenase complex (24) and has never been identified as a proteasome substrate in M. tuberculosis. Under routine culture conditions used in this work, we did not observe an accumulation of NuoG in the dop mutant, which we would expect if NuoG were a proteasome substrate (Table S1). In contrast, NuoE, which is also a part of this complex, is a confirmed pupylated substrate that accumulated in the dop-null mutant
Dop loop affects substrate selection

| Protein     | PSM | MW (kD) |
|-------------|-----|---------|
| Rv3151 (NuoG) | 9   | 85      |
| Rv2721c     | 8   | 72      |
| Pup         | 6   | 7       |
| ClpC1       | 6   | 94      |

| Parental | ΔnuoAN |
|----------|--------|
|          |        |
| 1 and 4  | empty  |
| 2 and 5  | wt     |
| 3 and 6  | ΔWDYEV |

Figure 3. NuoG was hyperpupylated in M. tuberculosis overproducing DopΔWDYEV. A, top peptide-spectrum match (PSM) hits identified from the Pup immunoprecipitations. B, whole cell lysates were collected from equivalent amounts of bacteria and separated by 10% SDS PAGE. Pupylated proteins were analyzed by IB for Pup. Ponceau S-stained membrane before IB is shown at the bottom as a loading control. MW standards are indicated on the left. IB, immunoblotting; MW, molecular weight in kD.

(Table 2) (9). Although we do not know which lysine in NuoG is pupylated, it is possible that access to this residue is affected by its location within the NADH dehydrogenase complex (Fig. 4).

Discussion

In this study, we sought to understand the in vivo function of a highly conserved loop sequence in the M. tuberculosis amidase Dop. We showed that the effect of the loop deletions depended on which residues were deleted, and deletion of as few as three amino acids from the Dop loop had global effects on pupylome levels. The overexpression of a specific dop allele, ΔWDYEV, resulted in the dramatic accumulation of Pup~NuoG, suggesting this substrate could not be efficiently depupylated by this mutant Dop. Thus, our data suggest highly conserved amino acids in the Dop loop regulate the ability of Dop to depupylate certain substrates in M. tuberculosis.

Previous work by two other groups worked to understand the function of the Dop loop. Both studies concluded that the loop affected the rate of catalysis by Dop but in contradictory ways (20, 22). In one study, deletion of the entire loop sequence or replacement of seven highly conserved residues in the loop with glycine or serine resulted in faster depupylation of three model substrates in vitro (22). In another study, deletion of the Dop loop resulted in slightly slower depupylation of a model substrate and a fluorescent probe (20). It is possible that the differences observed by the two groups were due to the use of Dop from different bacterial species.

It was also proposed that the highly conserved tryptophan of the WDYEV sequence in the Dop loop stabilizes ATP binding and hydrolysis, with the liberation of phosphate suggested to be required for depupylation (20). However, deletion of the loop, which includes WDYEV, does not abolish Dop activity as reported here and elsewhere (17, 22). Perhaps most relevantly, ATP hydrolysis is not required per se for depupylation given that ADP and Pi are sufficient for Dop to robustly catalyze depupylation in vitro (8, 25). Thus, it seems unlikely that the loop plays a role in nucleotide hydrolysis to promote depupylation.

Our M. tuberculosis results support a scenario observed in M. smegmatis in which the loop plays an inhibitory role in depupylation. However, this does not appear to be the only effect of the Dop loop on the pupylome. In addition to a model where hyperdepupylation by Dop loop mutants led to a reduced pupylome, it is possible that proteins were underpupylated due to a reduction in the overall Pup pool. This idea is based on the observation that Pup recycling by Dop is essential to maintain a robust pupylome in M. tuberculosis; if Pup is not constantly removed and reattached to substrates, the pupylome is substantially diminished (13). Thus, it is possible that if proteins like NuoG are inefficiently depupylated, the overall Pup pool would be insufficient to maintain a wt pupylome. Taken together, it is possible the loop can both restrict and promote depupylation depending on the substrate.

