

# *Mycobacterium tuberculosis* MutT4/RppH is an RNA pyrophosphohydrolase that forms condensate-like bodies and impacts mRNA degradation

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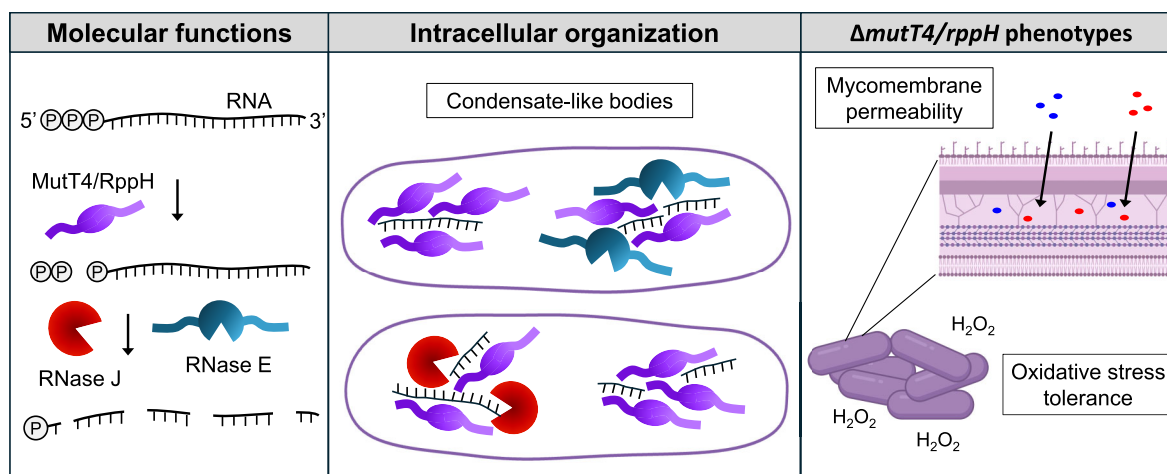
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## Abstract

Bacterial adaptation to stress involves changes in transcription and messenger RNA (mRNA) degradation. In *Escherichia coli*, the Nudix hydrolase RppH initiates mRNA degradation by removing pyrophosphate from mRNA 5'-ends, converting 5'-triphosphates to 5'-monophosphates. We aimed to identify the RppH homolog in the globally important pathogen *Mycobacterium tuberculosis* (Mtb). We identified the protein encoded by Rv3908, previously annotated as a nucleotide pool cleanser *mutT4*, as the predominant mycobacterial RppH. Deletion of *rppH<sub>Mtb</sub>* increased the relative abundance of 5'-triphosphates on myriad mRNAs across the transcriptome. Purified RppH<sub>Mtb</sub> converted mRNA 5'-triphosphates into monophosphates, and stimulated degradation by RNase E and RNase J *in vitro* to varying extents. Surprisingly, deletion of *rppH<sub>Mtb</sub>* had mixed impacts on mRNA degradation *in vivo*, suggesting that it may not sensitize most transcripts to degradation. RppH<sub>Mtb</sub> has intrinsically disordered regions (IDRs), which often participate in biomolecular condensate formation. Microscopy showed that RppH<sub>Mtb</sub> forms condensate-like bodies that localize with RNases and dissociate upon addition of rifampicin. The N-terminal IDR is sufficient for condensate-like body formation. Deletion of *rppH<sub>Mtb</sub>* leads to higher outer membrane permeability and resistance to oxidative stress. We conclude that MutT4 is the mycobacterial RppH, assembling in condensate-like bodies with RNases but having unexpectedly complex impacts on mRNA degradation rates.

## Graphical abstract



## Introduction

*Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), has reclaimed its position as the leading cause

of death from a single infectious agent, surpassing SARS-CoV-2 [1]. In 2023 alone there were 8.2 million reported TB cases and 1.25 million estimated deaths [1]. Standard treatment re-

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quires a 4- to 6-month course of multiple antibiotics. Mtb's success as a pathogen and recalcitrance to treatment are due in part to its abilities to overcome the plethora of stressors it encounters within the host, such as reactive oxygen and nitrogen species, transition metal stress ( $\text{Cu}^+$ ,  $\text{Zn}^{2+}$ ), low pH, and hypoxia, among others (reviewed in [2]).

Bacterial adaptation to stress involves changes in RNA expression levels. The steady-state abundance of any given RNA results from the balance between its rate of transcription by RNA polymerase and its rate of degradation by RNases and auxiliary proteins such as helicases. Messenger RNA (mRNA) degradation is a regulated process, but the mechanisms underlying its regulation in mycobacteria are not well understood. Severe energy stressors such as hypoxia and carbon starvation lead to global slowing of mRNA degradation rates in mycobacteria through largely unknown mechanisms [3–5]. Meanwhile, translation and stability of some specific transcripts can be regulated by small RNAs [6–13]. Developing a comprehensive understanding of the mechanisms governing regulation of mRNA degradation is hampered in part by gaps in knowledge about the machinery that carries out mRNA degradation. Mycobacteria encode a number of RNases, including the essential RNase E, which has a rate-limiting role in degradation of most mRNAs in *Mycobacterium smegmatis* (a non-pathogenic model mycobacterium) [14], and RNase J, which has a specialized role in mRNA degradation and affects drug sensitivity in Mtb [15]. The physical and functional interactions among these and other enzymes are largely undefined in mycobacteria. In *Escherichia coli* and some other bacteria, complexes of RNases called degradosomes have been defined and appear to increase the efficiency of mRNA degradation [16, 17]. In Mtb, some RNases and putative RNA-binding proteins were shown to co-precipitate, suggesting multiple possible degradosome structures, although the direct protein–protein interactions mediating these remain mostly undefined [18]. Interestingly, degradosome composition in *E. coli* and *Caulobacter crescentus* seems to vary in response to environmental conditions [19, 20], and RNA degradation proteins in *Bacillus subtilis* were proposed to interact in a transient degradosome-like network rather than as a stable complex with defined stoichiometry [21]. Recent evidence shows that in *C. crescentus*, the enzymes that form RNA degradosomes also assemble into large, dynamic protein condensates within the bacterial cytoplasm, in a process that involves liquid–liquid phase separation [22]. mRNA degradation therefore appears to be regulated at multiple levels, including specific protein–protein interactions, weak multivalent interactions, and subcellular localization.

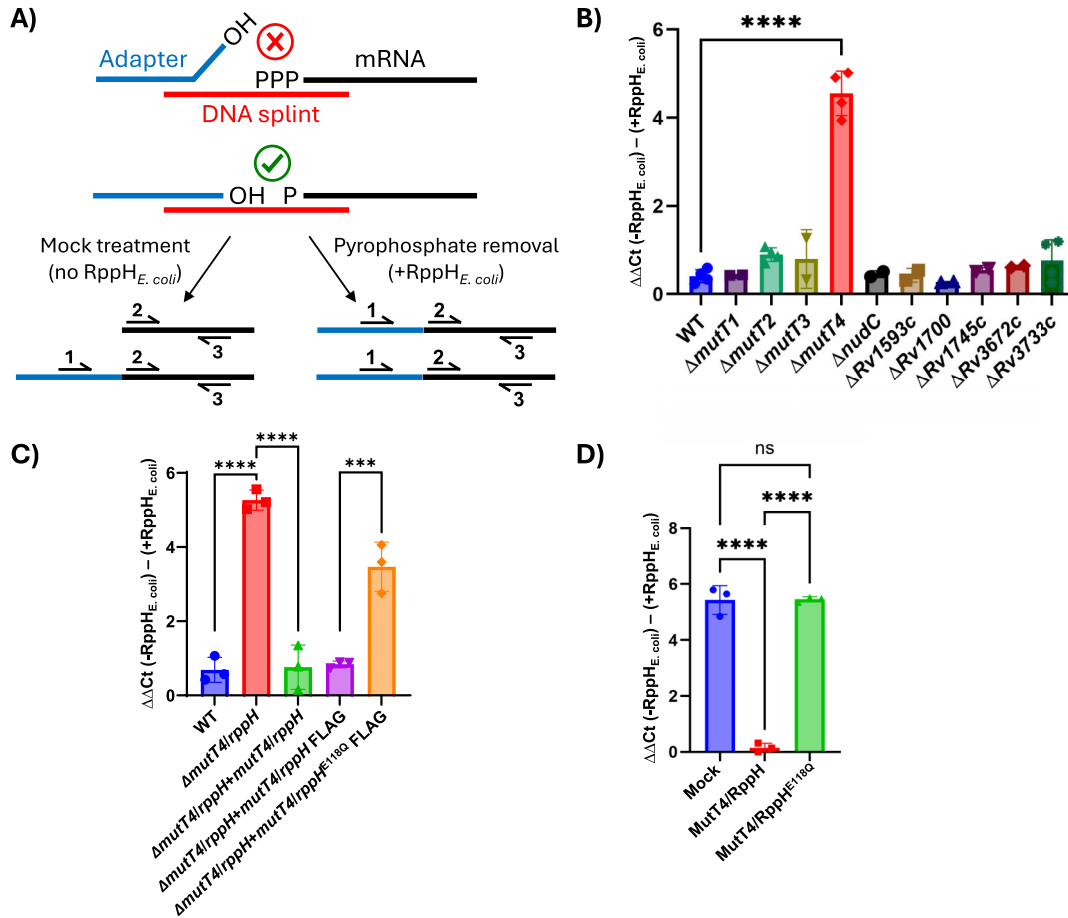
Some clinical strains of Mtb carry drug resistance-associated mutations in RNA processing and degradation enzymes [23]. For instance, loss of RNase J in Mtb increases drug tolerance by altering mRNA metabolism and impacting gene expression [15]. Although mRNA degradation pathways in Mtb are linked to drug resistance and have been proposed as targets for novel therapeutics [24], there is a key step in RNA degradation that has not yet been described in mycobacteria. Transcription of most bacterial mRNAs initiates with nucleoside triphosphates and primary transcripts therefore have triphosphates on their 5' ends. In *E. coli* and *B. subtilis*, these 5' triphosphates appear to have a role analogous to 5' caps on eukaryotic mRNAs; conversion of 5' triphosphates to 5' monophosphates by RNA pyrophosphohydrolase (RppH) sensitizes mRNAs to degradation [25, 26]. Further-

more, some RNAs are synthesized with non-canonical initiating nucleotides, leading to the presence of other caps such as NAD and dinucleoside polyphosphates ( $\text{Np}_n\text{Ns}$ ), which can also be removed to stimulate degradation (reviewed in [27]). 5' monophosphates stimulate the endonuclease activity of *E. coli* RNase E by an allosteric interaction [28, 29], while for RNase J, exonuclease activity is stimulated by these substrates [26, 30–34]. Pyrophosphate removal by RppH has been demonstrated as a rate-limiting step in RNA degradation for a subset of transcripts in *E. coli* [25, 35], *B. subtilis* [26, 36], and *Helicobacter pylori* [37]. No RppH ortholog has been identified or annotated in mycobacteria. However, 5'-end-directed RNAseq showed the presence of 5' monophosphate-bearing transcripts in both Mtb and *M. smegmatis* [14], indicating that there is a native enzyme in mycobacteria that converts RNA 5' triphosphates into monophosphates. Here, we show that MutT4 (Rv3908), previously thought to participate in nucleotide pool cleansing [38], is actually the RppH ortholog of Mtb (RppH<sub>Mtb</sub>). We show for the first time that conversion of 5' triphosphates to monophosphates moderately stimulates RNA cleavage by Mtb RNase E and RNase J *in vitro*, but surprisingly, deletion of *rppH*<sub>Mtb</sub> did not increase the stability of most of the apparent targets that we tested. Complementation of the  $\Delta$ *rppH*<sub>Mtb</sub> strain extended the half-lives of most of the tested transcripts, suggesting that the impact of RppH<sub>Mtb</sub> on mRNA degradation is complex and may differ substantially from what has been shown to date in other bacteria. Also, unlike the RppHs of *E. coli* and *B. subtilis*, mycobacterial RppH has intrinsically disordered regions (IDRs) that trigger formation of condensate-like bodies in the bacterial cytoplasm. Finally, deletion of *rppH*<sub>Mtb</sub> affects various clinically important phenotypes in Mtb by simultaneously increasing outer membrane permeability and resistance to oxidative stress.

## Materials and methods

### Bacterial strains and growth conditions

*Mycobacterium tuberculosis* mc<sup>2</sup>6030 H37Rv  $\Delta$ RD1  $\Delta$ panCD [39] and derivative strains were cultured in Middlebrook 7H9 supplemented with OADC (0.05 g/l oleic acid, 5 g/l bovine serum albumin fraction V, 2 g/l glucose, 0.85 g/l NaCl, and 4 mg/l catalase), 0.2% glycerol, 0.05% Tween 80, and 24  $\mu$ g/ml pantothenate at 37°C and 200 rpm shaking. Middlebrook 7H10 supplemented with 0.5% glycerol, OADC, and 24  $\mu$ g/ml pantothenate was used for growth on solid media. *Mycobacterium smegmatis* mc<sup>2</sup>155 (Msmeg) was grown in the same conditions but oleic acid and pantothenate were excluded from the media (7H9 DC). Apramycin (5  $\mu$ g/ml), hygromycin (50  $\mu$ g/ml for Mtb, 150  $\mu$ g/ml for Msmeg), or zeocin (25  $\mu$ g/ml) were added to the media when required. The recombineering system described in [40] was used to generate strains deleted for each of 10 non-essential genes predicted to encode Nudix proteins. Mutants were generated by replacing most of the coding sequence of each gene with a zeocin resistance gene under the EM7 promoter. The first 10–51 codons of each gene were left intact in order to prevent disruption of overlapping or divergently transcribed genes. These codons were followed by a stop codon, the EM7 promoter, 5' UTR, zeocin coding sequence, and 4–105 codons at the end of the Nudix gene as needed to prevent disruption of expression of downstream genes. Sequencing of polymerase chain reaction (PCR) amplification



**Figure 1.** MutT4 is an RppH that acts on Mtb mRNAs. **(A)** Schematic of splinted ligation. The adapter is ligated only if the target mRNA has a 5' monophosphate. Ligation is performed with and without treatment by *E. coli* RppH to convert 5' triphosphates to monophosphates. qPCR reveals the abundance of ligated mRNA (primers 1 and 3) relative to total mRNA (primers 2 and 3). **(B, C)** Screening of Mtb Nudix gene deletion mutants and complemented strains by splinted ligation of a transcript in the endogenous mRNA pool.  $\Delta\Delta Ct$  indicates the impact of *in vitro* treatment with *E. coli* RppH on the ligatability of the Rv3248c transcript, after normalizing to total transcript abundance. Higher  $\Delta\Delta Ct$  values indicate a higher proportion of the endogenous Rv3248c mRNA was triphosphorylated. Mean and standard deviation of three biological replicates are shown for panel (C). **(D)** MutT4/RppH has RppH activity *in vitro*. Splinted ligation of *in vitro* transcribed Rv3248c mRNA (5' triphosphorylated) treated with purified MutT4/RppH, MutT4/RppH E118Q, or no enzyme (mock). Each value represents results from the assay performed with an independent protein preparation. A one-way ANOVA was performed with Dunnett's test for panel (B) and Tukey's test for panels (C) and (D). \*\*\*  $P \leq .001$ , \*\*\*\*  $P \leq .0001$ .

products with primers flanking the deleted region of each strain was performed to confirm the expected sequence. A Giles-site integrating plasmid containing *rppH<sub>Mtb</sub>* (Rv3908) and its native promoter and 5' UTR (560 bp upstream of the start codon and 300 bp upstream of the TSS) was used for complementation. A C-terminal 3×FLAG tag was added when indicated. All DNA constructs were made with NEBuilder HiFi DNA Assembly (New England Biolabs, E2621). Primers, plasmids, and strains used in this study are listed in [Supplementary Table S1](#).

