INTRODUCTION

Despite more than 60 years of available treatments, multidrug regimens, and disease management strategies, Mycobacterium tuberculosis (Mtbc), the causative agent of tuberculosis (TB), remains one of the most medically important pathogens (WHO, 2021). It is widely accepted that a significant proportion of the world's population has been exposed to Mtbc. While most individuals either do not become infected or effectively control the infection long-term (Demissie et al., 2004), 5%-10% of these individuals will develop active TB disease in their lifetime and, thus, represent a significant source of new infections worldwide (Fox et al., 2017; Wood et al., 2011). The overall inability to control tuberculosis has been associated with the required lengthy multidrug regimens that often fail to effectively target all members of the phenotypically diverse bacterial populations and tolerant tubercle bacilli, resulting in latent tuberculosis...
infection (LTBI) that is refractory to treatment [https://www.niaid.nih.gov/diseases-conditions/tbdrugs]. Mtb is known to establish an adaptive persistent state in a host-activated immune environment, which is critical for establishing and maintaining a chronic Mtb infection (Dutta et al., 2010; Mariotti et al., 2013).

Studies have shown that Mtb type II toxin-antitoxin (TA) systems are significantly and differentially regulated in response to host-associated stresses encountered by tubercle bacilli during infection, implying that TA loci are involved in establishing and maintaining a persistent state (Agarwal et al., 2018, 2020; Gupta et al., 2017; Keren et al., 2011; Korch et al., 2009; Ramirez et al., 2013; Singh et al., 2010; Tiwari et al., 2015). It is presumed that during periods of active growth, such as the acute phase of Mtb infection, the transcription and translation of type II toxins and antitoxins are tightly coupled to ensure the production of equivalent stoichiometries of the toxin and its neutralizing antitoxin (Ramirez et al., 2013; Singh et al., 2010; Slayden et al., 2018; Tandon et al., 2019). However, under stressful conditions, such as host immune environments encountered by Mtb during chronic infection, cognate antitoxin and toxin protein levels are thought to be dysregulated by targeted degradation of the antitoxin, thereby freeing toxins (Yamaguchi et al., 2011). Accordingly, when the protein toxins become more abundant than the protein antitoxins, they induce bacteriostasis by cleaving translating mRNAs engaged with the ribosome, resulting in ribosomal stalling on truncated messages (Neubauer et al., 2009). This results in a transition from acute growth to a nonreplicating persistent state (NRP) characteristic of treatment tolerant infections involving bacterial adaptive responses and alternative and reduced metabolism (Ramirez et al., 2013). However, the regulation and coordination of the majority of Mtb TA loci are not defined.

A growing body of evidence has indicated that post-transcriptional regulation, including antisense transcription, which has been reported to be extensive in Mtb, is a hallmark of bacterial pathogenesis (Arnvig & Young, 2009; Dichiara et al., 2010; Dinan et al., 2014; Sesto et al., 2013). Mtb is known to transcribe complementary RNAs to approximately two-thirds of its annotated open reading frames (ORFs) during the exponential phase and more than 90% in the stationary phase (Arnvig et al., 2011). Such large numbers of antisense (as)RNAs are thought to modulate gene expression primarily and protein production levels by double-stranded (ds) RNA-dependent decay via the ribonuclease III protein, RNase III (Lasa et al., 2011). This has been further substantiated by specific reports that antisense regulation leads to a differential abundance of genes that are co-transcribed in polycistronic messages essential to the virulence (Arnvig et al., 2011; Arnvig & Young, 2009; Dichiara et al., 2010; Matsunaga et al., 2004; Movahedzadeh et al., 2004; Schnappinger et al., 2003). Interestingly, we have repeatedly observed significant differences in the abundance of type II cognate antitoxin and toxin mRNAs, including \( \text{relB2} \) and \( \text{relE2} \), under stress conditions that are presumably co-expressed as part of a single bicistron leading us to believe that select \( \text{Mtb} \) TA loci are post-transcriptionally regulated as part of broader adaptive responses to the host environment and immune stresses (Ramirez et al., 2013; Slayden et al., 2018).

