Molecular dissection of RbpA-mediated regulation of fidaxomicin sensitivity in mycobacteria

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RNA polymerase (RNAP) binding protein A (RbpA) is essential for mycobacterial viability and regulates transcription initiation by increasing the stability of the RNAP-promoter open complex (RPo). RbpA consists of four domains: an N-terminal tail (NTT), a core domain (CD), a basic linker, and a sigma interaction domain. We have previously shown that truncation of the RbpA NTT and CD increases RPo stabilization by RbpA, implying that these domains inhibit this activity of RbpA. Previously published structural studies showed that the NTT and CD are positioned near multiple RNAP-σf holoenzyme functional domains and predict that the RbpA NTT contributes specific amino acids to the binding site of the antibiotic fidaxomicin (Fdx), which inhibits the formation of the RPo complex. Furthermore, deletion of the NTT results in decreased Mycobacterium smegmatis sensitivity to Fdx, but whether this is caused by a loss in Fdx binding is unknown. We generated a panel of rbpA mutants and found that the RbpA NTT residues predicted to directly interact with Fdx are partially responsible for RbpA-dependent Fdx activity in vitro, while multiple additional RbpA domains contribute to Fdx activity in vivo. Specifically, our results suggest that the RPo-stabilizing activity of RbpA decreases Fdx activity in vivo. In support of the association between RPo stability and Fdx activity, we find that another factor that promotes RPo stability in bacteria, CarD, also impacts to Fdx sensitivity. Our findings highlight how RbpA and other factors may influence RNAP dynamics to affect Fdx sensitivity.

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**Results**

**RbpA E17 and R10 synergize to promote Fdx activity against *M. tuberculosis* RNAP-σ^A^ in vitro**

In vitro assays that monitor the production of a 3-nucleotide product as a proxy of RNAP-σ^A^ stability have shown that addition of Fdx to *M. tuberculosis* RNAP-σ^A^ holoenzymes reduces the amount of RNAP-σ^A^ formed following the subsequent addition of NTPs and a DNA template harboring the *M. tuberculosis* rrnA3P promoter (11). We used this assay with a range of Fdx concentrations to calculate the concentration of Fdx that inhibits 50% of RNAP-σ^A^ on the rrnA3P promoter in the presence or absence of different RbpA variants. Addition of WT RbpAWT (RbpAWT) to the RNAP-σ^A^ holoenzyme increases the sensitivity of the RNAP-σ^A^ holoenzyme to Fdx in this assay, and this is dependent on the presence of the NTT (deleted in the RbpAΔmb26–111 and RbpAΔmb26–111 mutants) (11) (Fig. 1, A–C). Deletion of both the RbpA NTT and CD resulted in an IC50 within the confidence interval of the IC50 when the NTT alone was deleted (Fig. 1, B and C), indicating that the presence of the RbpA CD does not affect Fdx activity against *M. tuberculosis* RNAP-σ^A^ in vitro. In contrast, an R88A substitution in the RbpA SID that weakens the interaction between RbpA and the RNAP resulted in an IC50 that was lower and outside the confidence interval, compared to RbpAWT, suggesting that domains outside of the NTT could increase Fdx sensitivity (Fig. 1C).

Importantly, a saturating concentration of RbpA protein was used in these assays, and therefore, the different effects of RbpA variants on Fdx sensitivity in this assay should not be a result of altered proportions of RbpA-bound RNAP-σ^A^ complexes.

Structural studies predicted that the NTT contributes contacts with Fdx when the antibiotic is bound to the *M. tuberculosis* RNAP-σ^A^ holoenzyme (PDB: 6BZO), specifically through a water-mediated interaction between RbpA E17 and Fdx (Fig. 1D) (11). To determine whether the predicted interaction between Fdx and RbpA E17 underpins NTT-dependent Fdx activity, we calculated the IC50 of Fdx in the presence of RbpAΔmb26–111El17A versus an RbpAΔmb26–111 mutant protein. The activity of Fdx against the *M. tuberculosis* RNAP-σ^A^ in the presence of RbpAΔmb26–111El17A was nearly equal to Fdx activity against the *M. tuberculosis* RNAP-σ^A^ in the presence of RbpAΔmb26–111WT, indicating that alterations in the size and charge of the amino acid side chain at RbpA NTT position 17 do not impact Fdx activity against the *M. tuberculosis* RNAP-σ^A^ (Fig. 1, B and C).

