Mycobacterium smegmatis HtrA Blocks the Toxic Activity of a Putative Cell Wall Amidase

Graphical Abstract

Highlights

- HtrA-LppZ is an essential protease-lipoprotein complex in *Mycobacterium smegmatis*
- HtrA-LppZ is required to negatively regulate Ami3, a putative cell wall amidase
- Without HtrA-LppZ, Ami3 can accumulate to toxicity when mannosylated by Pmt

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In Brief

Wu et al. show that in *Mycobacterium smegmatis*, the putative cell wall amidase Ami3 can accumulate to toxicity under the stabilizing influence of Pmt mannosylation. To control Ami3 levels, an essential complex between the periplasmic serine protease HtrA and the lipoprotein LppZ regulates Ami3 levels, maintaining cellular integrity.
**Mycobacterium smegmatis** HtrA Blocks the Toxic Activity of a Putative Cell Wall Amidase

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INTRODUCTION

*Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis, withstands diverse environmental stresses in the host. The periplasmic protease HtrA is required only to survive extreme conditions in most bacteria but is predicted to be essential for normal growth in mycobacteria. We confirm that HtrA is indeed essential in *Mycobacterium smegmatis* and interacts with another essential protein of unknown function, LppZ. However, the loss of any of three unlinked genes, including those encoding Ami3, a peptidoglycan muramidase, and Pmt, a mannosyltransferase, suppresses the essentiality of both HtrA and LppZ, indicating the functional relevance of these genes’ protein products. Our data indicate that HtrA-LppZ is required to counteract the accumulation of active Ami3, which is toxic under the stabilizing influence of Pmt-based mannosylation. This suggests that HtrA-LppZ blocks the toxicity of a cell wall enzyme to maintain mycobacterial homeostasis.

SUMMARY

*Mycobacterium tuberculosis*, the causative agent of tuberculosis, withstands diverse environmental stresses in the host. The periplasmic protease HtrA is required only to survive extreme conditions in most bacteria but is predicted to be essential for normal growth in mycobacteria. We confirm that HtrA is indeed essential in *Mycobacterium smegmatis* and interacts with another essential protein of unknown function, LppZ. However, the loss of any of three unlinked genes, including those encoding Ami3, a peptidoglycan muramidase, and Pmt, a mannosyltransferase, suppresses the essentiality of both HtrA and LppZ, indicating the functional relevance of these genes’ protein products. Our data indicate that HtrA-LppZ is required to counteract the accumulation of active Ami3, which is toxic under the stabilizing influence of Pmt-based mannosylation. This suggests that HtrA-LppZ blocks the toxicity of a cell wall enzyme to maintain mycobacterial homeostasis.

Despite this reason, the reasons for HtrA’s essentiality have remained unclear.

In *E. coli* and other organisms, HtrA is characterized as a non-essential, periplasmic protease with secondary chaperone function (Clausen et al., 2011). Although dispensable for normal growth, HtrA is crucial for the virulence of several intracellular pathogens, including *Shigella*, *Listeria*, and *Salmonella* (Ingmer and Brøndsted, 2009). In these species, HtrA is required to tolerate a common set of stressful conditions, including high temperature, oxidative stress, and macrophage survival. The well-characterized *E. coli* HtrA homolog DegP is induced in conditions of membrane stress and becomes essential during heat shock (Ingmer and Brøndsted, 2009). DegP contains a protease domain with a conserved Ser-His-Asp catalytic triad and two PDZ domains (Figure 1A) that regulate substrate binding and access to the proteolytic chamber. Although a handful of substrates have been identified in *E. coli* (Clausen et al., 2002), DegP appears to be mostly indiscriminate in its specificity, preferring denatured, unfolded substrates with hydrophobic C termini.

Like *E. coli* and many other organisms, virulent mycobacteria express three orthologs of HtrA. In *Mtb*, these are *htrA* (Rv1223), *htrA2/pepD* (Rv0983), and *htrA3/pepA* (Rv0125); of these, only HtrA is predicted to be essential. However, this essentiality appears to be conserved across all mycobacteria, regardless of pathogenicity, including in the fast growing *Mycobacterium smegmatis* (Msm), which encodes both *htrA* (MSMEG_5070) and the non-essential *pepD* (MSMEG_5486) (Lew et al., 2011). Nevertheless, *htrA*’s synteny in mycobacteria indicates that its transcription may be stress responsive, as it lies in an operon with *sigE*, a stress-responsive alternative sigma factor (Manganelli et al., 2004). Additionally, its high degree of sequence conservation with homologs in other species indicates that HtrA may be capable of recapitulating some stress-responsive or virulence functions. Although HtrA’s role in mediating the mycobacterial stress response may partially overlap with its homologs in other species, its essentiality indicates it must be involved in a unique regulatory pathway that supports mycobacterial growth and survival.

