Nonredundant functions of *Mycobacterium tuberculosis* chaperones promote survival under stress

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Supplementary Information

Table S1. Genes and intergenic regions with highest number of TA insertions for each transposon library.

RANK	wildtype 1	Δ*clpB* 1	wildtype 2	Δ*clpB* 2
1*	'IG_Rv0905' (5274)	'IG_Rv0905' (10123)	'IG_Rv0905' (4629)	'IG_Rv0905' (19444)
2	'Rv1151c' (2600)	'IG_Rv1072' (5115)	'Rv1151c' (2332)	'IG_Rv1072' (11475)
3	'IG_Rv1072' (2385)	'Rv0270' (3810)	'IG_Rv1072' (2234)	'Rv1151c' (10907)
4	'Rv0819' (2128)	'Rv1211' (3754)	'Rv0819' (2077)	'Rv0819' (9383)
5	'IG_Rv0002' (2082)	'Rv1549' (3479)	'IG_Rv0820' (2059)	'Rv1211' (8170)

Number of insertions is shown in parentheses.
***** For each library, the gene with the highest number of TA insertions (seen as the highest peak in **Figure S1**) is the intergenic (IG) region of Rv0905, a possible enoyl-CoA hydratase EchA6 that is predicted to be nonessential. This "hotspot" might be a preferred insertion point due to accessibility because of chromosome structure, although further experimentation would be needed to understand this possibility.

Table S2. Percent TA dinucleotides disrupted for each library

library	%TAs disrupted
wildtype 1	79.2
Δ*clpB* 1	60.3
wildtype 2	74.0
Δ*clpB* 2	51.4

Table S3. Pathway analysis of genes significantly underrepresented in Mtb Δ*clpB* libraries relative to wild-type (p < 0.001) that fall into indicated pathways.

pathway/Rv number	gene	GO Term Annotations
Mycobactin synthesis		
Rv2384	*mbtA*	GOA:P71716
RV2383c	*mbtB*	GOA:P9WQ63
Rv2382c	*mbtC*	GOA:P71718
Rv2381c	*mbtD*	GOA:P71719
Rv2380c	*mbtE*	GOA:I6Y0L1
Rv2379c	*mbtF*	GOA:O05819
Sulfur metabolism		
Rv2397c	*cysA1*	GOA:P9WQM1
Rv1286	*cysN*	GOA:P9WNM5
Rv1285	*cysD*	GOA:P9WIK1
Rv2847c	*cysG*	GOA:I6X5I7
Rv2398c	*cysW*	GOA:P71746
Rv2391	*sirA*	GOA:P9WJ03
Rv2400c	*subI*	GOA:P71744
Lipid metabolism		
Rv1185c	*fadD21*	GOA:P9WQ49
Rv1529	*fadD24*	GOA:O53903
Rv0404	*fadD30*	GOA:P9WQ57
Rv2505c	*fadD35*	GOA:I6Y0X0
Rv0551c	*fadD8*	GOA:O06417
Rv0904c	*accD3*	GOA:P9WQH9
Rv1183	*mmpL10*	GOA:P9WJU1
Rv0507	*mmpL2*	GOA:P9WJV7
Rv3823c	*mmpL8*	GOA:P9WJU5
Rv2339	*mmpL9*	GOA:P9WJU3
Rv1683	NA	GOA:O33185
Rv0101	*nrp*	GOA:Q10896
Rv2048c	*pks12*	GOA:I6XD69
Rv1181	*pks4*	GOA:L0T647
Rv1527c	*pks5*	GOA:O53901
Rv0405	*pks6*	GOA:O86335
Rv1661	*pks7*	GOA:P94996
Rv1662	*pks8*	GOA:O65933
Rv1664	*pks9*	GOA:O06586
Rv2931	*ppsA*	GOA:P9WQE7
Rv2932	*ppsB*	GOA:P9WQE5
Rv2933	*ppsC*	GOA:P96202
Rv2934	*ppsD*	GOA:P9WQE3
Rv2935	*ppsE*	GOA:P9WQE1
Rv1182	*papA3*	GOA:P9WIK5
Rv2518c	*ldtB*	GOA:I6Y9J2
Starch and sucrose metabolism		
Rv1328	*glgP*	GOA:P9WMW1
Rv3490	*otsA*	GOA:P9WN11
Rv2006	*otsB1*	GOA:P9WN15
Rv0946c	*pgi*	GOA:P9WN69
Nitrogen metabolism		
Rv2476c	*gdh*	GOA:O53203
Rv1161	*narG*	GOA:P9WJQ3
Rv1736c	*narX*	GOA:P9WJQ1
Carbon metabolism		
Rv0973c	*accA2*	GOA:P71538
Rv1552	*frdA*	GOA:P9WN91
Rv2967c	*pca*	GOA:I6YEU0
Rv1617	*pykA*	GOA:P9WKE5
Proteostasis		
Rv2299c	*htpG*	GOA:P9WMJ7
Rv2112c	*dop*	GOA:P9WNU9
Predicted protein synthesis		
Rv1300	*hemK*	GOA:P9WHV3
Rv0120c	*fusA2*	GOA:P9WNM9
Rv2404c	*lepA*	GOA:P9WK97

Additional genes that have p<0.001 are not shown because they do not group into known pathways. Pathways were assigned by KEGG analysis and/or through manual annotation based on that of Mycobrowser (https://mycobrowser.epfl.ch/). GO Terms can be found at: https://www.ebi.ac.uk/QuickGO/

Table S4. Fraction of TA sites hit in Rv2299c (*htpG*) (out of 30 TA sites total) and reads/TA site in indicated transposon libraries generated in this study.

library	# of hit TAs	fraction TAs	total reads	avg. read/TA
Wild-type 1	26	0.867	1397	46.6
Wild-type 2	27	0.900	1389	46.3
Δc*lpB* 1	21	0.700	668	22.3
Δc*lpB* 2	20	0.667	398	13.3

Table S5. Top five "hits" from proteomic analysis of excised band from His-HtpG pull-down in mycobacterial lysate.

Accession	Protein	M. *tuberculosis* peptides*	M. *tuberculosis* % coverage#	*E. coli* peptides	*E. coli* % coverage	MW (kD)
P0A5B9	DnaK	89	82.24	-	-	66.8
P0A5B9	ArnA	-	-	41	62.58	74.2
P63531	GlgE	35	62.34	-	-	78.6
O53475	Uncharacterized protein Rv2030c	36	61.09	-	-	74.9
P64411	HtpG	40	58.42	-	-	72.9

From Colloidal blue-stained replicate gel to that shown in **Figure S12A**; sorted by percent coverage values.
*peptides = number of peptides identified from indicated protein
#percent coverage = total percent sequence coverage
MW = molecular weight

Table S6. Minimum inhibitory concentrations (MICs) for 90% growth inhibition of geldanamycin against Mtb strains with and without indicated chaperones under standard growth (replicating) conditions.

Strains	Rifampicin	Geldanamycin
Wild-type	0.125	>100
Δc*lpB*	0.125	>100
Δc*lpB* comp	0.125	>100
Δ*htpG*	0.125	>100
Δ*htpG* comp	0.125	>100

Table S7. Plasmids used in this study.

NO.	PLASMID NAME	DESCRIPTION	SOURCE
1	pETHisSUMO	pET His6 Sumo TEV LIC cloning vector (ampR)	Addgene #29711
2	pHYRS52	His6-*S. cerevisiae* ulp1 (res. 403-621) (ampR)	Addgene #31122; (Muona, Aranko, & Iwai, 2008)
3	pET-47b(+)	N-terminal His6 (kanR)	Novagen
4	pET-27b(+)	C-terminal His6 (kanR)	Novagen
4	EcTL08	pET-His-SUMO-htpG (Mtb)	(Lopez Quezada et al., 2020)
5	EcTL21	pET47b-His-htpG (Mtb)	This work
6	EcTL30	pET27b-htpG-His(Mtb)	This work
7	pEVOLV-pBpF	tRNA synthetase/tRNA pair for the in vivo incorporation of p-benzoyl-L-phenylalanine into proteins in *E. coli* in response to the amber codon, TAG (camR)	Addgene # 31190; (Chin, Martin, King, Wang, & Schultz, 2002)
8	pAJF550	pET-SUMO-cys-grpE-cys	This work
9	pAJF551	pET-SUMO-cys-dnaK-cys	This work
10	pDE43-XSTS	temperature-sensitive plasmid for deletion in Mtb	Gateway*
11	pEN21A KO Mtb clpB	Gateway entry vector encoding part of Mtb *clpB*	(Vaubourgeix et al., 2015)
12	pEN41A KO Mtb clpB	Gateway entry vector encoding part of Mtb *clpB*	(Vaubourgeix et al., 2015)
13	pEN12A-kanR-lox2	Gateway entry made with a BP reaction using vector 14 and a kanR region amplified using primers 49 and 50 and plasmid 3 as a template	This work
14	pDO221A	Gateway entry vector	Gateway*
15	pDO23A	Gateway entry vector	Gateway*
16	pKO-XSTS-clpB-tb	Mtb *clpB* deletion vector (kanR) made through an LR reaction with plasmids 11, 12, PvuII-digested 13	This work
17	pMCH_pre500_SD_htpG	Plasmid that will introduce an extra copy of *htpG* into the Mtb chromosome at L5	This work
18	pTCS-mcs1	Plasmid to introduce a streptomycin resistance cassette into the Mtb chromosome at L5	(Venugopal et al., 2011)*
19	pLJR965	CRISPRi backbone for Mtb	(Rock et al., 2017)
20	pTL_965_htpG_crispr_1	Mtb *htpG* depletion vector (used primers 5,6) encoding sgRNA1	This work
21	pTL_965_htpG_crispr_2	Mtb *htpG* depletion vector (used primers 7,8) encoding sgRNA2	This work
22	pET27b_HtpG F10TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
23	pET27b_HtpG W197TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
24	pET27b_HtpG W274TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
25	pET27b_HtpG F303TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
26	pET27b_HtpG F559TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
27	pET27b_HtpG Y618TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
28	pET27b_HtpG Y423TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
29	pET27b_HtpG F395TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
30	pET27b_HtpG F337TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
31	pET27b_HtpG R53TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work

NO.	PLASMID NAME	DESCRIPTION	SOURCE
32	pET27b_HtpG T236TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
33	pET27b_HtpG E257TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
34	pET27b_HtpG R384TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
35	pET27b_HtpG D510TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
36	pET27b_HtpG H269TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
37	pET27b_HtpG W529TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
38	pET27b_HtpG F635TAG	Plasmid 6 with indicated residue's encoding codon mutated to TAG	This work
39	pACYC-T7 yHsp90	N-terminal His6 *S. cerevisiae* Hsp82	(Wayne & Bolon, 2007)

ampR = ampicillin resistant; kanR = kanamycin resistant; camR = chloramphenicol resistant
*We would like to acknowledge the Schnappinger lab (Weill Cornell Medicine) for the generous gift of these plasmids
Note: See "Experimental Procedures" for description of cloning of vectors that are not fully described in this table.