The structure in Boyaci et al. (11) also highlights potential van der Waals interactions between RbpA R10 and Fdx in the RNAP-σ^A^ holoenzyme bound to double stranded forked DNA (PDB: 6BZO) (Fig. 1D); however, given the distance between RbpA R10 and Fdx, one would predict this to be a weak interaction. In a separate structure of RbpA bound to *M. tuberculosis* RNAP-σ^A^ in complex with two double-stranded forked DNA molecules that mimics the RNAP-σ^A^, the RbpA R10 positively charged side chain is positioned within 2.4 Å of the negatively charged side chain of σ^A^ 3,2 D441, forming a polar interaction (11) (Fig. 1E). Fdx activity against *E. coli* RNAP-σ^A^ holoenzyme lacking σ^A^ 3,2 is attenuated approximately 20-fold (32), indicating that σ^A^ 3,2 contributes to Fdx inhibition of the *E. coli* RNAP. Therefore, if RbpA R10 interacts with σ^A^ 3,2, this may also affect Fdx activity.

To examine whether RbpA R10 contributes to *M. tuberculosis*
RNAP-σ^A Fdx sensitivity, we measured Fdx IC50 against the *M. tuberculosis* RNAP-σ^A in the presence of RbpA_Mtb^R10A^*. Similar to the RbpA_Mtb^E17A^* mutant, we observed no change in Fdx IC50s against the *M. tuberculosis* RNAP-σ^A in the presence of RbpA_Mtb^R10A^* compared to RbpA_Mtb^WT^ (Fig. 1, B and C), indicating that the R10 residue is not required for RbpA NTT-dependent Fdx activity. To determine the effect of disrupting the contacts made by the both RbpA E17 and R10,
we measured the Fdx IC50 against *M. tuberculosis* RNAP-σA in the presence of RbpA<sub>Mtb</sub><sup>R10A/E17A</sup>. Mutating both the R10 and E17 residues resulted in an approximately 3-fold increase in the Fdx IC50 compared to RbpA<sub>Mtb</sub><sup>WT</sup>, although this was still at least 5-fold lower than RbpA mutants lacking the entire NTT (RbpA<sub>Mtb</sub><sup>26–111</sup> and RbpA<sub>Mtb</sub><sup>72–111</sup>) (Fig. 1, B and C). These data indicate that loss of one of these residues increases the importance of the other for Fdx activity, but additional mechanisms also contribute to NTT-dependent Fdx activity in vitro.

**Multiple RbpA domains impact Fdx activity in vivo**

Previous work showed that truncation of the RbpA NTT decreases the sensitivity of *Mycobacterium smegmatis* to Fdx (11). To investigate the effect of mutations in RbpA on Fdx sensitivity in vivo, we used a strain we previously engineered that expresses rbpA<sub>Mtb</sub><sup>WT</sup> at the attB site of *M. smegmatis* and has the endogenous rbpA gene deleted (5). We then attempted to replace the rbpA<sub>Mtb</sub><sup>WT</sup> gene at the attB site in *M. smegmatis* with alleles encoding each of the RbpA mutants studied in Figure 1 using a gene swapping method (5, 33, 34). We have previously used this approach to generate an *M. smegmatis* strain expressing rbpA<sub>Mtb</sub><sup>72–111</sup>, which has a deletion of both the NTT and CD (Fig. 1A), as its only rbpA allele (5). However, we were unable to generate a viable strain expressing rbpA<sub>Mtb</sub><sup>26–111</sup> which deletes only the NTT (Fig. 1A), in place of rbpA<sub>Mtb</sub><sup>WT</sup>. In contrast, we were able to replace the rbpA<sub>Mtb</sub><sup>WT</sup> allele with the *M. smegmatis* allele rbpA<sub>Msm</sub><sup>28–114</sup>, which has previously been used to study the NTT in *M. smegmatis* (10, 11). Similar to our previous report with the *M. smegmatis* strain expressing RbpA<sub>Mtb</sub><sup>72–111</sup> (5), RbpA<sub>Msm</sub><sup>28–114</sup> and RbpA<sub>Msm</sub><sup>72–114</sup> strains also exhibited a slow growth phenotype (Fig. 2A), confirming that while the NTT and CD are not required for viability in *M. smegmatis*, they are important domains for RbpA activity. We have also
previously shown that *M. smegmatis* expressing RbpA\textsubscript{Mtb} R88A or RbpA\textsubscript{Mtb} R79A as its only *rpbA* allele also exhibits a slow growth phenotype due to the importance of RbpA’s interaction with the RNAP and DNA (5). Using the gene swapping approach, we found that the RbpA\textsubscript{Mtb} R10A, RbpA\textsubscript{Mtb} E17A and RbpA\textsubscript{Mtb} R10A/E17A point mutants could support viability in *M. smegmatis* and had no effect on growth rate compared to RbpA\textsubscript{Mtb} WT in LB media (Fig. 2A), indicating that these mutations do not affect RbpA’s essential role in *M. smegmatis*.