Our investigation uncovered a novel antisense RNA \( \text{asRelE2} \) encoded by \( \text{ncRv2866Ac} \) on the complementary strand of the type II \( \text{relBE2} \) locus (Rv2865-Rv2866 or RelFG). We determined that convergent transcription of this novel tripartite hybrid type II TA locus, \( \text{relBE2-asrelE2} \), is regulated by the essential stress-responsive transcription factor cAMP receptor protein, Crp, in a CAMP-dependent manner. Under host-associated environments such as low pH and nutrient limitation, we found that \( \text{relE2} \) mRNA expression levels were significantly and differentially upregulated relative to \( \text{relB2} \) and contrary to \( \text{asRelE2} \). Ex vivo survival studies with \( \text{relE2} \) and \( \text{asrelE2} \) knockout strains showed that \( \text{asRelE2} \) regulates \( \text{RelE2} \), and \( \text{RelE2} \) contributes to \( \text{Mtb} \) survival to low pH and nutrient limitation and activated macrophages (Mφs). To our knowledge, this is the first report of a unique tripartite type II TA locus we have termed a type IIb defined by co-regulation by the cognate antitoxin protein and antisense RNA to the toxin. This novel molecular mechanism ultimately implicates antisense-mediated differential regulation of TA systems in \( \text{Mtb} \) persistence and pathogenesis.

## RESULTS

### 2.1 Identification and mapping of a novel cis-encoded antisense RNA, asRelE2

By definition, type II toxins are encoded in bicistrons and regulated exclusively at the post-transcriptional level through protein–protein interactions with the type II antitoxins (Korch et al., 2009; Miallau et al., 2013; Riffaud et al., 2020; Wessner et al., 2015). Interestingly, we have repeatedly observed significant differences in the abundance of Mtb type II cognate antitoxin and toxin mRNAs, including \( \text{relB2} \) and \( \text{relE2} \), co-expressed in a single bicistron. This observation indicates an additional mechanism of regulation, likely at the post-transcriptional level, that alters the relative mRNA abundances of the \( \text{relE2} \) toxin relative to the \( \text{relB2} \) antitoxin. One common mechanism of post-transcriptional regulation of mRNA abundance involves antisense RNA (Sesto et al., 2013). Therefore, we investigated the presence of an asRNA as a possible co-regulatory mechanism that differentially controls \( \text{relB2} \) or \( \text{relE2} \) mRNA levels. Northern blot analysis was performed with total RNA isolated from \( \text{Mtb} \) at different growth phases using riboprobes designed to identify sense and antisense \( \text{relBE2} \) transcripts (Figure 1A). The \( \text{relB2} \)-specific riboprobe identified 282-nucleotide (NT) and 549-NT fragments corresponding to \( \text{relB2} \) and \( \text{relBE2} \) mRNAs. The \( \text{relE2} \)-specific riboprobe identified 264-NT and 549-NT length fragments corresponding to \( \text{relE2} \) and \( \text{relBE2} \) mRNAs. Notably, two novel RNAs, 512-NT and 264-NT in size, corresponding to \( \text{asrelE2-1} \) and \( \text{asrelE2-2} \), were also discovered.

Rapid amplification of cDNA ends (RACE) was applied to map the identified fragments using sense and antisense \( \text{relB2} \)-specific and \( \text{relE2} \)-specific riboprobes. Sequencing of the 5′/3′ RLM-RACE PCR products mapped the transcriptional start sites (TSSs) of the 282-NT and 549-NT fragments corresponding to \( \text{relB2} \) and \( \text{relBE2} \) to genome base A-3177537, the first NT in the start codon of \( \text{relB2} \), and...
the 3′ ends to genome bases C-3177820 and G-3178085, the third NT in the stop codon of relB2 and relE2, respectively (Figure 1b).

Sequencing the 5′/3′ RLM-RACE products mapped the 5′ and 3′ ends of the 512-NT fragment of asrelE2-1 to genome bases G-3178333 and C-3177822, respectively. Sequencing the 5′/3′ RLM-RACE products amplified from enriched cleaved RNAs containing 5′ monophosphorylated (PO₄) ends revealed that asRelE2-3 fully complements relE2 and is processed from the primary transcript asRelE2-1 (Figure 1c). Sequencing of additional clones of the 5′/3′ RACE PCR products with 5′ monophosphorylated RNAs identified 6-NTs directly upstream (5′ UGAGCG 3′) as the consensus 5′ end of processed relE2 mRNA, along with the corresponding 6-NTs on the complementary strand as the consensus 3′ end of asRelE2-2 (Figure 1c).

