Supporting Online Material for

Ubiquitin-Like Protein Involved in the Proteasome Pathway of *Mycobacterium tuberculosis*

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Supplementary Online Material

Materials and Methods

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table S1. *E. coli* strains used for cloning and expression were grown in LB Miller broth (Difco) at 37°C with aeration on an orbital shaker or on LB agar. *E. coli* strains were chemically transformed as previously described (*1*).

Minimal medium using E-salts (*2*) was supplemented with 1% lactose, maltose or glucose as needed. *Mtb* strains were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol, 0.05% Tween-80, 0.5% bovine serum albumin, 0.2% dextrose and 0.085% sodium chloride. *Mtb* cultures were grown without shaking in 75 cm^2 vented flasks (Corning) in humidified incubators with 5% CO_2 at 37˚C. 7H11 agar (Difco) supplemented with oleic acid, albumin, dextrose and catalase (BBL) was used for growth on solid medium. *Msm* strains were grown at 37˚C with shaking in 7H9 broth supplemented with 0.2% glycerol and 0.05% Tween-80 or on 7H11 agar with 0.2% glycerol. Mycobacteria were transformed as described elsewhere (*3*). The *Msm mpa* mutant was made by integrating a non-replicative plasmid, pWKS130-sm-*mpa*, into the chromosome of mc^2155, resulting in strain MsHD3.

The final concentrations of antibiotics used for *E. coli* were: ampicillin, 200 μg/ml; hygromycin, 150 μg/ml; and kanamycin, 100 μg/ml. For *Mtb* and *Msm*, hygromycin and kanamycin were each used at 50 μg/ml. Trimethoprim (Trim) was used at 50 μg/ml for *Msm*.

Antibodies, plasmids and primers. Plasmids and primers used in this study are listed in Table S1. For construction of the *Msm mpa* mutant, a 537 bp fragment was amplified from mc^2155 genomic DNA using primers smmpaf1 and smmpar1 (Table S1) and cloned into pSTBlue (Novagen) resulting in pWKS130-sm-*mpa*. The insert was then subcloned into

pWKS130 using EcoRI. For all other plasmids, *Mtb* sequences were used. For overexpression of native *Mtb mpa* in *E. coli*, PCR products were cloned into the NdeI and NotI sites of plasmid pET24b(+), maintaining the stop codon. For over expression from a plasmid with a different origin of replication, pET24b(+)-*mpa* was digested with XbaI and NotI and the fragment, including the consensus ribosome binding site (RBS) from pET24b(+), was sub-cloned into pWSK29.

pET24b(+) was used to express His_6-*pup* in *E. coli* for purification for in vitro interaction studies and for antibody production. *pup* was first cloned into the SacI and KpnI sites in pQE30 (which adds a His_6 tag to the 5' end of *pup*) via restriction sites included in the primers used to PCR amplify *pup*. Using pQE30-*pup* as a template, a second set of primers was used to amplify His_6-*pup* and clone it into the NdeI and HindIII sites of pET24b(+). This strategy included the nucleotides AGAGGATCG (RGS epitope) before the His_6 sequence and the nucleotides GGATCCGCATGCGAG between His_6 and *pup*.

The previously reported pMN-FLAG-*fabD*-His_6 (*4*) was used as a template for making additional *fabD* constructs. *fabD* was PCR amplified using Fab_NT_NdeI_F and Fab_CT_PstI_R, and cloned into the NdeI and PstI restriction sites of pMNL, which has the *Mtb hsp60* promoter for expression of the cloned gene (creating pMNL-*tbfabD*-UT). We made a new FLAG/His_6 epitope-tagged *fabD* because we found that two forms of FabD were being synthesized: one that had both FLAG and His_6 epitopes and one that had only the His_6 epitope. This was due to the presence of two recognizable RBS in the transcript expressed from the original pMN-FLAG-*fabD*-His_6 (*4*). To make an epitope-tagged *fabD* with only one start of translation, we used PCR using primers FabTBNdeIFlafF and HIII-PstI-HisFabR and cloned PCR products into the NdeI and the PstI sites of pMNL, creating pMNL-FLAG-tb*fabD*-$His_6$1S.

To make FabD with only the FLAG epitope tag, we subcloned the N-terminal fragment of pMNL-FLAG-tb*fabD*-$His_6$1S containing the FLAG epitope into the NdeI and PvuII restriction sites of pMNL-tbfabD-UT, resulting in pMNL-FLAG-tb*fabD*.

To make pMN-FLAG-tb*fabD*K173A-His_6, we mutated lysine 173 to alanine by splicing overlap extension PCR. We used pMNL-*tbfabD*-UT as a template and the primers FabD_TB_NdeI_Flag_F, Fab_K173_R, Fab_K173_F, FabD_NT33_Pst_R to construct pMNL-FLAG-tb*fabD*K173A. This plasmid was then used as the template for PCR with primers FabD_TB_NdeI_Flag_F and HIII_Pst_His_Fab_R to make pMN-FLAG-tb*fabD*K173A-His_6, which produces the mutant FabD with both FLAG and His_6 epitopes.

pMV306.kan-His_6-*pup* was made as follows: His_6-*pup* was sub-cloned from pET24b(+)-His_6-*pup* into the NdeI and HindIII restriction sites of the pUV15 mycobacterial expression vector creating pUV15-His_6-*pup*. The pUV15 vector contains the strong mycobacterial promoter from the *Msm rpsA* gene (*5*). This plasmid was digested with SpeI and ClaI to subclone the UV15 promoter with His_6-*pup* into the XbaI and ClaI restriction sites of pMV306.kan, which integrates into the *attB* site on the chromosome. To make pWKS30-His_6-*pup*, His_6-*pup* was sub-cloned from pET24b(+)-His_6-*pup* into the XbaI and HindIII restriction sites of pWKS30 downstream of the T7 promoter.

Pfu Polymerase (Stratagene) was used for all PCR. All primers were purchased from Invitrogen. Restriction enzymes were purchased from New England Biolabs. All clones were sequenced by GENEWIZ, Inc. to confirm the correct DNA sequence.

For antibody production, His_6-Pup was purified from *E. coli* ER2566 with pET24b(+)His_6-Pup under native conditions as described in the QIAexpressionist manual (Qiagen). Polyclonal rabbit antibodies were produced by Covance. Antibodies to His_6-Pup were

affinity purified as described elsewhere (*6*). FLAG antibodies were purchased from Sigma; antibodies to penta-His were purchased from Qiagen. Polyclonal antibodies to DlaT-His_6 were a gift from Ruslana Bryk and Carl Nathan and used as described elsewhere (*6-8*). Horseradish peroxidase (HRP) coupled anti-rabbit secondary antibodies were used according to manufacturer's instructions (GE Healthcare).

***E. coli* BTH screen.** A genomic *Mtb* DNA library was constructed by cloning 300-800 bp DNA fragments from a limited Sau3AI digestion of *Mtb* chromosomal DNA into the BamHI site of pKT25. 100 ligations were used to transform DH5α. Plasmid DNA was purified from all 100 pools, each representing ~1,000 clones. The BTH screen was performed using methods described elsewhere (*9*). Briefly, *mpa* was cloned into in the KpnI and XbaI sites of pUT18C and used to transform BTH101. This bait strain was then transformed with 1 µl of each library pool, totaling 100 transformations. Bacteria were plated on minimal media containing 1% lactose as the carbon source. Plates were incubated at 30°C for four days and then for an additional four days at 25°C. Growth indicated putative interactions between Mpa and another protein. Colonies were patched onto MacConkey indicator agar containing 1% maltose as the carbon source and incubated at 30°C for two days and then for an additional two days at 25°C. Red colonies indicated a positive interaction. We recovered the pKT25 library plasmids from these clones and transformed BTH101 with the bait plasmid or empty vector to either confirm interactions or eliminate false positives, respectively. Plasmids that conferred growth on minimal media with 1% lactose were sequenced. Full-length *pup* was cloned into the XbaI and KpnI sites of pKT25 and pUT18C to confirm interaction with the Mpa bait.