Another hypothesis we propose is that some pupylated proteins act as sources of Pup to be directly transferred from one substrate to another (8). This hypothesis arose from the observation that all enzymes encoded in the fatty acid synthase II (FASII) biosynthetic pathway operon are pupylated, but only FabD is robustly degraded under steady state conditions (3, 9, 10, 26). Given that PafA can transfer Pup from one substrate to another in vitro (8), it is possible PafA can transfer Pup from one or more of the other pupylated FASII enzymes to FabD to facilitate its degradation. Although NuoE did not accumulate in the ΔWDYEV strain, it is still possible that Pup~NuoG is a source of Pup to promote the degradation of NuoE or other nearby proteasome substrates under different conditions.

The selection mechanisms of proteins to be pupylated or depupylated remain to be determined. In particular, how do loop residues affect depupylation? It is notable that Pup~NuoG did not accumulate in the ΔWDYEV strain, it is still possible that Pup~NuoG is a source of Pup to promote the degradation of NuoE or other nearby proteasome substrates under different conditions.
intrinsically disordered proteins (IDPs) frequently achieve structure when interacting with a specific binding partner (reviewed in (27)). A relevant example in *M. tuberculosis* is the interaction of Pup, an IDP, with Mpa, the receptor and chaperone of Pup-dependent proteasomal degradation. In solution, Pup is mostly disordered but forms a robust interaction with Mpa in a hexamer (28–30). Thus, it remains to be determined if the Dop loop directly interacts with substrates or binds to one or more proteins that facilitate depyylation.

### Experimental procedures

#### Strains, plasmids, primers, and culture conditions

See Table 1 for strains, plasmids, and primers used in this work. Reagents used for making all buffers and bacterial media were purchased from Thermo Fisher Scientific, unless otherwise indicated. *M. tuberculosis* was grown on 7H9c (BD Difco Middlebrook 7H9 broth with 0.2% glycerol and supplemented with 0.5% bovine serum albumin, 0.2% dextrose, 0.085% sodium chloride, and 0.05% Tween-80). For solid media, *M. tuberculosis* was grown on Middlebrook 7H11 agar (“7H11”, BD Difco) containing 0.5% glycerol and supplemented with 10% final volume of BBL Middlebrook OADC Enrichment. For selection of *M. tuberculosis*, the following antibiotics were used as needed: kanamycin 50 μg/ml and hygromycin 50 μg/ml. *E. coli* was cultured in Luria-Bertani broth or on Luria-Bertani agar (both BD Difco). Media were supplemented with the following antibiotics as needed: kanamycin 100 μg/ml and hygromycin 150 μg/ml.

Dop loop deletions were made by splicing overlap extension PCR with Phusion polymerase (31). Dop encoding C-terminal hexahistidine and FLAG tags were cloned into the BamHI and HindIII sites of pOLYG for overexpression. To make integrative plasmids with the same mutant sequences, we used the overexpression plasmids as templates for PCR to add HindIII and XbaI cut sites and remove the affinity tag sequences. PCR products were cloned into plasmid pMV306. Calcium chloride–competent *E. coli* DH5α was used for transformations. Plasmids were purified from *E. coli* using the QIAprep Spin Miniprep Kit (Qiagen). All plasmids made by PCR cloning were sequenced by GENEWIZ, Inc to ensure the veracity of the cloned sequence. Primers used for PCR amplification or sequencing were purchased from Life Technologies and are listed in Table 1. DNA was PCR amplified using Phusion polymerase (New England Biolabs; NEB) according to the manufacturer’s instructions. PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen). Restriction enzymes and T4 DNA ligase were purchased from NEB.

*M. tuberculosis* was transformed by electroporation as previously described (32). All *M. tuberculosis* work was performed in the ABSL3 facility of NYU Grossman School of Medicine, in accordance with its Biosafety Manual and Standard Operating Procedures.