To examine the Fdx sensitivity of each *M. smegmatis* strain, we used a zone of inhibition assay, similar to previous studies (2, 11). By spreading approximately 2.5 × 10\textsuperscript{8} colony forming units of bacteria on an agar plate and spotting 10 μl of 100, 250, or 500 μM Fdx dissolved in dimethyl sulfoxide (DMSO) onto a disk placed onto the plate, the bacteria form a lawn after incubation at 37˚C for 2 days, and a zone absent of bacterial growth indicates growth inhibition by Fdx. DMSO had no effect on *M. smegmatis* growth and did not generate a zone of clearing on its own, whereas incubation of *M. smegmatis* with Fdx resulted in growth inhibition (Fig. 2B). We compared the radii of the zones of inhibition formed on each *M. smegmatis* mutant with Fdx and reproduced previous findings that deletion of the RbpA NTT results in resistance to Fdx in vivo (RbpA\textsubscript{Mtb} \textsuperscript{72–111}, RbpA\textsubscript{Msm} \textsuperscript{28–114}, and RbpA\textsubscript{Msm} \textsuperscript{72–114} mutants in Fig. 2, B and C) (11), which is consistent with the in vitro findings (Fig. 1, B and C). In contrast, the RbpA\textsubscript{Mtb} R10A, RbpA\textsubscript{Mtb} E17A, and RbpA\textsubscript{Mtb} R10A/E17A mutants were not more resistant to Fdx in vivo, despite the trend observed in vitro of RbpA\textsubscript{Mtb} R10A/E17A displaying decreased Fdx sensitivity compared to RbpA\textsubscript{Mtb} WT (Figs. 1, B and C and 2, B and C). Strikingly, the *M. smegmatis* RbpA\textsubscript{Mtb} R79A and RbpA\textsubscript{Mtb} R88A mutants, which have decreased affinity for DNA and the σ factor, respectively, were significantly more sensitive to Fdx treatment (Fig. 2, B and C). These in vivo data highlight the existence of other contributors to RbpA’s effect on Fdx activity that exist in the bacteria but are not recapitulated in the in vitro assay.

**Effects on RP\textsubscript{o} stability correlate to sensitivity to Fdx in *M. smegmatis* in vivo**

Although the RbpA SID and BL domains are not predicted to contact Fdx in structural models, mutations of residues within the SID (R88A) and BL (R79A) still affected Fdx sensitivity in vivo (Fig. 2, B and C). This suggests that the relationship between RbpA and Fdx sensitivity is not limited to the contribution of specific amino acids within the NTT for Fdx binding to RbpA-bound RNAP-σ\textsuperscript{A}. Therefore, we investigated whether RbpA’s functional role during transcription initiation contributed to its effects on Fdx sensitivity. During transcription initiation, RbpA stabilizes RNAP-σ\textsuperscript{A} (or σ\textsuperscript{B}) RP\textsubscript{o} (5, 8–10, 13, 14), which requires binding of the SID to the σ factor and binding of the BL to the DNA (5). In contrast, the NTT and CD antagonize RbpA’s RP\textsubscript{o} stabilizing activity (5, 10). Using the 3-nucleotide transcription assay to measure RP\textsubscript{o} stability in the absence of Fdx, we found that addition of RbpA\textsubscript{Mtb} WT to *M. tuberculosis* RNAP-σ\textsuperscript{A} and the rrnAP3 promoter increased RP\textsubscript{o} stability compared to no factor, and this effect was abolished with the RbpA\textsubscript{Mtb} R88A mutant (Fig. 3, A and B), consistent with previously published stopped flow fluorescence data (5). Addition of RbpA\textsubscript{Mtb} \textsuperscript{72–111} to *M. tuberculosis* RNAP-σ\textsuperscript{A} and the rrnAP3 promoter increased RP\textsubscript{o} stability compared to RbpA\textsubscript{Mtb} WT, while addition of the RbpA\textsubscript{Mtb} R10A/E17A mutant showed similar activity as compared to RbpA\textsubscript{Mtb} WT (Fig. 3, A and B), demonstrating that R10 and E17 are not involved in RbpA’s activity on RP\textsubscript{o} stability.

