PNAS
www.pnas.org

Supplementary Information for

Progression from remodeling to hibernation of ribosomes in zinc-starved mycobacteria

Yunlong Li[1], Jamie Corro[1,2], Christopher Palmer[3,4], Anil K. Ojha[1,2*]

*Corresponding authors:
anil.ojha@health.ny.gov

This PDF file includes:

Supplementary text
Figs. S1 to S11
Tables S1 and S2
References for SI reference citations

www.pnas.org/cgi/doi/10.1073/pnas.2013409117

Supplemental Text (Materials and Methods)

Bacterial growth medium

Unless specified, *M. smegmatis* (mc^2155) and *M. tuberculosis* strains (an attenuated mc^27000 strain and a virulent Erdman stain) were routinely maintained in Middlebrook 7H9 + 10%ADC + 0.05% Tween-80 and 7H9 + 10%OADC + 0.05% Tween-80, respectively. Pantothenate (100 µg/mL) was added for cultures of *M. tuberculosis* (mc^27000). 7H10ADC and 7H11OADC agar plates were used for recombinant selections and colony growth of *M. smegmatis* and *M. tuberculosis*, respectively. For experiments related to analysis of ribosomes, cells were cultured in Sauton's medium with either zinc or zinc-chelator, N,N,N,N′ -Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) added as specified. Pellicle biofilms of *M. tuberculosis* (mc^27000) were cultured in detergent-free Sauton's medium, either with 1mM $ZnSO_4$ or without supplemental zinc. Cells from log-phase planktonic cultures were inoculated at a 1:100 dilution into 4 mL of detergent-free Sauton's medium in a 12-well polystyrene plate and incubated without shaking for 7-weeks at 37 ºC in humidified conditions. *Escherichia coli* strains (GC5 and BL21) were grown in LB broth or on LB agar plates at 37 °C. As necessary, 25 µg/mL zeocin, 20 µg/mL kanamycin,150 µg/mL (for *M. smegmatis* and *E. coli*) or 50 µg/mL (for *M. tuberculosis*) hygromycin, 50 µg/mL carbenicillin, and 5 µg/mL apramycin were used for selection.

Construction of recombinant plasmids and strains

Recombinant plasmids and bacterial strains used in this study are listed in SI Appendix,

Table S1. All target genes in *M. smegmatis* were mutated with a *zeo*^{r} marker using a modified version of the PCR-based recombineering strategy as described previously (1). Oligonucleotides used for mutant constructions are provided in SI Appendix Table S2. Mutants were selected on 7H10ADC plate with 25 µg/mL zeocin, and the genotypes of the mutants were confirmed by PCR using primer pairs corresponding to sequences upstream or downstream of the corresponding target genes and the *zeo*^{r} cassette. Deletion mutants of Rv3241c (*mpy*$_{Mtb}$) in virulent (Erdman) and attenuated (mc^{2}7000) strains of *M. tuberculosis*, and Rv0106 (*mrf*$_{Mtb}$) in mc^{2}7000 were constructed by a phage-based gene-replacement method as described earlier (2). The phAE87-based cosmid containing allelic exchange substrates for Rv3241c and Rv0106 (a gift from Dr. William Jacobs) were electroporated into *M. smegmatis* (mc^{2}155) and incubated at 30 ºC to recover recombinant plaques. Phages recovered from the plaques were transduced into *M. tuberculosis* strains to recover recombinants on 7H11OADC plates with 50 µg/mL hygromycin. Recombinant colonies were confirmed by PCR and DNA sequencing for the expected genotype. Integrative plasmids for expression of recombinant genes used either pMH94 (*kan*^{r}*),* carrying the attachment site of L5 mycobacteriophage (1), or pTTPla (kan^r) (a gift from Dr. Graham Hatfull), carrying the attachment site for mycobacteriophage Tweety (3), as the vectors. The integrative vector pTTPla (*apr*^{r}) was constructed by replacing the *kan*^{r} cassette in pTTPla with an *apr*^{r} cassette using PstI and AsiI sites. A hygromycin resistant derivative of pJL37 – pJL37(hyg^r) – was constructed by replacing the *kan*^{r} cassette flanked by SpeI and NheI sites in pJL37, with the *hyg*^{r} cassette, excised using XbaI and NheI from pYUB854.

Plasmids for CRISPRi based depletion of Clp proteases, provided by Drs. Keith Derbyshire and Todd Gray, were constructed as a part of the Mycobacterial Systems Resource (Canestrari et al. manuscript under preparation). Briefly, small guide RNA (sgRNA) were designed 5' to the optimal PAM sites in *clpP1* and *clpP2*. Oligoncleotide templates based on the sgRNA were PCR amplified and cloned by in-fusion into the BsmB1 site of pJR962 (4), an L5-attP based integrative vector (*kan*^{r}) harboring tetracycline (Tet)-inducible sgRNA and dCas9 systems. The resulting plasmids (pYL205 and pYL206) were sequenced to confirm the sgRNA sequence, and electroporated into a Δ*zur* strain.

Recombinant protein expression, purification and analysis

For purification of His-tagged recombinant Mpy (rMpy), the *mpy* (Msmeg_1878) gene was cloned into pET21b vector using NdeI and XhoI (pYL140), and introduced into BL21 (DE3) pLysS cells for Mpy expression. The transformed BL21 cells were grown in 500 mL of LB medium with 50 µg/mL of carbenicillin until late log phase (OD_{600} of 0.8) at 37 °C, and rMpy expression was induced with 1 mM IPTG for an additional 4 hours at 37°C. Cells were harvested by centrifugation at 8000 rpm in a Thermo Scientific™ Fiberlite™ F12-6 × 500 fixed-angle rotor for 20 minutes, and were resuspended in 20 mL of N-I buffer (50 mM Tris pH 8.0, 300 mM NaCl, 5% glycerol, 10 mM imidazole, 1 mM PMSF) prior to sonication at the 30% amplitude for 20 cycles of 10 second pulse every 10 second interval. The sonicate was clarified by centrifugation (13000 rpm, 20 minutes, 4 °C, Thermo Scientific™ F21-8 x 50y fixed-angle rotor) and the supernatant was mixed with 2 mL Ni-NTA resin (pre-equilibrated with N-I buffer) for 1 hour at 4 °C. The protein-bound

95 resin was washed five times with 10 mL of N-II buffer (50 mM Tris pH 8.0, 300 mM NaCl,
96 5 % glycerol, 40 mM imidazole). Mpy_{6XHis} was eluted with 6 mL N-III buffer (50 mM Tris-
97 pH 8.0, 300 mM NaCl, 5% glycerol, 250 mM imidazole). Imidazole was removed by
98 dialysis against the storage buffer (50 mM Tris pH 8.0, 300 mM NaCl and 50% glycerol).

99 For purification of FLAG-tagged Mrf from high-zinc cultures, YL27 strain
100 expressing Mrf-FLAG from acetamide inducible promoter (pYL218) in CrisPRi-ClpP1
101 background was constructed. YL27 cells were grown in 500 mL Sauton's medium with 1
102 mM $ZnSO_4$ until an OD of 0.8, when 0.2 % (vol/vol) acetamide was added. Cells were
103 grown for a further 62 hours for induction of Mrf-FLAG before adding Atc (100 ng/mL) to
104 deplete ClpP1 for additional six hours. For purification of FLAG-tagged Mrf from a low-
105 zinc culture, YL18 cells were cultured in 500 mL Sauton's medium with 1 μM TPEN for
106 96-hour. Cells were harvested at 8000 rpm in Thermo Scientific™ Fiberlite™ F12-6 × 500
107 fixed-angle rotor for 20 minutes, and resuspended in 20 mL of buffer containing 50 mM
108 Tris pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF
109 and sonicated (amplitude-30% with 10 second pulses at 10 second intervals) for 60
110 minutes. The sonicate was clarified by centrifugation (13,000 rpm, 20 minutes, 4 °C,
111 Thermo Scientific™ F21-8 x 50y fixed-angle rotor) and the supernatant was mixed with 1
112 ml Pierce™ Anti-FLAG Magnetic Agarose overnight at 4 °C. The protein bound FLAG
113 agarose was washed five times with 10 mL buffer containing 50 mM Tris pH 8.0, 150 mM
114 NaCl, 10% glycerol and 1 mM EDTA. Mrf-FLAG was then eluted with 2 mL buffer
115 containing 50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM EDTA and 1.5 mg/mL
116 of 3XFLAG peptides (Pierce). The purified Mrf-FLAG proteins (20 μg) were transferred

on PVDF membrane for N-terminal sequencing by Edman degradation on ABI Precise 494HT instrument at the Tufts university proteomics core facility.

For purification of Mrf*, YL35 strain expressing Mrf*-FLAG was grown in Sauton's medium with 0.2% succinate and 1 μM TPEN for 28 hours, after which Mrf*-FLAG expression was induced by adding 0.2% acetamide for additional 16 hours. Mrf*-FLAG was then purified using the method described for purification of Mrf-FLAG.

Ribosome purification and analysis

Ribosomes were purified as described previously (1, 5), with a few modifications. As necessary, planktonic or biofilms of indicated strains of *M. smegmatis* (mc^2155) and Mtb (mc^27000) cells were grown in Sauton's medium, either with the specified amounts of zinc, without zinc, or with 1 mM TPEN. About 500 mL planktonic cells or four 12-well plates of Mtb (mc^27000) pellicle biofilms (spooled in PBS with 0.05% Tween-80) were harvested (8000 rpm, 20 minutes, 4 °C in Thermo Scientific™ Fiberlite™ F12-6 × 500 fixed-angle rotor) and flash frozen in liquid nitrogen. Frozen cells were pulverized 6 times for *M. smegmatis* or 8 times for Mtb at 15 Hz for 3 minutes in a mixer mill (Retsch MM400). The pulverized cell lysates were mixed with 20 mL of ice-cold HMA-10 buffer (20 mM HEPES-K pH 7.5, 30 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol) and centrifuged (15,000 rpm, 30 minutes, 4 °C, in Thermo Scientific™ F21-8 x 50y fixed-angle rotor). The supernatants were transferred into the Beckman PC ultracentrifuge tubes (Beckman 355618) and treated with 3 units/mL RNase-free DNase (Ambion) for one hour at 4 °C and then centrifuged (35000 rpm, 1 hour, 4 °C in a Beckman rotor Type

70Ti) to separate ribosomes from other low molecular weight cellular contents. The ribosome pellet (P1) was harvested and resuspended in 10 mL HMA-10 buffer and layered on top of a 10 mL 32% sucrose solution in HMA-10 buffer and centrifuged for 16 hours at 37,000 rpm in a Beckman Type 70Ti rotor. The supernatant was discarded and the ribosome pellet (P2) was briefly rinsed and then solubilized in HMA-10 buffer. The ribosomes were quantified by measuring absorbance at 260 nm. Ribosomes collected on the sucrose cushion were used for determining the relative abundance of FLAG-tagged Mrf, Mpy and other individual ribosomal proteins.