Table S8: Strains used in this study.

NO.	STRAIN NAME	DESCRIPTION	SOURCE
1	H37RvN	*M. tuberculosis* wild-type strain	Nathan lab stock
2	Rosetta2	*E. coli* BL21 derivative for rare codon usage	Novagen
3	Rosetta2(DE3) pLysS	Rosetta2 expressing T7 lysozyme	Novagen
4	His6-SUMO-HtpG	Rosetta2 transformed with EcTL08	(Lopez Quezada et al., 2020)
5	His6-HtpG	BL21 transformed with EcTL21	This work
6	Mtb Δ*clpB*	Mtb with *clpB* deleted	(Vaubourgeix et al., 2015)
7	Mtb Δ*clpB* complement	Strain 7 carrying an extra copy of *clpB* following the native promoter	(Vaubourgeix et al., 2015)
8	Mtb Δ*htpG*	Mtb with *htpG* deleted	(Lopez Quezada et al., 2020)
9	Mtb Δ*htpG* complement	Strain 8 carrying an extra copy of *htpG* following the *hsp60* promoter	(Lopez Quezada et al., 2020)
10	Mtb Δ*clpB* kanR	Mtb with *clpB* deleted and replaced with a kanamycin resistant cassette	This work
11	Mtb Δ*clpB* pre500htpG	Strain 10 transformed with plasmid 17; carries *hspR* SNP (A19V)	This work
12	Mtb Δ*clpB* kanR *hspR* SNP	Strain 11 transformed with plasmid 18; carries *hspR* A19V without an additional copy of *htpG*	This work
13	HtpG-His6	BL21 transformed with EcTL30	This work
14-30	HtpG_His6 BpF mutants	BL21 transformed with pEVOLV-BpF and with a single plasmid 22-38	This work
31	EcTL02	Rosetta2 expressing His6-SUMO-Mtb ClpB	(Lupoli, Fay, Adura, Glickman, & Nathan, 2016)
32	EcTL03	Rosetta2 expressing His6-SUMO-Mtb DnaJ1	
33	EcTL04	Rosetta2 expressing His6-SUMO-Mtb DnaJ2	(Lupoli et al., 2016)
34	EcTL05	Rosetta2 expressing His6-SUMO-Mtb GrpE	(Lupoli et al., 2016)
35	EcTL06	Rosetta2 expressing His6-SUMO-Mtb DnaK	Nathan lab stock
36	mc^2155	*M. smegmatis* wild-type strain	(Vaubourgeix et al., 2015)
37	*M. smegmatis* Δ*clpB*	*M. smegmatis* with *clpB* deleted	(Vaubourgeix et al., 2015)
38	*M. smegmatis* Δ*clpB* complement	Strain 36 carrying an extra copy of *clpB* following the native promoter	
39	*M. smegmatis* Δ*mbtE*	*M. smegmatis* with *mbtE* deleted	(LaMarca, Zhu, Arceneaux, Byers, & Lundrigan, 2004)
40	Mtb Δ*mbtK*	Mtb with *mbtK* deleted	(Xu et al., 2017)

Table S9. Primers used in this study.

NO	NAME	SEQUENCE
1	attB3-htpGrev	GGGGACAACTTTGTATAATAAAGTTGCTACAAGGTACGCGCGAGACGTTC
2	attB2r_500bp_pre_htpG	GGGGACAGCTTTCTTGTACAAAGTGGCTCACTTCGACTTCGACCACATC
3	Mtb_hspR_outside_fow	TCAGGAAGCTCTGGAAGCC
4	Mtb_hspR_outside_rev	ACCGGCTGGTGCCAACT
5	htpG_1_crispr_fow	GGGAGTTGGCGCGTCTCGCCGGTGGC
6	htpG_1_crispr_rev	AAACGCCACCGGCGAGACGCGCCAAC
7	htpG_2_crispr_fow	GGGAGTTCCTCCTCGCTGTACGTGGAGACAAAC
8	htpG_2_crispr_rev	AAACGTTTGTCTCCACGTACAGCGAGGAGGAAC
9	HtpG F10TAG fow	ATGTCGAGCAGTTGGAGTAGCAGGCGGAGGCCCGGC
10	HtpG F10TAG rev	GCCGGGCCTCCGCCTGCTACTCCAACTGCTCGACAT
11	HtpG W197TAG fow	CACGACTACACCTCGGAATAGAAGATCCGTAACCTGG
12	HtpG W197TAG rev	CCAGGTTACGGATCTTCTATTCCGAGGTGTAGTCGTG
13	HtpG W274TAG fow	CAAACACGTCGCGCACGCCTAGGACGACCCGCTCGAGATCATC
14	HtpG W274TAG rev	GATGATCTCGAGCGGGTCGTCCTAGGCGTGCGCGACGTGTTTG
15	HtpG F303TAG fow	CCCGTCTCATGCCCCGTAGGATCTGTTCGACCGGGACG
16	HtpG F303TAG rev	CGTCCCGGTCGAACAGATCCTACGGGGCATGAGACGGG
17	HtpG F559TAG fow	CTGATCACCGATGCCTAGGGGATGACACCCGCGCTCGC
18	HtpG F559TAG rev	GCGAGCGCGGGTGTCATCCCCTAGGCATCGGTGATCAG
19	HtpG Y618TAG fow	GAAACCGCGGAATTACTTTAGGGCACAGCGCTTCTCGCCG
20	HtpG Y618TAG rev	CGGCGAGAAGCGCTGTGCCCTAAAGTAATTCCGCGGTTTC
21	HtpG Y423TAG fow	TCTTCGTTTGTCTCCACGTAGAGCGAGGAGGAACCCACC
22	HtpG Y423TAG rev	GGTGGGTTCCTCCTCGCTCTACGTGGAGACAAACGAAGA
23	HtpG F395TAG fow	CGCACGTTCTGGACACAGTAGGGCAGGGTCCTCAAAGAGGG
24	HtpG F395TAG rev	CCCTCTTTGAGGACCCTGCCCTACTGTGTCCAGAACGTGCG
25	HtpG F337TAG fow	CATGCCCGAGTACTTGCGTTAGGTCAAGGGTGTGGTCGACGCGC
26	HtpG F337TAG rev	GCGCGTCGACCACACCCTTGACCTAACGCAAGTACTCGGGCATG
27	HtpG_R53_TAG_fow	GCGGATTGAGGCGCTGTAGAACAAGGACCTGGAGGTC
28	HtpG_R53_TAG_rev	GACCTCCAGGTCCTTGTTCTACAGCGCCTCAATCCGC
29	HtpG_T236_TAG_fow	GGCGGCGAGGAGTAGGTCACCATCGAAACC
30	HtpG_T236_TAG_rev	GGTTTCGATGGTGACCTACTCCTCGCCGCC
31	HtpG_E257_TAG_fow	TGGGCGAGGCCCAAAGAATAGGTGTCTGAGCAGGAG
32	HtpG_E257_TAG_rev	CTCCTGCTCAGACACCTATTCTTTGGGCCTCGCCCA
33	HtpG_R384_TAG_fow	GACGTGCAGTCCAGCTAGCCGGAGGACTA
34	HtpG_R384_TAG_rev	TAGTCCTCCGGCTAGCTGGACTGCACGTC
35	HtpG_D510_TAG_fow	CTTGAGTTCCGAAGAGTAGACCAGCGAGGCCGAG
36	HtpG_D510_TAG_rev	CTCGGCCTCGCTGGTCTACTCTTCGGAACTCAAG
37	HtpG H269_TAG fow	AAGGAGTTCTACAAATAGGTCGCGCACGCCTGG
38	HtpG H269_TAG rev	CCAGGCGTGCGCGACCTATTTGTAGAACTCCTT
39	HtpG W529_TAG fow	GCCGACCTGCTGACCTAGTTGCAGGAGACG
40	HtpG W529_TAG rev	CGTCTCCTGCAACTAGGTCAGCAGGTCGGC
41	HtpG F635_TAG fow	GAGGATCCGGCGAGGTAGGCCGAGCTGCTGG
42	HtpG F635_TAG rev	CCAGCAGCTCGGCCTACCTCGCCGGATCCTC
43	oAF842	GAACAGATTGGTGGATCCGGTTGCGCTCGTGCGGTCGGTATC
44	oAF845	GAACAGATTGGTGGATCCGGTTGCACTCAGGACGATTCGC
45	oAF846	GCTTCCTTTCGGGCTTTGTCAGCAATTGTCTGATTCTGCGGC
46	oAF847	GAGAAGCTTCAGCACTTGTTCTCCTTGCC
47	pET47b_htpG_fow	CGGCTCTTGAAGTCCTCTTTCAGGGACCCATGAACGCCCATGTCGAGCAG
48	pET47b_htpG_rev	TTCTTTACTACCGCGTGGCACCAGAGCGAGCTACAAGGTACGCGCGAGAC
49	attB1-lox2-SD-kanR	GGGGACAAGTTTGTACAAAAAAGCAGGCTATAACTTCGTATAATGTATGCTATA CGAAGTTATCGCTCAGTGGAACGAAAACTCACGTTAAGG
50	attB2-lox2-linker-kanR	GGGGACCACTTTGTACAAGAAAGCTGGGTAATAACTTCGTATAGCATACATTAT ACGAAGTTATGGAAATGTGCGCGGAACCCCTATTTG
51	pET27b_noleader_htpG_fow	AGGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGATGAACGCCCATGTCGAGCAG
52	pET27b_CHis_ htpG_rev	TTAGCAGCCGGATCTCAGTGGTGGTGGTGGTGGTGCAAGGTACGCGCGAGACGTTC