The effect of these RbpA alleles on RP\textsubscript{o} stability mirrors the pattern of Fdx sensitivity in vivo, where *M. smegmatis* strains expressing RbpA alleles conferring higher RP\textsubscript{o} stability (RbpA\textsubscript{Mtb} \textsuperscript{72–111}) in vitro were less sensitive to killing by Fdx. Conversely, *M. smegmatis* strains expressing RbpA alleles conferring decreased RP\textsubscript{o} stability (RbpA\textsubscript{Mtb} R25E) mutant allele, which has a weaker affinity for the RNAP and is defective in stabilizing RP\textsubscript{o} (2, 7, 35) would be more sensitive to Fdx than *M. smegmatis* expressing CarD\textsubscript{Mtb} WT. Indeed, when we performed the zone of inhibition assays on these strains, we found that the R25E mutation in CarD also increased the sensitivity of *M. smegmatis* to Fdx (Fig. 3, C and D). In summary, our experiments uncover a relationship between RP\textsubscript{o} stability and sensitivity to Fdx in *M. smegmatis* (Table 1), suggesting that the role of RbpA for Fdx sensitivity in mycobacteria may involve RbpA’s functional activity during transcription initiation in addition to the role of the RbpA NTT in Fdx binding. In addition, these studies highlight that other factors that regulate RP\textsubscript{o} stability, such as CarD, could also affect sensitivity to Fdx.

**Discussion**

Prior studies on RbpA have focused almost exclusively on the SID interaction with σ factor and the BL interaction with DNA, leaving the NTT and CD largely uncharacterized. Structural studies have provided tremendous insight into the potential interactions between the NTT and CD with multiple RNAP-σ\textsuperscript{A} holoenzyme domains as well as the antibiotic Fdx (10, 11, 37). Herein, we test the prediction that RbpA R10 and E17 contribute contacts with the antibiotic Fdx that are important for RbpA’s NTT-dependent activity against *M. tuberculosis* RNAP-σ\textsuperscript{A}. We find that in vitro, combined mutation of both residues affects the IC50 of Fdx activity against the *M. tuberculosis* RNAP-σ\textsuperscript{A} (Fig. 1, B and C); however, it is still not clear whether RbpA R10 and E17 promote RbpA NTT-dependent Fdx activity through direct interaction with Fdx or through an alternative mechanism. Maintenance of partial Fdx activity against *M. tuberculosis* RNAP-σ\textsuperscript{A} bound by RbpA\textsubscript{Mtb} R10A/E17A in vitro indicates that additional RbpA NTT residues, or perhaps the entire structural domain, mediate RbpA NTT-dependent Fdx activity. In addition, the
RbpA effects on RNAP and fidaxomicin activity

A

No Factor RbpA<sup>WT</sup> RbpA<sup>R10A/E17A</sup> RbpA<sup>R88A</sup>

B

Figure 3. RP<sub>P</sub> stability in associated with Fdx sensitivity in vivo. A, representative gels of three nucleotide transcripts produced by M. tuberculosis RNAP-σ<sup>A</sup> alone or in complex with RbpA<sub>Mtb</sub><sup>R</sup>−111, RbpA<sub>Mtb</sub><sup>R10A/E17A</sup>, or RbpA<sub>Mtb</sub><sup>R88A</sup> from a plasmid DNA template containing positions −39 to +4 of M. tuberculosis <i>rrn</i>AP3 relative to the +1 transcription start site. B, ratio of transcript produced as compared to the average of “No Factor” replicates included on the same gel. Results are plotted as individual values with the mean ± SD shown. Statistical significance of differences was determined by ANOVA and Tukey’s multiple comparison test. ‘ns’, not significant; **<i>p</i> < 0.01; ****<i>p</i> < 0.0001. C, zones of inhibition (ZOI) by Fdx on bacterial lawns of M. smegmatis expressing CarD<sub>Mtb</sub><sup>WT</sup> or CarD<sub>Mtb</sub><sup>R25E</sup> as the only copy of <i>carD</i>. D, mean radii of ZOI ± SD from at least two experiments with at least three replicates at 100 μM, 250 μM, and 500 μM Fdx is plotted. Statistical significance was analyzed by two-tailed Welch’s t test. *<i>p</i> < 0.05; **<i>p</i> < 0.01. Fdx, fidaxomicin; RbpA, RNA polymerase binding protein A; RP<sub>P</sub>, RNAP-promoter open complex.