To analyze individual subunits, the ribosome pellet P2 was loaded on a 12 mL of 10-40% sucrose density gradient (SDG) and centrifuged for 5 hours at 4 °C, at 35,000 rpm in a Beckman SW41 rotor. For purifying individual subunits, the ribosome pellet P2 was layered on top of 40 mL sucrose gradients (10%-40%) in the HMA-10 buffer and centrifuged for 16 hours at 24,000 rpm in a Beckman rotor SW28. The gradients were fractionated using the density gradient fractionation system (Brandel). Fractions corresponding to 30S, 50S and 70S ribosomes were pooled separately and further pelleted by ultracentrifugation at 42,800 rpm for 3 hours in Beckman rotor Type 70Ti and resuspended in HMA-10 buffer. For Western blot analysis, 200 µL of individual fractions were mixed with 800 µL methanol, 200 µL chloroform and 600 µL H_2O sequentially, followed by centrifugation at 13000 rpm, 5 minutes in a benchtop centrifuge (Eppendorf 5415D). The top aqueous layer was gently removed, leaving the interface. The interface containing protein precipitates and the organic layer were mixed with 800 µL methanol and the mixture was centrifuged at 13,000 rpm for 5 minutes. The pellet was resuspended

160 in 1X SDS loading buffer for analysis by immunoblot.

161 For *in vitro* analysis of rMpy binding to ribosomes, 24 pmoles of ribosomes
162 collected on sucrose cushion (P2) were incubated with 24, 48 or 96 pmoles of rMpy in
163 HMA-10 buffer for 1 hour at 37 °C. The reactions were then layered on top of 5 mL SDG
164 (5%-30%) in HMA-10 buffer and centrifuged at 35,000 rpm for 2 hours and 45 minutes in
165 a Beckman rotor SW55. The fractions corresponding to 70S were pooled, precipitated by
166 chloroform methanol method as described above, and analyzed by immunoblot.

167 For large-scale purification of 30S and 50S subunits for subunit reassociation
168 studies, low magnesium (1 mM $MgCl_2$) buffer was used for dissociating the 70S ribosome
169 prior to SDG. Briefly, the ribosome pellet (P1) was resuspended in HMA-1 buffer (20 mM
170 HEPES-K pH 7.5, 30 mM NH_4Cl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol) and layered on
171 top of 40 mL SDG (10%-40%) in HMA-1 buffer and centrifuged for 16 hours at 24,000
172 rpm in a Beckman rotor SW28. The gradients were fractionated by the Brandel
173 fractionation system. The pooled 30S, 50S fractions were further pelleted by
174 ultracentrifugation at 42,800 rpm for 3 hours in Beckman rotor Type 70Ti and
175 resuspended in HMA-10 buffer. The subunits were quantified and stored at -80 °C until
176 further use.

177 For the ribosome reassociation assay in the presence or absence of rMpy, 36
178 pmoles each of the 30S (from low-zinc culture of either YL4 *or* YL25 strain) and 50S (from
179 low-zinc culture of YL4 strain*)* subunits were mixed either with or without 72 pmoles of
180 rMpy in HMA-10 buffer with 30 mM KCl. The mixture was incubated for 1 hour at 37 °C,
181 then layered on top of 12 mL 10%-40% sucrose gradients in HMA-10 buffer and

centrifuged at 35,000 rpm for 5 hours in a Beckman rotor SW41. The gradients were then fractionated using the density gradient fractionation system (Brandel). The 70S fractions were pooled and precipitated as described above. The abundance of rMpy in 70S ribosomes was determined by immunoblot.

In vitro translation assay

A synthetic DNA template (a gift from Drs. Todd Gray and Keith Derbyshire) containing a T7 promoter and a Shine-Dalgarno sequence cloned upstream of the Nano-Luc reporter coding sequence *was* used for the *in vitro* translation assay. The assay was performed using a PURExpress® Δ Ribosome Kit (New England BioLabs Inc.). The protein synthesis reaction was set up according to manufacturer's instructions with few modifications. Briefly, 2.0 µL of Solution A and 0.6 µL of Factor Mix from the kit were mixed with 12.5 nM of ribosomes purified on a sucrose cushion and 18 ng of DNA template in a total volume of 5 µL. After 1 hour of incubation at 37 °C, 2 µL of the reaction was mixed with 18 µL of H_2O, 20 µL luciferase assay buffer and 0.4 µL luciferin substrate (Nano-Glo® Luciferase Assay system, Promega Inc.). After 5 minutes of incubation at room temperature, relative luminescence unit (RLU) produced from the translated luciferase was measured according to manufacturer's instructions using a Veritas Microplate Luminometer (Turner Biosystems).

Immunoblotting

Culture aliquots of indicated *M. smegmatis* strains – 5 mL for ≥96h or 10 mL for early time points – were harvested at 4000 rpm for 10 minutes. The cell pellets resuspended

in 800 µL of lysis buffer (PBS, 1 mM PMSF, 0.1% Triton X-100, 1 mM EDTA) were disrupted by bead-beater, and centrifuged at 13000 rpm for 10 minutes. The concentrations of the cell lysates were determined by Bradford assay. Proteins equivalent to 200 µg from each lysate were then mixed with 25 µL 4X SDS loading buffer (100 µL total volume) and heated for 10 minutes at 95 °C. Equal amounts (20 µg) of denatured proteins from each lysates were resolved in 8% SDS-PAGE gel (For Mrf) or 12% SDS-PAGE gel (for GroEL, Mpy or r-proteins) and proteins were transferred to PVDF membranes. The membranes were blocked, washed and probed with indicated antibodies [GroEL (Enzo, rabbit 1:5000); S14c- (in-house, rabbit, 1:1000); Mpy (in-house, rabbit, 1:5000); FLAG-HRP (Genescript, rabbit, 1:5000); S13 (DSHB, mouse, 5 µg/mL corresponding to 1:100); 6XHis-tag (Thermo, mouse, 1:2000)]. As necessary, the membranes were incubated with secondary HRP conjugated antibody (1:5000). The membranes were developed with ECL reagents (Thermo fisher) and exposed to chemiluminescence films, developed and scanned. Densitometric analysis of protein bands were performed by Image J, and signal in each lane was quantified relative to a reference after normalizing the signals with the corresponding loading control, as indicated for each sample.

ICP-MS analysis

M. smegmatis cells of were cultured in Sauton's medium containing either 1mM $ZnSO_4$ or 1µM TPEN. At indicated time points, 10-30 mL aliquots were drawn out and cells were harvested by centrifugation. Harvested cells were washed thrice with doubled-deionized ($\geq$ 18.0 MΩ.cm) zinc-free water in acid-washed, zinc-free polypropylene tubes (Sarstetd

225 Inc.), and heat-killed for 20 minutes at 95 °C. Cells equivalent to 0.1-0.4 mg of dry weight
226 of biomass were washed with 5% v/v double-distilled HNO_3, and analyzed for zinc (Zn)
227 using a Thermo Scientific™ X Series 2 Inductively Coupled Plasma-Mass Spectrometer
228 (ICP-MS) at the Wadsworth Center's Trace Elements Laboratory (New York State
229 Department of Health, Albany, New York, USA). Eight multielement intermediate
230 calibration standards (containing Zn) were prepared from a NIST-traceable stock solution
231 (High Purity Standards, Charleston, SC, USA). Working standards and working liquid
232 samples were prepared by performing a 1+49 dilution (200 μL of sample diluted into a
233 final 10 mL volume) of the intermediate standard/media sample into a diluent solution.
234 The diluent solution contained 0.5% v/v double-distilled nitric acid, 0.005% Triton™ X-
235 100, 1 μg/L Ga, and 1 mg/L Au. Gallium served as the internal standard for Zn, i.e., the
236 measured ^{66}Zn signal for each sample was corrected against the ^{71}Ga response to
237 compensate for any instrumental drift encountered during an analytical run. Harvested
238 cells of known weight were digested at room temperature in 5 mL double-distilled HNO_3
239 overnight, to ensure complete sample solubilization. The solubilized sample was then
240 diluted and analyzed. All of the plasticware used in this study were acid washed with 5%
241 v/v double-distilled HNO_3, to minimize any background Zn contamination prior to use.
242 Additionally, all of the samples were prepared for analysis in a SterilGARD® e3 Class II,
243 Type A2 Biological Safety Cabinet (The Baker Company, Sanford, MA) which was
244 determined to meet ISO 5 (Class 100) clean standards. All other preparation work was
245 performed under Class 100 clean room conditions or better (Terra Universal, Fullerton,
246 CA). Method accuracy was assessed throughout the study by analyzing three levels of

247 internal quality control material (IQC), with Zn target values established against National
248 Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs).

249 For determining zinc in Mrf, only polystyrene tubes and polypropylene beakers and
250 cylinders were used, which were rinsed with zinc-free water several times before use.
251 The contaminating zinc in the exchange buffer (B) (50 mM Tris pH 8.0, 150 mM NaCl,
252 10% glycerol) was removed by Chelex-100 (Sigma). YL18 strain expressing Mrf-FLAG
253 was grown in Sauton's medium with 1 μM TPEN for 4 days. The FLAG-tagged proteins
254 were purified as described earlier, and dialyzed in zinc-free exchange buffer. After
255 dialysis, 100μl exchange buffer (B0) and each protein sample (P0) were kept aside for
256 ICP-MS. A known quantity of the remaining protein in the sample was further dialyzed in
257 the exchange buffer containing 8-times molar excess of $ZnSO_4$ for 4 hours, followed by
258 three sequential dialysis with zinc-free exchange buffer for 4, 2 and 2 hours. After dialysis,
259 100μl of the exchange buffer (B1) from last dialysis was kept aside , and concentration of
260 protein in each sample (P1) after dialysis was determined. A second set of protein (P2)
261 for each sample was similarly treated with zinc. The zinc contents in all the buffer
262 (B0/B1/B2) and protein (P0/P1/P2) were determined by ICP-MS. The background value
263 in the buffers was subtracted from each corresponding sample before determining zinc
264 to protein ratio for the protein.