2.2 RelE2 is co-regulated by asRelE2-1, asRelE2-3, and the RelB2 antitoxin

Monitoring relE2 and asRelE2 expression after 6, 10, and 16 days of growth revealed that relE2 mRNA increased more than 20-fold relative to the constitutively expressed 5S rRNA (MTB00002 or Rrf), which is consistent with the greater abundance of relE2 in later growth phases observed via northern blotting (Figure 1a,d). In contrast, asRelE2-1 and asRelE2-3 declined sharply over time, decreasing by 20-fold in the stationary phase. Concomitant constitutive expression of relBE2 and relB2 mRNA levels were observed throughout in vitro growth, increasing only 2- to 4-fold over the same period (Figure 1d). These findings indicate that asRelE2-1 is processed, resulting in asRelE2-3, which directly interacts and differentially modulates relE2 mRNA expression levels in a growth phase-dependent manner.

To determine how asRelE2 targets and silences RelE2 production in situ, we characterized the co-overexpression of asrelE2-1, asrelE2-2, and asrelE2-3 on RelE2 production using a tandem ATc-inducible P₅myc-tetO fluorescent protein overexpression system. Nonfunctional RelE2 mutants, RelE2ΔR61L, and RelE2ΔR81L,Y85F with amino acid (AA) substitutions at arginine (R)61, or at R81, and tyrosine (Y)85 corresponding to essential catalytic residues in prototype E. coli RelE were engineered and utilized as functionally inactive positive production controls (Neubauer et al., 2009).

Induction of WT relE2, relE2ΔR61L, or relE2ΔR81L,Y85F alone resulted in protein production as indicated by fluorescence units (RFUs) over time in situ (Figure 2a). In contrast, no increase in RFUs
was observed with co-overexpression of relE2 and asRelE2-1 or asRelE2-3, demonstrating that the full-length primary transcript, asRelE2-1, and the processed transcript, asRelE2-3, directly inhibit the production of RelE2 (Figure 2a). In contrast, the RelE2 noncomplementary asRelE2-2 did not prevent RelE2 production, further supporting the direct inhibition of relE2 translation by asRelE2-1 or asRelE2-3.

Complementary co-expression and growth studies assessed the ability of asRelE2-1 or asRelE2-3 to rescue the growth inhibition phenotype seen with RelE2 production. As expected, the expression of WT relE2 alone inhibited Mtb growth (Figure 2b). In contrast, the expression of the inactivate mutants, relE2ΔR61L and relE2ΔR81L,Y85F, did not affect the growth over the 20 days (Figure 2b). Similarly, when asrelE2-1 or asrelE2-3 were co-overexpressed with WT relE2, logarithmic growth is comparable to the relE2 mutants. Again, asRelE2-2 had no regulatory effect on relE2 based on the observation that co-overexpression did not reverse the growth inhibition observed with WT relE2 alone. These observations were further supported by northern blotting of relE2 and the complementary asrelE2-3, which revealed that relE2 is downregulated ~100-fold by co-overexpression of asrelE2-3 (Figure 2c,d). These findings indicate that asRelE2 functions to silence relE2 translation in situ and that the inhibition of RelE2 production requires complementary base-pairing between relE2 and asRelE2-1 or asRelE2-3. An in silico analysis further supports our findings, which predicts that the complementary portion present in asRelE2-1 and asRelE2-2 interact with relE2 (i.e., ΔG = −226.525 kcal mole⁻¹ and p-value <0.0001).