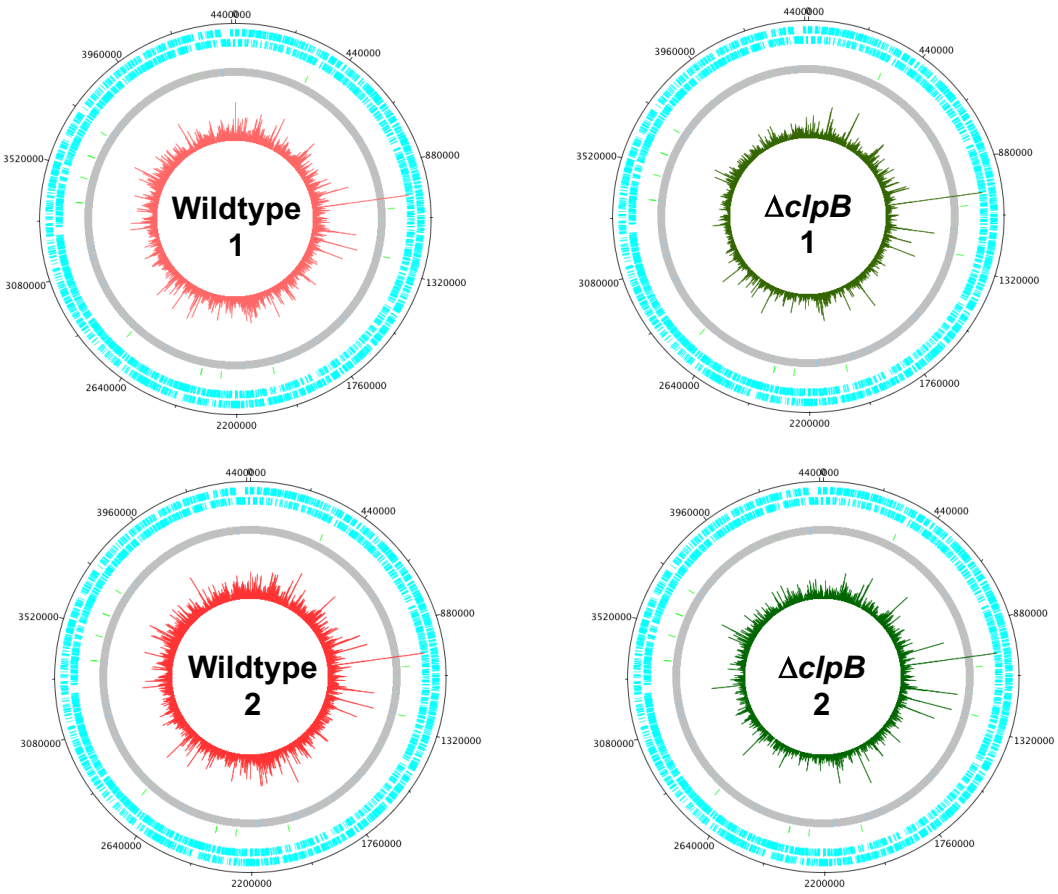

Figure S1. The sequence reads from replicate Mtb transposon libraries in wild-type (red) and Δ*clpB* (green) backgrounds visualized using DNA plotter. Insertions are represented as peaks in TA dinucleotides distributed throughout the Mtb genome. Peaks that have the highest number of insertions in each library (indicated by the height of the spike) are shown in **Table S1**.

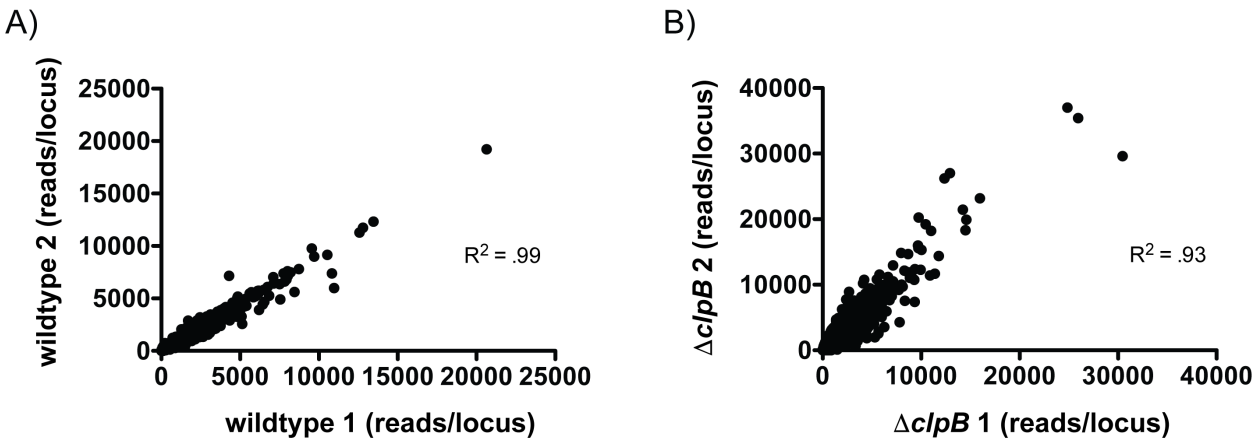

Figure S2. Comparison of replicate transposon libraries based on number of TA insertions (reads) per gene in (A) wildtype and (B) Δ*clpB* backgrounds. The R^2 values are indicated in each graph.

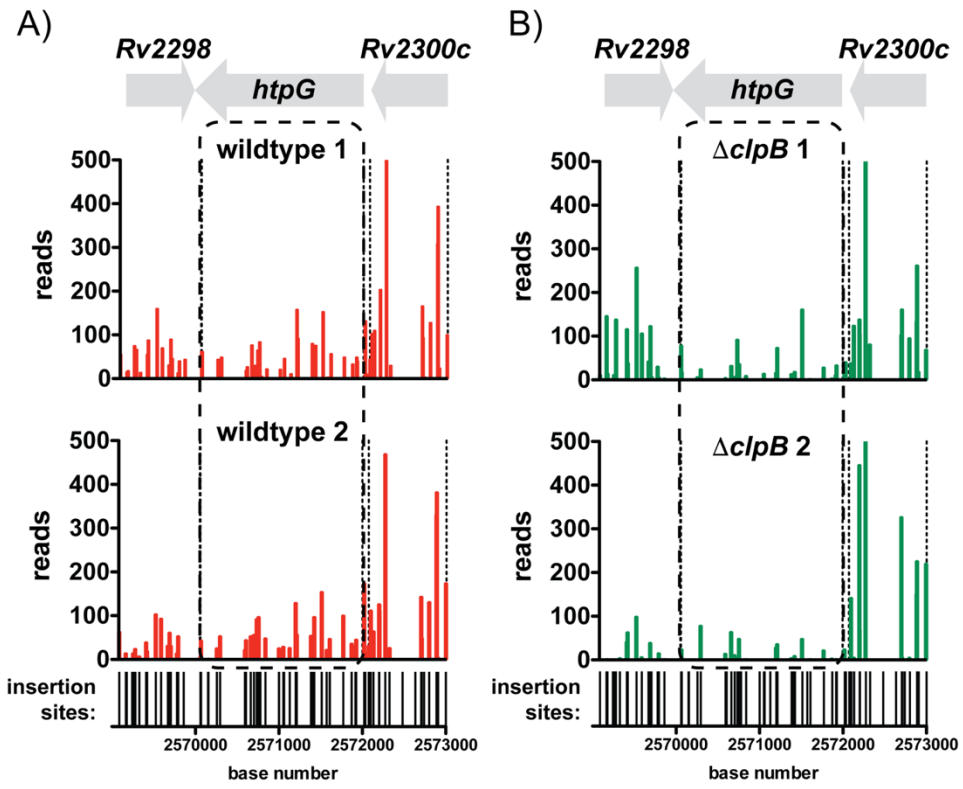

Figure S3. Mutations in *htpG* are underrepresented in the Mtb Δ*clpB* background relative to wildtype (H37RvN). Sequence reads at individual TA insertion sites (indicated at bottom) visualized in replicate transposon libraries in (A) wildtype and (B) Δ*clpB* backgrounds with relevant loci highlighted above. Note that *htpG* (boxed) contains less insertions in the Δ*clpB* compared to wild-type libraries. Dashed lines are cutoffs between indicated loci.

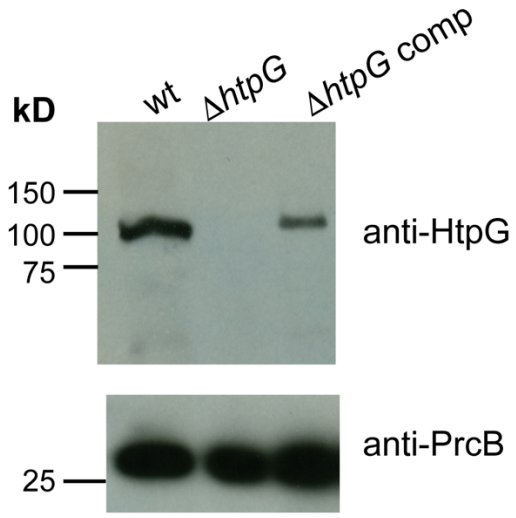

Figure S4. Western blot of lysates of Mtb H37RvN wild-type, Δ*htpG* and Δ*htpG* complemented strains (comp or Δ*htpG::hsp60-htpG*) illustrate deletion of *htpG* in Mtb. Strains were harvested at an OD580 of ~1.2 for wild-type, ~0.6 for Δ*htpG* and ~0.7 for the complemented strain. About 50 μg of total protein in lysate was laded per lane and was blotted using anti-HtpG and anti-PrcB (as a loading control). The *hsp60* promoter was chosen because the expected native promoter region of *htpG* did not produce signficant levels of protein (when compared to the wild-type strain).