265 **Immunoprecipitation**

266 The strains YL16 or YL17 used for testing interaction between Mrf and ClpS, were grown
267 in 500 mL Sauton's medium either with 1mM $ZnSO_4$ until an OD_{600} 0.8, or with 1μM

TPEN for 4 days. The zinc-rich cultures were induced for 6 hours with 100 ng/mL anhydrotetracycline (Atc) to inhibit ClpP1. The cells were harvested at 8000 rpm (Thermo Scientific™ Fiberlite™ F12-6 × 500 fixed-angle rotor) for 20 minutes, and resuspended in 20 mL of buffer A (50 mM Tris pH 8.0 150 mM NaCl, 10 % glycerol, 1mM PMSF and 10 mM imidazole. After sonication (amplitude 30% and 10 sec on/off cycle on ice) for 60 minutes, cellular debris was removed by centrifugation at 13000 rpm (Thermo Scientific™ Fiberlite™ F12-6 × 500 fixed-angle rotor) for 20 minutes, and the supernatants were incubated with 2 mL Ni-NTA resin (pre-equilibrated in buffer A) for 1 hour. The protein-bound Ni-NTA resin was washed with 10 mL buffer A containing 40 mM imidazole five times. The protein bound on the Ni-NTA resin was eluted with buffer A containing 250 mM imidazole. The eluted samples, along with lysates and washes as controls, were analyzed for $ClpS_{6XHis}$ and Mrf-FLAG by immunoblot using anti-FLAG and anti-6xHis antibodies.

For analysis of ^{35}S-labeled Mrf by immunoprecipitation (IP), cells of YL7 strain were grown in 12 mL Sauton's medium with 1 µM TPEN at 37°C for 4 days on a shaker at the speed of 200 rpm. Cells from 6 mL culture were harvested and the supernatant (S_0) was filter-sterilized and saved, while 60 µCi ^{35}S-Methionine (PerkinElmer) was added to the remaining 6 mL culture and cells were labeled for 2 hours. After 2 hours, labeled cells were washed with PBS + 0.05% Tween-80 three times, and resuspended in S_0 supernatant, and split into three 2 mL aliquots. Cells from one aliquot were immediately harvested (13000 rpm for 2 minutes) and frozen for downstream processing. Zinc was supplemented in one of the remaining two aliquots at the final concentration of 1 mM

290 $ZnSO_4$, while the other aliquot was untreated. Both aliquots were incubated for 37°C for
291 1 hour, after which cells were harvested (13000 rpm for 2 minutes) and processed for IP.
292 For IP, harvested cells were resuspended in 800 µL lysis buffer (50 mM Tris pH 8.0, 150
293 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF), and disrupted by
294 bead-beater. Cell lysates were centrifuged at 13000 rpm for 10 minutes, and supernatants
295 equivalent to 12000000 cpm were diluted to 500 µL with the lysis buffer and incubated
296 with either 100 µL FLAG magnetic beads (Thermal Fisher) or 5 µg of GroEL antibody
297 (Enzo Life Sciences) for 2 hours at room temperature. For IP with GroEL, the cell lysates
298 with GroEL antibody mixture were also mixed with 50 µL Protein A/G magnetic beads
299 (Thermo Fisher) and further incubated for 1 hour at room temperature. The magnetic
300 beads were collected with a magnetic stand and the supernatant was discarded. The
301 beads were washed three times with the wash buffer (50 mM Tris pH 8.0, 150 mM NaCl,
302 10% glycerol, 1 mM EDTA, 0.1% Triton X-100) and one time with distilled water. The
303 beads were resuspended in 30 µL 1X SDS protein loading buffer and heated at 95°C for
304 10 minutes. The supernatants were collected after separating the beads using a magnetic
305 stand. The collected supernatants were resolved on 8% SDS-PAGE gel, fixed in buffer
306 containing 10% glacial acetic acid, 20% methanol for 30 minutes, dried at 80 °C for 1
307 hour, and autoradiographed.

308 **Analysis of *in vivo* protein synthesis with ^{35}S-methionine**

309 YL2 strain was grown at 37 °C in Sauton's medium with 1 µM TPEN. Two mL aliquots of
310 cells were taken from 15-, 24- and 96-hour post inoculation and mixed with 20 µCi ^{35}S-
311 Methionine (PerkinElmer) at 37 °C for 10 minutes, after which aliquots were flash frozen

312 in dry ice. Frozen cells were harvested (13000 rpm for 2 minutes) and resuspended in
313 300 µL of lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM EDTA,
314 0.1% Triton X-100, 1 mM PMSF), and disrupted by bead-beater (5 cycles of 30 sec). Cell
315 lysates were centrifuged at 13000 rpm for 10 minutes, and protein concentration in each
316 sample was determined. Five micrograms of total proteins from each sample were
317 resolved on a 12% SDS-PAGE gel. The gel was first stained with Coomassie blue, then
318 fixed in buffer containing 10% glacial acetic acid and 20% methanol for 30 minutes, dried
319 at 80 °C for 1 hour, and autoradiographed.

320 **qRT-PCR**

321 Total RNA extraction and qRT-PCR were performed as previously described (1). Briefly,
322 10 ng total cDNA prepared from DNA-free RNA and 1µL of 5 µM primers were mixed with
323 10 µL 2X SYBR Green master mix. Real time PCR was performed on an ABI 7000
324 instrument at: 95 °C 10min, followed by 40 cycles of 95 °C 10 sec and 60 °C 1min. SigA
325 and non-template controls were set up for the endogenous and negative controls,
326 respectively.

327 **Zinc-binding assay using FluoZin-3**

328 To determine the linear range of FluoZin™-3 assay (cell impermeant, Invitrogen), 10 µM
329 of the probe was mixed with 0.1, 0.5, 1, 2, 4 or 6µM of $ZnSO_4$ in 100µl of zinc-free buffer
330 (buffer B: 50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol: chelated with Chelex-100) and
331 the mixtures were incubated at 37 °C for 10 minutes. The zinc-free buffer mixed with
332 FluoZin™-3 was used a background. After incubation, fluorescence was measured at

333 excitation/emission of 494 nm/516 nm. The background value was subtracted from the
334 experimental values, and the signal was plotted as a function of zinc concentration.

335 To determine zinc affinity of Mrf-FLAG and Mrf*-FLAG, proteins were purified in
336 the presence of EDTA from low-zinc cultures of YL18 and YL35, as described earlier in
337 this document. The purified proteins were dialyzed against the zinc-free buffer (buffer B)
338 to remove EDTA. A 2-fold serial dilutions of the proteins in 0.004 to 5 µM range were
339 mixed with 5 µM $ZnSO_4$ in 100 µL zinc-free buffer (buffer B) and the mixtures were
340 incubated at room temperature for 30 minutes. After incubation, the reactions were
341 transferred to Pierce™ Protein Concentrators PES (10K MWCO, 0.5 mL) and centrifuged
342 at 5000g for 3 minutes. The filtrates containing free zinc ions in collection tubes were
343 adjusted to 100 µL with zinc-free buffer (buffer B) and mixed 10 µM FluoZin™-3 before
344 incubated at 37°C for 10 minutes. A 100 µL reaction containing 5 µM $ZnSO_4$ and 10 µM
345 FluoZin™-3 in zinc-free buffer was set up as the control. After 10 minutes of incubation
346 fluorescence was measured as described above. The % Zn_{free}/Zn_{total} in a sample was
347 determined $fluorescence_{sample}/fluorescence_{control}$ x 100. The % Protein-Zn complex was
348 determined by 100% – (% Zn_{free}/Zn_{total}).

349 **Ribosome degradation assay**

350 The assay was performed as described earlier (1, 6). Briefly, pellicle biofilms of $mc^2$7000,
351 Δ*mpy*$_{Mtb}$ and a complementing strain were cultured in Sauton's medium with 1 µCi/mL of
352 [^{3}H]-uridine (Perkin Elmer), 0.1 mM uridine (Sigma-Aldrich), and either 1 mM $ZnSO_4$ or
353 without supplemental zinc for 7 weeks. The pellicles were harvested, washed three times
354 with PBS + 0.1% Tyloxapol, and resuspended in PBS containing 0.05% Tyloxapol, 0.1

355 mM uridine, 100 μg/mL pantothenate. 1mM $ZnSO_4$, was added to PBS for cells that were
356 cultured in zinc-rich medium. The acid-soluble radioactivity and the total radioactivity in
357 the culture were determined by Liquid Scintillation Analyzer (Perkin Elmer, Tri-
358 Carb@4910TR), as previously described (1, 6).

359 **Antibiotic sensitivity assay**

360 Pellicles of mc^27000 and derivative strains were cultured in Sauton's medium either with
361 1 mM $ZnSO_4$ (for high-zinc) or without supplemental zinc for 7 weeks. Cells in the pellicles
362 were washed three times with PBS + 0.1% tyloxapol. About 10^8 CFU/mL of cells were
363 resuspended in PBS with 0.05% tyloxapol and zinc (either 1 mM $ZnSO_4$ for high-zinc
364 cultures or no supplemental zinc for low-zinc cultures). Streptomycin (5 μg/mL) was then
365 added to the cultures and viable cells were enumerated by plating dilutions on
366 7H11OADC at the indicated time points.

367 **Animal infection and drug treatment**

368 All animal experiments were approved by the Institutional Animal Care and Use
369 Committee (IACUC) of the Wadsworth Center. Using a Glas-Col aerosolizer (Glas-Col
370 Inc.), 60-day old C3HeB/FeJ female and male mice were infected with aerosolized
371 particles of *M. tuberculosis* (Erdman) and its derivative mutants at a density of 10^6
372 CFU/mL. Default aerosolization settings of the instruments were used for each cycle of
373 exposure: 10 minutes nebulization, 15 minutes of cloud time and 10 minutes of exhaust.
374 Three mice from each group were euthanized after 24 hours of infection to determine the
375 initial bacterial burden, which ranged from 50-100 bacilli per lung. At 6-week post-

infection, four mice from each group were euthanized for the analysis of bacterial burden. Half of the mouse population from each group were administered with streptomycin subcutaneously @200 mg/kg/day, 5 days a week, for up to 8 weeks. Remaining mice from each group were administered with PBS as placebo. At 4- and 8-weeks post antibiotic treatment, five mice from each group were euthanized, and lung homogenates were plated on 7H11OADC to determine the bacterial burden.