RbpA<sub>Mtb</sub><sup>R10A/E17A</sup> mutant did not alter Fdx sensitivity in <i>M. smegmatis</i> (Fig. 2), indicating that those residues play less of a role in Fdx activity in <i>vivo</i>. The R88A substitution that weakens RbpA’s interaction with the RNAP in <i>vivo</i> (5), and thus would be expected to decrease <i>M. smegmatis</i> sensitivity to Fdx since less RbpA would be associated with RNAP-σ<sup>A</sup>, also unexpectedly increased <i>M. smegmatis</i> sensitivity to Fdx. Taken together, these observations reveal differences in the effects of RbpA mutants on Fdx sensitivity in <i>vitro</i> compared to in <i>vivo</i> and support a model where RbpA can impact Fdx activity independent of its direct contacts with the antibiotic.

These discrepancies between the measured sensitivities in <i>vitro</i> versus in <i>vivo</i> may be due in part to the limited scope of the in <i>vitro</i> assay used here and in previous studies to probe Fdx activity (11), where Fdx is added to RbpA and RNAP-σ<sup>A</sup> holoenzyme before DNA addition. Whereas in the cell, RNAP-σ<sup>A</sup> holoenzyme could be bound to DNA prior to Fdx binding. This limitation may bias the in <i>vitro</i> assay toward identifying the factors that affect Fdx binding to free RbpA-RNAP-σ<sup>A</sup> holoenzyme complex. In particular, our in <i>vivo</i> results support an association between effects on RP<sub>P</sub> stability and Fdx sensitivity. Our work indicates that RP<sub>P</sub> stability is a newly characterized way that RbpA contributes to Fdx activity. During transcription initiation, RP<sub>P</sub> stabilization involves closing of the RNAP clamp module around downstream nucleic acid as the transcription bubble is formed (38). Structural studies indicate that Fdx inhibits transcription initiation by trapping the mycobacterial transcription initiation complex in an open-clamp conformation (11). In addition, Fdx is predicted to be unable to bind the closed-clamp conformation (11). Therefore, mycobacterial transcription factors such as RbpA and CarD that favor RP<sub>P</sub> formation (7, 8, 10) may impact Fdx sensitivity by reducing the lifetime of open-clamp RNAP complexes that Fdx can bind. Conversely, Fdx has also been shown to decrease the affinity of CarD to RNAP in <i>vitro</i> (39). CarD has a lower affinity to the open-clamp RNAP complex compared to the closed-clamp RNAP complex (RP<sub>P</sub>) (7). Thus, it is possible that Fdx lowers the fraction Table 1

Summary of the effects of RbpA and CarD mutants on fidaxomicin (Fdx) sensitivity and open complex (RP<sub>P</sub>) stability, compared to WT protein

| RbpA construct | In vitro Fdx sensitivity | In vivo Fdx sensitivity | RP<sub>P</sub> stability |
|---------------|--------------------------|-------------------------|----------------------|
| RbpA<sup>R10A/E17A</sup> | Decrease | Decrease | Increase |
| RbpA<sup>R88A</sup> | No change | Increase | Decrease |
| RbpA<sup>R10A/E17A</sup> | Decrease<sup>a</sup> | No change | No change |
| CarD<sup>R25E</sup> | N/A | Increase | Decrease |

<sup>a</sup>The level of decrease in Fdx sensitivity in vitro with RbpA<sup>R10A/E17A</sup> is intermediate to that of RbpA<sup>R10A/E17A</sup> when both are compared to RbpA<sup>WT</sup>.
of CarD bound to RNAP-promoter complexes by reducing the amount of RPₖ formed at equilibrium. This work highlights the need to biochemically understand Fdx activity against the diversity of RNAP complexes that exist within the bacteria.