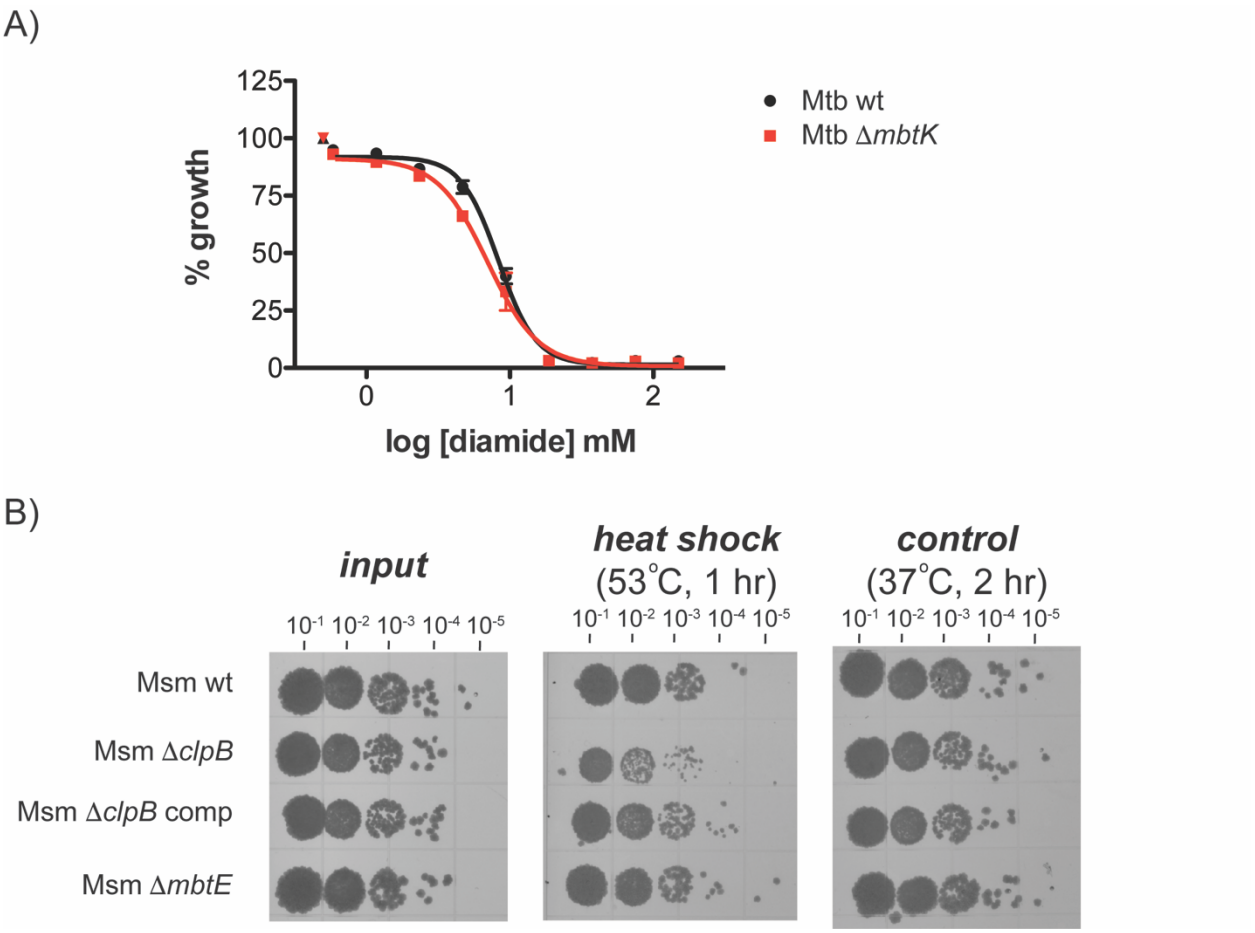

Figure S5. Mycobacterial *mbt* deletion mutants do not demonstrate increased sensitivity to diamide or heat stress. (A) Dose-response of diamide to Mtb wild-type (wt) and Δ*mbtK* strains illustrates similar MIC90 (minimum inhibitory concentration at 90%) values for wt (18.4 mM) and ΔmbtK (19.6 mM) (n = 3). OD580 values were read after t = 9 days at 37 °C to calculate percent growth. (B) Heat shock of indicated *M. smegmatis* (Msm) strains illustrates that cells lacking *clpB* are more senstive to heat than wild-type, which is complemented in the "comp" strain; Msm lacking *mbtE* behaves like wild-type. Note that Msm cells have greater heat tolerance than Mtb cells, as seen in other work (Gebhard, Hümpel, McLellan, & Cook, 2008). Plates shown are representative of n = 6 experiments.

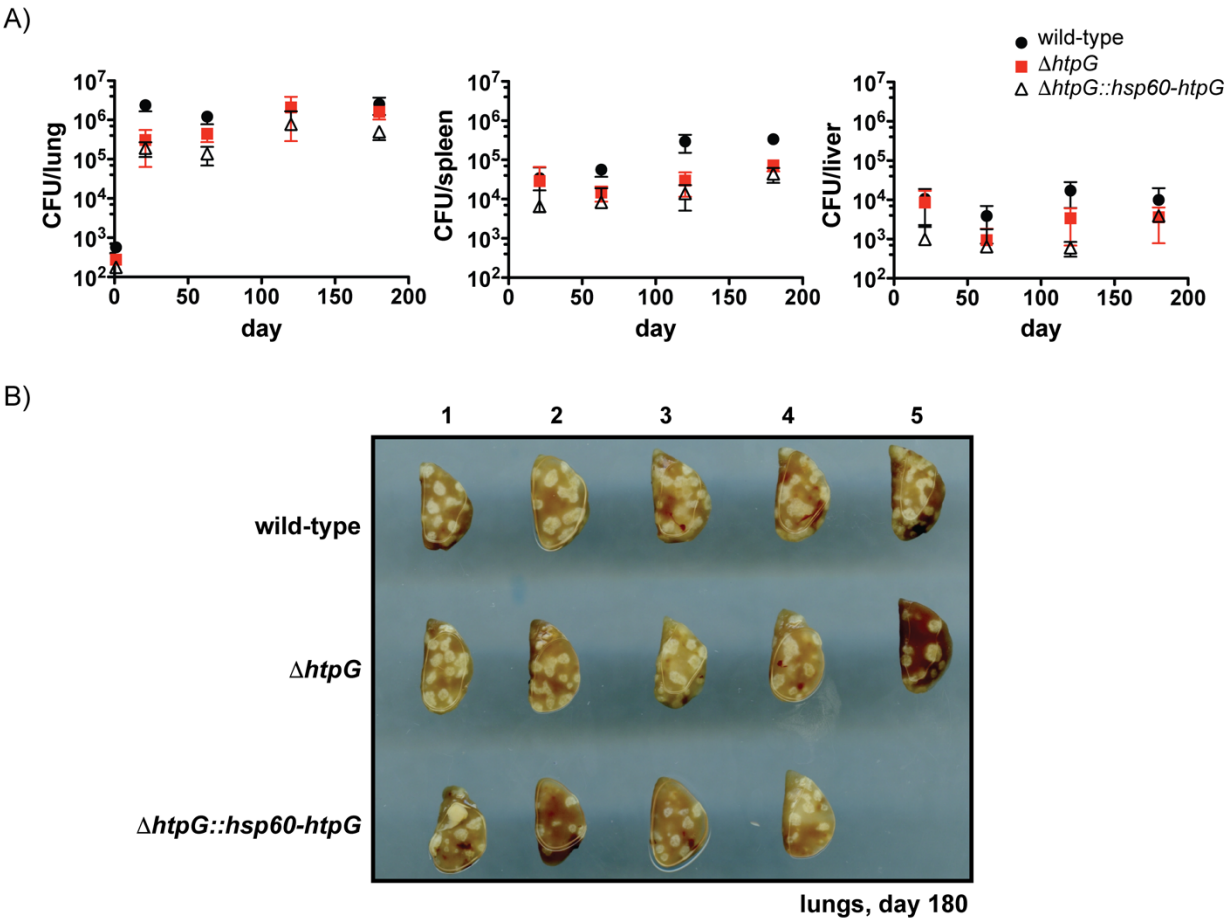

Figure S6. Mtb cells lacking *htpG* replicate and persist similarly to wild-type Mtb in a C57BL/6 mouse infection model. (A) Mtb wild-type (H37RvN), Δ*htpG* and Δ*htpG* complemented strains were used to infect mice over a 180 day time course as described in the supplemental methods section. At each time point, CFU/organ of bacteria were determined for 4-5 infected mice. (B) Lungs of sacrificed mice after 180 days of infection, with Mtb lesions shown in white.

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H37RvN                  GTTTTTGATCTCGGTAGCCGCCGAGCTAGCCGGCATGCATGCACAGACCC
KOhtpG                  GTTTTTGATCTCGGTAGCCGCCGAGCTAGCCGGCATGCATGCACAGACCC
KOclpB                  GTTTTTGATCTCGGTAGCCGCCGAGCTAGCCGGCATGCATGCACAGACCC
KOclpB(kanR)            GTTTTTGATCTCGGTAGCCGCCGAGCTAGCCGGCATGCATGCACAGACCC
KOclpBpre500htpG2       GTTTTTGATCTCGGTAGCCGTCGAGCTAGCCGGCATGCATGCACAGACCC
KOclpBpre500htpG1       GTTTTTGATCTCGGTAGCCGTCGAGCTAGCCGGCATGCATGCACAGACCC
KOclpBSNP(strepR)       GTTTTTGATCTCGGTAGCCGTCGAGCTAGCCGGCATGCATGCACAGACCC
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Figure S7. Alignment of sequenced genomic DNA (*hspR* region) of strains described in this study shows a SNP in Mtb Δ*clpB* kanR strains after genetic manipulation. While only a portion of the sequence is shown, only a single SNP exists, C→T, corresponding to mutation of Ala19Val in HspR. Above, H37RvN = wildtype; KOhtpG = Δ*htpG;* KOclpB = Δ*clpB* used in this study, which has resistant cassettes for hygromycin, nourseothricin and zeocin; KOclpB (kanR) is a new Mtb Δ*clpB* strain with a single kanamycin resistant cassette (and is the parent strain for the remaining strains listed); KOclpBpre500htpG2 and 1 are two clones of Mtb Δ*clpB* (kanR) containing an extra copy of *htpG* at the L5 site; KOclpBSNP (strepR) is the Mtb Δ*clpB hspR* SNP (A19V) strain characterized in **Figure 3** and is resistant to both kanamycin and streptomycin (has a streptomycin resistant cassette in place of *htpG* at the L5 site).

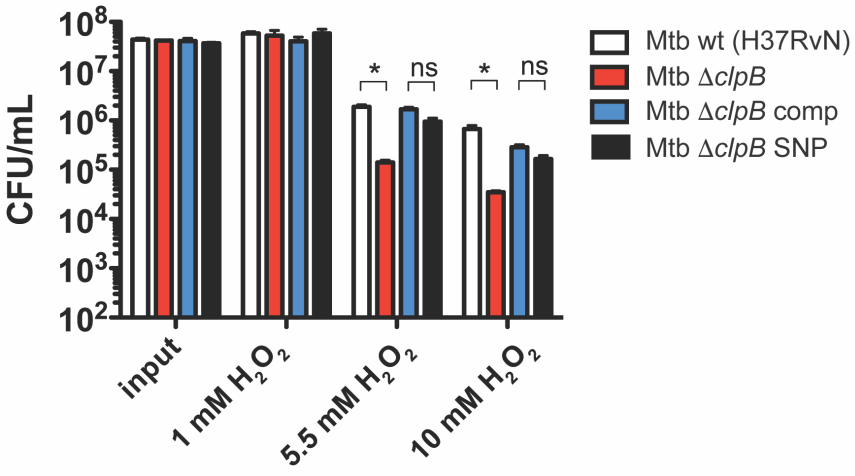


Figure S8. A suppressor mutation in Mtb *hspR* protects Mtb Δ*clpB* from oxidative stress. Indicated strains, including Mtb Δ*clpB hspR* A19V (SNP) were incubated in the presence of varying concentrations of H_2O_2 for t = 4 hr prior to plating as described for **Figure 3A**. While Mtb Δ*clpB* strains are more senstive to peroxide than wild-type, Δ*clpB hspR* A19V cells behave similar to the complemented strain. (n = 3, error bars represents SD, * $p<0.0332$, ns = non-significant Student's t-test was used for comparison.)