Here, we present evidence that HtrA serves a previously undescribed and essential role in the regulation of the growth of *Msm* by degrading a putative cell wall muramidase. HtrA engages in a periplasmic complex with another essential protein, LppZ, to control levels of the lethal hyperactivity of Ami3, an amidase.
Figure 1. HtrA-LppZ Are Essential Interacting Proteins in Mycobacterium smegmatis

(A) DegP/HtrA homolog domain architecture. E. coli DegP contains a protease domain and two C-terminal PDZ domains. In contrast, mycobacterial HtrA is anchored in the inner membrane and contains a single PDZ domain. Additionally, mycobacterial HtrA has a cytoplasmic domain with no homology to any known protein.

(B) HtrA and LppZ are essential by L5 swap. Top: a schematic of the L5 essentiality swap. Placing a second copy of htrA, along with a nourseothricin resistance cassette, at the L5 phage integration site allows replacement of endogenous htrA with a hygromycin resistance cassette. This copy of htrA can be swapped for another copy of htrA with a different antibiotic resistance marker but not for a functionally unrelated gene such as gfp. Bottom: quantification of htrA and gfp swaps. A total of 200 transformants were tested for antibiotic resistance. An equivalent swap was performed for lppZ and enumerated in the same manner.

(C) Cells depleted of HtrA grow at a slower rate. When regulated by an aTc-repressible promoter, htrA can be transcriptionally depleted from cells. Two strains were constructed: htrAHIGH, which used a strong promoter susceptible to aTc-based repression, and htrALOW, a weak promoter susceptible to aTc-based repression. These strains were grown, with or without aTc, in each of four conditions: rich media at 37°C, high temperature (42°C), carbon-nitrogen starvation (PBS-Tween 20), and oxidative stress (0.02% tert-butyl hydroperoxide). Error bars represent SD of the mean.

(D) HtrA and LppZ interact. HtrA-Strep and LppZ-FLAG were individually immunoprecipitated using anti-Strep and anti-FLAG magnetic beads, respectively, and the following fractions were analyzed using western blotting: L, lysate; FT, flow through; W1, wash 1; W2, wash 2; W3, wash 3; and E, elution. Control immunoprecipitations lacking the respective bait were performed simultaneously and lysate, flow through, and elution were analyzed.

(E) LppZ is not a substrate of HtrA. Strains expressing htrA or htrA(S354A) under htrA’s native promoter, htrAHIGH, or htrALOW, were grown to log phase, and cell lysates were analyzed using western blotting to detect levels of HtrA-Strep and LppZ-FLAG. In the case of htrAHIGH and htrALOW, strains were also grown with or without 100 ng/mL aTc.

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stabilized by Pmt-mediated O-mannosylation. Loss of either ami3 or pmt is sufficient to relieve the essentiality of both htrA and lppZ. These data expand upon a growing body of literature illustrating the complex ways in which bacteria regulate growth and division.

RESULTS

HtrA-LppZ Forms an Essential Complex in the Mycobacterial Periplasm

Previous transposon insertion-based (Tn-seq) screens have predicted the essentiality of htrA in both Msm and Mtb (Griffin et al., 2011; Zhang et al., 2012; DeJesus and loerger, 2013). To confirm htrA essentiality, we attempted to delete these genes from the genome of M. smegmatis through mycobacterial recombineering and homologous recombination (Figure 1B, top). Briefly, a second copy of htrA was transformed into the L5 phage integration site with a nourseothricin resistance cassette, allowing the replacement of the endogenous copy of htrA with a hygromycin resistance cassette. We then attempted to transform htrA or an empty vector with a kanamycin resistance cassette into the L5 integration site (an “L5 swap”). Three outcomes can result from these experiments. First, integrations resulting in nonviable cells will prevent the growth of transformants on antibiotic selection. Second, successful “swaps” are those that acquire kanamycin resistance at the expense of nourseothricin resistance, indicating the replacement of the original integrated vector with the transformed vector. Third, the second vector may recombine in alongside the original vector, creating a double integrant that retains resistance to both antibiotics. In these experiments, htrA swapped in at a rate of 63.5%, while the empty vector yielded only double integrants (Figure 1B, bottom), indicating that HtrA is essential.

To assess the phenotype of htrA hypomorphs, we constructed depletion strains in which the only copies of htrA were under the control of anhydrotetracycline (aTc)-repressible promoters of different strengths (htrALOW and htrAHIGH). With the addition of aTc, htrALOW cells clumped in liquid culture and exhibited a slower growth rate (Figure 1C) but remained viable, suggesting that a minimal amount of HtrA is sufficient to sustain growth. As HtrA homologs in other bacterial species are associated with protection against environmental stress, we tested the ability of depleted htrALOW cells to survive extreme conditions, including high temperature, oxidative stress, and carbon-nitrogen starvation (Figure 1C). All tested conditions impaired the growth of or killed depleted htrALOW cells, indicating that higher amounts of HtrA are required to sustain growth under environmental stress.