#### Preparation of *M. tuberculosis* extracts for immunoblotting

*M. tuberculosis* cultures were grown to an absorbance at 580 nm (*A* ≤ 0.1) of ~1. Equivalent cell numbers were collected based on the *A* ≤ 0.1 of the cultures. For example, an “*A* ≤ 0.1” indicates the *A* ≤ 0.1 of a 1 ml culture is 1.0. Five *A* ≤ 0.1 equivalents of bacteria were harvested by centrifugation at 3000g. washed in PBST (PBS, 0.05% Tween 80), resuspended in lysis buffer (100 mM Tris-Cl pH 8, 1 mM EDTA pH 8), and transferred to a tube containing 250 μl of 0.1 mm zirconia beads (BioSpec Products). Bacteria were lysed using a mechanical bead-beater (BioSpec Products). Whole-cell lysates were mixed with 4x reducing SDS sample buffer (250 mM Tris pH 6.8, 2% SDS, 20% 2-mercaptoethanol, 40% glycerol, 1% bromophenol blue) to a 1x final concentration, and samples were boiled for 10 min at 100°C.

For immunoblotting, whole cell lysates were separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose.
membranes (GE Amersham) by semidy transfer and blocked with 3% bovine serum albumin or 2% milk in 0.1× Tris-buffered saline with Tween-20 (TBST); use of 0.1× TBST allowed for a more sensitive detection of the pupylome. Membranes were incubated with monoclonal Pup antibody reported previously (13). Horseradish peroxidase– conjugated secondary antibodies to mouse and rabbit IgG were purchased from Pierce. Immunoblots were developed using SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific) and imaged using Bio-Rad ChemiDoc system and quantified using Fiji (33). Blots were stripped as previously described (34). The membrane was reblocked with 2% milk in 1× TBST, incubated with polyclonal Dop antibodies reported previously (6), and imaged as aforementioned.

**TMT-MS**

*M. tuberculosis* expressing wt dop, Δloop, and ΔWDYEV were grown as described previously. To prepare samples for TMT-MS, *M. tuberculosis* strains were grown to an A580 of 1 to 1.3. Nineteen A580 equivalents of bacteria were collected as stated previously. Insoluble debris was pelleted by centrifugation for 1 min at top speed in a microfuge. Lysates were filter sterilized using 0.2 μm nylon Spin-X columns (Costar). Sterilized samples were submitted to the NYUMC Proteomics Laboratory for proteome quantification by TMT-MS.

Samples were reduced using DTT for 1 h at 55 °C and reduced cysteines were alkylated with iodoacetamide. Each sample was loaded onto S-Trap microcolumns (Protifi) according to the manufacturer’s instructions. Samples were centrifuged at 4000g for 30 s. After three washes, proteins were trypsinized and peptides were eluted with 40% acetonitrile (ACN) in 0.5% acetic acid followed by 80% ACN in 0.5% acetic acid. Eluted peptides were dried and concentrated in a SpeedVac. The dried peptide mixture was resuspended in 1× TBST, incubated with polyclonal Dop antibodies reported previously. Immunoblots were developed using SuperSignal West premier reagent according to the manufacturer’s instructions. A-Sepharose slurry for 1 h at 4 °C, transferred to a tube with 200 μl of 7H9c (pH 5.5) and diluted to an A580 of 0.08. Bacteria were then aliquoted in triplicate to flat bottom 96-well plates, and a fresh sodium nitrite solution was added to each well at a ratio of proteins in loop mutant to wt Dop expressing strains.

**Nitric oxide sensitivity assay**

Assays were performed as described previously (1). Briefly, bacteria were grown to an A580 ~0.8 to 1 and resuspended in acidified 7H9c (pH 5.5) and diluted to an A580 of 0.08. Bacteria were then aliquoted in triplicate to flat bottom 96-well plates, and a fresh sodium nitrite solution was added to each well at a final concentration of 3 mM. Bacteria were incubated for 6 days at 37 °C before plating onto 7H11 OADC plates and incubated at 37 °C for enumeration 2 to 3 weeks later.