In addition to the initiation complexes formed following RNAP-σ²⁻₄ binding to DNA, one could envision other factors that exist in vivo and not in vitro that could impact Fdx activity. The in vitro assays of Fdx activity also exclude RNAP holoenzymes containing alternative σ factors and additional RNAP interacting proteins present in the bacteria. Fdx has been shown to be more active at inhibiting the E. coli RNAP-σ²⁻₄ holoenzyme compared to the E. coli RNAP-σ⁰ holoenzyme (32), suggesting that the presence of alternative σ factor-bound holoenzymes may also explain some discrepancies between our in vitro and in vivo findings. In addition to these direct effects on RNAP, truncation of the RbpA NTT and CD results in global dysregulation of gene expression in M. smegmatis (5, 10), which could also affect sensitivity to Fdx. Therefore, the effect of RbpA on Fdx activity in vivo is likely multifactorial. As such, analysis of RbpA mutants with substitutions in conserved residues within the NTT that are predicted to contact different domains in the RNAP-σ²⁻₄ holoenzyme revealed diverse effects of RbpA on the Fdx sensitivity of M. smegmatis (Fig. S1). The impact of these mutants on transcription initiation is unknown, but further investigation into this area could shed more light on how association of RbpA on transcription initiation complexes contributes to antibiotic susceptibility.

Collectively, our results demonstrate that the RbpA NTT domain is a significant contributor to the Fdx sensitivity of the mycobacterial transcription machinery, consistent with previous studies. However, we also discover that the role for RbpA involves more than simply providing amino acids to the Fdx binding site. Our data support a model where multiple RbpA domains, including the NTT, can impact Fdx sensitivity through modulation of transcription initiation kinetics. Our studies reveal a role for another factor that also regulates RPₖ stability, CarD, in Fdx sensitivity. Fdx is currently used to treat infections caused by C. difficile, a bacterium that does not encode an RbpA homolog but does encode CarD and other factors that will regulate transcription by modifying RPₖ lifetime (1). Therefore, these studies also shed light on pathways that can be targeted to improve Fdx activity in the clinic.

**Experimental procedures**

**Media and bacterial strains**

All *M. smegmatis* strains were derived from mc²155 and grown at 37°C in LB medium supplemented with 0.5% dextrose, 0.5% glycerol, and 0.05% Tween 80. *M. smegmatis* strains expressing RbpA_Mtb⁻¹⁻¹, RbpA_Mtb⁺⁻⁻, RbpA_Mtb⁻¹⁻⁴, RbpA_Mtb⁻¹⁻⁷, and RbpA_Mtb⁻¹⁻¹⁰, RbpA_Mtb⁻¹⁻¹⁷, RbpA_Mtb⁻¹⁻²⁶, RbpA_Mtb⁻¹⁻¹¹, RbpA_Mtb⁻¹⁻¹¹, RbpA_Mtb⁻¹⁻¹⁴, and RbpA_Mtb⁻¹⁻¹⁴ were engineered using pMSG430 plasmids that express each rbpA allele from a constitutive *Pmyc1-tetO* promoter and integrated into the attB site of the *M. smegmatis* Δ*rbpA attB::tet-rbpA* strain previously described (5, 33, 34). The primers used to make RbpA strains are in Table S1. RbpA_Mtb⁻¹⁻¹, RbpA_Mtb⁻¹⁻⁴, RbpA_Mtb⁻¹⁻⁷, and RbpA_Mtb⁻¹⁻¹⁰ have been previously described in (5). The *M. smegmatis* Δ*rbpA attB::tet-rbpA* strains expressing RbpA_Mtb⁻¹⁻⁴, RbpA_Mtb⁻¹⁻⁷, RbpA_Mtb⁻¹⁻¹⁰, and RbpA_Mtb⁻¹⁻¹⁴ were named csm455, csm461, csm456, csm457, csm458, csm451, csm462, csm450, csm498, csm510, csm511, csm322, and csm314, respectively.

**Protein preparation for biochemical assays**

Plasmids containing the *M. tuberculosis* H37Rv genomic DNA encoding the different *M. tuberculosis* RNAP holoenzyme subunits were a gift from Jayanta Mukhopadhyay (Bose Institute) (40). Expression and purification were carried out in accordance with the methods described previously (5). Recombinant *M. tuberculosis* RbpA proteins were purified from *E. coli* as previously described using the pET-SUMO vector (primers used to make RbpA constructs for protein purification are in Table S2) (5). RbpA was stored at −80°C in 150 mM NaCl, 20 mM Tris pH 8.0, and 1 mM β-mercaptoethanol. *M. tuberculosis* RNAP-σ⁰ holoenzyme was stored at −80°C in 50% glycerol, 10 mM Tris pH 7.9, 200 mM NaCl, 0.1 mM EDTA, 1 mM MgCl₂, 20 μM ZnCl₂, and 2 mM DTT.