***Msm* protein fragment complementation assay (M-PFC).** M-PFC takes advantage of the fact that murine dihydrofolate reductase (mDHFR) has a 12,000-fold lower affinity for the

antibiotic trimethoprim (Trim) than the bacterial DHFR (*10*). Like the *E. coli* BTH system, the interaction of two *Mtb* proteins fused to complimentary domains of mDHFR can restore mDHFR activity, allowing *Msm* to grow on selective (Trim-containing) media. Plasmids used in the assay are listed in Table S1. pUAB100 and pUAB200 containing the *Saccharomyces cerevisiae* GCN4 leucine zipper domain were used as a positive control. In some cases, we replaced GCN4 in pUAB200 with an *Mtb* gene. Pairs of plasmids were used to transform WT *Msm* and bacteria were grown on 7H11 agar for five days at 37°C. Single colonies were inoculated into 150 µl 7H9 broth and incubated for five days at 37°C. 10 µl of each culture was then inoculated onto 7H11 agar with or without Trim, and incubated at 37°C for three days.

Affinity chromatography. For in vitro validation of the Pup/Mpa interaction, 1 L cultures of *E. coli* ER2566 containing either pET24b(+)-His_6-*pup* or pET24b(+)-*sigE*-His_6 were induced with 1mM IPTG at an OD_{600} of ~0.5 for 5 h at 37°C. Cell lysates were made and proteins were purified at 4°C exactly as described in the QIAexpressionist manual. Purified proteins were dialyzed overnight in 4 l of 50 mM NaH_2PO_4/300 mM NaCl then quantified using a NanoDrop spectrophotomoter. For untagged Mpa, a 50 ml culture of *E. coli* strain ER2566 containing pWKS30-RBS-*mpa* was induced with 1 mM IPTG at an OD_{600} of ~0.5 for 5 h at 37°C ("Mpa lysate"). A cell lysate ("empty cell lysate") from an *E. coli* strain containing pET24b(+) was also prepared. To examine protein-protein interactions, 60 µg of purified His_6-tagged proteins or 900 µl of empty cell lysate were incubated with 25 µl of Ni-NTA agarose for 1.5 h at 4°C with agitation. The agarose was collected by centrifugation and the supernatant was discarded. 50 µl of Mpa lysate in 450 µl of lysis buffer was incubated with the agarose for 1.5 h at 4°C with agitation. The agarose was collected, the supernatant was saved ("flow through"), and the agarose was washed with 750 µl wash buffer (50 mM NaH_2PO_4/300 mM NaCl/30 mM

imidazole) three times. The agarose was resuspended in 200 µl of elution buffer (50 mM NaH_2PO_4/300 mM NaCl/250 mM imidazole) and collected by centrifugation. The supernatant was saved ("elution").

For analysis of the Pup~FabD interaction in *Msm*, 50 ml cultures of WT *Msm* containing either pMNL-FLAG-*fabD* + pMV306-kan-His_6-*pup*, pMNL-*fabD*UT + pMV306-kan-His_6-*pup* or pMNL-FLAG-*fabD* + pMV306-kan were grown to an OD_{580} of ~1.0. Bacteria were collected by centrifugation and resuspended in 4 ml of FLAG buffer (50 mM Tris HCl, 150 mM NaCl, pH 7.4). 1 ml aliquots of resuspended cells were transferred to bead beating tubes each with 250 µl of zirconia silica beads (BioSpec Products). Cells were lysed by bead beating four times for 1 m each in a BioSpec Mini Bead Beater. Samples were clarified by centrifugation and were then passed through a 0.2 µ filter. The soluble lysates were quantified using a NanoDrop spectrophotometer and 7.8 mg were incubated with 250 µl of ANTI-FLAG M2 Affinity Gel (Sigma) for 2 h at 4°C with agitation. The matrix was washed two times 5 ml with FLAG buffer in a Poly-Prep column (Bio-Rad). Proteins were eluted with two 300 µl fractions using 100 µg/ml FLAG peptide (Sigma) and then concentrated using a Microcon YM-30 concentrator (Millipore).

To test for the Pup~FabD interaction in *E. coli,* 50 ml cultures of WT *E. coli* containing either the combination pMNL-FLAG-*fabD* + pWKS30-His_6-*pup*, or pMNL-FLAG-*fabD* + pWKS30 were grown to an OD_{580} of ~1.0. Bacteria were processed and purified as described above except cells were lysed by bead beating three times, 1 m each.

To purify FLAG-FabD-His_6 from *Mtb*, 30 ml cultures were grown to an OD_{580} of ~0.8-1.0. Bacteria were collected by centrifugation and resuspended in 3 ml of Ni-NTA lysis buffer. Cells were lysed and quantified as described for *Msm* and 4.65 mg were incubated with 40 µl of

Ni-NTA agarose for 2 hours at 4°C with agitation. The agarose was collected by centrifugation, the supernatant was saved (flow through), and the agarose was washed three times with 750 µl Ni-NTA wash buffer. The agarose was resuspended in 100 µl of Ni-NTA elution buffer and collected by centrifugation, and the supernatant was saved (elution). Samples were boiled for 10 min, and proteins were detected by immunoblotting.

To purify Flag-PanB-His_6 proteins from *Mtb*, 90 ml cultures were grown to an OD_{580} of ~0.8-1.0. 40 OD_{580} cell equivalents were collected by centrifugation and resuspended in 4 ml of Ni-NTA lysis buffer. Cells were lysed as described for *Msm*, but not filtered, and equivalent amounts of protein lysates were incubated with 40 µl of Ni-NTA agarose for 2 hours at 4°C with agitation. The agarose was collected by centrifugation, the supernatant was saved (flow through), and the agarose was washed four times with 750 µl Ni-NTA wash buffer. The agarose was resuspended in 100 µl of Ni-NTA elution buffer and collected by centrifugation, and the supernatant was saved (elution). Samples were boiled for 10 min, and proteins were detected by immunoblotting.

MS analysis. To purify FLAG-FabD~His-Pup for MS analysis, 2 L of WT *Msm* containing pMNL-FLAG-*fabD* + pMV306(kan)-His_6-*pup* were grown to an OD_{580} of ~1.0. Bacteria were collected by centrifugation and resuspended in 66 ml of lysis buffer (see above) and lysed by sonication. Cellular debris was removed by centrifugation, the soluble lysate was filtered using a 0.2 µ syringe filter and the lysate was incubated with 1.6 ml of Ni-NTA agarose for 2 h at 4°C with agitation. The agarose was collected in a polypropylene column and washed once with 10 ml and twice with 5 ml of Ni-NTA wash buffer. Proteins were eluted twice with 1 ml Ni-NTA elution buffer. 500 µl of ANTI-FLAG M2 Affinity Gel was added directly to the elution and incubated for 2 h at 4°C with agitation. The matrix was washed twice with 5 ml with

FLAG buffer in a Poly-Prep column. Two 350 µl and one 250 µl elutions were collected using 100 µg/ml FLAG peptide, combined and concentrated using a Microcon YM-30 concentrator. The 45 kD band was excised from a 12% SDS-PAGE gel stained with CBB, de-stained and in-gel digested with trypsin. The resulting peptide mixture was subjected to LC-MS/MS in an LTQ-OrbitrapXL hybrid mass spectrometer (ThermoFisher, San Jose, CA). The instrument was operated in data-dependent mode using a setup similar to one described previously (*11*). Here, the six most abundant ions detected in the survey MS scan were selected for MS/MS in the Orbitrap. The resolution was set to 1.5×10^4 and AGC to 2×10^4 for the MS/MS scans. MS/MS spectra were assigned by searching them with the SEQUEST algorithm (*12*) against the *M. tuberculosis* sequence database.