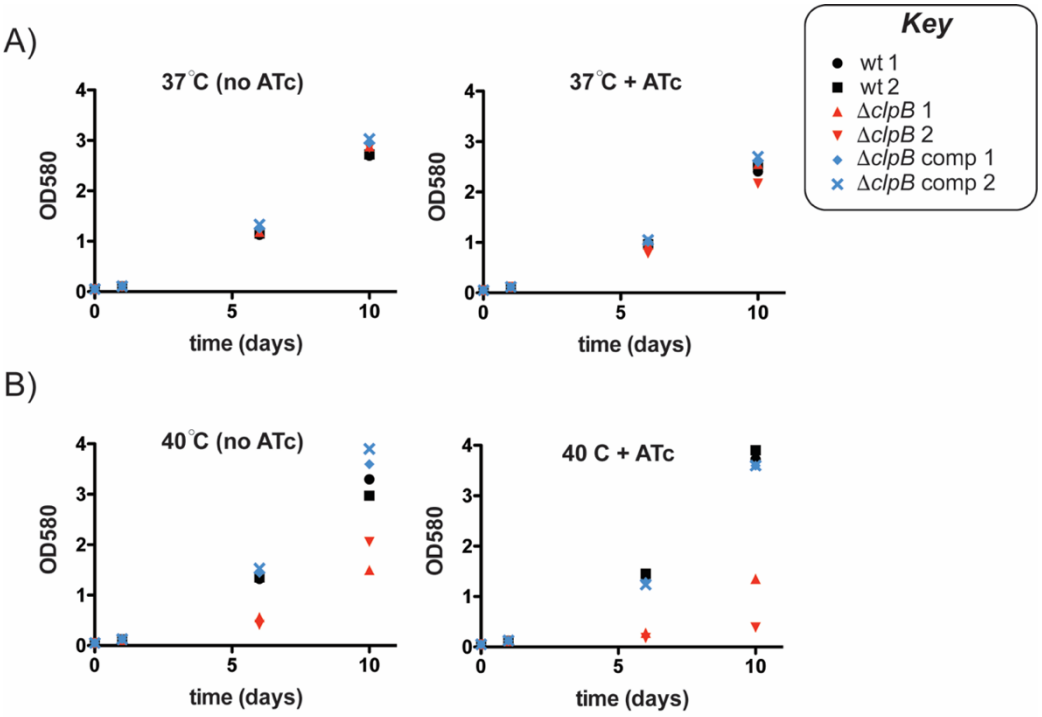


Figure S9. Evaluation of single guide RNA (sgRNA) sequences targetting *htpG* in Mtb cells with and without *clpB* grown at different temperatures. Indicated strains were diluted to OD = 0.05 on day 0 in the presence and absence of 100 ng/mL ATc and incubated at the indicated temperatures. For each strain, the number indicates the sgRNA sequence, each targeting the open reading frame (ORF) of *htpG* in wild-type, Δ*clpB* and Δ*clpB* complemented backgrounds. sgRNA1: GTTGGCGCGTCTCGCCGGTGGC; sgRNA2: GTTCCTCCTCGCTGTACGTGGAGA CAAAC (both adjacent to PAM sequences predicted to be "strong" according to previous work) (Rock et al., 2017). Since sgRNA2 showed the greatest defects with heat, experiments were continued with sgRNA2.

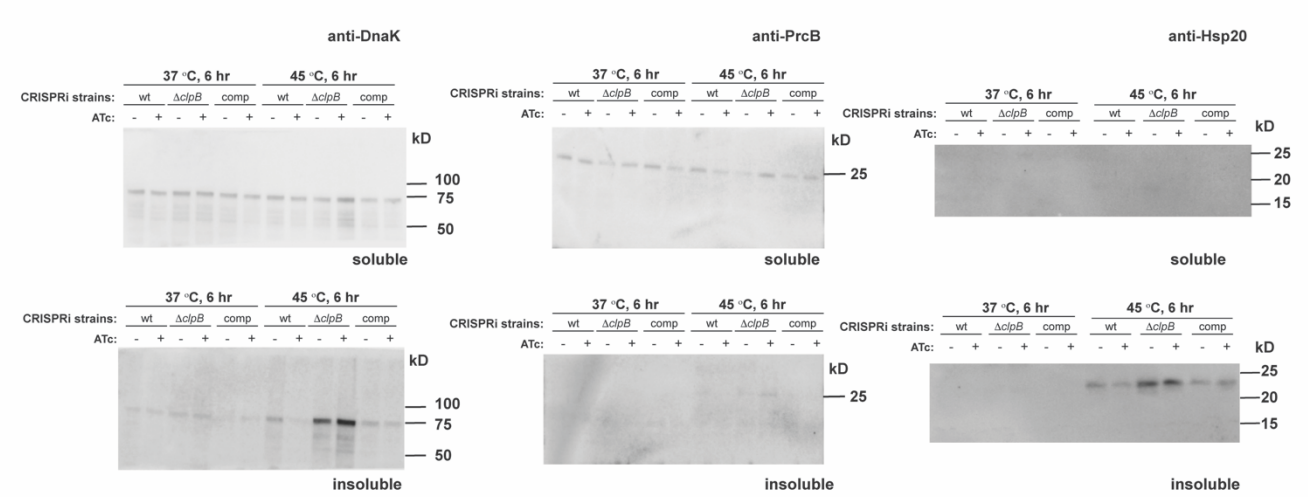


Figure S10. Mtb cells lacking two chaperones show increased co-aggregation of chaperone DnaK and co-chaperone Hsp20 with insoluble proteins after heat shock. Indicated Mtb CRISPRi strains were depleted of *htpG* upon addition of ATc as described in the main text, grown to late log phase and incubated at 37 or 45 ºC for 6 hr prior to purification of lysate and fractionation to separate insoluble and soluble fractions. Western blot analysis revealed that increased levels of DnaK accumulate in the insoluble fraction of Δ*clpB* strains, especially upon loss of HtpG under heat stress (left, plus ATc). DnaK is known to play an essential role in protein folding in mycobacteria (Fay & Glickman, 2014) and bacterial DnaK has been shown to associate with protein aggregates (Acebrón, Fernández-Sáiz, Taneva, Moro, & Muga, 2008). PrcB is a low molecular weight proteosomal subunit that is used as a loading control throughout this work, and does not appear to aggregate dramatically (middle). sHsp20 is a small heat shock protein, known to co-aggregate with misfolded proteins (Laskowska, Wawrzynów, & Taylor, 1996); we observe increased levels of sHsp20 in the insoluble fraction after heat shock, especially in strains lacking *clpB* (right). Coomassie staining was not used for analysis due to difficulties in visualizing changes in aggregated protein levels.

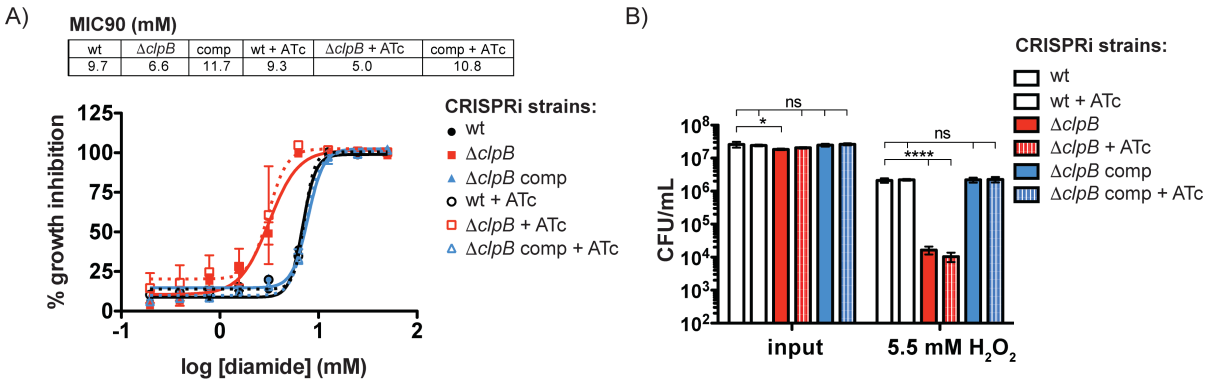


Figure S11. Depletion of *htpG* from Mtb cells lacking *clpB* does not exacerbate growth defects in the presence of select oxidants. (A) Dose response of indicated CRISPRi strains with and witout ATc (addition of ATc leads to depletion of *htpG*) to varying concentrations of diamide (n = 3). Loss of HtpG in addition to ClpB leads to only a minor increase in sensitivity to diamide compared to loss of ClpB alone. OD580 values were read after t = 11 days at 37 °C to calculate percent growth inhbition. (B) Colony forming units (CFU) per mL of indicated strains with and without ATc measured before and after t = 4 hours of peroxide treatment show that Δ*clpB* cells behave similarly with and without HtpG present. (n = 3, error bars indicate SD, * p<0.0332, **** p<0.0001, one-way ANOVA was used for group comparison.)