We reasoned that identifying binding partners could help define HtrA’s function. To find interactors, we immunoprecipitated Strep-tagged HtrA and identified eluted proteins using mass spectrometry. The most abundant protein, LppZ (MSMEG_2369, Rv3006), had no known function. LppZ is also predicted to be essential in both M. smegmatis and M. tuberculosis and is annotated as a putative secreted lipoprotein (Griffin et al., 2011; Zhang et al., 2012; DeJesus and loerger, 2013). We confirmed LppZ essentiality with an L5 swap (Figure 1B) and detected a stable interaction between HtrA and LppZ by co-immunoprecipitation of tagged proteins (Figure 1D).

On the basis of this interaction, LppZ could be an HtrA substrate or adaptor. To distinguish between these possibilities, we tagged LppZ with a FLAG sequence and monitored its stability during depletion of htrA. LppZ levels were completely insensitive to alterations in HtrA (Figure 1E). This suggests that LppZ may be an adaptor of HtrA proteolytic activity, analogous to interactions in E. coli between the periplasmic protease Prc and the lipoprotein NlpI (Singh et al., 2015; Tadokoro et al., 2004) and between DegP and recombinant mutants of the lipoprotein Lpp (Park et al., 2017).

If LppZ is an HtrA adaptor, LppZ mutants should phenocopy HtrA mutants. Indeed, both htrALOW and lppZLOW depletion strains exhibited increased sensitivity to antibiotics targeting cell wall crosslinking (but not other antibiotics such as isoniazid) (Figure 1F) and severe morphological defects (Figure 1G). Cells bulged and shortened upon htrALOW or lppZLOW depletion; however, a small subpopulation branched in only the htrALOW depletion. Both these morphologies are reminiscent of previously characterized strains in which dysregulation of cell wall machinery induces swelling and branching (Chao et al., 2013; Kieser et al., 2015). These data suggest that HtrA and LppZ act cooperatively as a complex in the same step of an essential genetic pathway that engages the cell wall.

Loss-of-Function Mutations in ami3, pmt, or mprB Suppress htrA and lppZ Essentiality

Unlike E. coli DegP, mycobacterial HtrA is predicted to be membrane anchored and have only one PDZ domain. Furthermore, it has a unique cytoplasmic domain at its N terminus that bears no homology to any known protein (Kappoupolou et al., 2011). To understand structure-function relationships in HtrA, we made a series of domain deletions and tested their viability using L5 swaps. We found that both the cytoplasmic and PDZ domains are essential for function (Figure S1A) but do not affect HtrA’s interaction with LppZ (Figure S1B). However, these experiments yielded a small number of survivors that grew despite loss of functional HtrA. We hypothesized that these might be due to mutations elsewhere in the chromosome and performed whole-genome sequencing of these strains to identify potential extragenic suppressors of htrA essentiality.

We sequenced nine strains in total and compared their genomes with that of the parental strains. Each swapped strain carried at least one of the following mutations (Figure S1C):

(F) Cells lacking HtrA or LppZ are highly sensitive to cell wall-targeting antibiotics. htrALOW and lppZLOW strains expressing the respective essential gene under a weak promoter susceptible to aTc-based repression, were grown in teicoplanin and vancomycin, antibiotics targeting D-ala-D-ala crosslinking in the mycobacterial cell wall, and isoniazid, an antibiotic that inhibits mycolic acid synthesis.

(G) Depletion of HtrA or LppZ induces morphological defects. htrALOW and lppZLOW were grown to log phase in aTc and observed under the microscope. Two representative images for each strain are shown. At least 100 cells were quantified in each condition. Dotted black lines indicate median values. Western blot images were cropped, but display all relevant lanes and reactive bands. ***p < 0.0001.
Singh et al., 2015). Because the htrA (Schwechheimer et al., 2015; Dörfler and Ioerger, 2013) double-deletion strains in all backgrounds exhibited reduced cell length and large polar bulges, all points of cell growth and division (Figure S3B). Ami3 exhibits some structural homology to E. coli AmiD, a zinc metalloprotease in the amidase_2 domain family of cell wall hydrolyzing amidases (Kelley et al., 2015; Senzani et al., 2017). Threading the Ami3 sequence into the AmiD structure using Phyre2 allowed us to identify a potential triad of zinc-coordinating residues in Ami3 consisting of two histidines (H226, H362) and a cysteine (C370). To test the contribution of Ami3 enzymatic activity to HtrA essentiality, we constructed mutants carrying amino acid substitutions in each of these residues and attempted to swap enzymatically inactive versions of am3 into a strain lacking the endogenous copies of both am3 and htrA and carrying a copy of htrA at the L5 integration site. We found that the inactive alleles could exchange with htrA and, in an analogous experiment, lppZ (Figure 3C). Thus, HtrA and LppZ are only essential in the presence of Ami3 catalytic activity.