### RNA purification

Mtb cultures in exponential phase ( $OD_{600nm} = 0.8-1.6$ ) were centrifuged at 3900 rpm for 7 min at 4°C. The bacterial pellets were resuspended in 1 ml of TRI Reagent (Molecular Research Center, TR118) and transferred to bead-beating tubes (OPS Diagnostics 100  $\mu$ m zirconium beads, PFMB 100-100-12). Cells were lysed with 3 cycles at 7 m/s, 30 s per cycle in a FastPrep-24 bead-beater (MP Biomedicals), and then 300  $\mu$ l of chloroform was added. Samples were centrifuged

15 000 rpm for 15 min at 4°C and RNA from the aqueous phase was purified using Direct-Zol RNA miniprep kit (Zymo Research, R2052) following the manufacturer's instructions. DNase treatment was performed on-column and the purified RNA was stored at -80°C.

### Splinted ligation

Splinted ligation was performed to quantify the relative amounts of RNA 5' tri- and diphosphates (henceforth referred to collectively as triphosphates, because they cannot be distinguished from each other by this method) or 5' monophosphates following the method described in [41] with modifications (Fig. 1A). Five micrograms of DNase-treated total RNA extracted from Mtb in exponential phase were incubated with 10 U of *E. coli* RNA 5' pyrophosphohydrolase (RppH<sub>E. coli</sub>) (New England Biolabs, M0356), or with no enzyme as a control, in NEBuffer 2 for 1 h at 37°C. RNA was purified with RNA Clean & Concentrator-5 (Zymo Research, R1014) following the manufacturer's instructions. One microgram of RppH<sub>E. coli</sub>-treated RNA (or no enzyme control) was

mixed with a chimeric DNA/RNA adapter oligonucleotide (SSS1016) and a DNA oligo splint (SSS3118) targeting the previously mapped +1 nucleotide of Rv3248c mRNA [14, 42] and incubated for 5 min each at 70°C, 60°C, 42°C, and 25°C. Next, 1000 U of T4 DNA Ligase (New England Biolabs, M0202), 40 U of RNase Inhibitor, Murine (New England Biolabs, M0314), and T4 DNA ligase buffer to a final 1× concentration were added and the sample was incubated at 15°C overnight. Ligation occurs at this step only in Rv3248c mRNA with a monophosphate at its 5' end. The splint increases the efficiency of ligation to the exact 5' end of the Rv3248c mRNA. RNA was purified with RNA Clean & Concentrator-5 and used for complementary DNA (cDNA) synthesis as follows. RNA was incubated 10 min at 70°C with 1.6 μM of a gene-specific reverse primer for Rv3248c (SSS2650) in Tris-HCl 12.8 mM pH 7.5. Then, the sample was mixed with 100 U of ProtoScript II Reverse Transcriptase (New England Biolabs, M0368), 10 U of RNase Inhibitor Murine, 0.5 mM dNTPs, 5 mM DTT, and 1× ProtoScript II Reverse Transcriptase Reaction Buffer. The sample was incubated for 10 min at 25°C and 2 h at 42°C for cDNA synthesis. Quantitative PCR (qPCR) with iTaq Universal SYBR Green Supermix (Bio-Rad, 1725124) was performed to quantify two amplicons derived from the cDNA: one internal amplicon of the Rv3248c gene to use for normalization, and one amplicon derived from the junction of the adapter and Rv3248c ligation product. qPCR was performed in a QuantStudio 6 Pro (Applied Biosystems, ThermoFisher Scientific) thermocycler with 0.25 μM each primer (Supplementary Table S1C) in 10 μl reaction mixtures, with a cycle consisting of: 2 min at 50°C, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 61°C. All primers used for qPCR had an amplification efficiency between 90% and 110%, as determined by linear regression of serial dilution of cDNA samples.

### RNA-seq

RNA was extracted from *Mtb* exponential growth phase cultures as described earlier. rRNA was depleted as stated in [43], using oligos shown in Supplementary Table S1C. Briefly, 1 μg of total RNA was used as an input to deplete rRNA for each library preparation. Biotinylated oligos for rRNA depletion (Supplementary Table S1C) were ordered from IDT and resuspended at 100 μM with water. Next, a mixture of all biotinylated oligos was made at an equimolar concentration (2.38 μM final concentration each). One microgram of input RNA was mixed with a master mix containing 0.38 μl SSC 20× buffer (ThermoFisher Scientific, AM9763), 0.08 μl 100 mM ethylenediaminetetraacetic acid (EDTA) (VWR, E177), and 0.77 μl biotinylated oligo mix. The sample was brought to 7.5 μl of final volume with TE 1× (Promega, V6231). Samples were incubated 5 min at 70°C and then cooled down to 25°C at −0.1°C/s. 191.4 μl NEB Streptavidin beads (New England Biolabs, S1420) were separated with a magnetic rack, washed with 191.4 μl SSC 1×, and resuspended in 7.5 μl of SSC 1× plus 1 μl RNase Inhibitor Murine (New England Biolabs, M0314). Beads were mixed with the RNA-oligo sample, incubated 5 min at RT, and then 5 min at 50°C. A magnetic rack was used to separate the beads, and the supernatant containing the rRNA-depleted RNA was transferred to a new tube. Two such depletions were performed for each sample/replicate, and the resulting rRNA-depleted RNA was pooled, purified with RNA Clean & Concentrator-

5, and then separated into two aliquots of the same volume to construct the +RppH *E. coli* and −RppH *E. coli* 5'-end-directed libraries. 5'-end-directed libraries were constructed as stated in [44] with the following modifications. rRNA was depleted as stated above. For normalization, 2.5 pg of *in vitro* transcripts (IVTs) (see below) were added at the adapter ligation step. Fragmentation was performed for 15 min at 94°C. PCR primers for adding full-length Illumina adapter sequences are listed in Supplementary Table S1C, and the primers used for each library are listed in Supplementary Table S1D. Selection of library fragments was done with a double-sided purification using HighPrep PCR Clean Up System (MagBio, AC-60005).

IVTs used as spike-ins for normalization were designed to include synthetic transcripts absent in *Mtb* (Supplementary Table S2). IVTs were synthesized by IVT with HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, E2050) using as templates purified PCR products containing a T7 promoter and the coding sequence of each transcript. IVT RNA products were separated in a 1% agarose TBE gel, and bands corresponding to the expected transcript size were excised and purified using the ZymoClean Gel RNA Recovery Kit (Zymo Research, R1011). IVTs were mixed in equimolar amounts and treated with 5 U of RppH (New England Biolabs) for 1 h at 37°C in NEBuffer 2 and purified with RNA Clean & Concentrator-5 (Zymo Research).

RNAseq expression libraries were constructed with an NEBNext Ultra II Directional Library Prep Kit for Illumina (New England Biolabs, E7760L) following rRNA depletion as described earlier. All libraries were pooled and sequenced at SeqCenter (Pittsburgh, PA).

### Bioinformatics tools and analyses

Reads from *Mtb* 5'-end-directed libraries were aligned to the NC\_000962 reference genome using Burrows-Wheeler Aligner [45]. Due to the library construction method, Read 1 corresponds to the sequence of an endogenous RNA, with its first nucleotide representing the 5'-most nucleotide of that RNA molecule, and Read 2 corresponds to the reverse complement of a downstream segment of the same RNA molecule. Samtools was used to remove Read 2 and separate Read 1s that mapped to plus and minus strands of the genome [46]. Bedtools was used to obtain the read depth for each coordinate at the 5' end of a Read 1, representing the 5' end of an RNA used to construct the library [47]. The total number of reads mapped to spike-in controls in each library was used to normalize the coordinate-based coverage of 5' ends. To identify biologically relevant 5' ends, those with a normalized coverage below 5 were excluded. The log<sub>2</sub> ratio of the filtered 5' end coverage in +RppH *E. coli* / −RppH *E. coli* conditions was then calculated for each strain. Only the 5' ends that were previously annotated as transcription start sites (TSSs) and cleavage sites were used for downstream analysis [14]. Normalized coverage at each of these coordinates in each library is reported in Supplementary Tables S4 and S5. The distribution of the log<sub>2</sub> ratio of 5' ends in +RppH *E. coli* / −RppH *E. coli* is expected to be bimodal, with one population centered around zero and another with higher log<sub>2</sub> ratios, representing TSSs [44]. The 20-nucleotide sequence, starting from the 5' end (the first nucleotide), was extracted and used to compute the minimum free energy (MFE) and unpaired probability using RNAfold [48]. RNAseq expression library reads were mapped in the same way, counted with FeatureCount, and differential



expression assessed by DESeq2 [49]. Gene cluster comparison figures were created with Clinker [50]. Intrinsically disordered protein regions were analyzed with IUPred3 [51].

### Measurement of mRNA half-lives

Fifty-five milliliters of biological quadruplicate cultures were grown to an OD<sub>600nm</sub> of 0.63–0.82. They were divided into 7 ml aliquots in 50 ml conical tubes and returned to the 37°C shaking incubator for 3.5–4.5 h. Batches of 18 tubes were placed in a 37°C water bath in a biosafety cabinet and shaken manually at least every 30 s to maintain homogenous oxygenation. Tubes were removed from the water bath for a maximum of 10 s for addition of rifampicin to a final concentration of 50 µg/ml. Cultures were snap-frozen by immersing the tubes in liquid nitrogen after 0, 2, 4, 6, 8, or 10 min. The “0 minute” samples were immersed in nitrogen a maximum of 10 s after addition of rifampicin. Frozen cultures were stored at –80°C until RNA extraction.

RNA was extracted as described earlier, except that cultures were first thawed by alternating manual warming, shaking, and placing on ice to thaw as quickly as possible without substantial warming above 4°C. cDNA was synthesized in 10 µl reactions as described earlier for splinted ligations, except for the following changes: 600 ng of total RNA were used for each cDNA synthesis reaction; 1 µl of 3 mg/ml random hexamer primers (Invitrogen) was used instead of a gene-specific primer; the 42°C incubation step was extended to ~18 h. Control reactions with addition of water instead of reverse transcriptase were performed in parallel to later confirm the absence of meaningful levels of genomic DNA contamination. RNA was degraded by addition of 5 µl of 1 N NaOH and 5 µl of 0.5 M EDTA and incubation at 65°C for 15 min, followed immediately by addition of 12.5 µl of 1 M Tris–HCl pH 7.5. cDNA was then purified with a Monarch PCR and DNA Cleanup kit (New England Biolabs, T1030), beginning with addition of 325 µl of binding buffer and following the manufacturer’s instructions thereafter. cDNA concentrations were measured by Nanodrop.

qPCRs were performed as described above for splinted ligations, except that they were done in 384-well plates in technical duplicate using 400 pg of cDNA per reaction. For a given primer set, all reactions were done on the same plate. Cq values of the technical duplicates were averaged and these values were used for subsequent analyses. Half-lives were calculated from linear regression of the –Cq values (which represent a log<sub>2</sub> measurement of abundance) over time following addition of rifampicin. Half-life = –1/slope. When biphasic curves were observed, the timepoints from the initial plateau period were omitted from the half-life calculation. These occur when the primer annealing sites are substantially downstream from the TSS, since rifampicin blocks initiation but not transcription elongation.

### Expression and purification of Mtb RppH

RppH<sub>Mtb</sub> was expressed by pET42-derived vectors with N-terminal 6×His and 3×FLAG tags (Supplementary Table S1B). *Escherichia coli* BL21(DE3) pLysS transformed with pET42 *rppH*<sub>Mtb</sub> or pET42 *rppH*<sub>Mtb</sub> E118Q (catalytically inactive mutant) were grown at 37°C with 200 rpm shaking in LB with 50 µg/ml kanamycin and 20 µg/ml chloramphenicol to OD<sub>600nm</sub> = 0.6. IPTG was added at 0.1 mM and bacteria were incubated at 30°C at 200 rpm for 3 h to induce pro-

tein expression. The culture was centrifuged, and the bacterial pellet from 1 L of culture was resuspended in 10 ml equilibration buffer [Tris–HCl 20 mM pH 8, NaCl 300 mM, glycerol 5% (v/v), IGEPAL 0.01% (v/v), imidazole 10 mM], supplemented with 20 U of Turbo DNase (ThermoFisher Scientific, AM2238) and 1.25 mg/ml of lysozyme (ThermoFisher Scientific, J60701.06). The sample was transferred to 100 µm zirconium beads tubes (~1.5 ml/tube; OPS Diagnostics, PFMB 100-100-12) and cells were lysed with a FastPrep-24 bead-beater (MP Biomedicals) using 4 cycles of 30 s at 6.5 m/s. Lysates were centrifuged for 20 min at 13 000 rpm at 4°C, and the supernatant was incubated with HisPur Ni-NTA resin (ThermoFisher Scientific, 88221) and Halt Protease Inhibitor Cocktail 1× (ThermoFisher Scientific, 78430) for 1 h at room temperature with end-to-end rotation. The resin was washed six times with wash buffer [Tris–HCl 20 mM pH 8, NaCl 1 M, glycerol 5% (v/v), IGEPAL 0.01% (v/v), imidazole 50 mM] and then elution buffer [Tris–HCl 20 mM pH 8, NaCl 300 mM, glycerol 5% (v/v), IGEPAL 0.01% (v/v), imidazole 500 mM] was added to elute the purified protein. The eluted samples were dialyzed in Slide-A-Lyzer 20 000 MWCO cassettes (ThermoFisher Scientific, 66003) against storage buffer [Tris–HCl 20 mM pH 8, NaCl 300 mM, glycerol 5% (v/v), IGEPAL 0.01% (v/v)]. Purified protein samples were flash frozen in liquid N<sub>2</sub> and stored at –80°C.