Immunoblotting. Total protein lysates were prepared from equivalent cell numbers. We harvested 11 OD_{580} equivalent cell numbers by centrifugation and washed in 5 ml of 0.05% Tween-80 in phosphate buffered saline, resuspended in 500 µl of lysis buffer (100 mM Tris-Cl, 100 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, pH 8) and transferred to bead beating tubes with 250 µl of zirconia silica beads. Cells were lysed by bead beating three times for 1 min each time. Lysates were clarified by centrifugation and 270 µl of soluble cell lysate was mixed with 90 µl of 4 × protein sample buffer. The samples were boiled at 100˚C for 10 min and equal volumes representing equivalent cell numbers were separated by 10% SDS-PAGE. Proteins were transferred to nitrocellulose and blocked in either 5% milk or 1% BSA. For immunoblots, experimental membranes were stripped and incubated with anti-DlaT to check equivalent loading of samples. Detection of HRP was performed using either SuperSignal West Pico or West Femto Chemiluminescent Substrate (ThermoScientific).

Pulse-Chase Analysis. For pupylated protein analysis, cultures of WT and *mpa Msm* containing pUV15-His_6-*pup* were grown to OD_{580} of ~1.0 and 55 OD equivalents were collected of each strain. 1.68 mCi of TRAN^{35}S-Label (MP Biomedicals, Inc.) was added and the bacterial cells were incubated with the radiolabeled amino acids at 37°C for 15 minutes (pulse). Cells were then collected by centrifugation, washed twice in Middlebrook 7H9 broth (Difco) supplemented with 10 mM methionine and 10 mM cysteine (chase media), and then resuspended in chase media to OD_{580} of 1. About 5 OD equivalents (5 ml) of bacteria were removed for the t = 0 time point and the chase was initiated by returning the bacterial cells to 37°C. Samples of 5 ml were taken every hour for nine hours. Bacteria from each sample were resuspended in 1 ml Ni-NTA lysis buffer. Cells were lysed as described above and equal volumes of soluble lysate were incubated with 40 µl of Ni-NTA agarose for 2 h at 4°C with agitation. The agarose was collected by centrifugation and washed four times with 750 µl Ni-NTA wash buffer. The agarose was resuspended in 150 µl of Ni-NTA elution buffer and collected by centrifugation, and the supernatant was saved (elution). Samples were boiled for 10 m, and proteins were analyzed on a 10% SDS-PAGE gel. Radioactivity was quantified using a STORM820 phosphorimager (Molecular Dynamics) and Quantity One v.4.5.0 (Bio-Rad).

For FabD analysis, cultures of WT *Msm* containing pMNL-FLAG-tb*fabD*-$His_6$1S and pMN-FLAG-tb*fabD*K173A-His_6 were grown to OD_{580} of ~1.0 and 45 OD equivalents were collected of each strain. 1.37 mCi of TRAN^{35}S-Label (MP Biomedicals, Inc.) was added and the bacterial cells were incubated with the radiolabeled amino acids at 37°C for 15 minutes (pulse). Cells were processed and proteins were purified as above except 35 µl of Ni-NTA agarose was used during the purification.

Figure legends for Supplementary Online Material

fig. S1. BTH controls. **(A)** All *E. coli* BTH strains grew on minimal E-salts agar supplemented with 1% glucose. **(B)** All *Msm* BTH strains grew on 7H11 lacking Trim. Also included are several plasmid test pairs that were negative (m-r) on Trim.

fig. S2. Genomic orientation of *pup* in *Mtb. pup* (Rv2111c) is upstream of the proteasome core genes *prcB* (Rv2110c) and *prcA* (Rv2109c). The stop and start codons of *pup/prcB* overlap, suggesting that they are co-transcribed and translationally coupled. Sequences were compiled from the NCBI server.

fig. S3. FLAG antibodies recognized two forms of FabD in mycobacteria but not *E. coli*. **(A)** Longer exposure of immunoblot of samples shown in Fig. 1D showed epitope-tagged FabD at the predicted size and a second species at ~45 kD (arrow). Samples shown in Fig. 1D were analyzed on a separate 10% SDS-PAGE gel to resolve the ~45 kD band from the cross-reactive background bands. **(B)** His_6-Pup~FLAG-FabD complex was not formed in *E. coli*. Equal amounts of soluble *E. coli* lysates from WT strains co-expressing FLAG-FabD and either His_6-Pup or empty vector were incubated with anti-FLAG M2 affinity matrix for enrichment of FLAG-tagged proteins. Samples were analyzed by 12% SDS-PAGE. Anti-FLAG immunoblots (left) of eluates recognized a protein at the predicted size of FLAG-FabD (arrow). Anti-His_5 immunoblots (right) of the same samples failed to detect a His_6-Pup~FLAG-FabD complex.

fig. S4. Evidence for isopeptide linkage between a FabD lysine and the C-terminus of Pup. The modified fragment ion nomenclature used here includes the charge (either + or 2+), subscript 1 or 2, referring to the FabD and Pup peptides respectively, and the sequential fragment number. The capitalized B/Y refers to fragment ions that include the isopeptide bond. **(A)** Detailed assignment of both singly- and doubly-charged *b*- and *y-type* ion fragments for the spectrum shown in Fig. 2C in the main text. Mass deviation information is included for ions matching within 10 ppm. **(B)** Tandem mass (MS/MS) spectrum of the same pupylated peptide as shown in Fig. 2C in the main text and derived by collision-induced dissociation of the $(M+3H)^{3+}$ precursor, m/z 580.312 (0.60ppm). **(C)** Tandem mass (MS/MS) spectrum of a FabD partially tryptic peptide derived from collision-induced dissociation of the $(M+2H)^{2+}$ precursor, m/z 673.344 (2.13 ppm). The peptide arises from cleavage at the weak Asp-Pro peptide bond.

fig. S5. MS/MS analysis of non-C-terminal Gln in Pup. Chromatographic traces of the C-terminal peptide of Asp-N-digested His_6-Pup. The traces correspond to *m/z* of MH_2^{2+} precursors (+/- 3 ppm). Q* denotes a de-amidated Gln, equivalent to Glu. See (*13*) for additional details.

fig. S6. Analysis of substrate pupylation in *Mtb*. **(A)** PanB is a pupylated substrate in *Mtb*. Epitope-tagged PanB was purified from *Mtb* with plasmid pMN-FLAG-*panB*-His_6. The construction of this plasmid resulted in the synthesis of two species of PanB: one with both FLAG and His_6 epitopes and one with just the His_6 epitope. Thus, purification using nickel affinity chromatography resulted in the isolation of two His_6-reactive PanB species (left), both of which were pupylated (right). The smaller PanB species was sequenced by Edman degradation (Columbia University Protein Core Facility) to confirm the nature of the PanB N-terminus. We

were unable to detect PanB in the WT strain as we have previously reported (*4*), suggesting that the protein has a short half-life. **(B)** Ponceau S staining of the nitrocellulose membrane analyzed by anti-Pup immunoblotting in Fig. 3A. All samples were separated by 12% SDS-PAGE.