Table S1: List of plasmids and strains

	Plasmids	Remarks	Reference
	pJl37	P_{hsp60}-based expression vector for mycobacteria; *kan*^{r}	(1)
	pMH94	L5-attp-based integrative vector for mycobacteria; *kan*^{r}	(1)
	pYUB854	Cosmid vector, *hyg*^{r}	(1)
	pTTPla	Tweety-attp-based integrative vector for mycobacteria*; kan*^{r}	(3)
	pJV53-SacB	Sucrose-sensitive marker SacB cloned in pJV53 @ SpeI site; *kan*^{r}	(1)
	pLAM12	$P_{acatamidase}$-based expression vector for mycobacteria; *kan*^{r}	(7)
	pYL3	*kan*^{r} cassette in pMH94 @EcoRV & NotI sites is replaced by *hyg*^{r} cassette from pYUB854; *hyg*^{r}	(1)
	pYL11	*kan*^{r} cassette in pJL37 @ NheI & SpeI is replaced by *hyg*^{r} cassette from pYUB854 @ NheI & XbaI; *hyg*^{r}	(1)
	pYL42	*MSMEG_6065-6070* including P^{zurbox} cloned in pYL3 @ SacI & XbaI; *hyg*^{r}	(1)
	pYL40	Internal deletion in *mrf (Msmeg_6069*) on pYL42 backbone @ SacI & XbaI, *hyg*^{r}	(1)
	pYL140	*mpy* (Msmeg_1878) cloned in pET21b @ NdeI & XhoI; *amp*^{r}	This study
	pYL141	C-terminally FLAG-tagged *mrf* and its 543 bp UPS with native promoter (P^{zurbox}) cloned in pJL37 @ XbaI & NheI sites; *kan*^{r}	(1)
	pYL148	*Rv_3241c* + 800 bp UPS cloned in pMH94 @ SacI & XbaI; *kan*^{r}	This study
	pYL149	*Rv_0106* + 542 bp UPS cloned in pMH94 @ SacI & XbaI; *kan*^{r}	This study
	pYL150	C-terminally FLAG-tagged *mrf,* and its 543 bp UPS with mutations changing P^{zurbox} into constitutive (P^{const}), cloned in pJL37 @ XbaI & NheI sites ; *kan*^{r}	This study
	pYL155	C-terminally FLAG-tagged *mrf* with P^{zurbox} cloned in pTTPla @ SacI & XbaI sites; *kan*^{r}	This study
	pYL156	C-terminally FLAG-tagged *mrf with* P^{const} cloned in pTTPla @ SacI & XbaI sites; *kan*^{r}	This study
	pYL178	pYL155 carrying $Mrf_{H36A/H41A/H62A/H81A/C64A/C67A}$ mutations (Mrf*); *kan*^{r}	This study
	pYL180	*kan*^{r} cassette in pTTPla replaced with the apramycin resistance (*apr*^{r}) cassette @ PstI and AsiSI sites; *apr*^{r}	This study
	pYL181	C-terminally FLAG-tagged *mrf* with P^{zurbox} cloned in pYL180 @ SacI & XbaI sites; *apr*^{r}	This study
	pYL205	L5-attp-based integrative vector carrying tet-inducible dCas9 and gRNA complementary to Clpp2; *kan*^{r}	This study
	pYL206	L5-attp-based integrative vector carrying tet-inducible dCas9 and gRNA complementary to Clpp1; *kan*^{r}	This study
	pYL210	*mrf* with FLAG tag at C- terminal cloned in plam12 @ NdeI & NheI; kanr	This study
	pYL212	*kan*^{r} cassette in pYL178 replaced with the *apr*^{r} cassette @ PstI and AsiSI sites; *apr*^{r}	This study
	pYL216	*Msmeg_4910* with HIS_6-tag at C- terminal cloned in pYL11 @ NdeI & NheI; *hyg*^{r}	This study
	pYL218	*kan*^{r} cassette in pYL210 @ SpeI & NheI is replaced by *hyg*^{r} cassette from pYUB854 @ NheI & XbaI; *hyg*^{r}	This study
	pYL222	pLAM12 carrying $Mrf_{H36A/H41A/H62A/H81A/C64A/C67A}$ mutations (Mrf*); *kan*^{r}	This study
Name	**Strains**	**Remarks**	**Reference**
	mc^2*155*	High-Frequency Transformation strain of *M. smegmatis* as parent wild-type	(1)
	Δ*c-*	Unmarked Δ*Msmeg_6065-6070* in mc^{2}155	(1)
WT	wild type	Unmarked Δ*c-* operon (*Msmeg_6065-6070)* harboring pYL42; *kan*^{r}	This study
YL1	Δ*mrf*	Unmarked Δ*c-* operon harboring pYL40; *hyg*^{r}	This study
YL2	Δ*mrf*comp	Unmarked Δ*c-* operon harboring pYL40 and pYL155; *hyg*^{r}*, kan*^{r}	This study
YL3	Δ*mpy*	Unmarked Δ*mpy* (*Msmeg_1878)* in mc^{2}155	(1)
YL4	Δ*mpy*/Δ*mrf*	Unmarked Δ*c-* operon /Δ*Msmeg_1878* harboring pYL40; *zeo*^{r}*, hyg*^{r}	This study
YL5	Δ*zur*	Unmarked Δ*zur (Msmeg_4487)* in mc^{2}155	(1)
YL6	Δ*zur*/Δmrf	Unmarked Δ*c- operon* Δ*zur* harboring pYL40; *hyg*^{r}	This study

YL7	Δ*mrf: pYL156*	Δ*mrf harboring pYL156; kan*^{r}	This study
YL8	Δ*mrf: pYL150*	Δ*mrf harboring pYL150; kan*^{r}	This study
YL9	CRISPRi-ClpP1	Δ*zur* harboring pYL181 and pYL206; *apr*^{r}*, kan*^{r}	This study
YL10	CRISPRi-ClpP2	Δ*zur* harboring pYL181 and pYL205; *apr*^{r}*, kan*^{r}	This study
YL11	Δ*zur*/Δ*mrf*/Δ*clp*S	Δ*c*- operon/Δ*zur*/Δ*clps* harboring pYL40; *kan*^{r}	This study
YL12	$Mrf_{H36A/H41A/H62A/H81A}$	Δ*zur* /Δ*mrf* harboring pYL185; *hyg*^{r}*, kan*^{r}	This study
YL13	$Mrf_{C64A/C67A}$	Δ*zur* /Δ*mrf* harboring pYL157; *hyg*^{r}*, kan*^{r}	This study
YL14	*Δclps/Δmrf*	Unmarked *Δmsm*$_c$*-/ΔMsmeg_4910* harboring pYL40*; hyg*^{r}	This study
YL15	YL14*:* pYL155	*Δclps/Δmrf* harboring pYL40 and pYL155; *hyg*^{r}*, kan*^{r}	This study
YL16	YL9: pYL216	Δ*zur* harboring pYL181, pYL206 and pYL216; *hyg*^{r} *apr*^{r}*, kan*^{r}	This study
YL17	CRISPRi-ClpP1-Mrf*	Δ*zur* harboring pYL206, pYL212, pYL216; *hyg,*^{r} *apr*^{r}*, kan*^{r}	This study
YL18	YL1:pYL141	Δ*c*- operon harboring pYL40 and pYL141; *hyg*^{r}*, kan*^{r}	This study
YL19	YL6:pYL141	Δ*c*- operon/Δ*zur* harboring pYL40 and pYL141; *hyg*^{r}*, kan*^{r}	This study
YL20	YL6:pYL155 (Mrf)	Δ*c*- operon/Δ*zur* harboring pYL40 and pYL155; *hyg*^{r}*, kan*^{r}	This study
YL21	YL6:pYL178 (Mrf*)	Δ*zur*/Δ*mrf* harboring pYL40 and pYL178; *hyg*^{r}*, kan*^{r}	This study
YL22	*Δclps*	Unmarked Δ*Msmeg_4910* in mc^{2}155	This study
YL24	YL11:pYL155	Δ*c*- operon/Δ*zur*/Δ*clps* harboring pYL40; *hyg*^{r} *kan*^{r}	This study
YL25	YL4:pYL155	Δ*c*- operon/Δ*mpy* harboring pYL40 and pYL155; *zeo*^{r}*, hyg*^{r}*, kan*^{r}	This study
YL26	YL4.pYL141	Δ*c*- operon /Δ*mpy* harboring pYL40 and pYL141; *zeo*^{r}*, hyg*^{r} *kan*^{r}	This study
YL27		mc^{2}155 harboring pYL218 and pYL206; *hyg*^{r}*, kan*^{r}	This study
YL28	YL1:Mrf*	Δ*mrf* harboring pYL178; *hyg*^{r} *kan*^{r}	This study
YL35	YL1:pYL222	Δ*mrf* harboring pYL222; *hyg*^{r} *kan*^{r}	This study
	mc^{2}7000	*M. tuberculosis* H37Rv:*ΔRD1*:*ΔpanCD* as parent wild type	(1)
	Mtb(Erd)	*M. tuberculosis* Erdman, ATCC 35801	(1)
YL29	*mc^{2}7000:Δmpy*$_{mtb}$	*ΔRv_3241c* in mc^{2}7000	This study
YL30	*Δmpy*$_{mtb}$comp	*ΔRv_3241c* harboring pYL148 in mc^{2}7000	This study
YL31	*Δmrf*$_{mtb}$	*ΔRv_0106* in mc^{2}7000	This study
YL32	*Δmrf*$_{mtb}$comp	*ΔRv_0106* harboring pYL149 in mc^{2}7000	This study
YL33	*Δmpy*$_{mtb}$ (Erd)	*ΔRv_3241c* in Mtb(Erd)	This study
YL34	*Δmpy*$_{mtb}$comp (Erd)	*ΔRv_3241c* harboring pYL148 in Mtb(Erd)	This study

Table S2: List of oligonucleotides

Name	Sequence	Used in:
p1878-NF	AGAAACGAGTTGCCACATATGTCAAGCCATTCGATG	pYL140
p1878-HR	GTGCTGGTCGGCAAGCTTGGCCAGGCGGATCAGCCC	
pRv3241c-F	ATGTCAAGGCTACATATGGATTCAGGTCAGGTTCTG	pYL146
pFlagRv3241c-NR	GGCCGGGCTAGCTCACTTATCGTCGTCATCCTTGTAATCCGCCAGACGGATCAA	
pRv3241c-SF	ATTTGTTCGCGGAGCTCCGCTACGCGGGCGTGCGTC	pYL148
pRv3241c-XR	GCGGCGCGCGCCTCTAGATCACGCCAGACGGATCAA	
pRv0106-SF	GAGTCCTTGATGGAGCTCCGGTCTTGCACTCGTGGG	