### RppH<sub>Mtb</sub> enzymatic characterization

Rv3248c mRNA was synthesized by IVT with HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, E2050) using as a template a purified PCR product (amplified with primers SSS3829 and SSS3830) containing a T7 promoter and the first 359 bp of Rv3248c mRNA starting from the +1 TSS (68 nt upstream of the annotated start codon [14]). The IVT RNA was purified using RNA Clean & Concentrator-25 (Zymo Research, R1018). A single RNA band was confirmed by running an aliquot of the purified RNA on a 1% agarose TBE 1× gel. 0.5 µg of purified RppH<sub>Mtb</sub> or RppH<sub>Mtb</sub> E118Q was mixed with 250 ng of Rv3248c mRNA in a buffer containing Tris HCl 40 mM pH 8, MgCl<sub>2</sub> 10 mM, DTT 2 mM, glycerol 2% (v/v), NaCl 600 mM and incubated for 1 h at 37°C. As a control, samples were incubated without the addition of protein. RNA was purified with RNA Clean & Concentrator-5 and the triphosphate-to-monophosphate ratio at the RNA 5′ end was measured with splinted ligation as stated previously.

### Expression and purification of Mtb RNase E and Mtb RNase J

Mtb RNase E (Rv2444c) and RNase J (Rv2752c) were cloned into pET vectors and overexpressed in *E. coli* with C-terminal 6×His tags (complete tag sequence: LEHHHHHH). The proteins were purified over Ni-NTA resin followed by size exclusion chromatography. Briefly, *E. coli* pellets were lysed in 20 mM Tris–HCl (pH 7.5), 500 mM NaCl, and 5% glycerol and loaded on Ni-NTA resin. The resin was washed with buffer containing 100 mM imidazole and 1 M NaCl, then eluted in a gradient from 75 to 500 mM imidazole. Proteins were then further purified with Superdex size-exclusion column in buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5% glycerol, and 2 mM DTT.

## RNA cleavage assay

Rv3248c mRNA was synthesized and treated with RppH<sub>Mtb</sub> as described in the “RppH<sub>Mtb</sub> enzymatic characterization” section earlier. RNA was then purified with RNA Clean & Concentrator-5 (Zymo Research). For each reaction 40 ng of RNA were incubated for 3 min at 65°C. Next, the RNA was diluted in a buffer containing Tris–HCl 20 mM pH 8.0, DTT 1 mM, IGEPAL 0.01% (v/v), glycerol 0.5% (v/v), MgCl<sub>2</sub> 10 mM, ZnCl<sub>2</sub> 10 µM, NaCl 150 mM, and 80 ng of purified Mtb RNase E, Mtb RNase J, or no enzyme added as a control in 10 µl of final volume. Samples were incubated for 30 min at 37°C. The reaction was stopped by addition of 1× RNA gel loading dye (Thermo Scientific, R0641) and incubation for 10 min at 70°C. Samples were analyzed in a 1× TBE Urea PAGE gel stained with SYBR Gold (Thermo Scientific, S11494). Quantification of band intensity was performed with ImageJ with Fiji plugin [52].

## Western blotting

Mtb samples grown to exponential phase were centrifuged for 7 min 3900 rpm at 4°C and washed with TBS (Tris–HCl 20 mM pH 7.6, NaCl 150 mM). Bacterial pellets were resuspended in lysis buffer [HEPES 50 mM pH 7.5, NaCl 150 mM, EDTA 1 mM, Triton X-100 0.5% (v/v), glycerol 10% (v/v), SDS 1% (w/v)], and transferred to tubes containing 100 µm zirconium beads (OPS Diagnostics, PFMB 100-100-12). Cells were lysed with a FastPrep-24 bead-beater (MP Biomedicals) using 4 cycles of 30 s at 6.5 m/s. Lysates were centrifuged for 10 min 13 000 rpm at 4°C and the supernatants were transferred to new tubes. Protein concentration in the lysates was quantified using Pierce BCA Protein Assay Kit (ThermoFisher Scientific, 23227). Equal amounts of protein from each sample were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and transferred to PVDF membranes. Membranes were stained with LiCor Revert 700 Total Protein Stain (LiCor, 926-11 021) following the manufacturer’s instructions and imaged to verify equal loading. The membrane was blocked with 3% TBS skim milk for 45 min at room temperature, washed with TBS, and incubated with monoclonal anti-FLAG M2 mouse antibody (1 µg/ml) (Sigma–Aldrich, F1804) in 3% TBS skim milk overnight at 4°C. Membranes were washed with TBS and then incubated with goat anti-mouse HRP antibody for 45 min at room temperature. Membranes were washed with TBS Tween-20 0.05% (v/v) and then developed with Azure Radiance ECL (AC2204) in an Azure 600 imager.

## Microscopy

Plasmids encoding RppH<sub>Mtb</sub> or RppH<sub>Msmeg</sub> with C-terminal Dendra2 fusions and *M. smegmatis* RNase E and RNase J with C-terminal mScarlet fusions are described in [Supplementary Table S1B](#). Microscopy images were acquired with a Nikon CSU W1 spinning disc confocal microscope equipped with a CFI 60 plan apochromat λ D 100× oil-immersion objective. *Mycobacterium smegmatis* cultures in exponential phase were washed once in 7H9 without DC and then spotted onto 1.2% agarose pads and imaged in a chamber at 37°C. Dendra2 was imaged using a 488 nm excitation laser and an ET525/36m emission filter. For mScarlet, a 561 nm excitation laser and an ET605/52m emission filter were used. For rifampicin treatment, exponential phase cultures were incubated with 100 µg/ml rifampicin

at 37°C and 200 rpm, and samples were taken after 30 min for imaging. A sample with DMSO (rifampicin vehicle) was included as a no-drug control. Images were acquired, denoised, and deconvoluted using Nikon Elements software (5.42.06). Image analysis was performed using ImageJ with Fiji plugin. The impact of rifampicin on RppH<sub>Msmeg</sub> localization was quantified as the coefficient of variation, as an unbiased measurement independent of cell length. To measure colocalization of RppH<sub>Msmeg</sub> signal with RNase E or RNase J signal, peaks in signal intensity in each channel were defined as local maxima in the gray value profile that exceeded the mean gray value of that cell. Candidate peaks were first identified as points rising above the mean and then refined by examining regions where the signal dipped below the mean on both sides. Within each such low–low interval, only the tallest peak was retained to avoid counting closely spaced fluctuations as separate peaks. For each cell, the peaks from both channels were sorted and compared in order, and a one-to-one matching was applied so that each peak could only be paired once. Two peaks from different channels were considered colocalized if their maxima were located within 0.2 microns. To assess the significance of the colocalization, the *x*-axis values for the signal intensity plots for one of the two colors were inverted to create a randomized set of peak positions with biologically appropriate spacing. The same colocalization procedure was performed, creating a null distribution of colocalization values. These were compared to the actual colocalization values by Mann–Whitney test.

## Antibiotic susceptibility testing

Susceptibility to antibiotics was determined using a resazurin microtiter assay. Mtb was grown to exponential phase and inoculated in 96-well plates with different concentrations of antibiotics in 200 µl of final volume and a bacterial OD<sub>600nm</sub> = 0.01. Plates were incubated for 10 days at 37°C and 125 rpm shaking. Resazurin 0.002% (w/v) was added to each well, incubated for 24 h, and absorbance of reduced resazurin was measured at 570 nm. The bacterial growth of each well containing antibiotic was normalized to growth in a control well without antibiotic.

## Hypoxic growth conditions

A variation of the Wayne model [53] was used to determine the bacterial viability in hypoxia as described in [54]. Vials were opened after 3 or 6 weeks, and cultures were diluted and plated on 7H10 to enumerate CFUs.

## Resistance to hydrogen peroxide

A disk diffusion assay was used to measure the resistance to H<sub>2</sub>O<sub>2</sub>. Mtb was grown to exponential phase in 7H9 without the addition of catalase (7H9 OAD), diluted to OD<sub>600nm</sub> = 0.01 in 7H9 OAD containing 0.6% (w/v) agar (12 ml final volume), and plated on top of 25 ml 7H10 OAD in a 100-mm round Petri dish. Paper disks with 10 µl of 3% H<sub>2</sub>O<sub>2</sub> were added on top of the solidified agar. Plates were incubated for 10 days at 37°C and the inhibition zone was measured. For acute H<sub>2</sub>O<sub>2</sub> treatment, Mtb was grown to exponential phase in 7H9 OAD, diluted to OD<sub>600nm</sub> = 0.1, and H<sub>2</sub>O<sub>2</sub> was added to a final concentration of 10 mM. Cultures were incubated for 24 h at 37°C with 120 rpm shaking and serial dilutions were plated on 7H10 to enumerate CFUs. Survival was ex-

pressed as the percentage of CFUs recovered at 24 h compared to before the addition of H<sub>2</sub>O<sub>2</sub>.

### Permeability assay (PAC-MAN) of azide–rifamycin on live Mtb

To evaluate the permeability of mycomembrane to rifamycin, we performed the previously described Peptidoglycan Accessibility Click-Mediated Assessment (PAC-MAN) assay [55–58]. Mtb wild type,  $\Delta rppH_{Mtb}$ , and the complemented strain  $\Delta rppH_{Mtb} + rppH_{Mtb}$  were diluted to OD<sub>600nm</sub> ~0.04 and grown in 7H9 ± 25 µM dibenzocyclooctyne (DBCO)-tetrapeptide (TetD [55] WuXi AppTec) for 3 days until OD<sub>600</sub> reached ~0.3–0.4. Bacteria were washed twice with polyphosphate buffer supplemented with Tween-80 0.05% (PBST) and then incubated with 50 µM of azide–rifamycin [59] in PBST in 96-well plates for 2 h at 37°C with shaking. Azide–rifamycin was removed by spinning, the supernatant was removed, and bacteria were then incubated in PBST containing 17 µM of the fluorogenic label CalFluor647-azide (Vector Laboratories, APC flow cytometry channel) for 1 h at 37°C with shaking. The fluorophore was removed by spinning and supernatant removal, and bacteria were fixed with freshly prepared 4% paraformaldehyde (PFA, Ted Pella) in PBS for 2 h at room temperature. Finally, bacteria were resuspended in physiological solution (0.9% NaCl in water) and analyzed by BD DUAL LSRFortessa flow cytometer.

### Fluorescent vancomycin permeability assay

Mtb wild type,  $\Delta rppH_{Mtb}$ , and the complemented strain  $\Delta rppH_{Mtb} + rppH_{Mtb}$  were grown to OD<sub>600</sub> ~0.1, concentrated to a working OD<sub>600nm</sub> of 0.4 in PBS, and incubated with BODIPY-vancomycin (FL-VAN, Thermo Fisher, FITC flow cytometry channel) 1:1 with native vancomycin, both 5 µg/ml, for 4 h in PBS at 37°C with shaking [60]. Bacteria were then washed three times with PBST, fixed with freshly prepared 4% PFA for 2 h at room temperature, resuspended in physiological solution, and analyzed by BD DUAL LSRFortessa flow cytometer. Fluorescence measurements were normalized to the wild-type (WT) strain.

## Results

### MutT4 is the Mtb RNA pyrophosphohydrolase (RppH<sub>Mtb</sub>)

Many of the RppH described so far in bacteria belong to the Nudix hydrolase family [25, 26, 37, 61]. We hypothesized that the RppH ortholog of Mtb also belongs to this protein family. The Mtb H37Rv genome has 11 genes with Nudix domains, only one of which is essential (Supplementary Table S3). To identify the Nudix protein that converts RNA 5' triphosphates to monophosphates in Mtb, we generated strains with single deletions of each non-essential Nudix gene. Next, we screened the mutant collection by splinted ligation to quantify the 5' end phosphorylation status of the mRNA target Rv3248c. We chose this target because in the WT background it is mainly monophosphorylated [14], suggesting that it is targeted by the native RppH. The splinted ligation method compares the relative abundance of adapter-ligated RNA with and without treatment of the RNA pool with *E. coli* RppH (Fig. 1A). Because ligation requires a 5' monophosphate, RNAs not treated with RppH<sub>*E. coli*</sub> are only ligated if they have 5' monophosphates when extracted from Mtb. Deletion