If Ami3 hyperactivity leads to cell death, mutating the catalytic residues of Ami3 might relieve toxicity. Indeed, overexpression of am3(H226A), am3(H362A), or am3(C370S) was no longer lethal to cells (Figure 3A). However, full wild-type viability was not restored, indicating that even inactive am3 still retains some toxicity despite being expressed at levels broadly similar to the wild-type allele (Figures 3C and S3C). Cells that overexpressed the mutant protein were still abnormal, albeit with a morphology distinct from that of wild-type Ami3 overexpression: overaccumulation of the catalytically inactive mutants of Ami3 resulted in long, branching cells (Figure 3B). These cells resemble a subpopulation of cells with increased length and branching observed upon htrA depletion (Figure 1G).

**HtrA-LppZ Regulates Ami3**

On the basis of the correlation in phenotypes between am3 overexpression and htrA and lppZ depletion, we hypothesized that HtrA-LppZ negatively regulates Ami3. To test this, we constructed strains in which the only copies of htrA and lppZ were expressed only in the presence of inducer and monitored levels of Ami3.

As expected, Ami3 levels decreased with HtrA induction (Figure 4A). We next mutated the catalytic serine of htrA, creating htrA(S334A). In PepD, another mycobacterial HtrA homolog, this mutation ablates up to 90% of proteolytic activity (White et al., 2010, 2011). As expected, Ami3 levels were less affected

**Figure 2. Loss-of-Function Mutations in am3, pmt, or mprB Suppress htrA Essentiality**

(A–C) Double-mutant phenotypes of htrA suppressor strains: am3 (A), pmt (B), and mprB (C). Wild-type Mycobacterium smegmatis (Msm), single suppressor gene deletions, suppressor deletions with htrA deletions, and htrA complemented strains (see Figures S1 and S6) were grown under conditions identical to those in Figure 1, including growth in rich media at 37°C, high temperature (42°C), carbon-nitrogen starvation (PBS-Tween 20), and oxidative stress (0.02% tert-butyl hydroperoxide). Error bars represent SD of the mean.

(D) htrA suppressor mutants exhibit reduced cell length. Single suppressor deletion strains (blue), htrA double-deletion strains (red), and htrA complemented strains (green) were grown to log phase and analyzed for total cell length (see Figure S2). At least 70 cells were quantified in each condition. Dotted black lines indicate median values. ***p < 0.0001.
by HtrA(S354A) induction; this is not due to a difference in stability between wild-type and catalytically inactive HtrA (Figure 1E). Increasing HtrA(S354A) expression further decreased Ami3 levels, but not to the levels achieved by wild-type HtrA (Figure 4B). Accordingly, strains expressing only HtrA(S354A) were more sensitive to Ami3 overexpression (Figure S3D). A similar but milder decrease in Ami3 protein levels was observed upon LppZ induction (Figure 4A), indicating that LppZ makes a less substantial contribution to Ami3 regulation.

Finally, we tested the dependency of the HtrA-LppZ interaction on the presence of its potential substrate Ami3. We found that HtrA and LppZ interacted even in a Δami3 background (Figure S1D). We conclude that the HtrA-LppZ complex negatively regulates Ami3, with HtrA likely serving as the limiting factor and catalytic agent.

**Pmt Stabilizes Ami3 through Mannosylation**

Because loss of pmt suppressed the essentiality of htrA and lppZ (Figures 2B and 2D), we hypothesized that it might be a positive regulator of Ami3 toxicity by driving its accumulation. Pmt has been previously characterized as a periplasmic mannosyltransferase with a catalytic aspartic acid at residue 68 (D68) (VanderVen et al., 2005; Liu et al., 2013); however, few of its targets have been identified. We tested the importance of this activity by mutating the catalytic aspartic acid D68 to an alanine, which decreases mannosyltransferase activity by more than 50%...
Ami3 toxicity (Figure S4C). To test whether mannosylation contributed to Ami3 stability, we monitored Ami3 levels by western blot in a Δpmt strain (Figure 5C, left). Adding wild-type pmt, but not pmt(D68A), restored wild-type levels of Ami3. In contrast, Ami3** was insensitive to the presence of Pmt (Figure 5C, right).

The simplest explanation for the effect of mannosylation on protein quantity is that modification affects protein stability. To test this, we assayed the half-life of Ami3 in wild-type and Δpmt strains after inhibiting translation with chloramphenicol. Ami3 levels decreased significantly faster in the Δpmt strain compared with wild-type cells (Figures 5D and 5E) demonstrating that mannosylation stabilizes Ami3.

Because Pmt increases Ami3 stability, we reasoned that overexpression of Ami3 in cells lacking pmt may be less toxic. Indeed, Ami3 overexpression in a Δpmt background and Ami3*** overexpression exhibit less severe morphological defects and 5-fold increased survival compared with wild-type Ami3 overexpression (Figures 6A–6C and S5) To determine whether abundance and catalytic activity make independent contributions to toxicity, we monitored the growth of strains overexpressing Ami3(H362A), the catalytic mutant of Ami3 with protein levels most closely mimicking those of wild-type Ami3 (Figure S3B) in the Δpmt background. As expected, growth of a strain overexpressing Ami3(H362A) was further rescued (Figure 6C) and its morphological defects were lessened (Figures 6A and 6B) by knocking out pmt. These results are consistent with a model in which Ami3 activity and stability are independent, and altering both in combination has an additive effect on relieving the toxicity of overexpression.