fig. S7. Pupylation leads to protein degradation. **(A)** Shorter exposure of film shown in Fig. 4. **(B)** Purified FLAG-FabD-K173A-His_6 was inefficiently pupylated. Epitope-tagged *Mtb* FabD-K173A was purified from *Msm* using NiNTA. Immunoblotting detected endogenous Msm Pup modification of the *Mtb* protein. **(C)** Total soluble cell lysates isolated from equivalent cell volumes analyzed over time. Left: WT *Msm*, right: *mpa* mutant. A significant global change in protein stability over 9 h in either strain was not observed, in contrast to the pupylated proteins, which decreased in the WT strain over the same period (Fig. 4C). **(D)** Quantification of radiolabeled proteins in (C) and Fig. 4B (*13*). The signal from t = 1 was set to 100% because there appeared to be an increase in pupylated proteins between t = 0 and t = 1 in both strains. Data presented are representative of at least two independent experiments.

fig. S8. Proposed model of substrate degradation by the *Mtb* proteasome. The C-terminus of Pup may be de-amidated before its conjugation to substrate lysines. PafA may play a role in the conjugation of Pup. Mpa may then recognize the pupylated substrate through non-covalent interactions with Pup. The substrate could then be unfolded and threaded into the proteasome core where it is degraded. It is unknown if Pup is also degraded, or if it is removed and recycled.

Table S1. Bacterial strains, plasmids and primers used in this work**.**

	Relevant genotype/sequence:	**Source or reference:**
Plasmids:		
pET24b(+)	Kan^r; for production of C-terminal His_6 epitope-tagged protein	Novagen
pET24b(+)-His_6-*pup*	Kan^r; for overexpression of His_6-*pup*	This work
pET24b(+)-*sigE*-His_6	Kan^r; for overexpression of *Salmonella enterica sigE*-His_6	(*14*)
pET24b(+)-*mpa*2	Kan^r; for overexpression of *mpa*-His_6	This work
pQE30	Amp^r; for production of N-terminal His_6 epitope-tagged protein	Qiagen
pQE30-*pup*	Amp^r; for overexpression of His_6-*pup*	This work
pWSK29	Amp^r; SC101 *E. coli ori*	(*15*)
pWKS30	Amp^r; SC101 *E. coli ori*	(*15*)
pWKS130	Kan^r; SC101 *E. coli ori*	(*15*)
pWSK29-RBS-*mpa*	Amp^r; for overexpression of *mpa*	This work
pWKS30-His_6-*pup*	Amp^r; for overexpression of His_6-*pup*	This work
pWKS130-sm-*mpa*	Kan^r; contains a 537 bp fragment of *Msm mpa* (nt 817-1353)	This work
pMV306.kan	Kan^r; mycobacterial plasmid that integrates at *attB* site on the *Mtb* chromosome	(*16*)

pMV306.kan-His$_6$-*pup*	Kan^r; pMV306.kan with pUV15 promoter and His$_6$-*pup*	This work
pUV15	Hyg^r; shuttle plasmid with *gfp* under the control of the *Msm rpsA* promoter	(*5*)
pUV15-His$_6$-*pup*	Hyg^r; pUV15 with GFP replaced by His$_6$-*pup*	This work
pMN402	Hyg^r; shuttle plasmid with *gfp* under the control of the BCG *hsp60* promoter	(*17*)
pMN-FLAG-*fabD*-His$_6$	Hyg^r; pMN402 with GFP replaced by FLAG-*fabD*-His$_6$ (produces both FabD-His$_6$ and FLAG-FabD-His$_6$)	(*4*)
pMN-FLAG-*panB*-His$_6$	Hyg^r; pMN402 with GFP replaced by FLAG-*panB*-His$_6$ (produces both PanB-His$_6$ and FLAG-PanB-His$_6$)	(*4*)
pMN-FLAG-*dlaT*-His$_6$	Hyg^r; pMN402 with *gfp* replaced by FLAG and His$_6$-tagged *dlaT*	(*4*)
pMNL	Hyg^r; pMN402 with *gfp* replaced by consensus RBS from pET24b(+) and a multiple cloning site.	This work
pMNL-*tbfabD*-UT	Hyg^r; pMNL with untagged *fabD*	This work
pMNL-FLAG-tb*fabD*-His$_6$1S	Hyg^r; pMNL with FLAG and His$_6$-tagged *fabD* (results in the synthesis of only FLAG-FabD-His$_6$)	This work
pMNL-FLAG-tb*fabD*	Hyg^r; pMNL with FLAG-tagged *fabD*	This work

pMNL-FLAG-tb*fabD*K173A	Hyg^r; pMNL with FLAG-tagged *fabD* K173A mutation	This work
pMN-FLAG-tb*fabD*K173A-His_6	Hyg^r; pMN with FLAG and His_6-tagged *fabD* K173A mutation	This work
pKT25	Kan^r; multiple cloning site downstream of T25 domain of adenylate cyclase gene from *B. pertussis* for BTH	(*9*)
pUT18C	Amp^r; multiple cloning site downstream of T18 domain of adenylate cyclase gene from *B. pertussis* for BTH	(*9*)
pUT18C-*mpa*	Amp^r; *mpa* cloned into pUT18C with XbaI and KpnI, translationally fused to the C-terminus of the T18 domain	This work
pKT25-*mpa*	Kan^r; *mpa* cloned into pKT25 with XbaI and KpnI, translationally fused to the C-terminus of the T25 domain	This work
pKT25-'*pup*	Kan^r; *Mtb* genomic library clone that contained base pairs 115-195 of Rv2111c translationally fused to the C-terminus of the T25 domain	This work
pKT25-*pup*	Kan^r; *pup* cloned into pKT25 with XbaI and KpnI, translationally fused to the C-terminus of the T25 domain	This work
pUT18C-*pup*	Amp^r; *pup* cloned into pUT18C with XbaI and KpnI, translationally fused to the C-terminus of the T18 domain	This work

pUAB100	Hyg^r; *Saccharomyces cerevisiae* GCN4 cloned upstream of F(1,2) domain of murine DHFR for M-PFC	(*10*)
pUAB200	Kan^r; *Saccharomyces cerevisiae* GCN4 cloned upstream of F(3) domain of murine DHFR for M-PFC	(*10*)
pUAB300	Hyg^r; F(1,2) domain of murine DHFR with downstream multiple cloning site for M-PFC	(*10*)
pUAB400	Kan^r; F(3) sdomain of murine DHFR with downstream multiple cloning site for M-PFC	(*10*)
pUAB200-*pup*	Kan^r; *pup* cloned into pUAB200 with MunI and ClaI translationally fused to the N-terminus of the F(3) domain	This work
pUAB200-*fabD*	Kan^r; *fabD* cloned into pUAB200 with MunI and ClaI translationally fused to the N-terminus of the F(3) domain	This work
pUAB300- *pup*	Hyg^r; *pup* cloned into pUAB400 with HindIII and PstI translationally fused to the C-terminus of the F(1,2)	This work
pUAB300- *fabD*	Hyg^r; *fabD* cloned into pUAB400 with HindIII and PstI translationally fused to the C-terminus of the F(1,2)	This work

pUAB400-*pup*	Kan^r; *pup* cloned into pUAB400 with MunI and ClaI translationally fused to the C-terminus of the F(3)	This work
pUAB400-*fabD*	Kan^r; *fabD* cloned into pUAB400 with MunI and ClaI translationally fused to the C-terminus of the F(3)	This work
E. coli:		
DH5α	F-, p80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (r_k-m_k+) *phoA supE44* λ- *thi-1 gyrA96 relA1*	Gibco, BRL
BTH101	F-, *cya-99*, *araD139*, *galE15*, *galK16*, *rpsL1* (Strr), *hsdR2*, *mcrA1*, *mcrB1*	(*18*)
ER2566	F- λ- *fhuA2* [lon] *ompT lacZ::T7 geneI gal sulA11* Δ(*mcrC-mrr*)114::IS10 R(*mcr-73*::miniTn10)2 R(*zgb-210*::Tn10)1 (Tets) *endA1* [dcm]	(*16*)
Mtb:		
H37Rv	wild type American Type Culture Collection 25618	ATCC
MHD2	Kan^r; *pafA*::ΦMycoMarT7 (Kan^r)	(*19*)
MHD5	Kan^r; *mpa*::ΦMycoMarT7 (Kan^r)	(*19*)
MHD75	Kan^r; *pafB*::ΦMycoMarT7 (Kan^r)	(*20*)