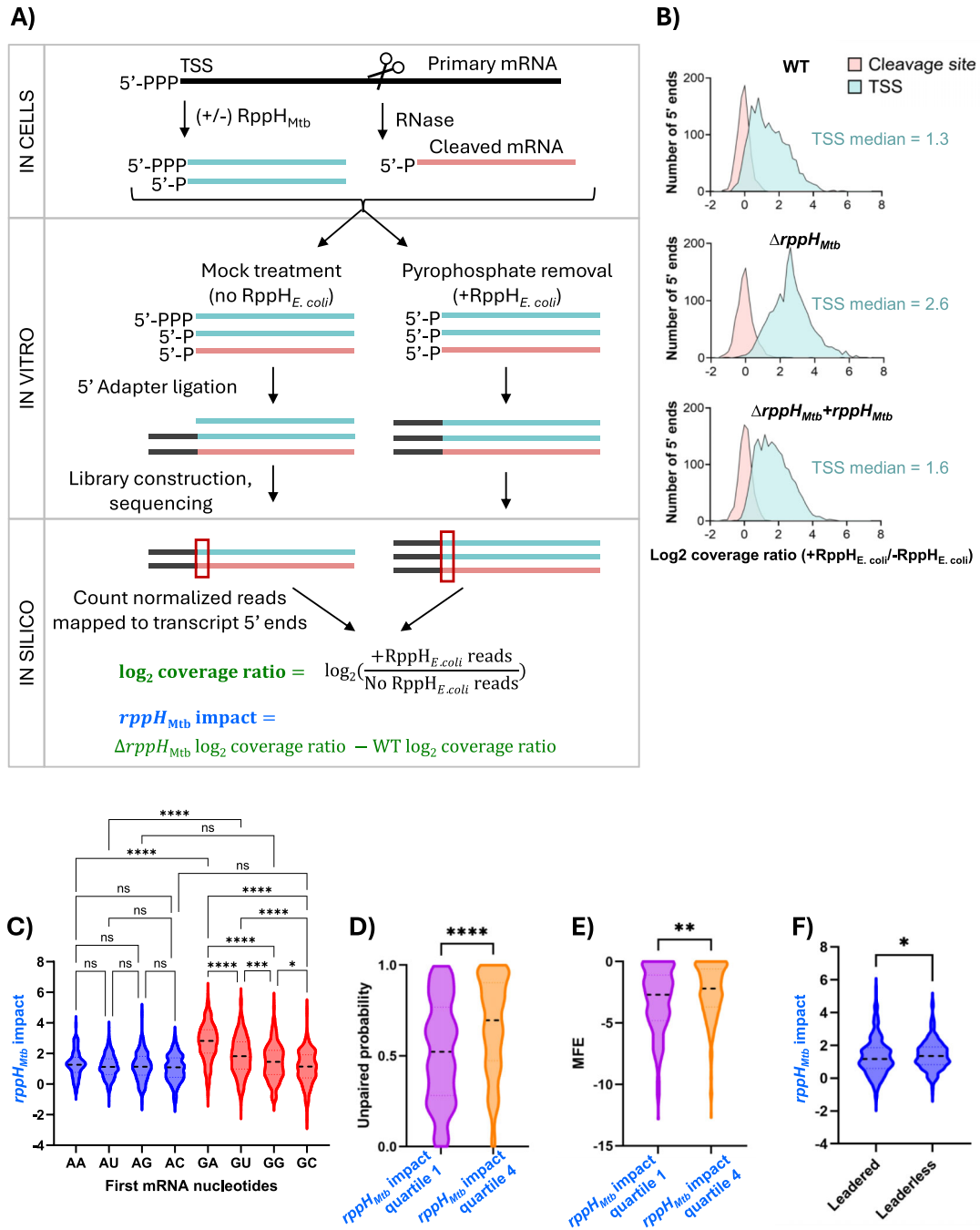
of *mutT4* (Rv3908) significantly increased the 5' end triphosphates of the target mRNA as compared to the WT strain (Fig. 1B), suggesting that in the WT strain *mutT4* is involved in conversion of 5' triphosphates to monophosphates on this transcript. Integration of a single copy of *mutT4* under its native promoter in the  $\Delta mutT4$  mutant strain reverted the 5' end triphosphates of target Rv3248c to WT levels (Fig. 1C). Analysis of gene expression by qPCR showed that the WT and complemented strains expressed *mutT4* at similar levels (Supplementary Fig. S1). *mutT4* is conserved among the mycobacteria, and it is coded downstream of *pcnA*, recently described as the tRNA 3'-end CCA-adding enzyme of Mtb [62], indicating that this genomic region codes for RNA modifying enzymes (Supplementary Fig. S2).

To confirm the enzymatic activity of MutT4, we complemented the  $\Delta mutT4$  mutant strain with a *mutT4* variant harboring a point mutation in the predicted catalytic site in the Nudix domain to abrogate its enzymatic function (Glu 118 to Gln, E118Q). Point mutations in this key residue have been reported to inactivate the catalytic activity of RppH in *E. coli*, *B. subtilis*, and *H. pylori* [25, 26, 37]. Western blotting revealed that FLAG-tagged WT and E118Q MutT4 had equivalent protein abundance in complemented strains (Supplementary Fig. S3). However, complementation of the  $\Delta mutT4$  mutant strain with the E118Q allele failed to revert the 5' end phosphorylation status of Rv3248c to WT levels (Fig. 1C), suggesting that the Nudix motif is required for the pyrophosphohydrolase activity. To further confirm that MutT4 acts directly upon mRNAs, we expressed and purified Mtb MutT4 protein (either WT or E118Q) from *E. coli* and tested its RppH activity *in vitro* using as a substrate Rv3248c mRNA synthesized by IVT with 5' triphosphates. Incubation with MutT4<sup>WT</sup> significantly converted the 5' end triphosphates to monophosphates as compared to a control where the mRNA was incubated without enzyme (Fig. 1D). No reduction in 5' end triphosphates was observed when incubating the RNA with the catalytically dead MutT4<sup>E118Q</sup> (Fig. 1D), indicating that this activity was not from a contaminant present in the protein preparation. These results show that MutT4 is a *bona fide* RppH and support the designation of this gene locus as *rppH* and the protein as RppH. In this manuscript we will henceforth refer to the gene as *rppH<sub>Mtb</sub>* and the protein as RppH<sub>Mtb</sub> for clarity.

### RppH<sub>Mtb</sub> acts on Mtb mRNAs transcriptome-wide

To determine which mRNAs are RppH<sub>Mtb</sub> substrates transcriptome-wide in Mtb, we next performed 5'-end-directed RNA-seq (Fig. 2A). This method compares the abundance of adapter-ligated RNA 5' ends with and without treatment of the RNA pool with *E. coli* RppH *in vitro* prior to ligation and library construction. The difference in abundance of a given RNA 5' end with and without RppH<sub>*E. coli*</sub> treatment therefore reflects the extent to which it was triphosphorylated prior to extraction from Mtb. We examined the relative phosphorylation states of RNA 5' ends corresponding to TSSs previously mapped in Mtb [14, 42] (Supplementary Table S4). Deletion of *rppH<sub>Mtb</sub>* dramatically increased the relative abundance of 5' triphosphates on myriad transcripts (Fig. 2B), and these were reverted to WT levels by complementation. This result shows that RppH<sub>Mtb</sub> has a transcriptome-wide role in converting RNA 5' triphosphates to monophosphates. We also examined 5'-end reads corresponding to previously





**Figure 2.** *RppH<sub>Mtb</sub>* has a transcriptome-wide role as an RppH, with a greater steady-state impact on transcripts that start with guanosine and have minimal secondary structure near their 5' ends. **(A)** Schematic of 5'-end-directed RNA-seq. Total RNA was used to construct 5'-end-directed libraries with or without pretreatment with *E. coli* RppH to convert 5' triphosphates to monophosphates. Only transcripts with 5' monophosphates (whether endogenous or produced by *E. coli* RppH) can be ligated to adapters and therefore be captured in the libraries. Comparison of the number of reads obtained in libraries made from *RppH<sub>E.coli</sub>*-treated versus untreated RNA indicates the extent to which the RNA was endogenously monophosphorylated. The “log<sub>2</sub> coverage ratio” (green) and “*rppH<sub>Mtb</sub>* impact” (blue) are metrics used in subsequent panels. **(B)** log<sub>2</sub> coverage ratio of  $\pm RppH_{E.coli}$  libraries for reads derived from RNA 5' ends previously classified as TSSs (teal curve,  $n = 1687$ ) or RNase cleavage sites (pink,  $n = 590$ ) (Zhou and Sun *et al.* 2023). The RNase cleavage sites produce RNAs with monophosphorylated 5' ends and are therefore expected to have log<sub>2</sub> coverage ratios of  $\sim 0$ , while TSSs produce RNAs with triphosphorylated 5' ends and are therefore expected to have log<sub>2</sub> coverage ratios  $> 0$ . The median TSS log<sub>2</sub> coverage ratio for each strain is shown in teal. **(C–F)** For each TSS quantified in panel (B), the impact of *rppH<sub>Mtb</sub>* on 5' end phosphorylation status was quantified by calculating the difference in log<sub>2</sub> coverage ratio  $\pm RppH_{E.coli}$  between the deletion strain and the WT strain (see panel A). A higher number indicates a larger impact by *rppH<sub>Mtb</sub>*. **(C)** Transcripts with 5' ends mapping to previously published TSSs were grouped according to their first two nt. As most transcripts initiate with A or G, those initiating with C or U are not shown. **(D, E)** Transcripts with 5' ends mapping to published TSSs that initiated with G were binned into quartiles according to the impact of *rppH<sub>Mtb</sub>* on their 5' end phosphorylation status. Secondary structure characteristics of the quartile least affected by *rppH<sub>Mtb</sub>* (Q1) and the quartile most affected by *rppH<sub>Mtb</sub>* (Q4) were compared. The first 20 nt of each transcript were computationally folded and the probability that the first 5 nt were unpaired in these structures **(D)** as well as the MFE of the structures **(E)** were determined. **(F)** The impact of *rppH<sub>Mtb</sub>* on leadered versus leaderless transcripts was compared. \*  $P \leq .05$ , \*\*  $P \leq .01$ , \*\*\*\*  $P \leq .0001$ . A one-way ANOVA was performed with Tukey's test for panel (C). Mann-Whitney test was performed for panels (D–F).



mapped RNase cleavage sites as a normalization control, since cleavage by the RNases most active in Mtb produces 5' monophosphates [14]. As these reads derived from exclusively monophosphorylated 5' ends, *in vitro* treatment with RppH<sub>E. coli</sub> should not increase their coverage ratio, which is what we observed (Fig. 2B and Supplementary Table S5).

We further analyzed the 5' ends corresponding to TSSs to gain insights into the sequence and secondary structure preference of RppH<sub>Mtb</sub> for its mRNA targets. First, we quantified the impact of *rppH*<sub>Mtb</sub> on each 5' end by comparing the  $\pm$ RppH<sub>E. coli</sub> coverage ratios in the WT strain to those in the  $\Delta$ *rppH*<sub>Mtb</sub> strain. We then grouped the 5' ends based on their first two nucleotides. As most mycobacterial mRNAs initiate with guanosine or adenosine, we focused on A- or G-starting transcripts. The impact of *rppH*<sub>Mtb</sub> was generally greater for G-starting than A-starting transcripts, but this was affected by the identity of the second nt (Fig. 2C). Transcripts starting with “GU” or “GA” were more impacted by *rppH*<sub>Mtb</sub> than transcripts starting with “AU” or “AA,” but the identity of the first nt seemed not to matter when the second nt was C or G. Stated another way, the identity of the second nt seemed to affect sensitivity to *rppH*<sub>Mtb</sub> when the first nt was G but not when the first nt was A.

An important caveat to these analyses is that they were limited to steady-state measurements. Changes in the degree of steady-state phosphorylation of a given transcript could be due to changes in the dephosphorylation process (RppH<sub>Mtb</sub> activity) or changes in rates of transcript degradation. Our measurement of “*rppH*<sub>Mtb</sub> impact” therefore potentially includes both the direct impact of RppH<sub>Mtb</sub>'s activity as well as subsequent indirect impacts of transcript degradation rates. Additionally, despite the apparent preferences for specific dinucleotides, RppH<sub>Mtb</sub> impacts the 5' end phosphorylation state of a majority of transcripts; over half of the TSS-derived 5' ends that we captured had an *rppH*<sub>Mtb</sub> impact >1 (Supplementary Table S4), and the transcript we used to identify this enzyme's RppH activity (Rv3248c, Fig. 1) begins with the dinucleotide “AA” [14].

To increase our sensitivity for detecting relationships between mRNA secondary structure and *rppH*<sub>Mtb</sub> impact, we divided the G-starting transcripts into quartiles based on the extent to which they were impacted by deletion of *rppH*<sub>Mtb</sub>. We then compared predicted secondary structure features of the RNAs for Q1, which represents the transcripts with the smallest increase in triphosphorylation when *rppH*<sub>Mtb</sub> was deleted, and Q4, which represents transcripts with largest increase in triphosphorylation when *rppH*<sub>Mtb</sub> was deleted. We used two metrics of secondary structure: the MFE of folding for the 5'-most 20 nt of each transcript and the probability that the first 5 nt in the RNA are unpaired when considering the folding of the first 20 nt. Both metrics indicated that RppH<sub>Mtb</sub> has a greater impact on mRNA 5' ends with less secondary structure (Fig. 2D and E), suggesting that structured 5' ends are less permissive for RppH<sub>Mtb</sub> binding or catalytic activity.

Previous studies showed that roughly 25% of the Mtb transcripts are leaderless, i.e. they lack a 5' untranslated region (UTR) and thus a Shine–Dalgarno sequence for ribosome binding [42, 63]. We wondered if RppH<sub>Mtb</sub> had a preference for leaderless versus leadered transcripts and found that indeed *rppH*<sub>Mtb</sub> had on average a greater impact on leaderless transcripts (Fig. 2F). The difference was subtle but statistically significant. This did not appear to be attributable to the starting nucleotides of the transcripts, because a larger propor-

tion of leadered transcripts than leaderless transcripts were G-starting (62.4% versus 48.8%, respectively; data from [42]).