Finally, mannosylation does not appear to affect the interaction of HtrA and LppZ (Figure S1D). Additionally, HtrA still reduces Ami3 levels in a Δpmt strain (Figure S4D), demonstrating that mannosylation is not required to elicit the activity of HtrA-LppZ against Ami3.

**Figure 4. Induction of HtrA-LppZ Decreases Cellular Ami3 Levels**

(A) HtrA and LppZ expression decrease Ami3 levels to varying degrees. Strains in which the only copy of htrA or lppZ was under an aTc-inducible promoter were constructed and grown with or without 100 ng/mL aTc for 12 hr. HtrA(S354A) is an allele of htrA presumed to exhibit reduced catalytic activity in HtrA homologs due to a mutation in its catalytic serine (see Figure S3). Whole-cell lysate was analyzed using western blotting using anti-Strep and anti-RpoB as a loading control. Western blot images were cropped but display all relevant lanes and reactive bands.

(B) Higher expression of HtrA(S354A) further decreases Ami3. A second copy as a loading control.

Using an L5 swap, we found that pmt(D68A), but not wild-type pmt, permitted growth of a strain missing either htrA or lppZ (Figure 5A).

To test if Ami3 is itself mannosylated by Pmt, we immunoprecipitated epitope-tagged Ami3, analyzed it using mass spectrometry, and found hexose modifications on three threonines: T122, T130, and T138 (Figures S4A and S4B; Table S1). These modifications were no longer detected when an Ami3 sample was isolated from a Δpmt strain (Figure S4A; Table S1). Given that diverse hexose modifications emerge at similar molecular weights by mass spectrometry, we were not able to chemically confirm mannosylation; however, given our genetic data, we proceeded under the assumption that we had found a putative mannosylation. An allele substituting alanines for these residues (Ami3[T122A/T130A/T138A] or Ami3***) allowed growth of a strain lacking htrA (Figure 5B). We assessed the contribution of each mannosylated residue to Ami3 toxicity and found that T122 and T130 were critical, while T138 did not affect
DISCUSSION

Peptidoglycan hydrolases must be carefully regulated in a spatiotemporal manner to coordinate the complex processes of cell expansion and division. Dysregulation of cell wall enzymes has been shown to be toxic in species from \textit{E. coli} to the various mycobacteria (van Heijenoort, 2011; Chao et al., 2013). The rapid turnover of cell wall requires an equally fast disposal of unwanted enzymes, and in the extracellular compartment, fewer mechanisms are available than in the cytoplasm. Proteolysis is one of the more rapid ways to effect change in the periplasm (Mukherjee et al., 2009; Festa et al., 2010). For instance, the hydrolase RipA must be cleaved by the serine protease MarP to process peptidoglycan (Botella et al., 2017). Cleavage can also efficiently halt the activity of enzymes: PBPB is cleaved by the metalloprotease Rv2869 in \textit{Mtb} during oxidative stress (Mukherjee et al., 2009). Finally, several factors involved in cell wall assembly have been shown to be targeted for pupylation and subsequent proteasomal degradation, including MurA, KasB, and MtrA (Festa et al., 2010). However, none of these proteolytic or processing systems are essential for viability and only take on critical roles under environmental stress.

Figure 5. Pmt Mannosylation Stabilizes Ami3

(A) Mutating the catalytic activity of Pmt suppresses the essentiality of htrA and lppZ. Top: the endogenous copies of \textit{pmt} and \textit{htrA} were replaced with zeocin and hygromycin resistance cassettes, respectively, and a copy of \textit{htrA} was integrated at the L5 phage integration site. \textit{pmt} or \textit{pmt(D68A)}, a catalytically inactive variant of \textit{pmt}, was transformed into this background. Full swaps that acquire kanamycin resistance at the expense of nourseothricin resistance render strains devoid of \textit{htrA} (or \textit{lppZ}) and must thus carry a suppressor mutation. Bottom: quantification of \textit{pmt} and \textit{pmt(D68A)} swaps. A total of 60 transformants were tested for antibiotic resistance. An equivalent swap was performed for \textit{lppZ} and enumerated in the same manner.

(B) Removing the mannosylation residues of Ami3 suppresses the essentiality of \textit{htrA} and \textit{lppZ}. Top: the endogenous copies of \textit{ami3} and \textit{htrA} were replaced with zeocin and hygromycin resistance cassettes, respectively, and a copy of \textit{htrA} was integrated at the L5 phage integration site. \textit{ami3} or \textit{ami3(T122A/T130A/T138A)} (\textit{ami3***}), an allele of \textit{ami3} in which all mannosylation sites have been mutated (see Figure S4), was transformed into this background. Full swaps that acquire kanamycin resistance at the expense of nourseothricin resistance render strains devoid of \textit{htrA} (or \textit{lppZ}) and must thus carry a suppressor mutation. Bottom: quantification of \textit{ami3} and \textit{ami3***} swaps. A total of 120 transformants were tested for antibiotic resistance. An equivalent swap was performed for \textit{lppZ} and enumerated in the same manner; a total of 60 transformants were tested for antibiotic resistance.