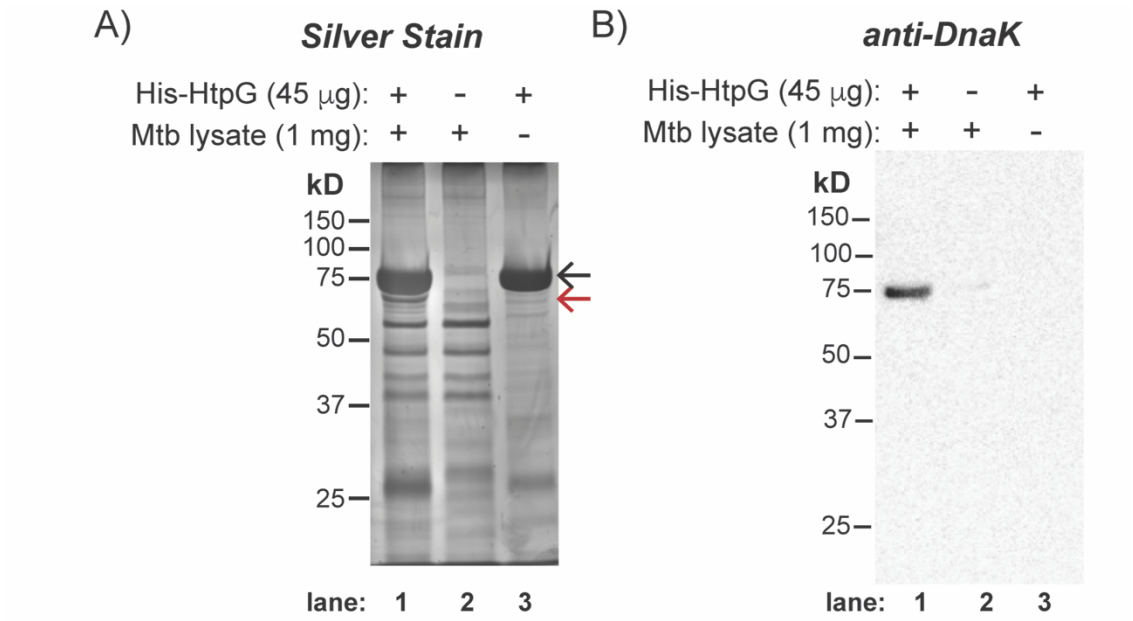


Figure S12. Mtb His-HtpG interacts with Mtb DnaK in cellular lysates. (A) Pull-down of mycobacterial proteins by Mtb-HtpG as analyzed by SDS-PAGE followed by silver staining of Ni-NTA elution fractions of indicated amounts of His-HtpG, Mtb lysate, or both co-incubated in the presence of excess ATP. In lane 1, His-HtpG (highlighted by black arrow) co-elutes with a band immediately underneath (red arrow) that is enriched relative to the lysate only sample (lane 2) and His-HtpG sample (lane 3). (B) Western blot analysis, using anti-DnaK, of a replicate experiment to that described in part A. There is enrichment of Mtb DnaK from lysate when His-HtpG is present (lane 1) compared to when it is absent (lane 2). This experiment was repeated for mass spectrometry analysis (**Table S5**) of the band indicated by the red arrow in part A.

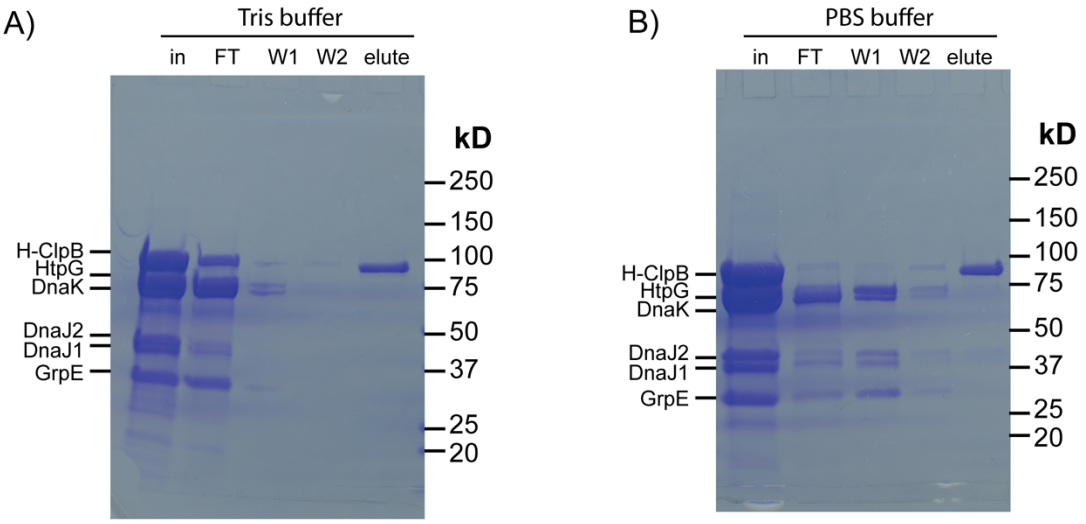
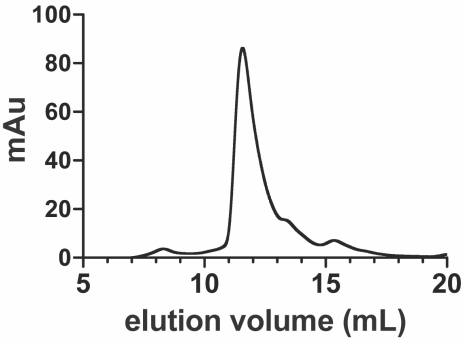


Figure S13. Mtb His-ClpB does not interact with Mtb HtpG in vitro under conditions tested. (A) His-ClpB was incubated with untagged HtpG, and known mycobacterial chaperone/cofactors DnaK, DnaJ1, DnaJ2, and GrpE for 2 hr at 37 °C in the presence of 1 mM ATP in 50 mM Tris (pH 8.0), 20 mM $MgCl_2$, 100 mM NaCl (Tris buffer). The proteins were then incubated with Ni-NTA, which was washed twice with 20 mM imidazole in Tris buffer followed by elution in 200 mM imidazole in Tris buffer. Samples were analyzed by SDS-PAGE followed by Coomassie staining. (B) The experiment was performed as described in part A except using PBS buffer (pH 7.4) with 2 mM $MgCl_2$ in place of Tris buffer. In both parts A and B, His-ClpB eluted alone in the final elution step. (FT = Flow through, W = Wash, E = Elute).

A)



B)

stand.	MW (kD)	elution vol. (mL)
1	440	10.4
2	158	12.5
3	75	14.1

sample	elution vol. (mL)	pred. MW (kD)	pred. HtpG monomers
Mtb HtpG	11.6	244.6	3.3

Figure S14. Mtb HtpG elutes as an oligomer when analyzed by gel filtration chromatography. After extensive dialysis to remove any bound nucleotide, 1 mg of Mtb HtpG was incubated in elution buffer prior to injection on a Superdex 200 10/300 GL column. Standard protein samples (ferritin, aldolase, conalbumin) (GE Healthcare) were analyzed under the same conditions and the resulting elution volume values were used to calculate the predicted molecular weight (MW) values of Mtb HtpG. Notably, the oligomeric state of Hsp90s cannot be accurately calculated by gel filtration analysis, as discussed in the main text.

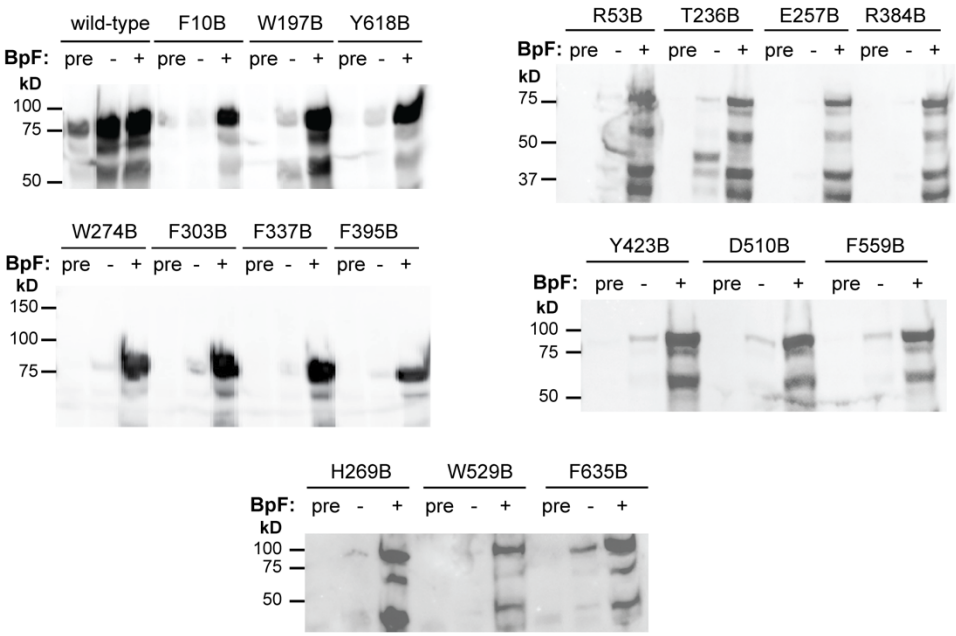


Figure S15. A UV-activatable crosslinking unnatural amino acid can be incorporated site-specifically into Mtb HtpG. Mtb HtpG with TAG mutations express as full-length proteins only in the presence of para-benzoyl-phenylalanine (BpF), otherwise there is premature termination of translation. Wild-type HtpG expresses whether or not BpF is present in the media (upper left), as expected. Briefly, LB (5 mL) supplemented +/- 0.8 mM BpF were inoculated with single colonies and incubated with shaking at 37 °C. At an OD600 > 0.2, cultures were induced with 1 mM IPTG and 0.2% arabinose for 3 hours at 37 °C with shaking. Samples were centrifuged and normalized by resuspension in a volume of 2x laemmli buffer (+0.05% beta-mercaptoethanol) that was based on the OD600 value of the culture prior to centrifugation. Samples (20 μL) were analyzed by SDS-PAGE (9% polyacrylamide) prior to immunobloting using anti-His. Note that "pre" samples are those from +BpF media prior to induction.

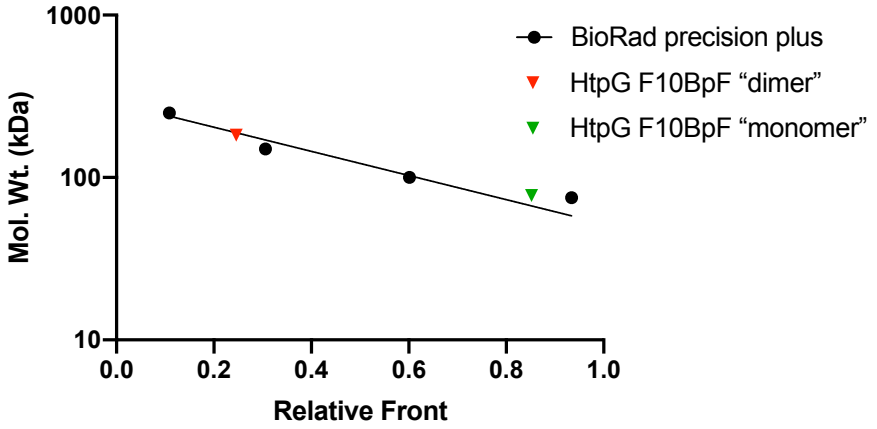


Figure S16. Analysis of molecular weight standards by SDS-PAGE for prediction of “monomer” and “dimer” molecular weight values following UV crosslinking. In the example above, Image Lab software (Bio-Rad) was used generate a standard curve with Precision Plus protein molecular weight standard ladder (black, Bio-Rad) to estimate the molecular weight of Mtb HtpG F10BpF before (~77.4 kDa, green) and after (~182.1 kDa, red) crosslinking. Actual molecular weight of wildtype HtpG is ~73.0 kD.