MHD77	Kan^r; *pafC*::ΦMycoMarT7 (Kan^r)	(*20*)
MHD84	Hyg^r; H37Rv, pMN-FLAG-*dlaT*-His_6	(*4*)
MHD216	Hyg^r; H37Rv, pMNL-FLAG-*fabD*-$His_6$1S	This work
MHD217	Kan^r, Hyg^r; MHD5 with pMNL-FLAG-*fabD*-$His_6$1S	This work
MHD218	Kan^r,Hyg^r; *pafA*::ΦMycoMarT7, pMNL-FLAG-*fabD*-$His_6$1S	This work
Msm:		
mc^2155	wild type	(*21*)
MsHD3	Kan^r; mc^2155*mpa*::pWKS130	This work
MsHD98	Kan^r,Hyg^r; mc^2155, pMNL-*tbfabD*-UT + pMV306.kan-His_6-*pup*	This work
MsHD99	Kan^r,Hyg^r; mc^2155, pMNL-FLAG-tb*fabD* + pMV306.kan	This work
MsHD100	Kan^r,Hyg^r; mc^2155, pMNL-FLAG-tb*fabD* + pMV306.kan-His_6-*pup*	This work
MsHD101	Hyg^r; mc^2155, pUV15-His_6-*pup*	This work
MsHD102	Kan^r,Hyg^r; MsHD3 pUV15-His_6-*pup*	This work
MsHD103	Hyg^r; mc^2155, pMN-FLAG-tb*fabD*K173A-His_6	This work

Primers **(all from this work except where noted):**

2111c_T25_F	gctctagacatggcgcaagagcagaccaa	
2111c_T25_R	ggggtacctcactgtccgccctttgga	
Rv2115c-NdeI-f	ggaattccatatgggtgagtcagagcgttctgag	(*6*)
Rv2115c-NotI-r	ctcgagtgcggccgcgccctacaggtactggccg	
Rv2115c-XbaI-f1	gctctagagatgggtgagtcagagcgttctg	
Rv2115c-KpnI-r1	ggggtacctcgctggtgaccgcctgagc	
smmpaf1	gagctgccgttcctgcacaaggacc	
smmpar1	gtcctgcgcggcctcggcatc	
pup_pQE30_S_F	ccgagctcatggcgcaagagcagaccaa	
pup_pQE30_K_R	ggggtacctcactgtccgccctttgga	
pQE_His_Nde_F	gggaattccatatgagaggatcgcatcaccatca	
pQE_His_R	atcaacaggagtccaagctc	
FabD_NT_NdeI_F	ggaattccatatgattgcgttgctcgcaccc	
FabD_CT_PstI_R	aaaactgcagttataggtttgccagctcgtccaggtc	
FabD_NT33_Pst_R	aaaactgcagttataggtttgccagctcgtccaggtc	

FabD_TB_NdeI_Flag_F	ggaattccatatggattacaaggatgacgacgataagatcattgcgttgctcgcaccc
HIII_Pst_His_Fab_R	cccaagcttactgcagtcagtggtggtggtggtggtgtaggtttgccagctcgtccaggtc
Fab_K173_F	gccggctgaccgcgttggagGCGctcgccgaagacccgccgg
Fab_K173_R	ccggcgggtcttcggcgagCGCctccaacgcggtcagccggc
Mfe_pup_p200_F	ccccaattgttatggcgcaagagcagaccaa
Cla_pup_p200_R	ccatcgatctgtccgccctttgga
Mfe_FabD_p200_F	ccccaattgttatgattgcgttgctcgcaccc
Cla_FabD_p200_R	ccatcgattaggtttgccagctcgtccaggtc
PstI_pup_p300_F	aaaactgcagagcgcaagagcagaccaa
HIII_pup_p300_R	cccaagctttcactgtccgccctttgga
PstI_fabD_p300_F	aaaactgcagaattgcgttgctcgcaccc
HIII_fabD_p300_R	cccaagcttttataggtttgccagctcgtccaggtc
Mfe_pup_p400_F	ccccaattggcgcaagagcagaccaa
Cla_pup_p400_R	ccatcgattcactgtccgccctttgga
Mfe_FabD_p400_F	ccccaattgattgcgttgctcgcaccc
Cla_FabD_p400_R	ccatccatttataggtttgccagctcgtccaggtc

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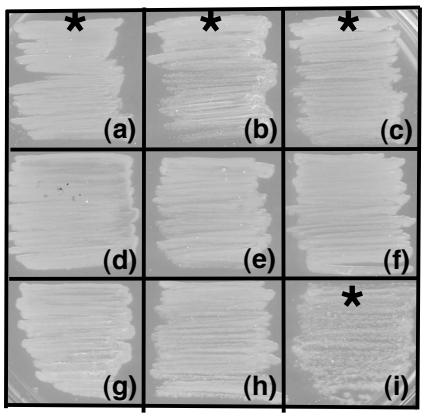
fig. S1

A.

***E. coli* 2-hybrid test pairs**
plasmid 1 + plasmid 2

(a)	**T25-'Pup + T18C-Mpa**
(b)	**T25-Pup + T18C-Mpa**
(c)	**T25-Mpa + T18C-Pup**
(d)	T25-Pup + T18C
(e)	T25 + T18C-Pup
(f)	T25 + T18C
(g)	T25 + T18C-Mpa
(h)	T25-Mpa + T18C
(i)	**T25-Mpa + T18C-Mpa**

minimal + glucose

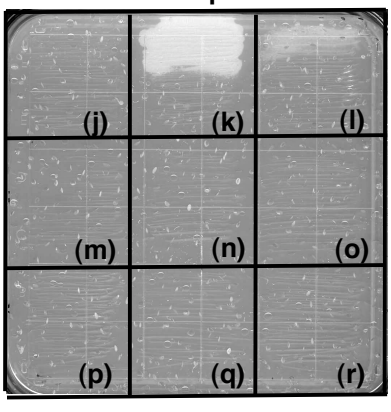


B.