**Immunoprecipitations**

To identify protein X, nProtein A Sepharose Fastflow beads (GE Healthcare) were incubated with Pup mAb rotating for 3 h at 4 °C. *Mtb dop* null strain with pOLYG-dopΔWDYEV was grown to A580 of 1.6 and 30 A580 equivalents were harvested by centrifugation. The pellet was washed with lysis buffer (PBS with DNase, complete Mini EDTA free protease inhibitor tablets (Roche)), transferred to a tube with 200 μl zirconia beads, and bead beat for 3 × 30 s. The lysates were centrifuged for 10 min 10,000g at 4 °C and then filtered with 0.22 μM cellulose Spin-X filters by centrifugation for 5 min at the same settings. The lysates were precleared to minimize nonspecific binding proteins by incubating them with the protein A-Sepharose slurry for 1 h at 4 °C. The beads were centrifuged to pellet and the lysate was transferred to a new tube with 50 μl
of ProteinA Sepharose beads and incubated overnight. Unbound protein was removed by centrifugation and then beads were washed with 1× PBS. The beads were resuspended in 50 μl 2 × SDS sample buffer and boiled for 10 min. The proteins in the eluate were separated by SDS-PAGE and the gel stained with Coomassie brilliant blue. We excised a region of the gel just below and above 100 kD marker and submitted the sample to the NYUMC Proteomics Laboratory for identification.

Data availability

All data supporting these findings are available within the article and/or its supplementary materials.

Supporting information—This article contains supporting information Table S1.

Acknowledgments—We thank the Bill Jacobs lab for the ΔnuoAN M. tuberculosis strain. We thank the A. Darwin lab for helpful lab meeting discussions. The Proteomics Laboratory is supported in part by the NYU Grossman School of Medicine. We thank the Office of Science & Research High-Containment Laboratories at NYU Grossman School of Medicine for their support in the completion of this research.

Author contributions—J. H. Y., S. C. K., and K. H. D. conceptualization; J. H. Y., S. C. K., and K. H. D. methodology; J. H. Y. and S. C. K. investigation; J. H. Y., S. C. K., and K. H. D. writing—review and editing.

Funding and additional information—This work was supported by NIH grant AI088075 awarded to K. H. D. S. C. K. was supported in part by a Public Health Service Institutional Research Training Award T32 AI007180. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ACN, acetonitrile; MS, mass spectrometry; PPS, Pup-proteasome system; TB, tuberculosis; TBST, Tris-buffered saline with Tween-20; TMT, tandem-mass tag.