(C) Ami3 levels decrease in cells missing Pmt. Strains expressing Ami3-Strep or Ami3*** in a wild-type, \textit{Dpmt}, or \textit{Dpmt+pmt(D68A)} background were grown to log phase. Whole-cell lysate was analyzed using western blotting using anti-Strep and anti-RpoB as a loading control.

(D) Pmt mannosylation increases the stability of Ami3 protein. At indicated time points after adding chloramphenicol, aliquots of cells were lysed, and levels of Ami3-Strep were monitored using western blot. Results are representative of three independent experiments.

(E) Quantification of Ami3 stability. Independent values of RpoB-normalized levels of Ami3 from (D) quantified using densitometry analysis. Western blot images were cropped but display all relevant lanes and reactive bands. *\(p < 0.05\) and **\(p < 0.01\). Error bars represent SD of the mean.
Figure 6. Removal of Mannosylation Partially Rescues Morphology and Survival of Ami3 Overexpression

(A) Loss of pmt relieves morphological defects in Ami3 overexpression. Wild-type ami3, ami3(H362A), or ami3*** was overexpressed in either Δami3 or Δami3 Δpmt cells (see Figure S5).

(B) Quantification of cellular morphology of Ami3 overexpression. Cells were observed by microscopy and measured for total cell length, maximum cell width, and the proportion of the cell bulging (length of bulge over total cell length). At least 80 cells were quantified in each condition. Dotted black lines indicate median values.

(C) Survival and morphological quantification of Ami3 overexpression. Strains expressing wild-type ami3, ami3(H362A), or ami3*** under an aTc-inducible promoter were grown in the presence or absence or 100 ng/mL aTc. Aliquots were taken at the indicated time points for CFU analysis. The uninduced wild-type Ami3 strain is representative of all uninduced strains. The growth of various strains was compared, and demarcated by asterisks (comparing the overexpression of ami3 in a wild-type or Δpmt background), stars (comparing the overexpression of wild-type ami3 or ami3***), or X’s (comparing the overexpression of ami3(H362A) in a wild-type or Δpmt background).

*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Error bars represent SD of the mean.
The muramidase Ami3, under the stabilizing influence of Pmt, potentially accumulates to toxic levels over the course of the cell cycle. The HtrA-LppZ complex restores normal Ami3 levels to abrogate this lethality and is thus essential for viability.

Figure 7. Model of HtrA-LppZ-Mediated Regulation of Ami3
The muramidase Ami3, under the stabilizing influence of Pmt, potentially accumulates to toxic levels over the course of the cell cycle. The HtrA-LppZ complex restores normal Ami3 levels to abrogate this lethality and is thus essential for viability.

We propose a model in which HtrA and LppZ are essential to degrade Ami3, thereby controlling its toxicity in Msm (Figure 7). Over the course of the cell cycle, preexisting cell wall material must be cleaved to make room for newly synthesized precursors. Cell wall hydrolases such as Ami3 are double-edged swords: although they are important for proper growth and septation, overactivity can destabilize the cell wall and lead to autolysis. Without spatiotemporal regulation, sustainable cellular growth cannot occur, thus necessitating the activity of a periplasmic protease such as HtrA that can regulate a highly active enzyme like Ami3 in Msm.

Notably, because Ami3 is still not completely stable even upon htrA deletion (Figures 5D and 5E), other proteases may have a more minor effect on Ami3 levels and will be the subject of future work.

The finding that HtrA requires a second protein is consistent with other observations of periplasmic proteases that rely on partners for fine-tuned regulation. Lipoproteins have been shown to be allosteric modulators of proteases in a variety of contexts (Tadokoro et al., 2004; Singh et al., 2015; Park et al., 2017) and have also been shown to directly activate the activity of cell wall enzymes (Paradis-Bleau et al., 2010; Typas et al., 2010; Egan et al., 2014; Lupoli et al., 2014). It is possible that LppZ facilitates the interaction between HtrA and Ami3, primes Ami3 for regulation by HtrA, or some combination of the above.

Although we were unable to measure amidase activity directly, Ami3 is likely a bona fide amidase on the basis of the phenotype of overexpression and requirement of catalytic residues for full toxicity, which mirror prior work in this field (Heidrich et al., 2001; Chao et al., 2013). In our work, all three N-acetylmuramoyl-L-alanine amidases in Msm are largely dispensable, though the importance of different amidases may vary in different Msm strains (Bavesh Kana, personal communication).