Mycobacterial 2-hybrid test pairs
plasmid 1 + plasmid 2

(a)	**GCN4-F(1,2) + GCN4-F(3)**
(b)	F(1,2) + F(3)
(c)	F(1,2) + Pup-F(3)
(d)	**F(1,2)-FabD + Pup-F(3)**
(e)	GCN4-F(1,2) + Pup-F(3)
(f)	F(1,2)-Pup + GCN4-F(3)
(g)	**F(1,2)-Pup + FabD-F(3)**
(h)	F(1,2)-Pup + F(3)
(i)	**F(1,2)-Pup + F(3)-FabD**
(j)	F(1,2) + F(3)-Pup
(k)	**F(1,2)-FabD + F(3)-Pup**
(l)	GCN4-F(1,2) + F(3)-Pup
(m)	GCN4-F(1,2) + FabD-F(3)
(n)	F(1,2) + FabD-F(3)
(o)	F(1,2)-FabD + GCN4-F(3)
(p)	F(1,2)-FabD + F(3)
(q)	GCN4-F(1,2) + F(3)-FabD
(r)	F(1,2) + F(3)-FabD

+Trimethoprim



- Trimethoprim

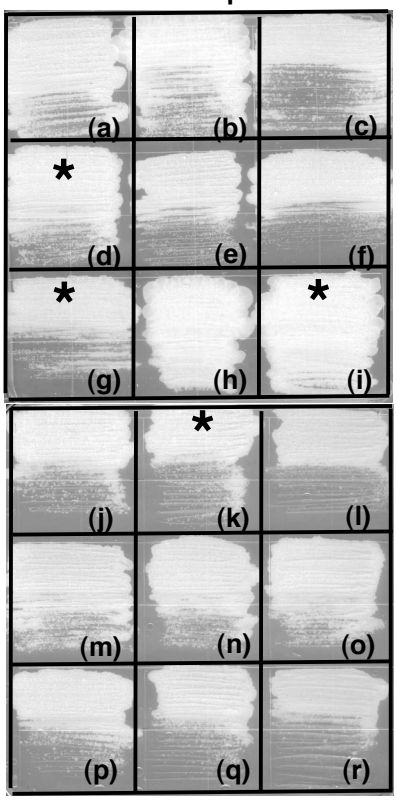


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fig. S2

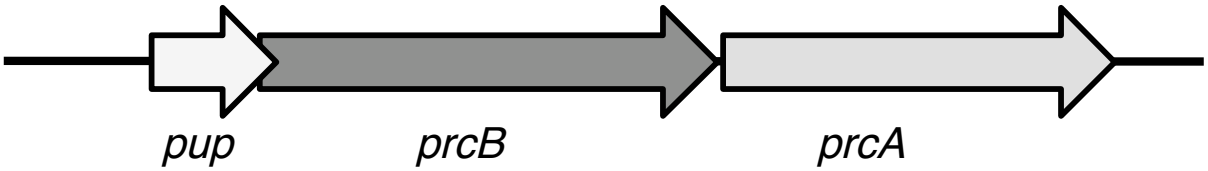

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fig. S3

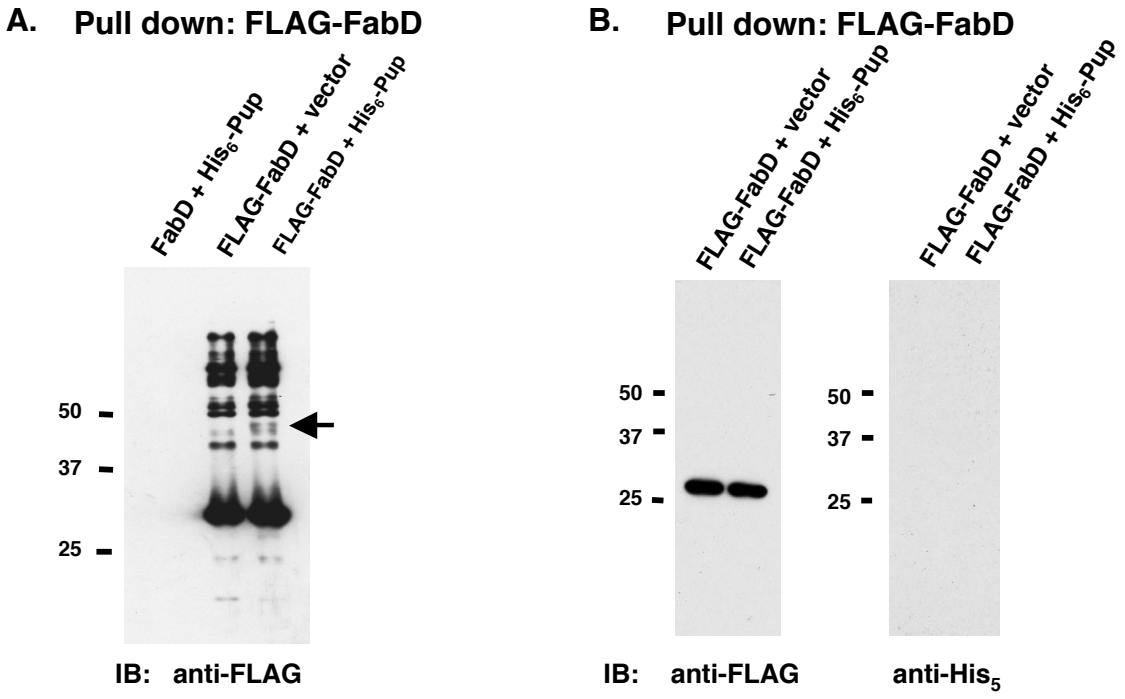

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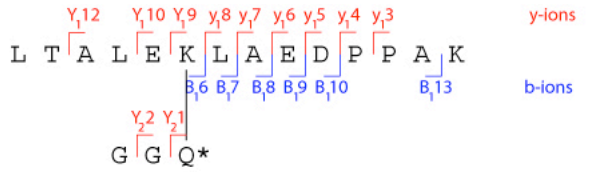
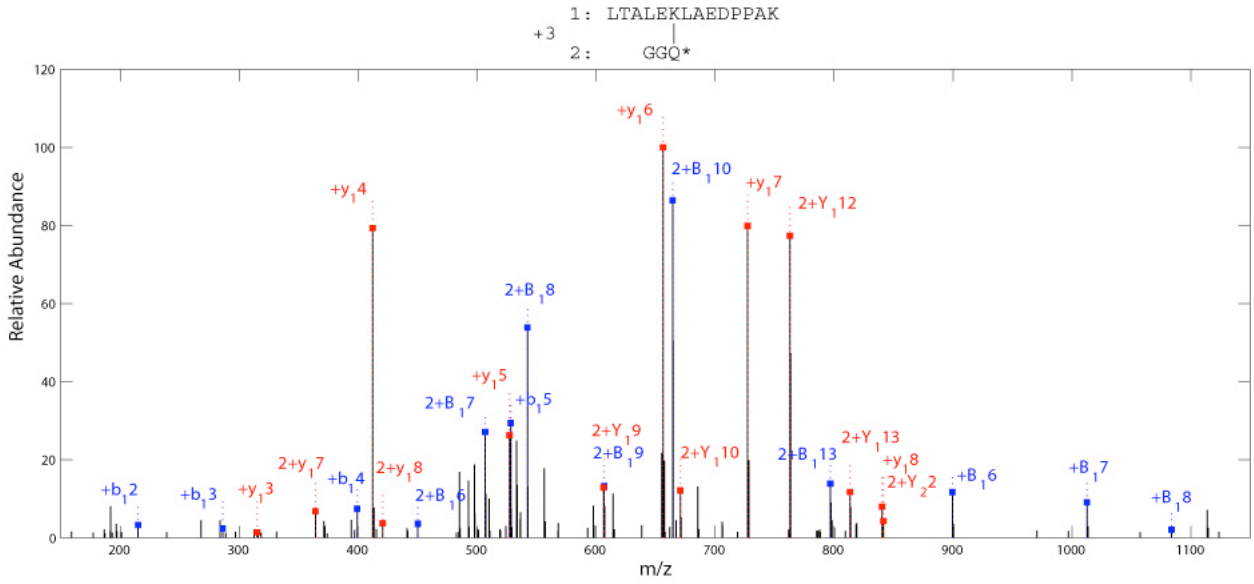
fig. S4