Although RelBE2 has been annotated and reported as a type II TA loci, we sought to confirm the functionality and physical interaction of RelB2 and RelE2 in Mtb (Yang et al., 2010). When relE2 is expressed, it induces a bacteriostatic phenotype (Figure 2b, Figure S1a). The observed bacteriostatic phenotype caused by relE2 expression can be rescued by co-expression of relB2, demonstrating a functional interaction of the cognate toxin and antitoxin proteins in vivo (Figure S1b). Co-purification and western blotting were performed to visualize direct physical interaction between RelB2 and RelE2 in Mtb (Yang et al., 2010). When relE2 is expressed, it induces a bacteriostatic phenotype (Figure 2b, Figure S1a). The observed bacteriostatic phenotype caused by relE2 expression can be rescued by co-expression of relB2, demonstrating a functional interaction of the cognate toxin and antitoxin proteins in vivo (Figure S1b). Co-purification and western blotting were performed to visualize direct physical interaction between RelB2 and RelE2 in Mtb (Yang et al., 2010). When relE2 is expressed, it induces a bacteriostatic phenotype (Figure 2b, Figure S1a). The observed bacteriostatic phenotype caused by relE2 expression can be rescued by co-expression of relB2, demonstrating a functional interaction of the cognate toxin and antitoxin proteins in vivo (Figure S1b). Co-purification and western blotting were performed to visualize direct physical interaction between RelB2 and RelE2 in Mtb (Yang et al., 2010). When relE2 is expressed, it induces a bacteriostatic phenotype (Figure 2b, Figure S1a).
addition to cognate protein–protein interactions that define type II TA systems.

2.3 Complementary asRelE2 mediates Rnc-dependent decay of relE2 mRNA in vitro

To determine if relE2 is degraded by the Mtb RNase III, Rnc, in an asRelE2-dependent manner, relE2 and asRelE2-3 in vitro transcribed RNAs were incubated in the presence of purified recombinant Mtb HIS-Rnc (Figure 3a). Negative control reactions containing either full-length relE2 or asRelE2-3 with HIS-Rnc showed no degraded product. When relE2 and asRelE2-3 were incubated together, full-length relE2 and asRelE2-3 were found to decrease concomitantly with the apparent appearance and accumulation of degraded low molecular weight RNA products. Moreover, these decay products increased in a magnesium activation-dependent manner characteristic of Rnc, with ~75% of the corresponding full-length RNA species being degraded by HIS-Rnc in 50 mM MgCl₂ (Figure 3b). This observed Rnc-dependent decay of relE2 mediated by the complementary asRelE2-3 demonstrates that relE2 undergoes targeted degradation by Rnc in an asRelE2-dependent manner.

2.4 Crp, relBE2, and asrelE2 are differentially regulated under low pH and nutrient limitation in a cAMP-dependent manner

To assess the expression of Crp, relBE2, and asrelE2 under host-associated conditions, Mtb was exposed to pH 4.5 and 5.5 and nutrient-limitation (NL). The transcription of Crp was transiently up-regulated at 24 h of exposure to pH 4.5 and NL (Figure 4a). Similarly, relB2 and relE2 expression increased within 24 h of exposure to pH 4.5 or pH 5.5 and NL (Figure 4c-e). In contrast, asRelE2 was repressed at pH 4.5 or pH 5.5 and NL throughout the 48 test period (Figure 4c-e). Notably, the increased but differential expression between relB2 and relE2 correlated with the observed decreased expression of asRelE2. The known pH-responsive adenyl cyclase (cya) transcriptional response steadily decreased to steady-state levels within 48 h (Figure 4a). Quantitation of total cAMP confirmed that intracellular cAMP levels peaked at 80 and 20 pmol at pH 4.5 and 5.5, respectively, and remained elevated for 24 to 48 h compared to cAMP levels at pH 6.5 (Figure 4b). This analysis revealed that Crp, relB2, relE2, and asrelE2 are regulated in response to the host-associated stresses of acidic pH and nutrient limitation, correlating with altered cAMP levels.