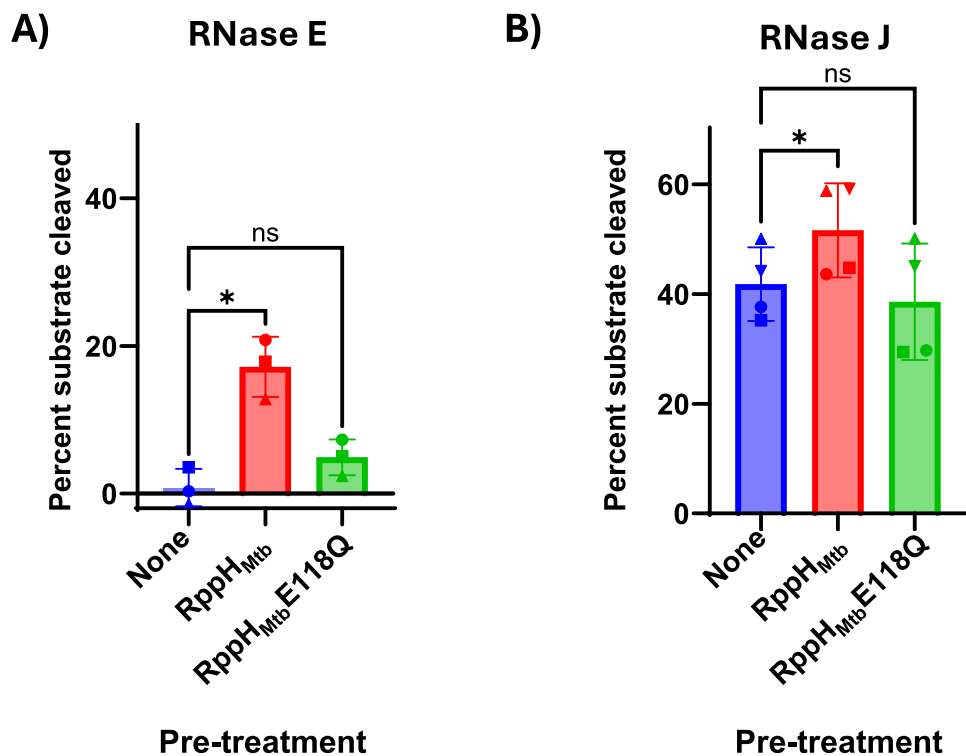
### Pyrophosphate removal by RppH<sub>Mtb</sub> stimulates degradation by RNase E and RNase J *in vitro*

Conversion of RNA 5' triphosphates to monophosphates has been proposed to be a rate-determining step for degradation of a substantial subset of transcripts in *E. coli* [25], where RNase E is a major mRNA degradation protein that is stimulated by binding to monophosphorylated 5' ends [28, 29]. Whether this is the case in mycobacteria is an open question; Mtb RNase E was shown to preferentially cleave a monophosphorylated substrate compared to a substrate with a 5' hydroxyl [64], but to our knowledge, a direct comparison of triphosphorylated versus monophosphorylated substrates has not been reported. RNase J also has important roles in mRNA degradation in some bacteria, and in some of these cases is known to be stimulated by 5' monophosphates, including in *M. smegmatis* [26, 30–34]. To test the impact of mRNA 5' end phosphorylation state on degradation by RNase E and RNase J, we treated *in vitro* transcribed Rv3248c mRNA with purified RppH<sub>Mtb</sub>, then tested the ability of Mtb RNase E and RNase J to cleave this mRNA. We found that pretreatment of the mRNA with WT RppH<sub>Mtb</sub> (but not the E118Q catalytically dead version) resulted in increased degradation by both RNase E and RNase J (Fig. 3 and Supplementary Fig. S4). This result confirms that mRNA degradation by both RNase E and RNase J is stimulated by 5' monophosphates in Mtb. The impact of 5' end phosphorylation seemed to be greater for RNase E, for which the Rv3248c mRNA appeared to be a relatively poor substrate (Fig. 3A), and smaller for RNase J, for which the Rv3248c mRNA appeared to be a relatively good substrate (Fig. 3B).

### *rppH*<sub>Mtb</sub> has unexpectedly complex impacts on mRNA degradation rates *in vivo*

To assess the impact of *rppH*<sub>Mtb</sub> on mRNAs *in vivo*, we first used RNAseq to identify differences in steady-state transcript abundance in the  $\Delta$ *rppH*<sub>Mtb</sub> strain compared to the WT strain. The apparent impact of *rppH*<sub>Mtb</sub> was small; only 19 genes met our criteria for differential expression ( $\log_2$  FC > 1 or < -1, adjusted *P* < .05; Supplementary Table S6), and several of these were transcribed near the *rppH*<sub>Mtb</sub> locus, suggesting that their differential expression was due to the gene deletion strategy rather than to loss of RppH<sub>Mtb</sub> activity. While interpretation of these data was limited by the lack of inclusion of a complemented strain, they allowed us to identify candidate RppH<sub>Mtb</sub> targets for further testing; in *E. coli* and *H. pylori*, genes that were overexpressed in strains lacking *rppH* activity were found to also have longer half-lives in those strains, indicating that RppH had a rate-limiting role in their degradation [25, 37].

To assess the impact of *rppH*<sub>Mtb</sub> on mRNA degradation rates directly, we used qPCR to quantify abundance of selected transcripts following treatment with rifampicin to block transcription elongation. We examined three transcripts for which deletion of *rppH*<sub>Mtb</sub> caused larger-than-average increases in 5' end triphosphorylation (Fig. 4A), three transcripts that were differentially expressed in the  $\Delta$ *rppH*<sub>Mtb</sub> strain (Fig. 4B), and four transcripts that did not meet either of these criteria (Fig. 4C). Surprisingly, only one of these ten transcripts had a longer half-life in the  $\Delta$ *rppH*<sub>Mtb</sub> strain (Fig. 4B and D). This transcript, *esxD*, was one of the very few that had



**Figure 3.** RppH<sub>Mtb</sub> sensitizes transcripts to cleavage by RNase E and RNase J *in vitro*. Pre-treatment with RppH<sub>Mtb</sub> stimulates degradation of an *in vitro* transcribed RNA (Rv3248c) by Mtb RNase E (A) and RNase J (B). Values represent the mean and standard deviation of three or four independent replicates. Cleavage was expressed as percentage decrease in substrate band intensity after incubation with RNase E or RNase J compared to the corresponding untreated control. A representative gel is shown in [Supplementary Fig. S4](#). \*  $P \leq .05$ , RM one-way ANOVA.

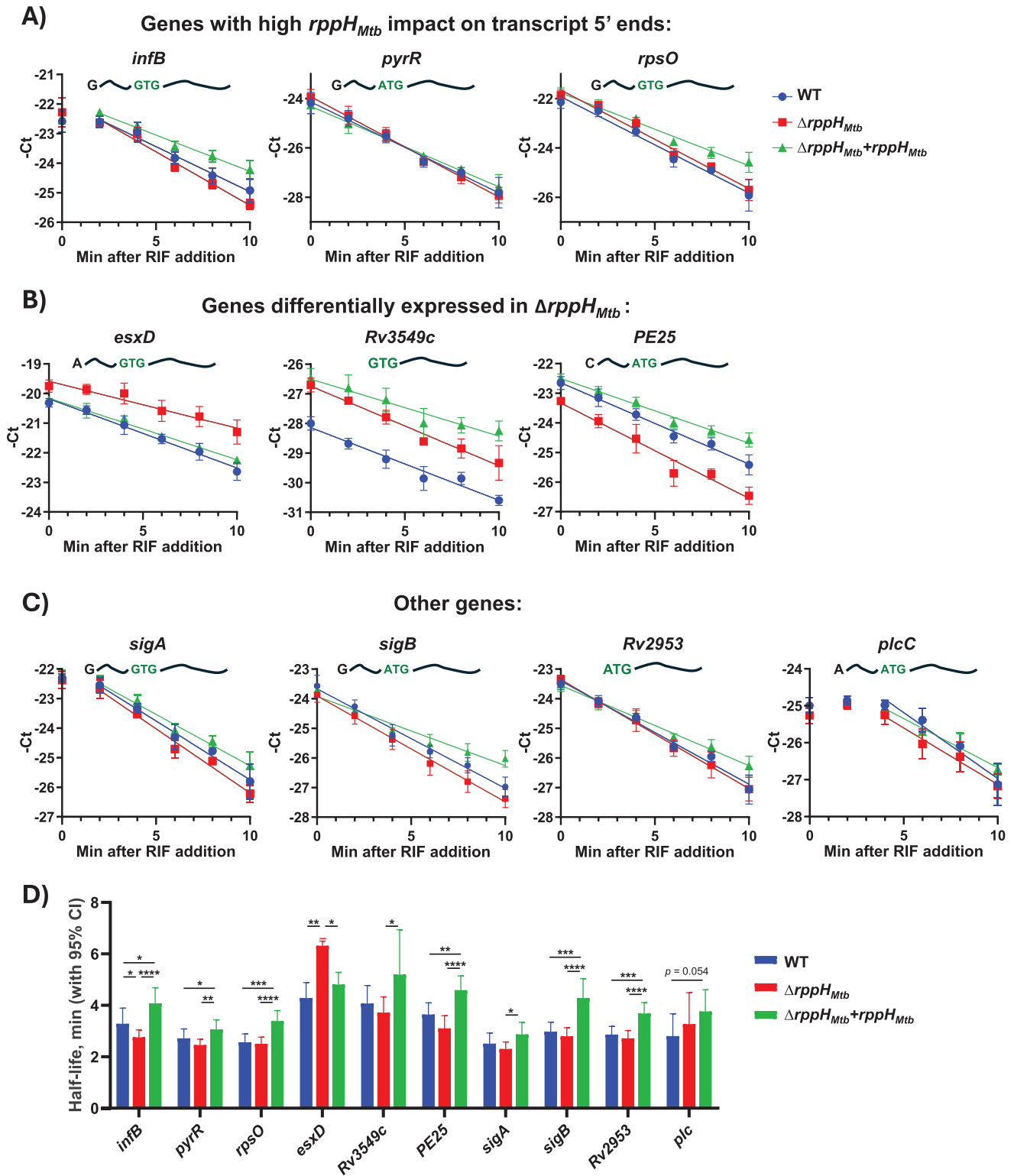
higher steady-state abundance in the  $\Delta rppH_{Mtb}$  strain. One transcript had a shorter half-life in the  $\Delta rppH_{Mtb}$  strain compared to the WT strain, and several others showed trends in this direction although they were not statistically significant (Fig. 4A and D). This result is the opposite of what would be expected if conversion of 5' triphosphates to monophosphates stimulated degradation. Also surprisingly, six of the ten transcripts had longer half-lives in the complemented strain compared to both the WT and  $\Delta rppH_{Mtb}$  strains (Fig. 4A–D). We did not observe any patterns with respect to the impact of *rppH*<sub>Mtb</sub> and the starting nt of the transcript or whether it was leadered/leaderless (Fig. 4A–C). Collectively these results support the idea that RppH<sub>Mtb</sub> impacts mRNA degradation in Mtb, but appear inconsistent with the expected model in which 5' triphosphate-to-monophosphate conversion is a rate-limiting step for many transcripts within the cellular milieu; see the “Discussion” section.

Another notable finding was that the half-lives of the transcripts that we measured were substantially shorter than those previously reported to be typical of Mtb [5]. Half-lives were previously reported for seven of the ten transcripts we studied, and the median was 7.8 min, similar to the reported transcriptome-wide median of 9.3 min [5], while in our experiment, the median was 2.9 min. This discrepancy may be due to methodology. The previous study used a virulent Mtb strain, which must be handled in BSL3 containment, and the cultures therefore were exposed to room temperature for periods of time during the addition of rifampicin. Here, we used an attenuated strain, which allowed us to keep the cultures in a 37°C water bath with manual agitation throughout the experiment, with periods of exposure to room temperature

not exceeding 10 s. Extended lengths of time at room temperature were shown to halt mRNA degradation completely [5]. Our result challenges the idea that the slow growth of Mtb correlates with slower mRNA degradation; the median half-life of the ten genes we assessed was 2.9 min, and thus no different from the transcriptome-wide median half-life of 3.3 min that we previously measured for the fast-growing *M. smegmatis* using the same methodology [14]. This result also highlights the exquisite sensitivity of bacterial mRNA degradation to environmental conditions and the importance of carefully controlling such conditions when measuring half-lives.

### RppH<sub>Msmeg</sub> forms dynamic condensate-like bodies that appear to localize with RNase E and RNase J

Analysis of the RppH<sub>Mtb</sub> protein sequence by IUPred3 and AlphaFold3 showed that it has a central folded Nudix domain and intrinsically disordered regions (IDRs) at both its N- and C-termini ([Supplementary Fig. S5](#)). The amino acid composition of the N- and C-terminal regions is consistent with expectations for IDRs ([Supplementary Fig. S6](#)). Interestingly, this protein architecture is similar to that of mycobacterial RNase E, an essential RNase and key component of the mycobacteria mRNA degradation machinery [18]. Many IDRs have been reported to be involved in the formation of biomolecular condensates by phase separation through both protein–protein and protein–RNA interactions (reviewed in [65]). Previous studies showed that IDRs are required for the formation of RNase E biomolecular condensates in some bacteria, and we found that *M. smegmatis* RNase E has rifampicin-sensitive



**Figure 4.** Deletion of  $rppH_{Mtb}$  does not lead to slower degradation of most transcripts *in vivo* during log phase growth. Half-lives of ten transcripts were measured by blocking transcription with rifampicin (RIF) and quantifying mRNA abundance by qPCR. (A–C) Transcript abundance over time following RIF treatment is displayed on a  $\log_2$  scale by plotting  $-C_t$  values from reactions performed with 400 pg of cDNA. Points and error bars represent the means and SDs of biological quadruplicate samples. Slopes were calculated by linear regression (strain lines), and half-life =  $-1/\text{slope}$ . Because RIF does not block transcription elongation, there is a plateau before the observed decrease in mRNA levels when the qPCR primers are distal from the transcription start site (*infB*, *sigA*, *plcC*); in these cases, only the timepoints after the plateau were used for linear regression. The first nt of each transcript is indicated, and the start codon is shown in green. (A) Three transcripts for which deletion of  $rppH_{Mtb}$  had an above-average effect on 5' end phosphorylation. (B) Three transcripts for which deletion of  $rppH_{Mtb}$  impacted steady-state abundance. (C) Four transcripts for which deletion of  $rppH_{Mtb}$  did not have an above-average impact on 5' status or impact abundance. (D) Half-lives calculated from the linear regressions shown in panels (A–C). Error bars denote 95% confidence intervals. The upper error bar for *esxD* was clipped for visualization purposes.  $P$ -values were determined by pairwise linear regression comparisons. Those not shown were  $> .05$ . \*  $P \leq .05$ ; \*\*  $P \leq .01$ ; \*\*\*  $P \leq .001$ ; \*\*\*\*  $P \leq .0001$ .

punctate localization in cells, a hallmark of phase separation (Supplementary Fig. S7). The localization of mycobacterial RNase E as apparent dynamic condensates is in agreement with a previous report [66]. As the formation of biomolecular condensates is emerging as an important regulatory mechanism in bacteria, we assessed if mycobacterial RppH could form this type of structure, using *M. smegmatis* as a model to allow us to do live-cell microscopy. A fusion of the fluorescent protein Dendra2 to the C-terminus of the *M. smegmatis* MutT4/RppH homolog (RppH<sub>Msmeg</sub>, MSMEG\_6927) formed puncta that partially colocalized with RNase E and RNase J condensates (Fig. 5A). These results suggest that there could be interactions between RppH<sub>Msmeg</sub> and RNase E or RNase J, and are consistent with the idea that mycobacterial RppH has a role in mRNA degradation despite its unexpectedly complex impact on mRNA half-lives. Expression of an RppH<sub>Mtb</sub>-Dendra2 construct in *M. smegmatis* showed focus formation similar to that of RppH<sub>Msmeg</sub>-Dendra2, indicating that the formation of these structures is conserved across mycobacteria (Fig. 5B).