A



	+1b		Δ mass ppm	Δ mass ppm	+1y			+2b		Δ mass ppm	Δ mass ppm	+2y	
L	+$b_1$1	114.0913			---	+$Y_1$14	L	2+$b_1$1	57.5493			---	2+$Y_1$14
T	+$b_1$2	215.1390			1625.8381	+$Y_1$13	T	2+$b_1$2	108.0731			813.4227	2+$Y_1$13
A	+$b_1$3	286.1761			1524.7904	+$Y_1$12	A	2+$b_1$3	143.5917		0.54	**762.8988**	2+$Y_1$12
L	+$b_1$4	399.2602			1453.7533	+$Y_1$11	L	2+$b_1$4	200.1337			727.3803	2+$Y_1$11
E	+$b_1$5	528.3028		2.50	**1340.6692**	+$Y_1$10	E	2+$b_1$5	264.6550			670.8383	2+$Y_1$10
K--	+$B_1$6	**899.4833**	1.56	2.27	**1211.6266**	+$Y_1$9	K--	2+$B_1$6	450.2453			606.3170	2+$Y_1$9
L	+$B_1$7	**1012.5673**	0.52	0.15	**840.4462**	+$Y_1$8	L	2+$B_1$7	506.7873			420.7267	2+$Y_1$8
A	+$B_1$8	**1083.6045**	0.71	2.65	**727.3621**	+$Y_1$7	A	2+$B_1$8	542.3059			364.1847	2+$Y_1$7
E	+$B_1$9	**1212.6470**	1.98	0.62	**656.3250**	+$Y_1$6	E	2+$B_1$9	606.8272			328.6661	2+$Y_1$6
D	+$B_1$10	**1327.6740**	0.12	0.89	**527.2824**	+$y_1$5	D	2+$B_1$10	664.3406			264.1448	2+$y_1$5
P	+$B_1$11	1424.7268		0.70	**412.2554**	+$y_1$4	P	2+$B_1$11	712.8670			206.6314	2+$y_1$4
P	+$B_1$12	1521.7795		1.00	**315.2027**	+$y_1$3	P	2+$B_1$12	761.3934			158.1050	2+$y_1$3
A	+$B_1$13	**1592.8166**	0.30		218.1499	+$y_1$2	A	2+$B_1$13	**796.9120**	3.11		109.5786	2+$y_1$2
K	+$B_1$14	---			147.1128	+$y_1$1	K	2+$B_1$14	---			74.0600	2+$y_1$1
G	+$b_2$1	58.0287			---	+$y_2$3	G	2+$b_2$1	29.5180			---	2+$y_2$3
G	+$b_2$2	115.0502			1681.9007	+$Y_2$2	G	2+$b_2$2	58.0287		5.52	**841.4540**	2+$Y_2$2
Q*	+$b_2$3	---			1624.8792	+$Y_2$1	Q*	2+$b_2$3	---		0.58	**812.9433**	2+$Y_2$1