A dual transcriptional reporter was engineered to examine further the cAMP-dependent transcription of relBE2 and asrelE2 and regulation by Crp. This dual reporter was constructed with unstable gfp and mcherry variants that are transcriptionally controlled by the 105-NTs and 120-NTs, including the CBSs upstream IGRs of relBE2 and asrelE2, respectively. Site-directed mutagenesis was utilized to change the WT PrelBE2-CBS (tGAGacgccgcgCACa) and the WT P asrelE2-CBS (cGACgtcctgtgCACg) to create noninducible mutant PrelBE2-CBS (tGGAacgccgcgCACa) and mutant P asrelE2-CBS (cGCAggtcctgtgCACg) controls for direct comparison. Midexponential phase recombinant Mtb H37Rv cultures were exposed to dibutyryl (db)-cAMP for 48 h. GFP RFUs driven from the WT PrelBE2-CBS increased significantly by approximately 10-fold at 24 h and 40-fold at 48 h (Figure 5a). In contrast, mCHERRY RFUs from the WT P asrelE2-CBS...
decreased 10-fold and 20-fold at 24 and 48 h, respectively. To further assess the complexities of cAMP-dependent regulation of \( \text{relBE2}/\text{asrelE2} \) transcription in situ, changes in \( \text{relB2} \), \( \text{relE2} \), and \( \text{asRelE2} \) levels were evaluated in tandem in WT \( \text{Mtb} \) cultures exposed to increasing amounts of \( \text{db-cAMP} \) for 4 h using RT-qPCR. This quantitative analysis showed that \( \text{relB2} \) and \( \text{relE2} \) transcripts were regulated in a dose-dependent manner following exposure to 100- to 10,000 \( \mu \text{M} \) of \( \text{db-cAMP} \). Specifically, \( \text{relB2} \) and \( \text{relE2} \) were increased 5–10-fold and 10–50-fold, respectively (Figure 5b). In contrast, \( \text{asrelE2} \) levels decreased with increasing intracellular cAMP concentrations (Figure 5c).

### 2.5 RelE2 contributes to survival due to limited nutrient and low pH exposure and activated macrophages

To discern the importance of \( \text{RelE2} \) and \( \text{asRelE2} \) for survival in host-associated conditions, \( \text{Mtb}\Delta\text{relE2} \) and \( \text{Mtb}\Delta\text{asrelE2} \) deletion strains were constructed (Figure 6a,b) and assessed in low pH and limited nutrient conditions. Differences in growth were observed for \( \text{Mtb}\Delta\text{relE2} \) and \( \text{Mtb}\Delta\text{asrelE2} \) strains compared to WT \( \text{Mtb} \) at pH 4.5. In particular, it was observed that the \( \text{Mtb}\Delta\text{asrelE2} \) strain grew slower, and the \( \text{Mtb}\Delta\text{relE2} \) strain reached the stationary
phase earlier than the WT control. The most significant difference between WT Mtb and the mutant strains was that the survival of MtbΔrelE2 was found to steadily decrease during extended periods at pH 4.5 and NL, resulting in a nearly 20-fold reduction compared to that of WT Mtb (Figure 6c). An intermediate survival phenotype was observed at pH 5.5 and NL (Figure 6d).

Macrophage ex vivo assays were also performed to assess further the role of RelB2, RelE2, and asRelE2 in survival to conditions encountered during infection. Differentiated resting and IFN-gamma/LPS-activated THP-1 cells were infected with Mtb WT and ΔrelB2, ΔrelE2, or ΔasrelE2 strains and monitored over 4 days. No significant differences in CFUs were observed in resting or activated...
macrophages during infection with the WT and ΔrelB2 and ΔasrelE2 knockout strains (Figure 6e,f). In contrast, a substantial decrease in CFUs was observed in activated macrophages for the ΔrelE2 knockout strain (Figure 6f). These observations substantiate that RelE2 is essential for the survival of Mtb under host-associated low pH and limited nutrient stress conditions.

3 | DISCUSSION

Adaptive responses and the bacterial NRP state involved in LTBI require alternative metabolic pathways coordinated by multiple regulatory mechanisms, including TA loci (Betts et al., 2002; Ramage et al., 2009; Ramirez et al., 2013; Salina et al., 2009; Slayden et al., 2018). We have observed that TA loci are differentially regulated in the lungs and spleen after 20 days of infection in an immune-competent murine model of tuberculosis (Ramirez et al., 2013). The observation that cognate type II toxin and antitoxin components are differentially regulated under host-associated stress conditions and during infection in animal models suggested the involvement of regulatory mechanisms beyond toxin-antitoxin interactions. Accordingly, we investigated the presence of a regulatory antisense RNA, which resulted in discovering the novel asRelE2 that maps to asrelE2 complementary to relE2 encoded in the type II TA loci, relBE2. RACE studies showed that asRelE2 complements the entire relE2 coding sequence and 248 nucleotides in the 3’ direction of the relBE2 loci. Identifying an antisense RNA that only maps to the relE2 portion of the relBE2 loci is consistent with the vast regulatory antisense RNAs previously identified in Mtb (Arnvig et al., 2011; Arnvig & Young, 2009; Coskun et al., 2021; Schwenk & Arnvig, 2018).