To determine if RppH<sub>Msmeg</sub> puncta were dynamic condensate-like bodies affected by the presence of mRNA, we treated *M. smegmatis* with rifampicin to block transcription and thus reduce the bacterial mRNA levels. We found that RppH<sub>Msmeg</sub> puncta were disassembled in the presence of rifampicin (Fig. 5C), confirming that they are dynamic and that they might be RNA-dependent.

### The N-terminal IDR of RppH<sub>Msmeg</sub> is sufficient for condensate-like body formation

We next examined if the IDRs present in mycobacterial RppH participate in formation of condensate-like bodies. To do so, we fused either the RppH<sub>Msmeg</sub> N- or C-terminal IDR, or both IDRs, to Dendra2 as shown in the schematic in Fig. 6A. As expected, a Dendra2 control showed diffuse cytoplasmic distribution without the formation of puncta (Fig. 6B and C). Fusion of the RppH<sub>Msmeg</sub> N-terminal IDR, but not the C-terminal IDR, was sufficient for the formation of puncta. Finally, a construct with both IDRs fused to Dendra2 (resembling the domain architecture of RppH<sub>Msmeg</sub>) also formed puncta. This result shows that the N-terminal IDR of RppH<sub>Msmeg</sub> is sufficient for localization into condensate-like bodies, while the role of the C-terminal IDR is unclear.

To investigate the functional roles of the RppH IDRs in Mtb, we complemented Mtb  $\Delta rppH_{Mtb}$  with *rppH<sub>Mtb</sub>* harboring deletions of the IDRs individually and together. Analysis of the protein expression of these constructs showed that deletion of either IDR decreased the protein abundance, with an additive effect when both IDRs were deleted (Supplementary Fig. S8). This suggests that the IDRs are involved in the stability of the RppH<sub>Mtb</sub> protein in Mtb and complicates further analysis of their roles since we cannot disentangle the impact of IDR truncation from the impact of altered protein abundance.

### The RppH<sub>Mtb</sub> R48G polymorphism found in Lineage 2 has no effect on RppH activity or formation of condensate-like bodies

An Arg-to-Gly point mutation at residue 48 (R48G) in RppH<sub>Mtb</sub> was previously identified in Mtb strains belonging to Lineage 2 (L2, Beijing lineage) [38]. As at the time RppH<sub>Mtb</sub>

was considered to be a DNA repair enzyme based on sequence homology, these studies postulated that the R48G polymorphism could affect the mutation rates of L2 strains. As our results indicate that Mtb RppH<sub>Mtb</sub> is an RppH, we sought to analyze if the R48G polymorphism affected this process. We complemented the Mtb  $\Delta rppH_{Mtb}$  mutant strain with either an *rppH<sub>Mtb</sub>* WT or R48G allele (nucleotide C142G) and used splinted ligation to quantify *in vivo* RppH activity on the Rv3248c transcript. We found no significant differences between the strains, indicating that the R48G polymorphism does not affect RppH<sub>Mtb</sub>'s enzymatic activity (Supplementary Fig. S9A). Both of the RppH<sub>Mtb</sub> versions were expressed at similar protein levels, indicating that R48G does not change the protein stability (Supplementary Fig. S9B). Finally, as the R48G mutation is located in the N-ter IDR, we heterologously expressed RppH<sub>Mtb</sub>-Dendra2 in *M. smegmatis* and quantified the CV of fluorescence signal within each cell as a metric of condensate-like body formation, and found no significant differences between the WT and R48G alleles (Supplementary Fig. S9C and D). Overall, these results indicate that the R48G point mutation does not affect the activity of RppH<sub>Mtb</sub>.

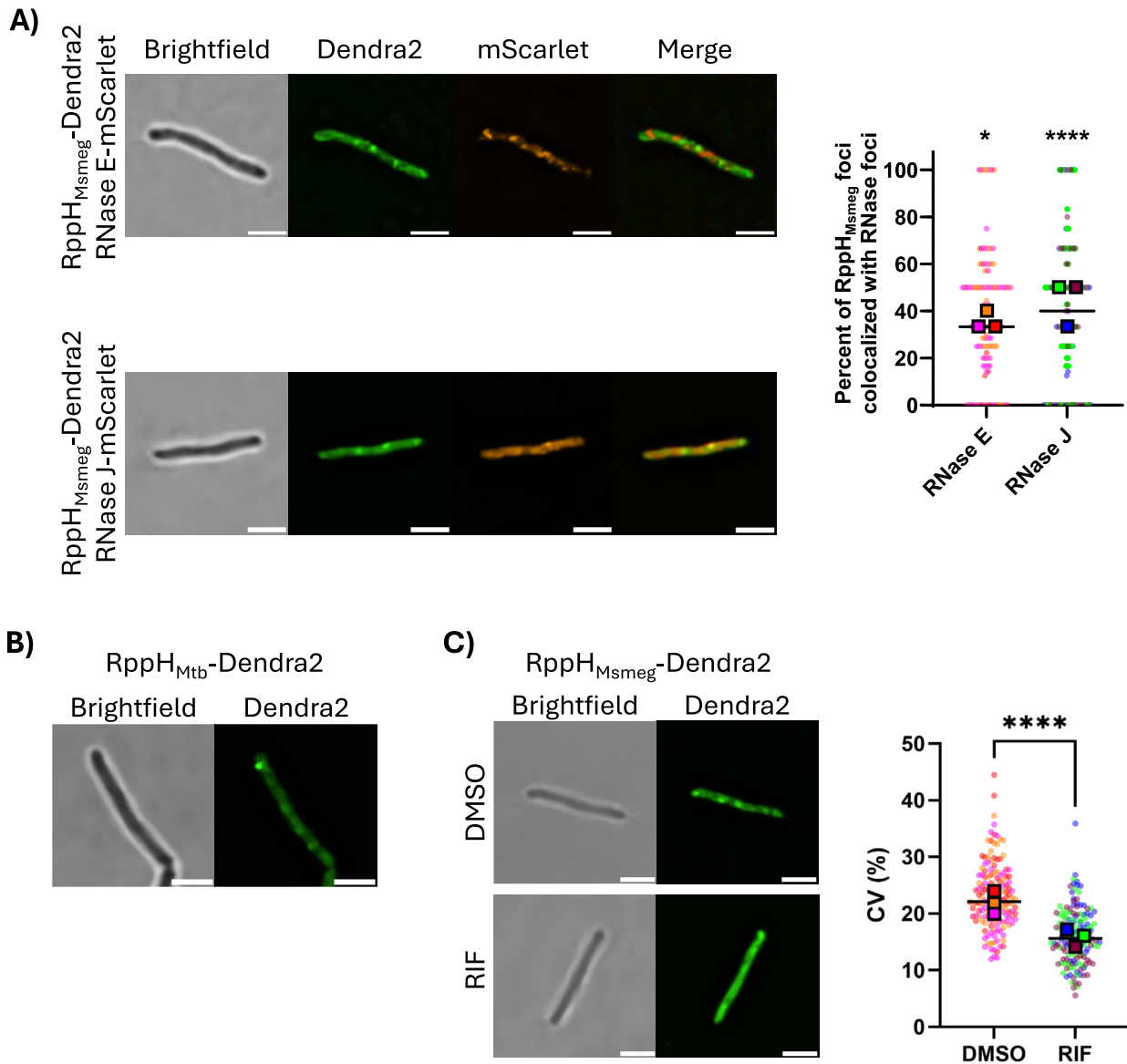
### RppH<sub>Mtb</sub> is not required for Mtb to survive in hypoxia

Adaptation and survival of hypoxic conditions is critical for a successful infection by Mtb [67]. We therefore compared the survival of Mtb WT and  $\Delta rppH_{Mtb}$  strains in hypoxic conditions using a modified Wayne model. We detected no differences in the time of methylene blue discoloration (an indirect measurement of oxygen consumption) or the overall viability in hypoxia, indicating that *rppH<sub>Mtb</sub>* is not involved in adaptation to, or survival of, hypoxia (Supplementary Fig. S10).

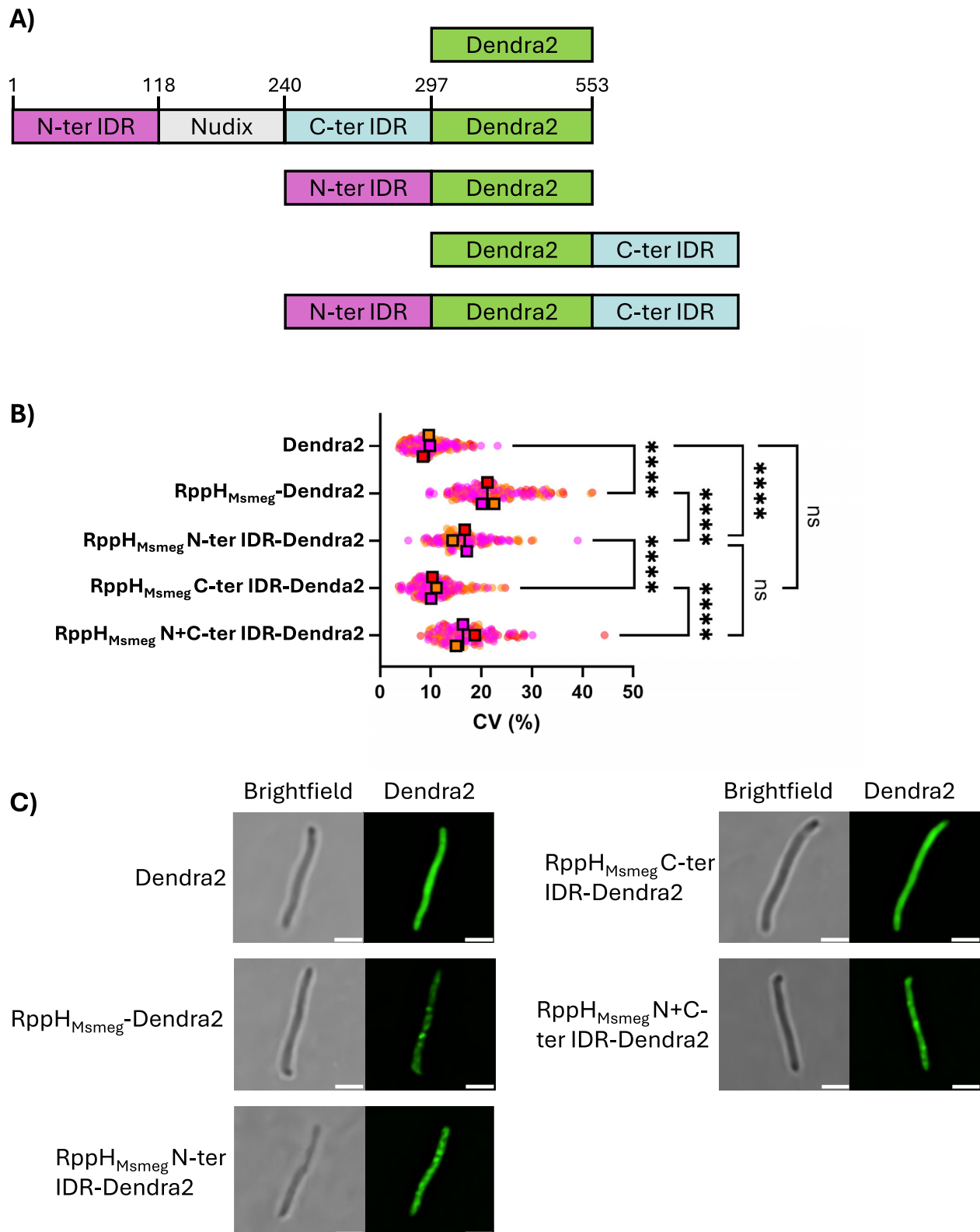
### Absence of RppH<sub>Mtb</sub> results in increased vancomycin sensitivity and outer membrane permeability

A previous CRISPRi [68] study indicated that repression of *rppH<sub>Mtb</sub>* led to increased vancomycin and rifampicin sensitivity. To test these predictions, and determine if they were due to *rppH<sub>Mtb</sub>* itself and not polar effects on downstream genes, we tested the resistance of  $\Delta rppH_{Mtb}$  to a panel of antibiotics. We found that deletion of *rppH<sub>Mtb</sub>* increased the sensitivity of Mtb to vancomycin (Fig. 7A) and a weak trend in the same direction was seen for rifampicin (Supplementary Fig. S11). Interestingly, we found no differences in sensitivity to bedaquiline, chloramphenicol, isoniazid, kanamycin, or ofloxacin, suggesting that this increased sensitivity caused by the absence of *rppH<sub>Mtb</sub>* is specific to vancomycin and rifampicin. As vancomycin and rifampicin have the highest molecular weights of the antibiotics tested, and the site of action of vancomycin is outside of the cell membrane, we hypothesized that the deletion of *rppH<sub>Mtb</sub>* may increase the permeability of Mtb's mycomembrane. To evaluate this, we measured the uptake of a fluorescent vancomycin derivative and found that it was increased in the *rppH<sub>Mtb</sub>* deletion strain (Fig. 7B). We then measured mycomembrane permeability in an orthogonal fashion by assessing uptake of an azide-rifamycin conjugate into the Mtb periplasm (Fig. 7C) [55, 58, 69]. Uptake of the azide-rifamycin conjugate was greater in the *rppH<sub>Mtb</sub>* deletion strain than in the WT or