B



C

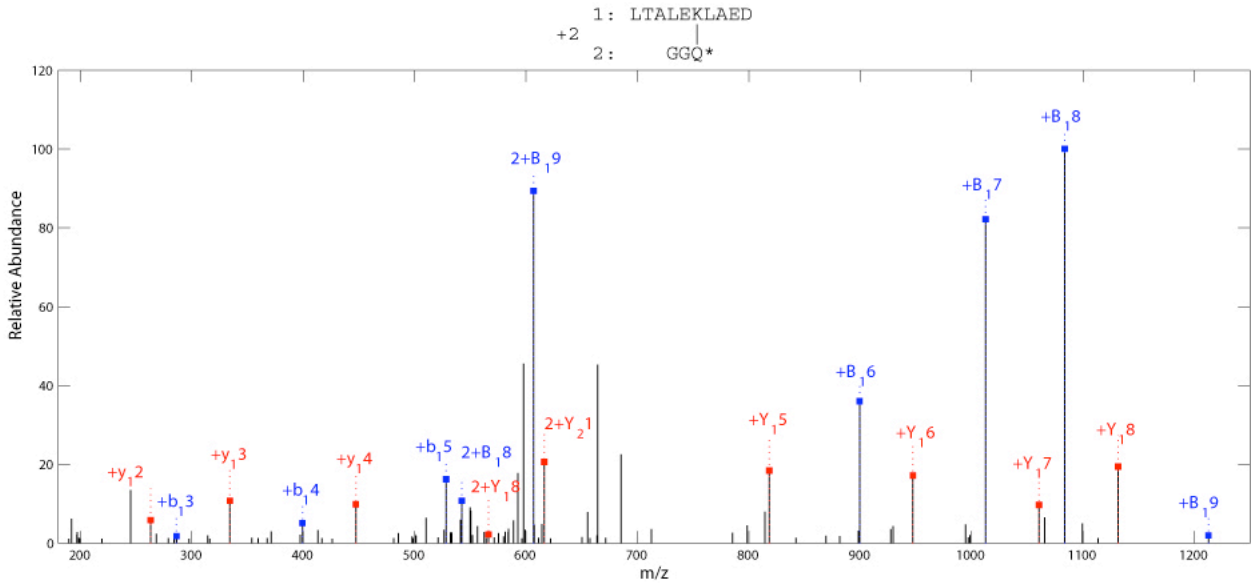


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fig. S5

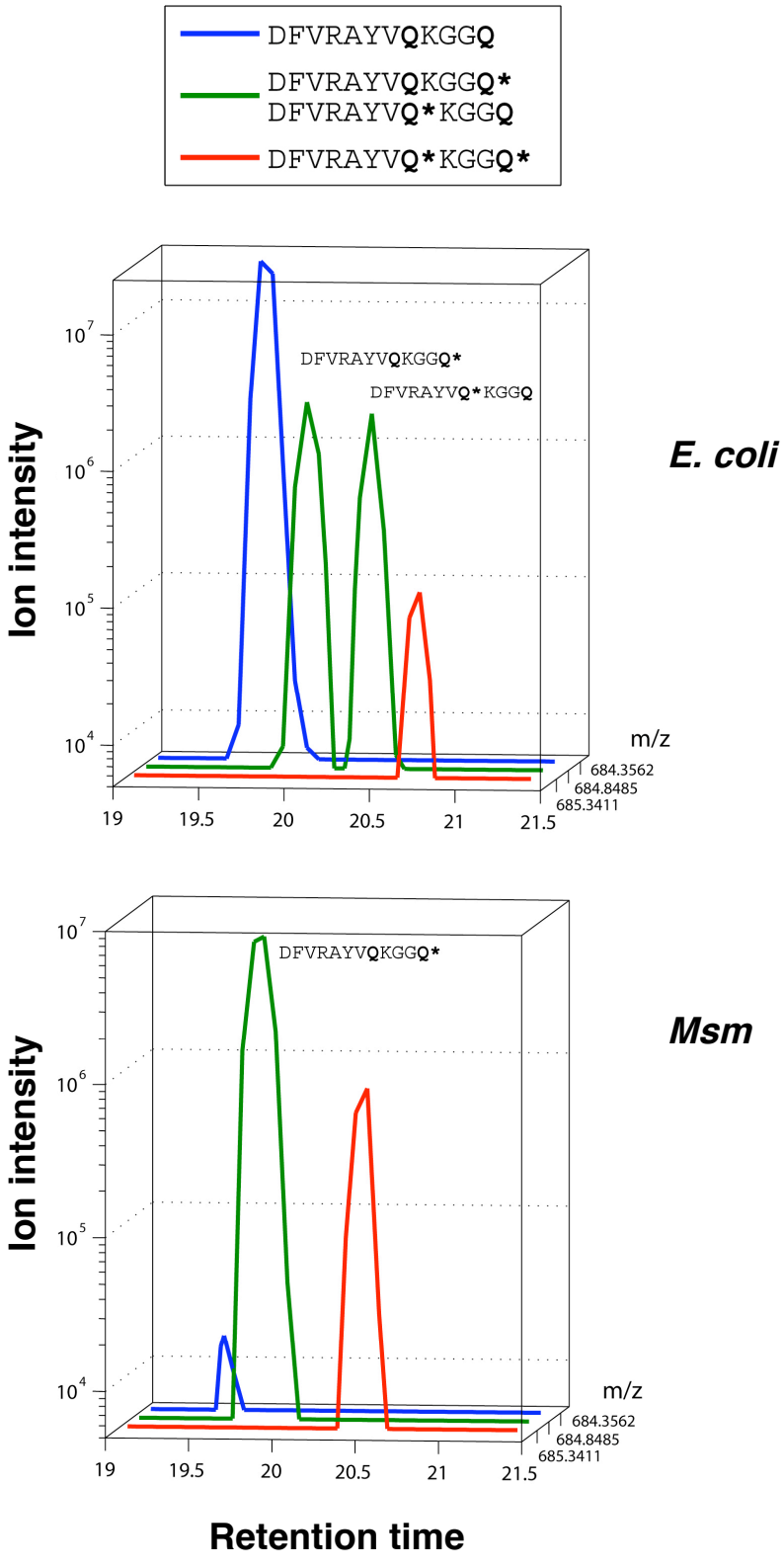

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fig. S6

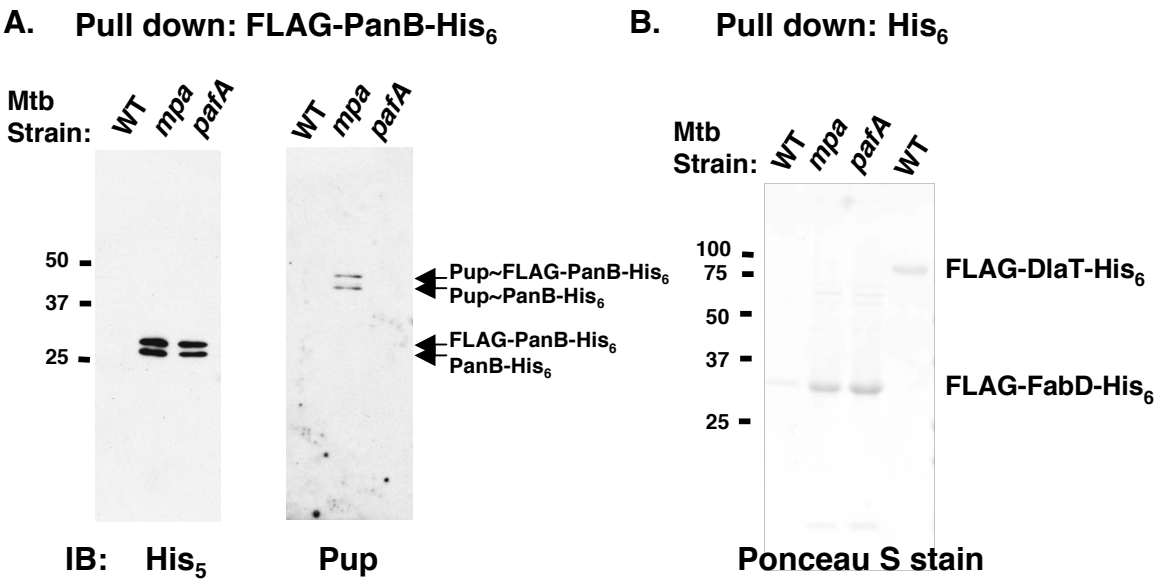

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fig. S7

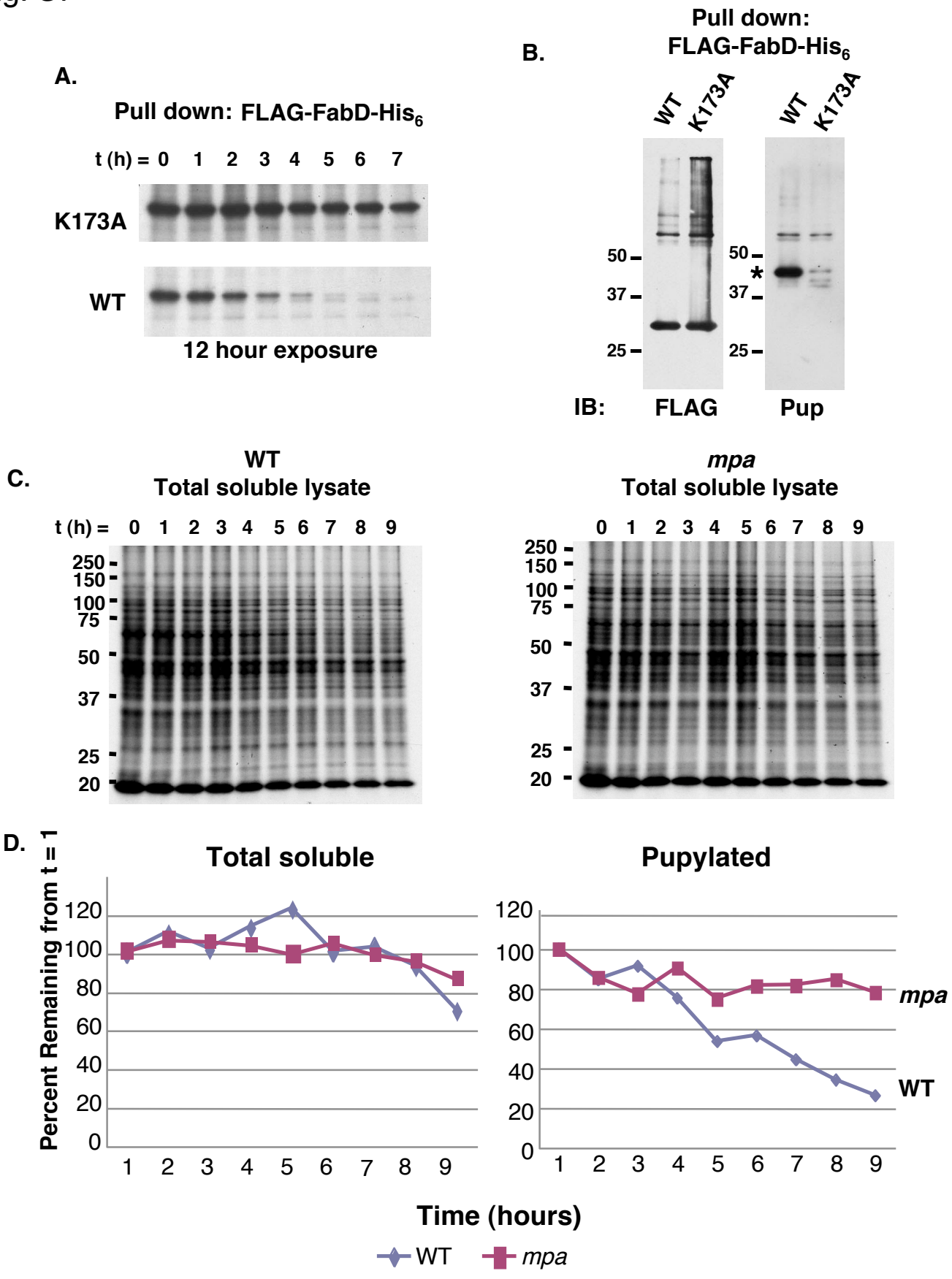

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fig. S8