Several alternative enzymes able to cleave peptidoglycan cross-links likely operate in our strain under the growth conditions that we assayed, compensating for the loss of this family of amidases. Moreover, we simply may not know the growth condition in which these amidases play a critical role.

If Ami3 is indeed an active amidase, it is perhaps surprising that even a catalytically inactive variant of Ami3 can arrest cell growth. There are multiple possibilities to explain our observations. First, these mutants might retain a small amount of activity that stunts growth when enough protein is produced. Second, amidase activity may account for only a portion of ami3 toxicity, which can still impair growth when expressed at high levels, perhaps by aggregating to levels the cell cannot tolerate. Third, Ami3 may engage with another cell wall enzyme that depends on Ami3 catalytic activity to execute its function. Enzymatically inactive Ami3 may thus lock its binding partner in a futile interaction that results in a failure to divide when the complex is sequestered or it fails to dissociate from peptidoglycan (Figure 3B). These models are not mutually exclusive.

In this context, mannosylation could alter protein folding or recognition by proteolytic enzymes. Post-translational modifications play an important role in the activation or stabilization of cell wall enzymes in mycobacteria. For instance, the peptidoglycan synthase PonA1 and MurA regulator CwlM must be phosphorylated to be active (Kieser et al., 2015; Boutte et al., 2016).

Although this could also be a possible consequence of Ami3 mannosylation, it is unlikely given that ami3 overexpression in a Δpmt background does not phenocopy overexpression of catalytically inactive ami3. Additionally, pmt mannosylation does not appear to affect localization, as Ami3-mRFP is recruited to the same locales regardless of background (Figure S3B).

Furthermore, Pmt might play a broad role in cell wall maintenance, as it contributes to the virulence, immunogenicity, permeability, and antibiotic susceptibility of Mtb and M. abscessus (Becker et al., 2017; Liu et al., 2013; Deng et al., 2016; Harriff et al., 2017). In fact, a Δpmt mutant in M. abscessus is more susceptible to several antibiotics including rifampin (Becker et al., 2017).

Proteases are attractive drug candidates, and in fact, an inhibitor of mycobacterial MarP and HtrA has been described (Zhao et al., 2015). However, at least for Msm, our results suggest that blocking HtrA could lead to high rates of resistance, as there are multiple mechanisms to escape drug-mediated killing. This might be less true for Mtb, especially during infection, when it is possible that Ami3 plays a larger role to maintain cellular integrity or HtrA, LppZ, and Pmt contribute to the pathogen’s virulence in additional ways (Liu et al., 2013; Deng et al., 2016; Harriff et al., 2017). Notably, because Mtb and Msm differ in many ways, including the existence of a third htrA homolog, pepA, in Mtb only, our findings may not be entirely generalizable. However, given the retention of all three HtrA family proteins in multiple pathogenic mycobacteria (Lew et al., 2011), it is likely that these proteases serve critical roles worthwhile of future study. In this light, targeting of the essential HtrA-LppZ pathway remains possible.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:
cell wall synthesis. Elife
A cytoplasmic peptidoglycan amidase homologue controls mycobacterial
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STAR METHODS

KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Eric J. Rubin (erubin@hsph.harvard.edu).

EXPERIMENTAL MODEL DETAILS

Msm mc2155 was grown in liquid media containing 7H9 salts (Becton Dickinson) supplemented with 5 g/L albumin, 2 g/L dextrose, 0.85 g/L NaCl, 0.003 g/L catalase, 0.2% glycerol, and 0.05% Tween80, or plated on LB agar. For all oxidative stress experiments, strains were grown in Hartmans-de Bont (HdB) media, which was made as described (Hartmans and De Bont, 1992) with 0.05% Tween80. E. coli TOP10 was used for cloning. Antibiotic selection concentrations for M. smegmatis were as follows: 25 μg/mL kanamycin, 50 μg/mL hygromycin, 20 μg/mL zeocin, 20 μg/mL nourseothricin, and 5 μg/mL gentamicin. Antibiotic concentrations for E. coli were as follows: 50 μg/mL kanamycin, 100 μg/mL hygromycin, 50 μg/mL zeocin, and 40 μg/mL nourseothricin. Anhydrotetracycline was used at 100 ng/mL for gene induction or repression. All strains were grown at 37°C unless otherwise indicated.

METHOD DETAILS

Strain construction
Deletions of htrA and lppZ were generated by first transforming in a second copy of the gene at the L5 and Tweety phage integration sites (Lewis and Hatfull 2003; Pham et al. 2007), then using mycobacterial recombineering as previously described (van Kessel and Hatfull 2008) to delete the endogenous copy. Where indicated, htrA and lppZ were cloned into vectors with a tetON repressor to make their expression tetracycline inducible, or into vectors with a tetOFF repressor to make their expression tetracycline repressible.
For growth curves, strains were grown to mid-log phase, diluted to OD600 0.005 in triplicate, and measured every 15 min in a Bioscreen growth curve machine (Growth Curves USA) at 37°C or 42°C, where indicated.