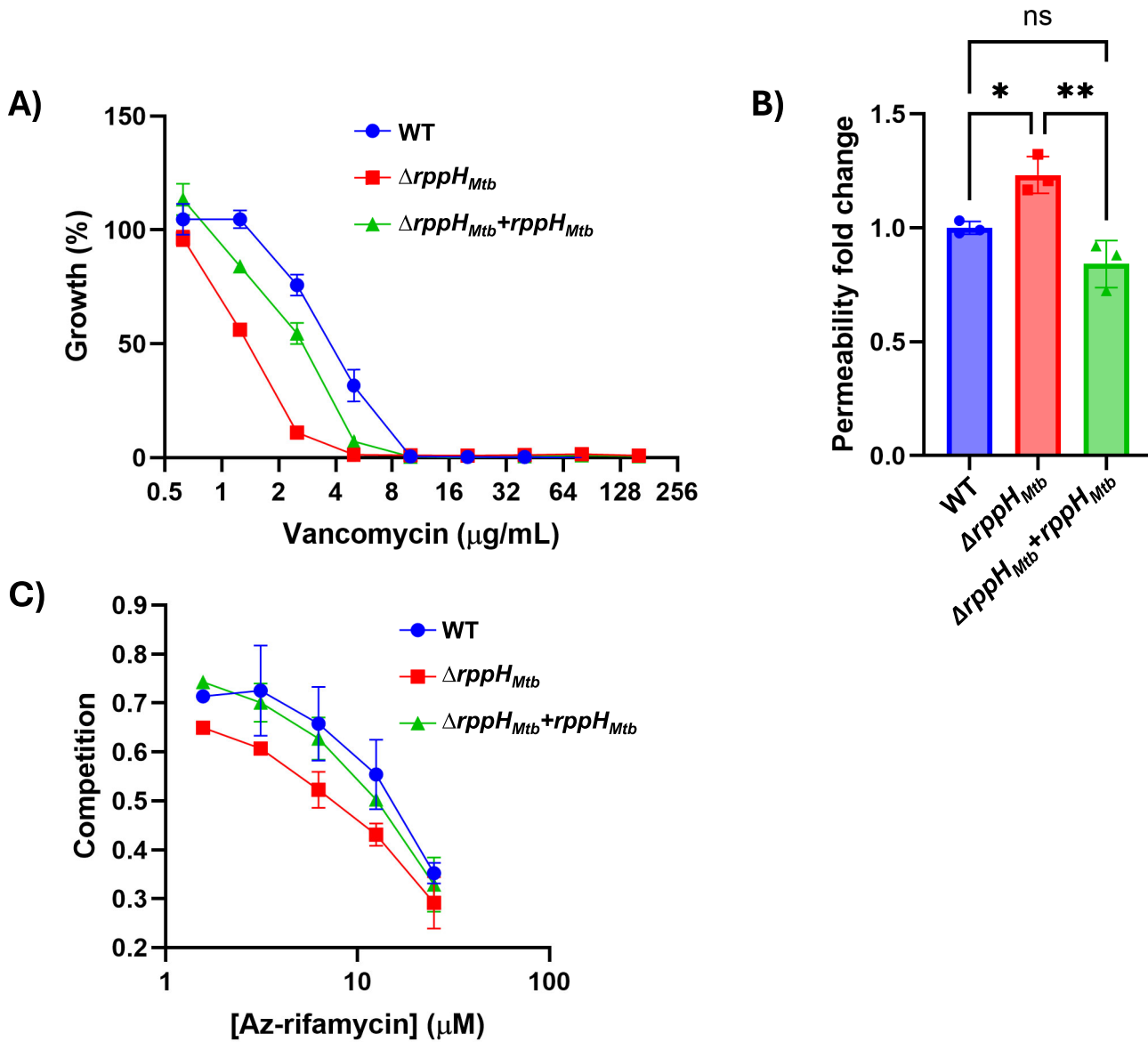




**Figure 5.** RppH<sub>Mtb</sub> forms dynamic condensate-like bodies that appear to localize with RNase E and RNase J. Genes encoding the indicated fluorescent protein fusions were integrated in single copy into phage integration sites in the genome of otherwise WT *M. smegmatis* strain mc<sup>2</sup>155. Live cells were imaged during mid-log phase. All genes are from *M. smegmatis* except in panel (B). **(A)** RppH<sub>Msmeg</sub> localizes with RNase E and RNase J condensates. Protein localization was analyzed using ImageJ by tracing a line along the center of each bacterium. Fluorescence intensity profiles for the red and green channels were plotted, and peaks within 0.2 μm were counted as colocalized. Percentage localization for each bacterium was calculated as RppH<sub>Msmeg</sub> colocalizing peaks divided by total RppH<sub>Msmeg</sub> peaks. The significance of the colocalization was assessed by flipping the x-axis values for one of the two colors, calculating percentage localization, and comparing those values to the real data by Mann-Whitney test. \*  $P < .05$ , \*\*\*\*  $P < .0001$  **(B)** RppH<sub>Mtb</sub> forms condensate-like bodies when expressed heterologously in *M. smegmatis*. **(C)** Treatment with rifampicin disassembles RppH<sub>Msmeg</sub> condensates. *Mycobacterium smegmatis* expressing RppH<sub>Msmeg</sub>-Dendra2 was treated with 100 μg/ml rifampicin (RIF) or DMSO as a control for 30 min and imaged. The coefficient of variance (CV) of fluorescence signal within each cell was used as a metric of condensate formation. More punctate signal results in a higher CV. Each dot indicates the CV of fluorescence intensity along a straight line from one cell pole to the other. Scale bar 2 μm for all images. In the plots in panels (A) and (C), data are from three biological replicates, 50 bacteria each. Medians from each replicate culture are shown as squares and data from individual bacteria as circles. Mann-Whitney test was performed for panel (C). \*\*\*\*  $P \leq .0001$ .



**Figure 6.** The N-terminal intrinsically disordered region of RppH<sub>Msmeg</sub> is sufficient for focus formation. **(A)** Schematic representation of Dendra2 constructs (not to scale). Amino acids are indicated in the RppH<sub>Msmeg</sub> full-length Dendra2 construct. **(B)** CV of fluorescence signal within each cell was used as a metric of condensate formation. More punctate signal results in a higher CV. Each dot indicates the CV of fluorescence intensity along a straight line from one cell pole to the other. Data are from three biological replicates, 50 bacteria each. Medians from each replicate are shown as squares and data from individual bacteria as circles. **(C)** Representative microscopy images. Scale bar 2  $\mu$ m. Kruskal-Wallis test was performed for panel (B). \*\*\*\*  $P \leq .0001$ .

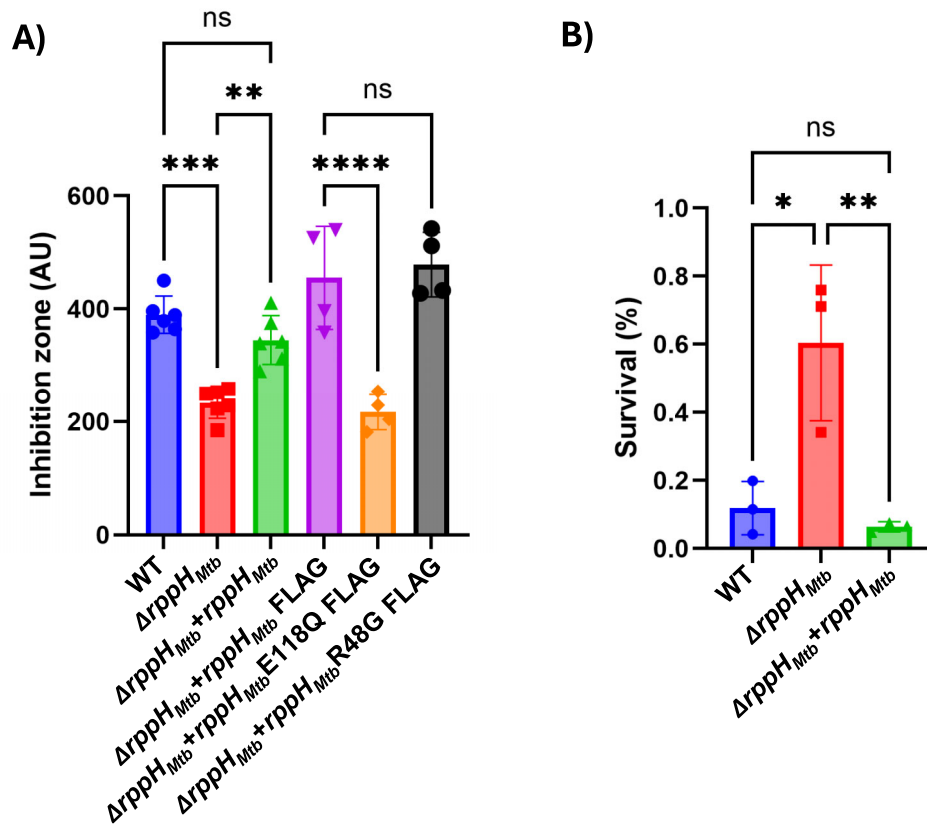


**Figure 7.** Deletion of *rppH<sub>Mtb</sub>* leads to increased vancomycin sensitivity and mycomembrane permeability. **(A)** Antibiotic susceptibility testing of Mtb strains against vancomycin. A representative result performed with technical duplicates of three biological replicates is shown. **(B)** Permeability to a fluorescent derivative of vancomycin (FL-VAN). Mtb was incubated with FL-VAN, and permeability to this molecule was measured by flow cytometry and normalized to the WT strain. Higher values indicate higher permeability. Mean and standard deviation of three biological replicates. **(C)** Permeability to an azide-rifamycin conjugate measured by PAC-MAN competition assay. Mtb peptidoglycan layer was tagged with DBCO groups. Next, cells were incubated with an azide-rifamycin conjugate that covalently reacts with the DBCO groups via SPAAC. Finally, unreacted DBCO epitopes were linked to a highly permeable azide fluorophore and total cellular fluorescence was measured by flow cytometry. Fluorescence was normalized to the control untreated with azide-rifamycin. Higher competition values indicate lower permeability. Mean and standard deviation of three biological replicates. A one-way ANOVA was performed with Tukey's test for panel (B). \*  $P \leq .05$ , \*\*  $P \leq .01$ .

complemented strains. Together, these data suggest that the *rppH<sub>Mtb</sub>* deletion strain has increased mycomembrane permeability that is not due to altered expression of genes encoded near the *rppH<sub>Mtb</sub>* locus. While identifying the mechanistic basis of this observation is beyond the scope of the current study, we found that the genes whose transcript 5' ends were most affected by deletion of *rppH<sub>Mtb</sub>* were enriched for the GO term “extracellular region,” suggesting that genes encoding cell envelope functions may be affected by loss of *rppH<sub>Mtb</sub>* (Supplementary Fig. S12; see the “Discussion” section).

#### Deletion of *rppH<sub>Mtb</sub>* increases Mtb oxidative stress resistance

In *M. smegmatis*, *rppH<sub>Msmeg</sub>* is required for resistance to oxidative stress [70]. To test if this was also true for Mtb, we analyzed resistance to  $\text{H}_2\text{O}_2$  by a disk diffusion assay. Our results show that in contrast to what was previously reported for *M. smegmatis*, deletion of *rppH<sub>Mtb</sub>* in Mtb increases the resistance to oxidative stress compared to the WT strain (Fig. 8A). This difference was reverted by complementation with a WT *rppH<sub>Mtb</sub>* allele, but not with the E118Q catalytically dead ver-



**Figure 8.** Deletion of *rppH*<sub>Mtb</sub> leads to increased resistance to H<sub>2</sub>O<sub>2</sub> in Mtb. **(A)** Resistance to H<sub>2</sub>O<sub>2</sub> measured by disk diffusion assay. **(B)** Acute treatment of Mtb with H<sub>2</sub>O<sub>2</sub> in liquid media. Mtb was exposed to 10 mM H<sub>2</sub>O<sub>2</sub> for 24 h and plated to enumerate CFU/ml. Data from three biological replicates are expressed as survival normalized to CFU/ml at time 0. A one-way ANOVA was performed with Tukey's test for panels (A) and (B). \*  $P \leq .05$ , \*\*  $P \leq .01$ , \*\*\*  $P \leq .001$ , \*\*\*\*  $P \leq .0001$ .

sion, suggesting that RppH activity is required for this phenotype. Complementation with the *rppH*<sub>Mtb</sub> R48G allele showed no difference compared to the *rppH*<sub>Mtb</sub> WT version. We further confirmed the impact of *rppH*<sub>Mtb</sub> on H<sub>2</sub>O<sub>2</sub> sensitivity with an orthogonal approach, by measuring the survival of Mtb after an acute exposure to H<sub>2</sub>O<sub>2</sub> in 7H9 liquid media. A higher number of viable bacteria were recovered in the *rppH*<sub>Mtb</sub> deletion strain compared to the WT and complemented strains (Fig. 8B).

## Discussion

Adaptation to changes in the environment by altering the transcriptome is critical for bacterial survival under stressful conditions. In *E. coli*, RNA degradation can be initiated by RppH pyrophosphate removal and this in turn modulates transcript abundance [25]. Here, we identify that *mutT4* (Rv3908) encodes the mycobacterial RppH enzyme. While we found that RppH<sub>Mtb</sub> processes the 5' ends of a significant proportion of the transcriptome, its impact on the steady-state transcriptome is small, and its deletion did not extend the half-life of most of the transcripts we tested, as discussed further below. This suggests that transcript sensitivity to RppH<sub>Mtb</sub> is not a major determinant of half-life under the conditions tested. Moreover, *rppH*<sub>Mtb</sub> is a non-essential gene and its deletion does not affect the overall growth rate of Mtb. This result

implies that in mycobacteria under optimal growth conditions, 5' end processing is likely a rate-limiting step for only a small subset of transcripts, and that degradation of the majority of transcripts may rely on mechanisms independent of 5' end chemistry, such as endonucleolytic cleavage by direct entry, 3'-to-5' exonucleolytic degradation, or 5'-to-3' exonucleolytic degradation by an RNase J that is active on some triphosphorylated transcripts. Alternatively, Mtb could code for other RNA pyrophosphohydrolase(s) that act in coordination with RppH<sub>Mtb</sub> and were not identified by our screening method. The presence of additional enzymes involved in the conversion of RNA 5' ends from triphosphates to monophosphates other than RppH was also suggested for *E. coli* [71] and *B. subtilis* [26]. Despite the apparently limited role of RppH<sub>Mtb</sub> on the steady-state transcriptome under the conditions tested, its deletion impacted both cell envelope permeability and sensitivity to oxidative stress, suggesting that it may have a larger role in other conditions and/or that it may have impacts on processes such as translation that are not captured by RNAseq.