References

1. Darwin, K. H., Ehrt, S., Gutierrez-Ramos, J. C., Weich, N., and Nathan, C. F. (2003) The proteasome of Mycobacterium tuberculosis is required for resistance to nitric oxide. Science 302, 1963–1966
2. Komander, D., and R ape, M. (2012) The ubiquitin code. Annu. Rev. Biochem. 81, 203–229
3. Pearce, M. J., Mintseris, I., Ferreyra, J., Gygi, S. P., and Darwin, K. H. (2008) Ubiquitin-like protein involved in the proteasome pathway of Mycobacterium tuberculosis. Science 322, 1104–1107
4. Guth, E., Th ommen, M., and Weber-Ban, E. (2011) Mycobacterial ubiquitin-like protein ligase PaFA follows a two-step reaction pathway with a phosphorylated pup intermediate. J. Biol. Chem. 286, 4412–4419
5. Striebel, F., Imkamp, F., Sutter, M., Steiner, M., Mamedov, A., and Weber-Ban, E. (2009) Bacterial ubiquitin-like modifer Pup is deamidated and conjugated to substrates by distinct but homologous enzymes. Nat. Struct. Mol. Biol. 16, 647–651
6. Burns, K. E., Cerda-Maira, F. A., Wang, T., Li, H., Bishai, W. R., and Darwin, K. H. (2010) Depupylation* of prokaryotic ubiquitin-like protein from mycobacterial proteasome substrates. Mol. Cell 39, 821–827
7. Imkamp, F., Striebel, F., Sutter, M., Ozcelik, D., Zimmermann, N., Sander, P., et al. (2010) Dop functions as a depuplyase in the prokaryotic ubiquitin-like modification pathway. EMBO Rep. 11, 791–797
8. Zhang, S., Burns-Huang, K. E., Janssen, G. V., Li, H., Ovaa, H., Hedstrom, L., et al. (2017) Mycobacterium tuberculosis proteasome accessory factor A (PaFA) can transfer prokaryotic ubiquitin-like protein (pup) between substrates. MBio 8, e00122-17
9. Festa, R. A., McAllister, F., Pearce, M. J., Mintseris, J., Burns, K. E., Gygi, S. P., et al. (2010) Prokaryotic ubiquitin-like protein (Pup) proteome of Mycobacterium tuberculosis [corrected]. PLoS One 5, e58589
10. Watrous, J., Burns, K., Liu, W. T., Patel, A., Hook, V., Bafna, V., et al. (2010) Expansion of the mycobacterial PUPylome. Mol. Biosyst. 6, 376–385
11. Schubert, O. T., Mouritsen, J., Ludwig, C., Rost, H. L., Rosenberger, G., Arthur, P. K., et al. (2013) The Mtb proteome library: a resource of assays to quantify the complete proteome of Mycobacterium tuberculosis. Cell Host Microbe 13, 602–612
12. Darwin, K. H., Lin, G., Chen, Z., Li, H., and Nathan, C. F. (2005) Characterization of a Mycobacterium tuberculosis proteasomal ATPase homologue. Mol. Microbiol. 55, 561–571
13. Cerda-Maira, F. A., Pearce, M. J., Fuortes, M., Bishai, W. R., Hubbard, S. R., and Darwin, K. H. (2010) Molecular analysis of the prokaryotic ubiquitin-like protein (Pup) conjugation pathway in Mycobacterium tuberculosis. Mol. Microbiol. 77, 1123–1135
14. Gandotra, S., Schnappinger, D., Monteleone, M., Hillen, W., and Ehr, S. (2007) In vivo gene silencing identifies the Mycobacterium tuberculosis proteasome as essential for the bacteria to persist in mice. Nat. Med. 13, 1515–1520
15. Samanovic, M. I., Tu, S., Novak, O., Iyer, L. M., McAllister, F. E., Aravind, L., et al. (2015) Proteasomal control of cytokinin synthesis protects Mycobacterium tuberculosis against nitric oxide. Mol. Cell 57, 984–994
16. Cerda-Maira, F. A., McAllister, F., Bode, N. J., Burns, K. E., Gygi, S. P., and Darwin, K. H. (2011) Reconstitution of the Mycobacterium tuberculosis pupylation pathway in Escherichia coli. EMBO Rep. 12, 863–870
17. Ozcelik, D., Baranudan, J., Schmitz, N., Sutter, M., Guth, E., Damberger, F. F., et al. (2012) Structures of Pup ligase PaFA and depupylase Dop from the prokaryotic ubiquitin-like modification pathway. Nat. Commun. 3, 1014
18. Iyer, L. M., Burroughs, A. M., and Aravind, L. (2008) Unraveling the biochemistry and provenance of pupylation: a prokaryotic analog of ubiquitination. Biol. Direct 3, 45
19. Burns, K. E., McAllister, F. E., Schwedtfeger, C., Mintseris, J., Cerda-Maira, F., Noens, E. E., et al. (2012) Mycobacterium tuberculosis prokaryotic ubiquitin-like protein-deconjugating enzyme is an unusual aspartate aminase. J. Biol. Chem. 287, 37522–37529
20. Cui, H., Muller, A. U., Leibundgut, M., Tian, J., Ban, N., and Weber-Ban, E. (2021) Structures of prokaryotic ubiquitin-like protein Pup complex with depupylase Dop reveal the mechanism of catalytic phosphate formation. Nat. Commun. 12, 6635
21. Hecht, N., Monteil, C. L., Perriere, G., Vishkautzan, M., and Gur, E. (2021) Exploring protein space: from hydrolyse to ligase by substitution. Mol. Biol. Evol. 38, 761–776
22. Hecht, N., Becher, M., Kormann, M., Vishkautzan, M., and Gur, E. (2020) Inter- and intramolecular regulation of protein depupylation in Mycobacterium smegmatis. FEBS J. 287, 4389–4400
23. Vilchez, C., Weinrick, B., Leung, L. W., and Jacobs, W. R., Jr. (2018) Plasticity of Mycobacterium tuberculosis NADH dehydrogenases and their role in virulence. Proc. Natl. Acad. Sci. U. S. A. 115, 1599–1604
24. Schimpf, J., Oppermann, S., Gerasimova, T., Santos Seica, A. F., Hellwig, P., Grishkovskaya, I., et al. (2012) Structure of Pup ligase PaFA and depupylase Dop from Mycobacterium tuberculosis substrate. Structure 20, 80–94.e84
25. Bolten, M., Vahlensieck, C., Lipp, C., Leibundgut, M., Ban, N., and Weber-Ban, E. (2017) Depupylase dop requires inorganic phosphate in the active site for catalysis. J. Biol. Chem. 292, 4044–4053
Dop loop affects substrate selection