pRv0106-XR	GGATGGATACCGTCTAGATCATCGAGATTCTCCTGA	pYL149
*P*zurbox 6069-XF	CGCGCCGTTCGTCTAGACGCTGCACCAGTTCTCGCC	
p6069FLAGNR	GGCCGGGCTAGCTCACTTATCGTCGTCATCCTTGTAATCCGATTGCTCTCCTGT	pYL150
*P*zurbox 6069-SF	CGCGCCGTTCGGAGCTCCGCTGCACCAGTTCTCGCC	pYL155;156,157;178;181,185
p6069FLAGXR	GGCCGGTCTAGATCACTTATCGTCGTCATCCTTGTAATCCGATTGCTCTCCTGT	
*P*zurbox 6069-XF	CGCGCCGTTCGTCTAGACGCTGCACCAGTTCTCGCC	
p6069-HR	GGTGGATGCCGGAAGCTTCGATTGCTCTCCTGTCTG	
His1-R	GGTGCGGACGACGACAGCGCCGTCGAAGGTGGCGCTGACGAGCAGGGT	pYL157; pYL178; pYL185
His1-F	ACCCTGCTCGTCAGCGCCACCTTCGACGGCGCTGTCGTCGTCCGCACC	
His2-R	GGCCGAGACGGCTCCGGCCACCAGCTCAAGTAC	
His2-F	GTACTTGAGCTGGTGGCCGGAGCCGTCTCGGCC	
His3-R	CACGTCGGCGCGCCGGGCCAGACGGCGAAGCAGGAT	
His3-F	ATCCTGCTTCGCCGTCTGGCCCGGCGCGCCGACGTG	
p6069-CXXCR	ATCGTCACGCACGGTGGCCGAGACGGCTCCGTGCACCAGCTC	
p6069-CXXCF	GAGCTGGTGCACGGAGCCGTCTCGGCCACCGTGCGTGACGAT	
pApr-PF	ATAACTTCGTATCTGCAGCATTATACGAAGTTATAG	pYL180
pApr-SR	GTTACAATTCGCGATCGCTCGACTCGTTCTCCGCTC	
P6069(ATG)-NF	AAACCGGGAGGTCATATGCGTACGCCGGTAGTGCTG	pYL210, pYL222
p6069-NFLAGR	GGATGCGCTAGCTCACTTATCGTCGTCATCCTTGTAATCCGATTGCTCTCCTGT	
p4910-NF	GGGTATTGTGGCCATATGGTTACACCGGCGAAGGCG	
p4910-HISNR	CTTGCGCACGCTAGCTCAGTGGTGGTGGTGGTGGTGGCGGTCCTGTTGCAT	pYL216
p4910ZeoA	TGCGTCAGGGCTTCGAGGAACCGCG	Δ*clpS*
p4910ZeoB	TGGTGAGGGAGATGAGGTCTGAAGGACATCGACTTCTTCT	
p4910ZeoC	GTTGAGGTGTGAGGTGTGCTGAAGTCCAAACTGCACGCCG	
p4910ZeoD	GGCCGGAAGAAATCCGGCAGCAGAC	
p4910SA	GTAGTCGAGCGTGTCGGCGTCGAGA	
p4910SD	TTGAGGCTCTCCGCGGTGCCCGA	
RT-6069F	TCCAGCGAGCTTGCCTACACGGA	RT-PCR_MsMrf
RT-6069R	ACGCAGTGCGTCGAGGATGTCGT	
RT-6068F	CCGAGACGCTGGGACCCCAATA	RTPCR_MsL28$_{C-}$
RT-6068R	GGTCGATCACCTTGATGCCTT TTGC	
RT-sigA(MS)F	AAGGCGTCCGGCGACTTCGTGT	RTPCR_MsSigA
RT-sigA(MS)R	TCCACCTCTTCTTCGGCGTTGAGC	
RT-2058F	GTCCGCCCACTGCCAAGTCACC	

RT-2058R	CGTGGACACCCGCAGCCGAATGC	RTPCR_MtL28$_{C-}$
RT-sigA(TB)F	TGGCAGCGACCAAAGCAAGCACG	RTPCR_MtbSigA
RT-sigA(TB)R	GGAGCCACTAGCGGACTTCGCC	
RT-clpp1F	CTCGGTGTACGAGCGGCTGC	RTPCR_MsClpp1
RT-clpp1R	CAGGTGGATGTCCTTGGTCGGAT	
RT-clpp2F	TCCGTCACTGGACGCCCGGCTG	RTPCR_MsClpp2
RT-clpp2R	CACGCCGAGGAAGATGATGCGT	

388

Supplemental References

1. Y. Li *et al.*, Zinc depletion induces ribosome hibernation in mycobacteria. *Proc Natl Acad Sci U S A* **115**, 8191-8196 (2018).
2. Y. Yang *et al.*, A hydrolase of trehalose dimycolate induces nutrient influx and stress sensitivity to balance intracellular growth of Mycobacterium tuberculosis. *Cell host & microbe* **15**, 153-163 (2014).
3. G. F. Hatfull, Mycobacteriophages. *Microbiol Spectr* **6** (2018).
4. J. M. Rock *et al.*, Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nat Microbiol* **2**, 16274 (2017).
5. P. Mehta, P. Woo, K. Venkataraman, A. W. Karzai, Ribosome purification approaches for studying interactions of regulatory proteins and RNAs with the ribosome. *Methods in molecular biology* **905**, 273-289 (2012).
6. M. A. Zundel, G. N. Basturea, M. P. Deutscher, Initiation of ribosome degradation during starvation in Escherichia coli. *RNA* **15**, 977-983 (2009).
7. J. C. van Kessel, G. F. Hatfull, Recombineering in Mycobacterium tuberculosis. *Nature methods* **4**, 147-152 (2007).

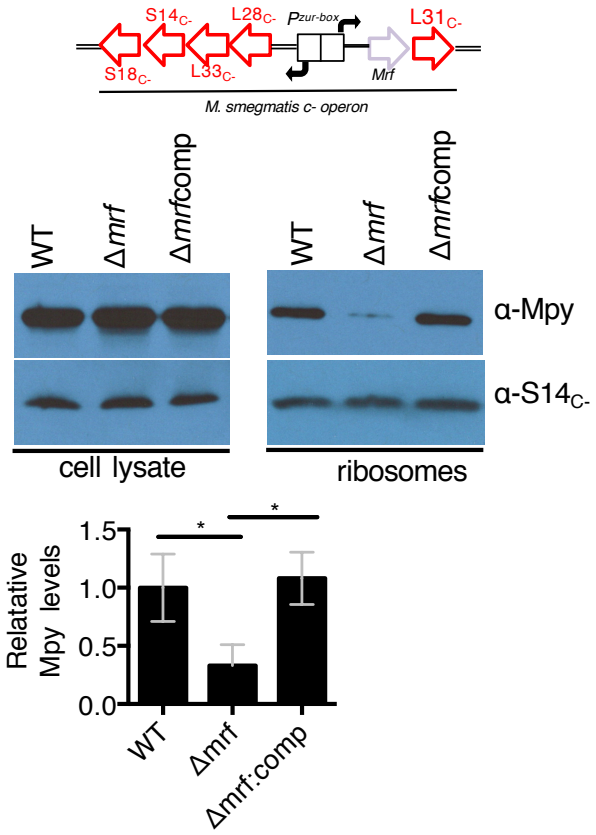


Figure S1: FLAG-tagged Mrf complements Δ*mrf* mutant. Levels of Mpy associated with the ribosome in WT, Δ*mrf* , and complemented strains (YL2). WT denotes the parent strain with wildtype phenocopy, carrying unmarked deletion in the *c-* operon (top) and expressing the *c-* operon on an integrative plasmid (pYL42). Δ*mrf* strain is a derivative of WT carrying an in-frame deletion in *mrf* in the operon (pYL40); the complemented strain (YL2) is Δ*mrf* expressing FLAG-tagged Mrf (called Mrf in the manuscript) from the native promoter on an integrative plasmid. The indicated strains were cultured in low-zinc Sauton's medium (1μM TPEN) for 96 hours prior to Mpy analysis in cell lysates and purified ribosomes. $S14_{C-}$ was probed as control. The plot below shows average Mpy levels in ribosomes (normalized to S14c-) in three biologically independent experiments; * denotes p (t-test) < 0.05.