For environmental stress kill curves, strains were grown to mid-log phase, washed twice with PBS-Tween80, diluted to OD600 0.05, and rolled at 37°C in PBS-Tween80 or HdB + 0.02% tert-butyl hydroperoxide. At the indicated time points, 200 μL aliquots were removed, serially diluted in PBS-Tween80, and plated for CFUs.

For aTc-inducible overexpression kill curves, all steps were the same except cells were not washed before subculturing into 7H9 with kanamycin to maintain the episomal plasmid, with or without 100 ng/mL aTc. Tert-butyl hydroperoxide and aTc were added once at the beginning of each experiment. Each kill curve was performed at least thrice, in triplicate, with similar results.

**MIC determination**
The MIC of *M. smegmatis* strains were performed using the Alamar Blue assay as previously described (Kieser et al. 2015).

**Calcein staining and flow cytometry**
Mid-log-phase cultures were stained with 0.5 μg/mL calcein for 1 hour at 37°C. These cells were then analyzed by flow cytometry (MACSQuant YB excitation: 488 nm; emission filter: 525/50).

Total cell length, bulge length, maximum cell width, and mean fluorescence intensity (MFI) were measured manually where indicated.
Hexose modification analysis by LC-MS/MS

To identify potential hexose modifications on Ami3, a 200 mL mid-log phase cultures of strains expressing Ami3-Strep in a wild-type or Δpmt background were spun down and resuspended in 2 mL of Buffer W (100 mM Tris, 150 mM NaCl, 1 mM EDTA) with an EDTA-free protease inhibitor cocktail (Roche, Switzerland). The cells were lysed by bead beating and SDS was added to the lysate to a final concentration of 1%. The lysate was pre-cleared of endogenously biotinylated proteins using Pierce Avidin Agarose for 1 hour at room temperature. The cleared supernatant was then added to MagStrep “type3” XT beads (IBA Lifesciences) and incubated overnight at 4°C. The beads were then washed three times with Buffer W and eluted using Buffer BX (IBA Lifesciences). The eluted samples were separated on a 4%–12% NuPAGE Bis-Tris precast gel (Invitrogen Novex) and stained with Coomassie Blue. A single band running at the predicted molecular weight of Ami3-Strep was excised from each sample.

The samples were reduced with 1 mM DTT for 30 minutes at 60°C, then alkylated with 5mM iodoacetamide for 15 minutes at room temperature in the dark. Samples were then subjected to a modified in-gel trypsin digestion procedure (Shevchenko et al. 1996). Gel pieces were washed and dehydrated with acetonitrile for 10 minutes, followed by removal of acetonitrile and complete drying in a speed-vac. Rehydration of the gel pieces was performed with an 50 mM ammonium bicarbonate solution containing 12.5 ng/μl modified sequencing-grade trypsin (Promega, Madison, WI) at 4°C. Samples were then incubated at 37°C overnight. Peptides were later extracted by removing the ammonium bicarbonate solution, followed by one wash with a solution containing 50% acetonitrile and 1% formic acid. The extracts were then dried in a speed-vac for about an hour. The samples were then stored at 4°C until further analysis, upon which samples were reconstituted in 5 to 10 μl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 2.6 μm C18 spherical silica beads into a fused silica capillary (100 μm inner diameter x ~30 cm length) with a flame-drawn tip (Peng and Gygi 2001). After the column was equilibrated, each sample was loaded onto the column via a Famos auto sampler (LC Packings, San Francisco, CA). A gradient was formed and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid).

As each peptide was eluted, it was subjected to electrospray ionization and then entered into an LTQ Orbitrap Velos Pro ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Eluting peptides were detected, isolated, and fragmented to produce a tandem mass spectrum of specific fragment ions for each peptide. Peptide sequences were determined by matching the acquired fragmentation pattern with the Ami3 amino acid sequence, using a software program, Sequest (ThermoFinnigan, San Jose, CA) (Eng et al. 1994). The modification of 180.156 mass units to serine, and threonine was included in the database searches to determine hexose-modified peptides. Modifications were scored from 0 to 1000. Scores over 19 were considered confidently assigned. Scores of 1000 were considered unequivocal. All databases include a reversed version of all the sequences and the data was filtered to between a one and two percent peptide false discovery rate.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical details of experiments can be found in the figure legends. All experiments were performed at least twice. Means were compared using a two-sided Student’s t test, and all error bars indicate standard deviation around the mean. p < 0.05 was considered significant. For most microscopy experiments, roughly 100-200 cells were analyzed and data medians are shown. No statistical methods were used to predetermine sample size, and the researchers were not blinded to sample identity.

DATA AND SOFTWARE AVAILABILITY

Full, uncropped Western blots for relevant figures have been deposited to the Mendeley database under the following DOI: https://doi.org/10.17632/r7mm9mtt3v.1#folder-26c6e562-7d8d-44c8-a65a-21fa0ae692eb.