26. Pearce, M. J., Arora, P., Festa, R. A., Butler-Wu, S. M., Gokhale, R. S., and Darwin, K. H. (2006) Identification of substrates of the Mycobacterium tuberculosis proteasome. EMBO J. 25, 5423–5432

27. Chakrabarti, P., and Chakravarty, D. (2022) Intrinsically disordered proteins/regions and insight into their biomolecular interactions. Biophys. Chem. 283, 106769

28. Wang, T., Darwin, K. H., and Li, H. (2010) Binding-induced folding of prokaryotic ubiquitin-like protein on the Mycobacterium proteasomal ATPase targets substrates for degradation. Nat. Struct. Mol. Biol. 17, 1352–1357

29. Chen, X., Solomon, W. C., Kang, Y., Cerda-Maira, F., Darwin, K. H., and Walters, K. I. (2009) Prokaryotic ubiquitin-like protein pup is intrinsically disordered. J. Mol. Biol. 392, 208–217

30. Sutter, M., Striebel, F., Damberger, F. F., Allain, F. H., and Weber-Ban, E. (2009) A distinct structural region of the prokaryotic ubiquitin-like protein (Pup) is recognized by the N-terminal domain of the proteasomal ATPase Mpa. FEBS Lett. 583, 3151–3157

31. Horton, R. M. (1993) In vitro recombination and mutagenesis of DNA: SOEing together tailor-made genes. Met. Mol. Biol. 15, 251–261

32. Hatfull, G. F., and Jacobs, W. R. J. (2000) Molecular Genetics of Mycobacteria, ASM Press, WA, DC

33. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012) Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682

34. Howland, J. (1996) Short Protocols in Molecular Biology, Third ed., John Wiley & Sons, NY, NY

35. Cox, J., Michalski, A., and Mann, M. (2011) Software lock mass by two-dimensional minimization of peptide mass errors. J. Am. Soc. Mass Spectrom. 22, 1373–1380

36. Garbe, T. R., Barathi, J., Barnini, S., Zhang, Y., Abou-Zeid, C., Tang, D., et al. (1994) Transformation of mycobacterial species using hygromycin resistance as selectable marker. Microbiology 140, 133–138

37. Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., et al. (1991) New use of BCG for recombinant vaccines. Nature 351, 456–460