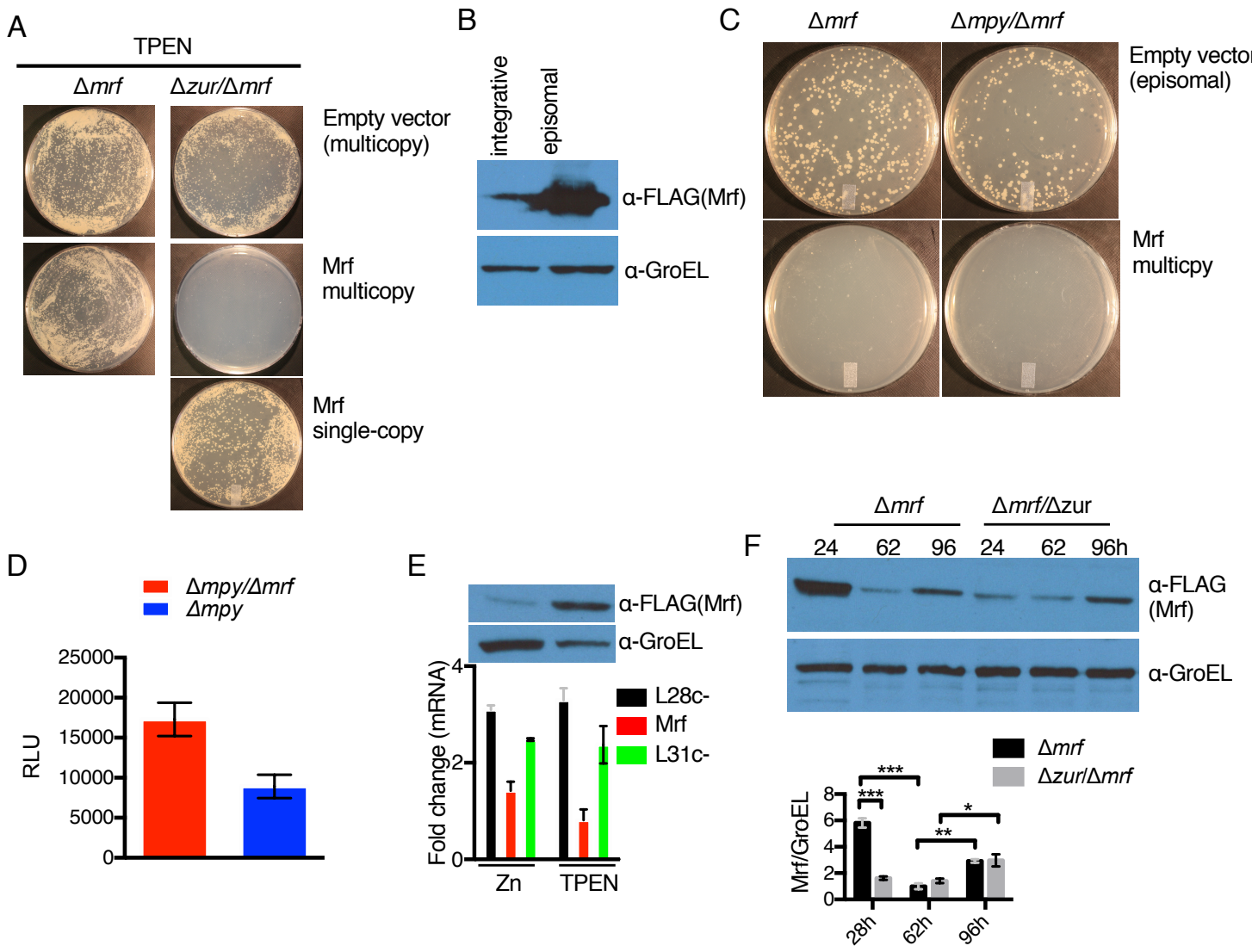


Figure S2: Post-transcriptional regulation of Mrf. A. Plating efficiency of the indicated mutants of *M. smegmatis,* transformed either with an integrative (YL155) or a multicopy episomal (pYL141) plasmid expressing Mrf from the native (P^{zurbox}) promoter, on Sauton's agar plates containing 1μM TPEN. **B.** Levels of Mrf expressed from P^{zurbox} on either an integrative (pYL155) or episomal (pYL141) plasmid in a Δ*mrf* strain cultured in low-zinc medium for 96-hour. GroEL level was probed as loading control. **C.** Mpy-independent toxicity of multicopy Mrf expressed from episomal plasmid pYL150. Transformants were plated on Sauton's agar plates containing 1μM TPEN . Empty vector (pJL37) was used as control. **D.** Effect of Mrf on the translation activity of purified C- ribosomes at a sub-saturating concentration (12.5nM). The activity was determined by a nano-luciferase based *in vitro* transcription-translation assay, which reports relative luminescence unit (RLU). Data represents mean ± SD of two biologically independent preparation of ribosomes. **E.** Zinc-dependent loss of stability of constitutively expressed Mrf is independent of C- ribosomes. Constitutive expression was achieved from pYL155 in Δ*mrf*/Δ*zur* mutant. The plot shows relative mRNA levels of Mrf and $L31_{C-}$ and $L28_{C-}$ of the *c-* operon, from either high- or low-zinc cultures after 96 hours of growth. The immunoblot above shows corresponding protein level of Mrf in the same samples used for the mRNA analyses. **F.** Levels of Mrf expressed from pYL155 in Δ*mrf* and Δ*mrf*/Δ*zur* strains at the indicated timepoints of growth. GroEL was probed as loading control. The plot below shows average Mrf levels (normalized to GroEL) in each sample.

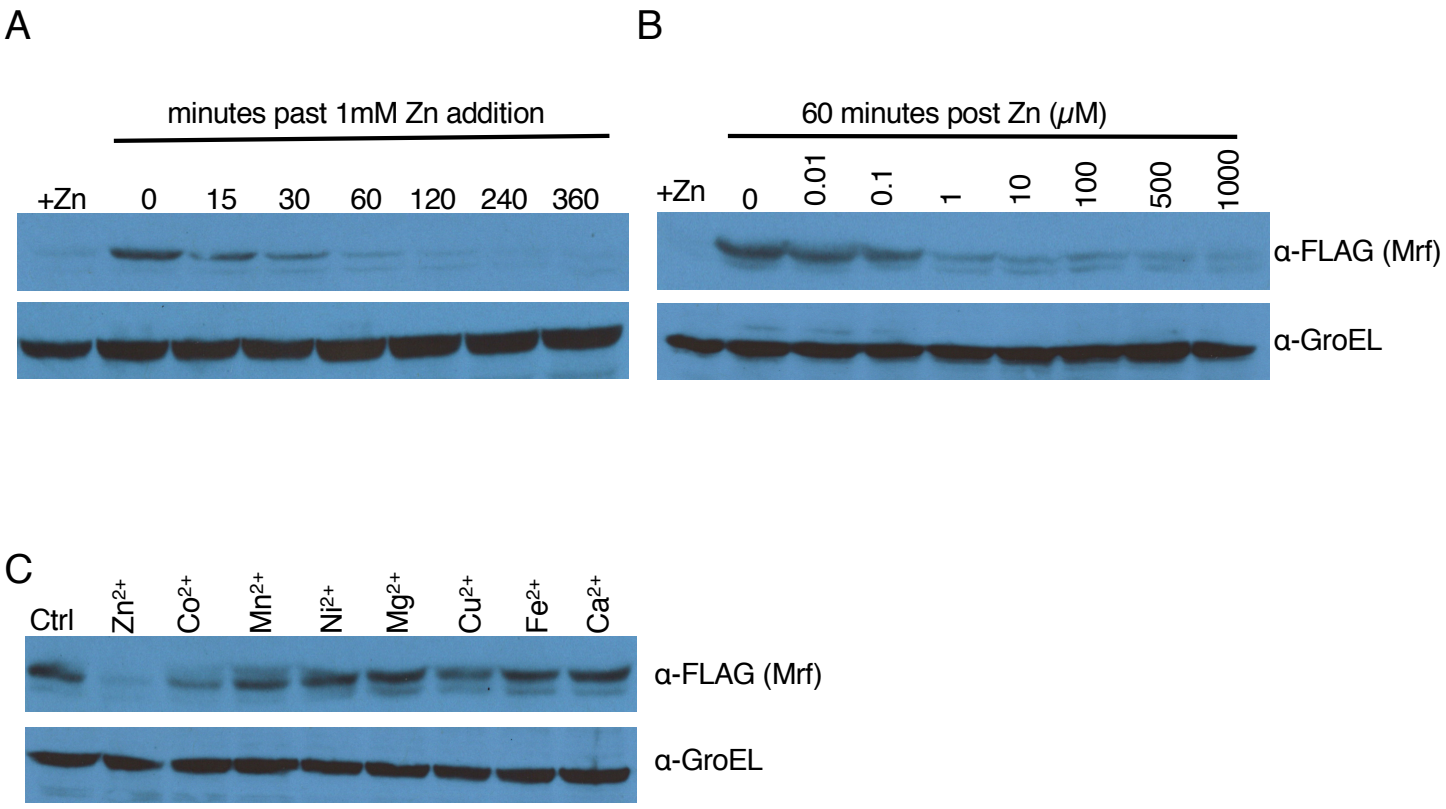


Figure S3: Stability of Mrf is reversed by reintroduction of zinc. A-B. Time (A) and concentration (B) dependent restoration of Mrf instability upon addition of zinc. *M. smegmatis* strain YL23 (Mrf expressed from pYL155 in Δ*zur*/Δ*mrf)*, was cultured for 96 hours in Sauton's medium with 1μM TPEN. Subsequently, zinc was added to the culture either at a fixed concentration for indicated time periods (A), or for one hour at the indicated concentration (B). A parallel culture grown in 1mM $ZnSO_4$ (denoted as +Zn) was used as control in both the experiments. **C.** Zinc-specific degradation of Mrf. 96-hour old cultures of the *M. smegmatis* strain used for panels A and B experiments were exposed to indicated metal ions at 10 μM concentration for 60 minutes and Mrf in cell lysates was monitored. GroEL was probed as the loading control.

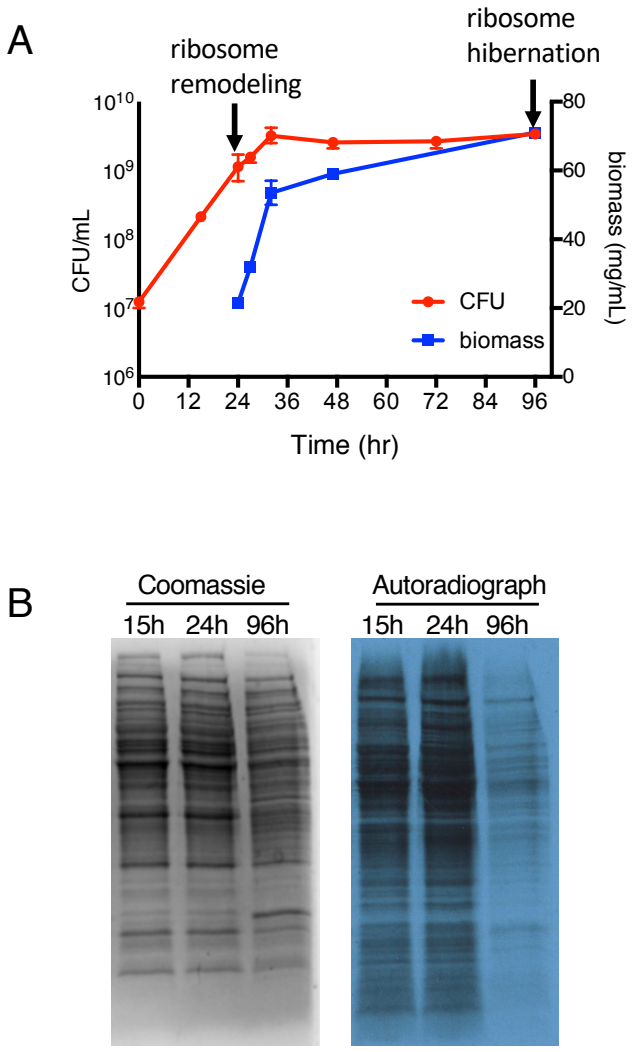


Figure S4: Ribosome remodeling maintains cellular growth and protein synthesis. A. Growth of *M. smegmatis* cells in Sauton's medium containing 1μM TPEN measured over 96 hours. Both colony forming unit (CFU) and biomass of the culture were determined at the indicated timepoints. Data represent average of three biologically independent experiments. The timepoints at which expression of C- ribosomes (remodeling) and recruitment of Mpy (hibernation) are indicate by arrows. **B.** Incorporation of ^{35}S-methionine over a 10-minute period in 15-, 24-, and 96-hour cells, representing the growth phases associated with pre-remodeling, remodeled and hibernating ribosomes, respectively. YL2 cells at the indicated stages were labeled with 10 μCi/mL of ^{35}S-methionine, and 5 μg of total proteins from the lysates were resolved on SDS-PAGE and visualized by Coomassie blue stain as well as autoradiography.