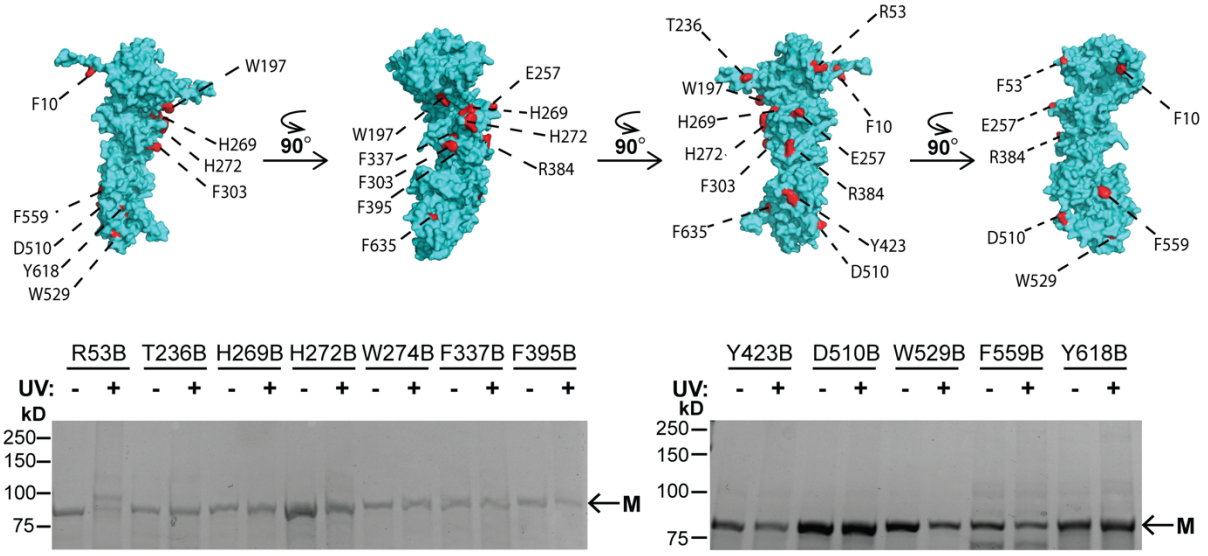


Figure S17. Mtb HtpG monomers form a defined dimer interface. SDS-PAGE analysis (bottom) of additional Mtb HtpG mutants (2 μM) containing BpF (B) at indicated positions (top) in buffer containing 5 mM ATP shows that residues on faces other than that shown in **Figure 5A-B** do not form homodimers following UV irradiation. M = HtpG monomer.

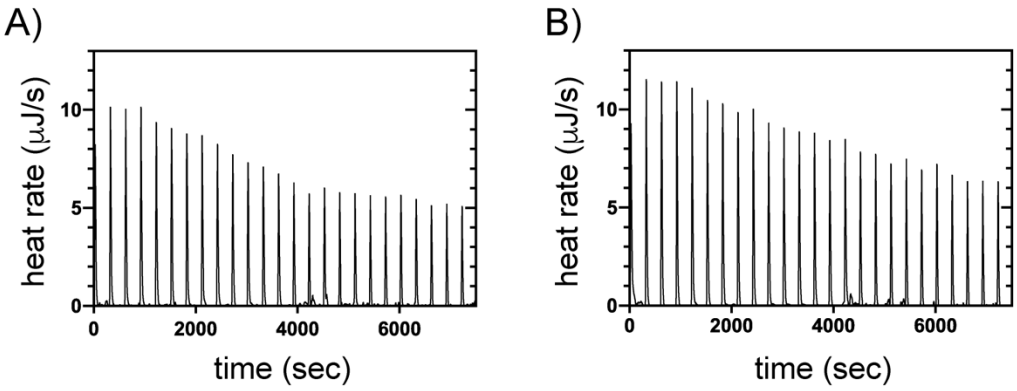


Figure S18. Replicate isothermal titration calorimetry data for addition of geldanamycin (GA) to Mtb HtpG. GA (750 μM) was titrated into Mtb HtpG (47 μM) at 25 °C. The K_D average ± standard deviation (SD) values were calculated based on addition of GA into Mtb HtpG under identical conditions, described in the Methods section, performed in triplicate.

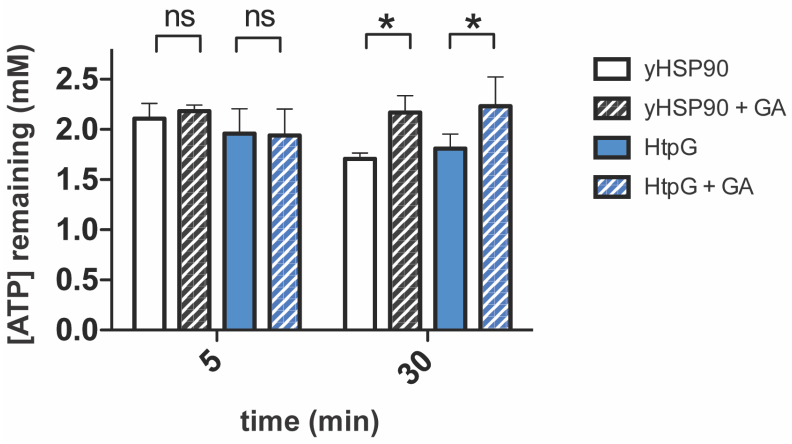


Figure S19. Similar to yeast Hsp90, Mtb HtpG exhibits weak ATPase activity that is inhibited by GA. Yeast Hsp90 or Mtb HtpG was incubated with or without GA (180 μM) and 2 mM ATP in buffer (40 mM HEPES-KOH, pH 7.5, 2 mM $MgCl_2$) at 30 °C and remaining ATP was calculated at indicated time points using Kinase-Glo (Promega) reagent. (n = 4, error bars indicated SD, * $p<0.0332$, one-way ANOVA was used for comparison.)

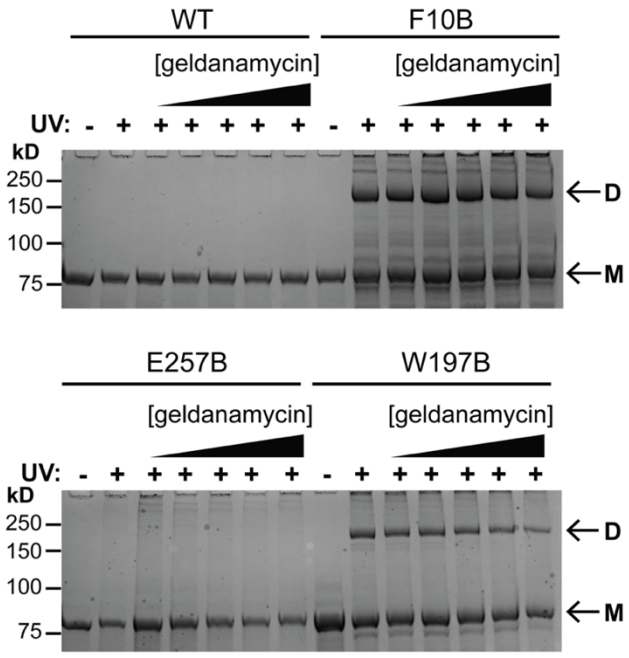


Figure S20. Titration of GA into indicated Mtb HtpG wild-type and select BpF mutants does not produce an observable change in dimer formation. Mtb HtpG wild-type and BpF mutants were incubated with geldanamycin (0, 10, 50, 100, 200, 400 μM), exposed to UV irradiation (365 nm), and compared to samples without GA in the absence of UV treatment. M = HtpG monomer; D = HtpG dimer.

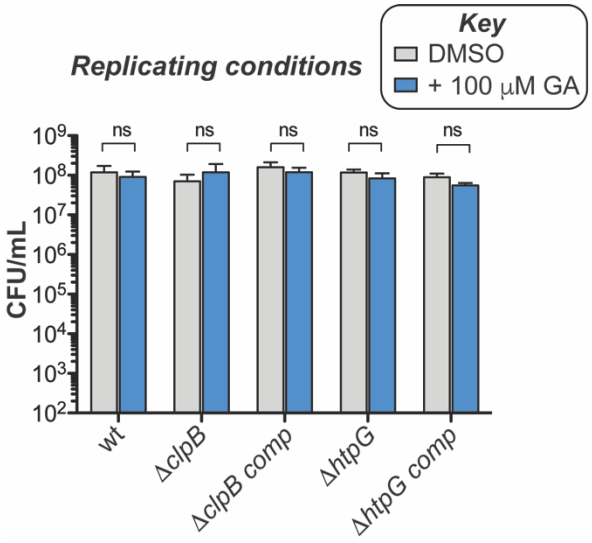


Figure S21. Addition of GA does not affect survival of cells under replicating conditions. CFU analysis of strains under replicating conditions demonstrates that there is no significant change in survival of indicated strains with or without GA added under replicating conditions, as expected. ns indicates not significant, two-way ANOVA was used for group comparison (n = 3, error bars represent SD).

Supplemental Methods:

Infection of mice with Mtb. C57BL/6 8-week-old female mice (Jackson Laboratories) were infected by aerosol using a Glas-Col inhalation exposure system with log phase *Mtb* cultures prepared as single-cell suspensions in PBS to deliver 100–200 bacilli per mouse. To quantify bacteria, serial dilutions of lung, spleen and liver homogenates were cultured on 7H10 plates containing 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) (Difco) and 0.5% glycerol. After 21 days of incubation of the plates at 37 °C, the CFUs were numerated.

The left lobe of the mouse lungs was fixed in 10% formalin in PBS and used for staining with hematoxylin and eosin. Procedures involving mice were performed as according to National Institutes of Health guidelines for housing and care of laboratory animals and were reviewed and approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College.

Gel filtration chromatography analysis of Mtb HtpG. Nucleotide free HtpG (2 mg/mL) (see Methods section), in storage buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, pH 8) (0.5 mL) was analyzed by an AKTA Pure FPLC 15L instrument (GE Healthcare) using a Superdex 200 10/300 GL gel filtration column (GE Healthcare) equilibrated in the storage buffer at a flow rate of 0.3 mL/min. The elution volume of protein sample was monitored using absorption at 280 nm. High molecular weight standards (GE Healthcare), ferritin (440 kDa, 0.4 mg/mL), aldolase (158 kDa, 4 mg/mL) and conalbumin (75 kDa, 3 mg/mL), were analyzed by gel filtration chromatography under the same conditions. The void volume was determined by applying bromophenol blue to the column. The elution volume of the void volume and standards were used to estimate the molecular weight and oligomerization state of HtpG following the manufacturer's protocol.