Oxidation of dGTP generates the mutagenic nucleotide 8-oxo-dGTP. In *E. coli* the MutT Nudix hydrolase degrades 8-oxo-dGTP, preventing its misincorporation into DNA [72]. In a search for mycobacterial genes involved in DNA repair, RppH<sub>Mtb</sub> was identified by sequence homology to MutT and initially named accordingly [38]. As Mtb clinical strains of the



L2-Beijing lineage carry a point mutation in *rppH*<sub>Mtb</sub> at codon 48 that results in an Arg-to-Gly substitution, it was speculated that this variant could lead to an increased mutation rate, a selective advantage for infection and drug resistance [38]. However, assigning RppH<sub>Mtb</sub> a function as a mutagenic nucleotide cleansing enzyme due to the presence of a Nudix domain is misleading, as this motif is involved in coordination of divalent cation(s) in the active site but does not participate in substrate specificity [73, 74]. Further, deletion of *rppH*<sub>Mtb</sub> in Mtb did not lead to an increase in spontaneous mutation frequency [75], suggesting that its role is not related to DNA repair. In *M. smegmatis*, deletion of *rppH*<sub>Msmeg</sub> affected bacterial growth and conferred sensitivity to H<sub>2</sub>O<sub>2</sub> stress and the antibiotics rifampicin and ciprofloxacin [70]. All these phenotypes were complemented by expressing *rppH* of either *M. smegmatis* or Mtb, but not by an *M. smegmatis* *rppH*<sub>Msmeg</sub> E162A catalytic mutant (equivalent to our Mtb *rppH*<sub>Mtb</sub> E118Q point mutation). These results confirm that RppH serves the same function in both species and that its catalytic activity is essential for this role. Our results here indicate that RppH<sub>Mtb</sub> is involved in RNA degradation due to its RppH activity. We hypothesize that the phenotypes observed by the deletion of *rppH* in Mtb and *M. smegmatis* are downstream effects due to changes in mRNA 5' end chemistry that modify transcript stability, and therefore gene expression, during the adaptation to stress. Interestingly, the growth disadvantage caused by the deletion of *rppH* in *M. smegmatis* [70] implies that it could be the only RppH present in that species. RppH in *H. pylori* is also involved in the resistance to oxidative stress, indicating a common link between these two pathways [76]. Regarding the RppH<sub>Mtb</sub> R48G point mutation in L2-Beijing lineage strains, our results show that this variant has no effect on the RppH activity, formation of condensate-like bodies, or resistance to oxidative stress related to RppH<sub>Mtb</sub>. It remains to be determined if this R48G variant has an undetected impact on RppH<sub>Mtb</sub> that enhances Mtb pathogenicity, or if its prevalence is due to linkage with another mutation that was advantageous and thus clonally expanded.

A significant number of Mtb RNA transcripts are leaderless, as they lack 5' UTRs [77]. This absence of a ribosome binding sequence does not impair their ability to be translated [42, 78, 79]. Previous studies have not, however, considered the possible impact of 5' end phosphorylation state on translation of leaderless transcripts. It could be the case that changes in the 5' end chemistry of the mRNA could alter not the transcript stability itself, but its ability to be translated. In this context, the deletion mutant of *rppH*<sub>Mtb</sub> is a useful model to test this kind of hypothesis, as in this background there is increased triphosphorylation of a large array of mRNAs, including leadered and leaderless transcripts.

Our 5' end RNA-seq results suggest that RppH<sub>Mtb</sub> may be most active on transcripts with unstructured 5' ends that begin with GA dinucleotides, while GU- and GG-starting transcripts were also affected more than transcripts beginning with GC or A. *B. subtilis* RppH requires at least two unpaired nucleotides at the 5' end of its RNA substrates and strongly prefers G as a second nucleotide, while for the first nucleotide A is slightly preferred over G [36, 80]. Studies on *E. coli* RppH showed that it also requires two unpaired nucleotides at the 5' end and has a less strict sequence preference, with A being modestly favored as a first nucleotide [81]. Differences in the active sites of these enzymes that shape their substrate preferences could impact the mRNAs they target, adding a layer of control to

tune mRNA levels by selective degradation. Biochemical characterization of RppH orthologs has also shown two distinct mechanisms of catalysis on 5'-triphosphorylated mRNA substrates: while *E. coli* RppH predominantly cleaves a pyrophosphate from the mRNA 5' end [25], *B. subtilis* RppH cleaves one phosphate at a time [26]. Further studies are needed to determine if catalysis by RppH<sub>Mtb</sub> resembles that of the *E. coli* or *B. subtilis* orthologs, as well as to determine if the observed relationships between 5' end nucleotide identity and RppH<sub>Mtb</sub> impact are due to RppH<sub>Mtb</sub>'s preferences, or to the preferences of RNases whose degradation activities are influenced by 5' end phosphorylation state, or to a mixture of both.

Unlike *E. coli* and *B. subtilis*, mycobacteria encode for both the endoribonuclease RNase E and the 5' exo-/endoribonuclease RNase J. *Escherichia coli* RNase E has been shown to prefer 5' monophosphorylated over triphosphorylated RNA substrates for degradation [28]. Studies on RNase J in *B. subtilis*, *Thermus thermophilus*, and *Streptomyces coelicolor* have shown that its exonuclease activity is enhanced by 5' monophosphates and its endonuclease activity predominates with 5' triphosphorylated RNA substrates [26, 30–33]. A study of *M. smegmatis* RNase J also found that exonucleolytic degradation of a tested transcript was greater when the 5' end was monophosphorylated compared to when it was triphosphorylated [34]. Structural analysis of RNase E and RNase J support these results by showing the presence of a monophosphate binding pocket, favoring degradation of these substrates [29, 32]. Here, we found that the activity of Mtb's RNase E and RNase J on a single tested substrate was greater following conversion of 5' triphosphates to monophosphorylates. Zeller *et al.* [82] characterized Mtb RNase E with an N-terminal truncation, comparing its activity on RNA substrates with either a 5' monophosphate or a 5' hydroxyl group, and found a preference for the 5' monophosphorylated substrates. Here, we extend these results by comparing monophosphorylated and triphosphorylated substrates, and by using full-length RNase E. In contrast to the previous report on *M. smegmatis* RNase J, our results suggest that Mtb RNase J's exonucleolytic activity may also be efficient on some triphosphorylated substrates, as we observed substantial disappearance of the substrates in all RNase J reactions without appearance of any clear bands. The impact of RppH<sub>Mtb</sub> pre-treatment on cleavage by RNase J was statistically significant but modest in magnitude, suggesting that for this substrate at least, the 5' end phosphorylation state has only a small impact on RNase J activity. Testing additional substrates with different 5' end sequences and secondary structure tendencies would be required to determine if this is a general property of RNase J or if the impact of 5' end phosphorylation is greater on some substrates; the study on *M. smegmatis* RNase J also tested only a single substrate [34]. Notably, the transcript we used for the *in vitro* cleavage assays was Rv3248c, which we had selected for use in our initial screen because it had relatively high levels of steady-state 5' monophosphorylation in Mtb. One possible explanation for the relatively high steady-state 5' monophosphate levels could be that for this particular transcript, conversion of 5' triphosphates to monophosphates does not greatly increase susceptibility to degradation by RNases. In sum, our *in vitro* results indicate that RNA degradation in mycobacteria can be enhanced by conversion of mRNA 5' ends to monophosphates, although likely to different extents depending on the substrate and enzyme.

Despite the observed impact of RppH<sub>Mtb</sub>'s activity on RNase E and RNase J activity *in vitro*, deletion of *rppH*<sub>Mtb</sub> increased the half-life of only one of the ten transcripts we tested in Mtb cells. Surprisingly, *rppH*<sub>Mtb</sub> deletion led to a shorter half-life for one gene and trends toward shorter half-lives for several others. Thus, the prevailing trend was the opposite of what we expected. Furthermore, complementation of the deletion strain increased the half-lives of most of the transcripts we tested. The complemented strain expressed the *rppH*<sub>Mtb</sub> from its native promoter, producing transcript abundance slightly lower than that of the WT strain (Supplementary Fig. S1). It is therefore unclear why the complemented strain would have a phenotype opposite that of the deletion strain. It is possible that the transcript produced by the complementation strain is translated more efficiently than that of the WT strain, resulting in greater RppH<sub>Mtb</sub> protein levels despite having slightly lower mRNA levels. While use of the native promoter is expected to produce a transcript with the same 5' end as the WT strain, the 3' end is different. Comparison of *rppH*<sub>Mtb</sub> expression patterns to those of the downstream gene, Rv3909, suggests that they may be co-transcribed in at least some conditions. This operon structure was not captured in the complementation plasmid, where the *rppH*<sub>Mtb</sub> coding sequence was followed by a short synthetic 3' UTR and synthetic transcription terminator. Secondary structure or translation dynamics of the two-gene polycistron may impact the translation of RppH<sub>Mtb</sub>. It is also possible that the location of the gene within the chromosome has an impact, as it could affect the spatial localization of RppH<sub>Mtb</sub> translation relative to that of other proteins that may interact with it and affect its activity.

While increased RppH<sub>Mtb</sub> protein levels would explain why the complemented strain has an impact on mRNA degradation opposite that of the deletion strain, it does not explain why the complemented strain has generally slower mRNA degradation than the WT strain. This seemingly paradoxical impact of RppH<sub>Mtb</sub> on mRNA degradation could result from compensatory regulation of RNases in response to the absence or overabundance of RppH<sub>Mtb</sub>. However, such an effect would have to be post-transcriptional, since we did not observe any changes in steady-state abundance of transcripts encoding RNases in the *rppH*<sub>Mtb</sub> deletion strain. It is also possible that overabundance of RppH<sub>Mtb</sub> could impact the localization of interacting RNAs or RNases in ways that inhibit their activity (discussed further below). Clearly, further work beyond the scope of the current study is needed to fully understand the role of RppH in mycobacterial mRNA degradation.

Our results show that the deletion of *rppH*<sub>Mtb</sub> in Mtb leads to an increase in vancomycin sensitivity. We further confirmed that the increased vancomycin sensitivity was due at least in part to a disruption of the mycomembrane permeability in the absence of *rppH*<sub>Mtb</sub>. This result is in line with what has been reported for *E. coli*, as the deletion of *rppH* leads to a higher envelope permeability and increased sensitivity to antibiotics [83]. This phenotypic convergence is remarkable given the substantial differences in cell envelope composition between mycobacteria and proteobacteria. The emerging link between RNA metabolism and cell envelope homeostasis is intriguing. RppH<sub>Mtb</sub> is coded in an operon that may include the gene encoding the putative lipid II flippase MurJ (Rv3910), which is expected to be required for peptidoglycan biosynthesis. Deletion of *rppH*<sub>Mtb</sub> appears to increase the mycomembrane permeability, and this phenotype is complemented by an ectopically expressed copy of

*rppH*<sub>Mtb</sub>, indicating that it is unrelated to any possible polar effects on *murJ*. The mechanistic link between RppH<sub>Mtb</sub> and cell envelope composition is therefore unknown. However, the genes with 5' ends most affected by *rppH*<sub>Mtb</sub> were enriched for those associated with the extracellular region. These include *fbpA* (an Ag85 component), *emhA* (arabinoxyltransferase), *mas* (mycosideric acid synthase), *eccB5* (ESX-5 secretion system ATPase), *rfe* (decaprenylphosphate N-acetylglucosaminephosphotransferase), *wbbL1* (N-acetylglucosaminyl-diphospho-decaprenol L-rhamnosyltransferase), *glf* (UDP-galactopyranose mutase), and *ppsE* (phenolphthiocerol/phthiocerol polyketide synthase). Additionally, several impacted transcripts encode membrane or secreted proteins of unknown function as well as predicted transcriptional regulators. While none of these transcripts appeared to have altered steady-state abundance, it is possible that their 5' end chemistry could impact their translation.

Interestingly, RNase J is encoded in an operon together with the gene encoding DapA, a protein involved in biosynthesis of an intermediate for mycobacterial peptidoglycan synthesis [84]. *Escherichia coli* RppH activity is stimulated by DapF [85], a metabolic enzyme involved in peptidoglycan biosynthesis [86]. Functional links between mRNA degradation and cell envelope biosynthesis therefore seem to be conserved across the bacterial domain. It is tempting to speculate that this functional link could facilitate the rapid changes in bacterial gene expression in response to stressors that impact the cell envelope; more work is needed to investigate this hypothesis.

Biomolecular condensates have recently gained recognition as mechanisms for bacterial cells to organize their cytoplasm into membraneless compartments with distinct roles as well as to stimulate chemical reactions by increasing local concentrations of enzymes and substrates [65, 87]. Several biomolecular condensates have been described in bacteria, such as BR-bodies (composed of the RNase E-based RNA degradosome and its substrates) [22], RNA polymerase clusters [88], and condensates of the transcription termination factor Rho [89], among others [90]. In Mtb, RNase E has been described as forming condensates [66], as well as the ABC transporter Rv1747, which phase separates depending on its phosphorylation status [91]. The overlap of RppH<sub>Mtb</sub> puncta with RNase E and RNase J puncta would be consistent with a model in which degradation proteins form condensates that serve as mRNA degradation hubs. However, this idea is inconsistent with our observation that the presence of RppH<sub>Mtb</sub> appears to decrease rather than increase mRNA degradation rates. It seems likely that the role of RppH<sub>Mtb</sub> is more complex; for example, those RppH<sub>Mtb</sub> condensates that do not include RNases could conceivably sequester mRNAs away from RNases and thus protect them from degradation.

Mycobacterial RppHs are the only RppH orthologs described so far that have large IDRs, and our results support a model where the N-terminal IDR is required for condensate formation. *Escherichia coli* RppH is predicted to have a very short IDR at its C-terminus consisting of only 11 amino acids, and the same is true for the first 10 amino acids of *Bdellovibrio bacteriovorus* RppH. It would be interesting to determine if other RppH orthologs are capable of forming biomolecular condensates, either by an IDR-independent pathway or by being recruited by an IDR-containing protein, as this compartmentalization could be a mechanism that bacteria use

for regulating mRNA degradation. Intriguingly, the eukaryotic mRNA decapping enzyme Dcp2 (a Nudix hydrolase) has a large IDR at its C-terminus involved in the formation of P-bodies, a biomolecular condensate enriched in mRNA decay proteins [92]. The parallelism of condensate formation by RNA 5' end modification enzymes (RppH in mycobacteria and Dcp2 in eukaryotes) shows that compartmentalization of this RNA degradation step is broadly conserved.

The results presented here support the designation of MutT4 (Rv3908) as the RppH enzyme in mycobacteria. Further studies on the proteins that interact with RppH<sub>Mtb</sub> and the dynamics and role of condensate formation will shed light on its surprisingly complex role in mycobacterial mRNA degradation.

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## Supplementary data

Supplementary data is available at NAR online.

## Conflict of interest

None declared.

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## Data availability

RNA-seq data are available in the NCBI Gene Expression Omnibus (GEO) repository (Accession number GSE292713).

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