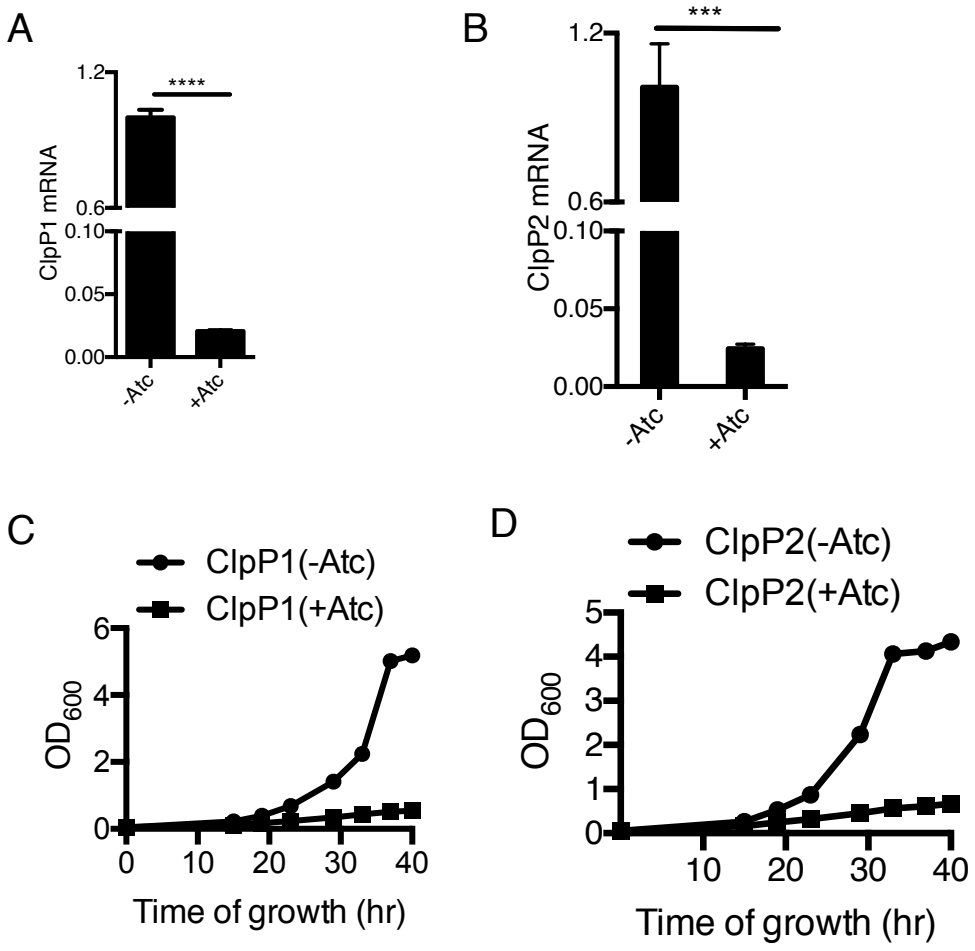


Figure S5: CRISPRi mediated depletion of ClpP1 and ClpP2 in *M. smegmatis*. An integrative plasmid carrying tetracycline-inducible dCas9 and gRNA complementary to either ClpP1 in YL9 strain (panel A and C) or ClpP2 in YL10 strain (panel B and D). **A-B.** Transcription inhibition of ClpP1 (A) and ClpP2 (B) upon induction of CRISPRi by anhydrotetracycline (Atc). mRNA levels were normalized with SigA as an endogenous control. **C-D.** Growth inhibition of the strains in medium containing Atc.

A

Mrf_{Msm}	MRTPVVLV….
Mrf_{Mtb}	MRTPVILV….
Mrf_{Mbo}	MRTPVILV….
Mrf_{Mab}	MRTPVLLV….
Mrf_{Mma}	MRTPVVLV….

B

Raw pmol table (Mrf from low Zn culture)

	ASP	ASN	SER	GLN	THR	GLY	GLU	HIS	ALA	ARG	TYR	PRO	MET	VAL	TRP	PHE	ILE	LYS	LEU
1	1.9	0.0	3.1.	7.9	4.0	8.3	1.3	0.6	2.854	4.154	0.900	2.749	134.9	0.000	0.184	0.417	0.406	1.462	1.245
2	4.704	1.269	2.892	2.918	4.856	6.913	63.13	0.571	7.929	58.38	1.259	5.299	3.561	4.698	0.354	1.430	1.781	1.226	4.856
3	10.49	46.56	3.446	3.937	81.34	8.337	11.21	0.836	13.96	28.08	1.133	7.120	0.568	9.642	1.154	3.049	2.701	2.085	12.10
4	12.65	7.278	4.585	5.876	14.03	11.90	9.742	1.519	21.35	21.24	1.717	53.02	0.695	14.35	1.982	3.494	3.438	65.85	20.44
5	14.46	1.411	5.849	6.894	10.40	14.82	11.36	2.151	27.91	22.14	1.949	59.53	0.773	72.58	3.147	4.467	4.529	9.904	22.71

C

Raw pmol table (Mrf from high Zn culture)

	ASP	ASN	SER	GLN	THR	GLY	GLU	HIS	ALA	ARG	TYR	PRO	MET	VAL	TRP	PHE	ILE	LYS	LEU
1	3.355	0.430	2.647	0.000	0.000	4.646	2.009	1.131	4.041	4.502	1.430	3.898	60.67	0.000	0.334	0.740	1.121	1.502	2.036
2	5.524	0.485	2.717	2.999	2.616	4.668	3.249	0.788	6.201	70.88	1.114	5.086	2.414	3.447	0.149	1.398	1.466	0.870	5.037
3	8.249	0.652	3.302	3.487	78.66	5.977	4.743	0.776	9.102	28.66	1.615	5.827	0.651	5.981	0.621	2.379	2.029	1.130	8.845
4	11.41	0.840	4.092	4.912	12.04	8.110	6.075	0.908	12.70	16.88	2.303	44.65	0.630	8.055	0.980	2.647	2.289	1.889	10.19
5	12.18	1.024	5.104	5.780	7.038	9.740	7.144	1.157	16.05	15.60	2.780	15.01	0.802	57.40	1.506	3.491	2.698	2.482	13.09

Figure S6: Characterization of N-terminal sequence of Mrf in high- and low-zinc cultures of *M. smegmatis*. A. Predicted first eight amino acid sequence of Mrf from *M. smegmatis* (Msm), *M. tuberculosis* (Mtb), *M. bovis* (Mbo), *M. abscessus* (Mab) and *M. marinum* (Mma). **B&C.** Edman degradation-based sequencing of first five amino acids of Mrf purified from either high-zinc cultures of YL27 or low-zinc culture of YL18. ClpP1 in YL27 was depleted by 16-hour Atc treatment to obtain Mrf in high-zinc culture. The values correspond to pmoles of each amino acid detected at the indicated cycle. Values for predicted amino acids are marked in red.

A

	Protein (μM)	Zn (μM)	Ratio (Zn/Mrf)
Mrf - Zn	22.4	1.82 (0.08)	0.08
Mrf + Zn	6.79	18.07 (0.13)	2.6
Mrf + Zn	5.2	12.83 (0.07)	2.5

B

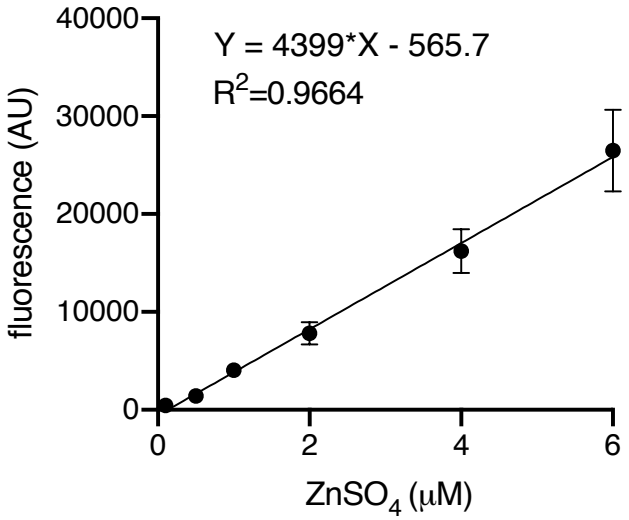


Figure S7: Zinc binding in Mrf and FluoZin-3. A. ICP-MS based analysis of zinc binding in Mrf. Mrf was purified from YL18 in zinc-depleted buffer and dialyzed with 8X molar excess of $ZnSO_4$, followed by three sequential dialysis with zinc-depleted buffer. Zinc was measured in the protein before (Mrf -Zn) and after (Mrf + Zn) the dialysis with $ZnSO_4$, along with exchange buffers (indicated in parenthesis). Background zinc in the exchange buffer was subtracted prior to calculating Zn/Mrf ratio. Values from two independent zinc treatments of the protein are provided. **B.** A linear relationship between fluorescence signal from 10 μM FluoZin-3 and free zinc in the concentration range of 0.1- 6 μM of $ZnSO_4$.

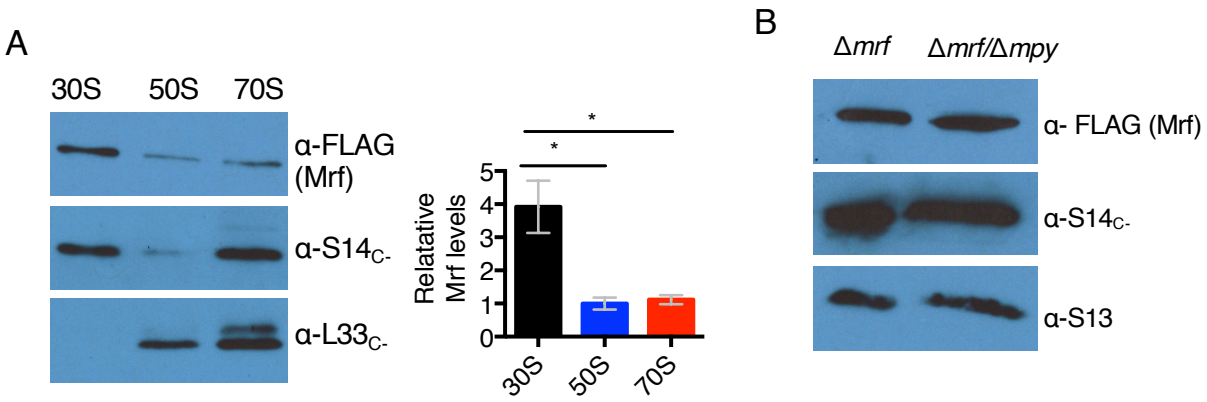


Figure S8: Mpy-independent binding of Mrf to the 30S subunit of the ribosome . A. Analysis of Mrf in 2.4 pmoles of the 30S, 50S and 70S particles from YL18 (*Δmrf* expressing Mrf from the native promoter on a multicopy plasmid) strain described in figure 4A. The plot shows average Mrf density (normalized to the levels in the 50S) from three biologically independent experiments; * denotes p (t-test) < 0.05. **B.** Mrf levels in 2.4 pmoles of the 30S ribosome in low-zinc cultures of YL18 and its isogenic *Δmpy* mutant strain YL26.