Assessment of library saturation and visualization using DNA plotter. The complexity of each transposon library was evaluated by calculating the number of transposon cassette insertions into TA dinucleotides as has been previously described using custom Python and MATLAB scripts (Kieser et al., 2015; Pritchard et al., 2014). DNAPlotter was used to generate

images of circular mycobacterial genomes sites of transposon insertions as described in a previous report (Carver, Thomson, Bleasby, Berriman, & Parkhill, 2009).

Minimum inhibitory concentration (MIC) assessment of Mtb strains. Briefly, for CRISPRi strains, cells were inoculated in 7H9 complete at a final OD580 = 0.05 in triplicate and incubated at 37 °C with or without ATc (100 ng/μL). On day 5, cells were centrifuged (3100 × g, 5 min), resuspended in fresh 7H9 complete and diluted to an OD580 of 0.06. For other strains, cells were grown to log-phase prior to dilution to OD580 = 0.06. Cells were added to clear 96-well plates (Costar) to a final OD580 = 0.03 in 200 μL final volume 7H9 complete +/- ATc (100 ng/mL) (some MIC experiments were done at an initial OD580 = 0.05). Indicated concentrations of compound were added to each well, with water used as a control. Cells were incubated without shaking at 37 °C for t = 11 days and the absorbance at 580 nm values were measured using a SpectraMax M2 microplate reader (Molecular Devices) and analysis was performed with SoftMax Pro software. Curve fitting was performed using Prism (GraphPad).

Cloning, Expression and Purification of Mtb His_6-HtpG. An overexpression plasmid for His-HtpG was constructed using overlap extension PCR cloning techniques (Bryksin & Matsumura, 2010) with pET47b plasmid (Novagen) and indicated primer pairs 47 and 48 (**Table S9**) following amplification from chromosomal H37RvN DNA. Following PCR steps and DpnI treatment, DNA samples were purified using a PCR purification kit (Qiagen) and transformed into Mach1 competent cells (Invitrogen). Following confirmation of gene insertion by DNA sequencing, the selected plasmid was transformed into BL21(DE3) competent cells (Novagen) for overexpression. A 5 mL overnight culture of cells was used to inoculate 500 mL LB (kanamycin 25 μg/mL), which was grown at 37 °C with shaking to OD600 ~ 0.2 prior to lowering temperature to 18 °C. Cells were induced with 0.01 mM IPTG (isopropyl-β-D-thiogalactoside) for 20 hr at 18 °C and were then harvested by centrifugation (3100 × g, 10 min, 4 °C). Pellets were resuspended on ice in 15 mL of lysis buffer (25 mM Tris (tris(hydroxymethyl)aminomethane) (pH = 8.0), 400 mM NaCl, 10%

glycerol) supplemented with 100 µg/mL lysozyme and 3 µg/mL DNaseI. Cells were rocked for at least 20 min at 4 °C and lysed by sonication on ice using a 30 sec interval program at an amplitude of 5 for 10 min total. Samples were then ultracentrifuged at 39,191 × g for 30 min at 4 °C. Resulting supernatants of each were added to 1.5 mL washed Ni-NTA agarose resin (Qiagen) with 2 mM added imidazole and rocked at 4 °C for 1 hr. Resin was then washed with 30 mL wash buffer (30 mM imidazole in lysis buffer) and His_6-tagged proteins were eluted with 10 mL elution buffer (200 mM imidazole in lysis buffer). The eluate fractions were dialyzed against 1 L lysis buffer overnight using a 10 kD MWCO Slide-A-Lyzer dialysis cassette (Pierce). Protein samples were concentrated to <1 mL using 10 kD MWCO Amicon Ultra Centrifugal Filter Device (Millipore) at 4 °C. Concentration was determined using BSA as a standard in the *DC* protein assay (Bio-Rad). Protein aliquots were flash frozen in $N_2(l)$ and stored at -80 °C.

Purification of Mtb lysates for pull-down experiments. Mtb lysate was purified using a standard mycobacterial protocol. Briefly, a 50 mL culture of H37RvN was grown to log phase (OD580 = 0.6-1.0) in 7H9 complete. Cells were centrifuged (3100 × g, 5 min) and washed with 30 mL of PBS + 0.05% tween-80 twice followed by centrifugation. The pellet was then resuspended in 1 mL of lysis buffer B (25 mM Tris (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8), 1 mM phenylmethylsulfonyl fluoride (PMSF)). Cell suspensions were added to sealed 2.0 mL microtubes tubes (Sarstedt) with approximately 250 µL of zirconia/silica beads (1 mm, BioSpec) prior to lysis by three rounds (high setting for 30 sec) of bead beating (Mini-BeadBeater-1, BioSpec) with cooling on ice in between each round. Samples were centrifuged at 20,000 x g (30 sec) to pellet beads, and resulting supernatants were transferred to microfuge tubes and spun at 20,000 × g (1 min) to pellet remaining whole cells. Finally, supernatant was added to a Spin-X centrifuge tube with a 0.22 µm nitrocellulose filter (Corning, Costar) and spun at 10,000 × g for 5 min. Total protein concentration was estimated using BSA as a standard in PBS buffer with the *DC* protein assay (Bio-Rad). For pull-down experiments, indicated amounts

of His-HtpG, Mtb lysate, or both were co-incubated in the presence of 1 mM ATP in PBS (pH 7.4) and 2 mM $MgCl_2$ for 1 hr at RT prior to incubation with Ni-NTA (Qiagen), washing with 20 mM imidazole twice and elution with 200 mM imidazole. Silver staining was performed using common biochemical protocols and western blotting was carried out as detailed in the main text.

Fractionation of Mtb CRISPRi strain lysates following heat shock prior to western blot analysis. Indicated Mtb CRISPRi strains were diluted in duplicate to a final volume 20 mL 7H9 complete to an OD580 = 0.05 in T75 flasks (Corning) and incubated at 37 °C with or without ATc (100 ng/μL). On day 5, OD580 values were measured (values were in the range of 1.2-2) and cells were incubated at 37 °C or 45 °C for 6 hours. Cells were then centrifuged (3100 × g, 5 min) and lysates were purified as described in the section above, except that 10% glycerol was added the lysis buffer (lysis buffer B). Total protein concentration was estimated using BSA as a standard in PBS buffer with the *DC* protein assay (Bio-Rad). In order to fractionate these samples, we modified a previously published protocol (Fay & Glickman, 2014). Briefly, 2.5 mg/mL of each sample was added to lysis buffer to a final volume of 120 μL. Samples were ultracentrifuged (200,000 × g, 2 hours, 4 °C), and resulting soluble and insoluble fractions were treated separately. The soluble fraction was removed and 20 uL of sample was added to 10 uL 2x laemmli sample buffer (+0.05% beta-mercaptoethanol). Insoluble fractions were resuspended in 60 uL lysis buffer supplemented with 1% Triton X-100, vortexed, and sonicated for 30 minutes. Following sonication, 20 uL 2x laemmli sample buffer (+0.05% beta-mercaptoethanol) was added to each insoluble fraction. Soluble and insoluble samples were boiled for 1 hour and subjected to western blotting. For western blot analysis, 30 μL of each soluble sample and 40 μL of each insoluble sample were loaded on 4-20% polyacrylamide gels and run at 150 V for 65 min. Immunoblotting was performed as described before with the following dilutions of primary antibodies in 2.5% milk fat: anti-DnaK (1:20K), anti-Acr2 (Mtb Hsp20 antibody, Novus Biologicals) (1:1K), and anti-PrcB

(1:10K). Anti-rabbit HRP (1:10K) in the same buffer was used as a secondary antibody in each experiment.

Purification of recombinant Mtb chaperones and in vitro pull-down experiment. Mtb DnaK, DnaJ1, DnaJ2, GrpE and ClpB were overexpressed and purified as described previously (Lupoli et al., 2016). Proteins were aliquoted, flash frozen in $N_2(l)$ and stored at minus 80 °C for use in the experiments shown here. In vitro pulldown experiments were performed using indicated conditions and a previously described method (Lupoli et al., 2016).

Expression and purification of yeast Hsp90 (yHsp90). Expression constructs of yHsp90 (pACYC-T7 yHsp90) (Wayne & Bolon, 2007) were transformed in *E. coli* BL21(DE3) cells. A 5 mL overnight culture of cells was used to inoculate 500 mL LB (50 μg/mL carbenicillin), which was grown at 37 °C with shaking to OD600 ~ 0.8 prior to lowering temperature to 25 °C. Cells were induced with 0.25 mM IPTG for 3 hr. Following harvesting, cells were resuspended and sonicated in lysis buffer (25 mM Tris (pH 8.0), 400 mM NaCl, 10% glycerol), supplemented with 1 mM PMSF, protease inhibitor cocktail (EDTA free), 100 μg/mL lysozyme and 3 μg/mL DNaseI. The insoluble fraction was removed by centrifugation at 10,976 × g for 30 min. Resulting cell lysates were purified using 2.0 mL washed Ni-NTA agarose resin. Resin was then washed with 30 mL wash buffer (30 mM imidazole in lysis buffer) and His_6-tagged proteins were eluted with 10 mL elution buffer (200 mM imidazole in lysis buffer). The elution fraction was dialyzed against 4 L buffer (25 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol) and further purified using a Superdex 200 10/300 GL gel filtration column (GE Healthcare). Concentration was determined using BSA as a standard in the *DC* protein assay (Bio-Rad). Protein aliquots were flash frozen in $N_2(l)$ and stored at -80 °C.

ATPase experiments with Mtb HtpG and yeast Hsp90. Recombinant Mtb HtpG (5 μM) or yHSP90 (5 μM) were incubated at 30 °C in 40 mM HEPES-KOH, pH 7.5, 2 mM $MgCl_2$ with or without 180 μM GA. Reactions were initiated by the addition of 2 mM ATP and were quenched

by the addition of 0.05% formic acid at indicated time points. Concentration of ATP was calculated using ATP standards followed by addition of Kinase-Glo (Promega) reagent according to manufacturer's instruction. Luminescence was read on a FlexStation 3 Microplate Reader (Molecular Devices) and analysis was performed with SoftMax Pro software. Curve fitting was performed using Prism.

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