**Fdx zone of inhibition**

*M. smegmatis* cultures were grown to OD₆₀₀ = 0.4 to 0.8. Based on the approximation that OD₆₀₀ = 1.0 is equivalent to 5 × 10⁸ mycobacteria, 2.5 × 10⁸ cells were collected, resuspended in 100 μl of LB, and plated on LB agar plates. Whatman filter paper disks were applied to the plates, and 10 μl of 100 μM, 250 μM, or 500 μM Fdx (Selleck Chemicals) resuspended in DMSO or DMSO alone were added to the Whatman filter paper disks. The plates were incubated at 37°C for 48 h, and the zones of inhibition were measured. The zone of inhibition for each replicative at each drug concentration is the average of four measurements approximately 90° apart.

**3-Nucleotide in vitro transcription assay**

For the Fdx studies in Figure 1, a linear 150 bp dsDNA template containing the *M. tuberculosis* rrrAP3 promoter was prepared by annealing and extending 85-mer oligonucleotide primers (Integrated DNA Technologies) with a 20 nucleotide overlap ranging from nucleotides 1,471,577 to 1,471,726 in the *M. tuberculosis* H37Rv genome (9) and HPLC purified as previously described (7). For the RPₖ stability assays in Figure 3, a plasmid DNA template containing the *M. tuberculosis* rrrAP3 promoter from the -39 to +4 positions relative to the +1 transcription start site, ranging from nucleotides 1,471,618 to 1,471,660 in the *M. tuberculosis* H37Rv genome, was used. Plasmid DNA was isolated by Midi-prep (Qiagen) and cleaned by alcohol precipitation. For all 3-nucleotide transcription assays, RbpA, *M. tuberculosis* RNAP-σ⁰ holoenzyme, and dsDNA template were incubated at 37°C for 10 min.
Reactions were initiated by adding 2.5 μl of a substrate mixture containing GpU, UTP, and $^{32}$P radiolabeled UTP and incubating at 37°C for 10 min to allow for production of a 3-nucleotide product in 20 μl reactions that included a final concentration of 2 μM RbpA (saturating concentration based on [5, 8]), 100 nM M. tuberculosis RNAP-σA holoenzyme, 10 nM dsDNA template, 1 mM DTT, 0.1 mg/ml BSA (NEB), 200 μM GpU, 20 μM UTP, 0.2 μl of $^{32}$P radiolabeled UTP, 75 mM NaCl, 10.1 mM MgCl$_2$, 2 μM ZnCl$_2$, 18 mM Tris pH 8.0, 0.01 mM EDTA, 5% glycerol, and 0.1 mM β-mercaptoethanol. Reactions were stopped with 2X formamide stop buffer (98% [vol/vol] formamide, 5 mM EDTA and 0.05% w/v bromophenol blue). Reaction products were resolved by 22% polyacrylamide-urea gel electrophoresis and exposure to autoradiography film. Products were quantified using ImageJ.

Dose–response curves were carried out the same way with the exception that Fdx was added to RbpA and M. tuberculosis RNAP-σA holoenzyme, incubated for 10 min at 37°C, at which point linear dsDNA template was added and allowed to incubate at 37°C for 15 min before initiating the reactions with the substrate mixture. The in vitro transcription reaction conditions are slightly different than those used in previously published work [11], including different salts in the buffers, different type of holoenzyme preps, and a different dsDNA template, all likely contributing to overall differences in the Fdx IC50 values. Nonetheless, the trends between samples are consistent between this manuscript and previously published work, and therefore, the different reaction conditions do not change the data interpretations or conclusions.

**Data availability**

All data are contained in the manuscript and the supporting information file.

**Supporting information**—This article contains supporting information.

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**Abbreviations**—The abbreviations used are: BL, basic linker; CD, core domain; DMSO, dimethyl sulfoxide; Fdx, fidaxomicin; NTT, N-terminal tail; RNAP, RNA polymerase; RbpA, RNA polymerase binding protein A; RP$_{\sigmaA}$, RNAP-promoter open complex; SI, sigma interaction domain.

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