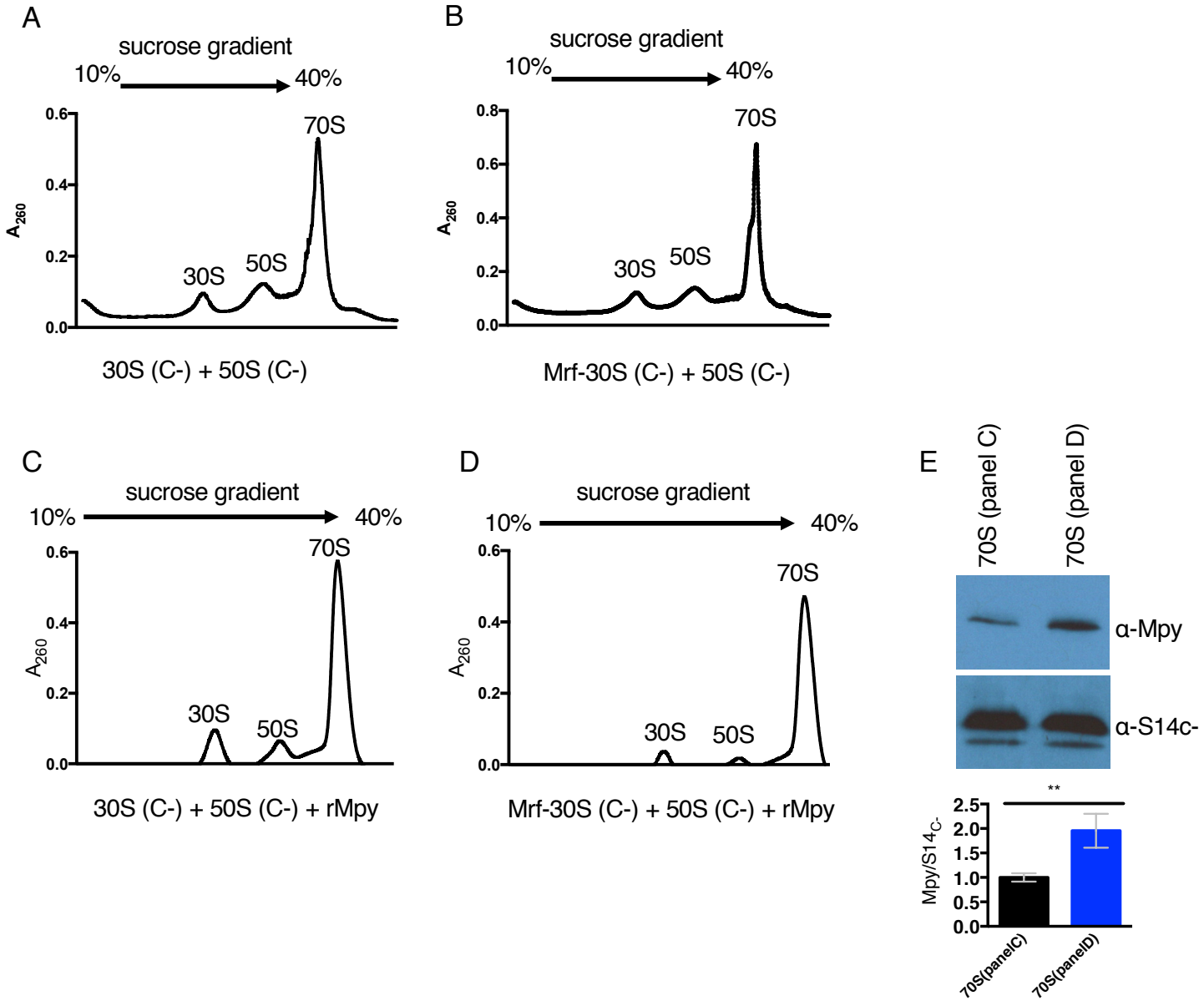
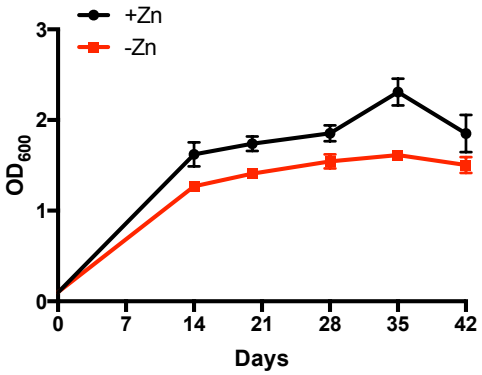
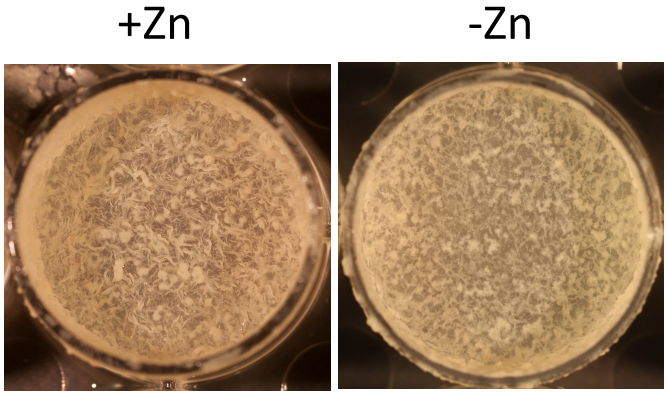


Figure S9: *In vitro* reconstitution of hibernating ribosomes with purified rMPY, Mrf-30S and 50S subunits. A-B. Spontaneous joining of the C- 50S subunit to either the C- 30S (A) or the C- Mrf-30S (B) subunit. 36 pmoles of the 50S subunit, purified from 96-hour old low-zinc culture of YL4 (*Δmpy/Δmrf)* were mixed with 36 pmoles of the 30S subunits purified from either YL4 (panel A) or YL25 (derivative of YL4 expressing Mrf from the native promoter on an integrative plasmid) (panel B). After 60-minutes of incubation, the mixture was resolved on the 10-40% SDG. **C-D.** Analysis of 70S upon simultaneous mixing for 30 minutes of rMpy (72 pmoles) with 36 pmoles each of the 50S and 30S subunits, either with (panel D) or without (panel C) Mrf. **E.** Western blot showing the level of rMpy in 70S collected from panels C and D. The plot below shows average Mpy density (relative to S14c-) from three biologically independent assays; ** denotes p (t-test) < 0.01.

A



B



C

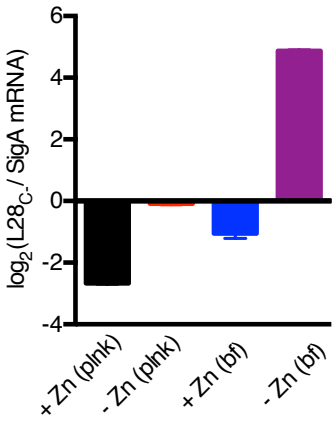


Figure S10: Growth in biofilm is necessary for high-level induction of C- ribosomes in Mtb. A-B. Planktonic (A) and pellicle (B) growth of Mtb in Sauton's medium with either 1mM $ZnSO_4$ (+Zn) or without any zinc supplement (-Zn). Pellicles were grown for 7 weeks. Further depletion of zinc with 1μM TPEN caused severe growth defect in both cultures (not shown). **C.** Analysis of transcripts of *mtb*$_{c-}$ operon (represented by L28c-) in total RNA isolated from saturated planktonic cultures or 7-week pellicles described in panels A and B.

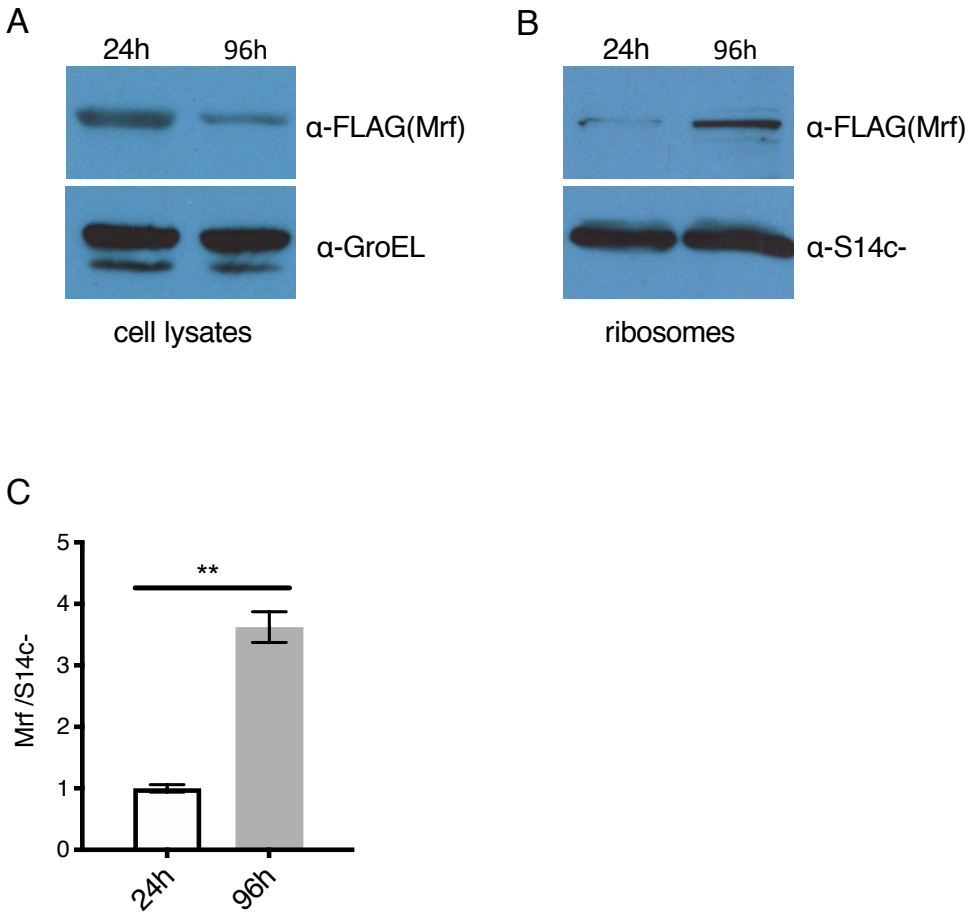


Figure S11: Comparison of Mrf-ribosome interaction in 24- and 96-hour cells of YL2 strain. A-B. Levels of Mrf in 10 μg of cell lysates (A) and 12 pmoles of ribosomes (B) of YL2 cells from 24- and 96-hour cultures in low zinc medium. GroEL and S14c- were probed as loading controls for cell lysates and ribosomes, respectively. Crude ribosomes were prepared on 32% sucrose cushion for analysis. **C.** The plot shows average Mrf signal (relative to S14c-) in the ribosome from three biologically independent assays; ** denotes p (t-test) < 0.01.