The discovery of a potential regulatory antisense RNA that complements relE2 offered the possibility that RelE2 is co-regulated by post-transcriptional processing at the antisense RNA level, whereby asRelE2 is involved with RNase dependent processing and alters the translation of relE2. Using the type I TA loci regulation mechanism of antisense RNA translational inhibition of toxins as a model, we found that the complementary portion of asRelE2 interacted with relE2, thus forming the required relE2::asRelE2 dsRNA hybrid. We showed that the Mtb RNase III enzyme Rnc processed the relE2 in an asRelE2-dependent mechanism resulting in significantly decreased relE2::asRelE2 dsRNA hybrids. The observed processing mediated by Rnc resulted in an overall reduction in relE2, demonstrating that the Mtb RNase III post-transcriptionally regulates relE2 in an asRelE2 dependent manner. The ability of asRelE2 to functionally regulate relE2 was obtained from relE2 and asrelE2 co-induction studies. These studies demonstrated that asRelE2 alone could rescue the observed bacteriostasis associated with RelE2. The extent of asRelE2 to regulate RelE2 was determined using our engineered recombinant fluorescence tagging system that showed co-induction of asrelE2 alone could comprehensively control and suppress RelE2 production. We have also verified a functional and physical interaction between RelB2 and RelE2 in live cells, thus confirming that the cognate RelB2 antitoxin could inhibit the bacteriostasis state by the toxin RelE2. These data show that asRelE2 can directly and independently control the production of the RelE2 toxin providing evidence that relE2 is uniquely co-regulated by an antisense mechanism and the previously defined cognate protein interactions.

There is emerging evidence that the phenotypic and adaptive diversity observed in bacterial populations is the result of multiple regulatory systems that couple bacterial cell cycle progression and metabolism with the changing growth environment (Crew et al., 2015; England et al., 2011; Ramirez et al., 2013; Schwenk & Arnvig, 2018). Many Mtb TA loci are induced by various adaptive responses to stress conditions (Ramage et al., 2009). This is consistent with identifying cell cycle regulators coupled with TA loci expression and adaptive responses (Crew et al., 2015; England et al., 2011; Ramirez et al., 2013). Because the regulation of relBE2 has been associated with the stress-responsive alternative transcription factor Crp (Kahramanoglu et al., 2014), bioinformatics searches for potential promoters and operators in proximal intergenic regions were performed. This identified a Crp binding site (CBS) in the upstream promoter region of relBE2 as anticipated, confirming previous reports (Kahramanoglu et al., 2014). This analysis also revealed a putative convergent CBS downstream of relBE2 on the complementary strand, indicating that Crp transcriptionally regulates asrelE2. We have shown that Crp, relB2, and relE2 are concordantly regulated by intracellular cAMP in a pH-dependent manner. In contrast, the transcription of asrelE2 was slightly repressed in response to reduced pH, suggesting that asRelE2 provides proportionally greater regulation of relE2 under conditions of increased growth. These findings establish a molecular link between the Crp regulation of relBE2 and asrelE2 to changes in environmental pH and intracellular cAMP levels involved in regulating Mtb adaptive responses and virulence pathways. The demonstration that the MtbΔrelE2 mutant strain had impaired survival under low pH and nutrient-limitation and activated macrophages substantiate that RelBE2 is necessary for survival. These data are consistent with molecular studies that have linked adaptive responses and survival of Mtb with increasing intracellular cAMP levels and secondary transcriptional responses (Choudhary et al., 2014; Gazdik et al., 2009; Rebollo-Ramirez & Larrouy-Maumus, 2019).

4 | CONCLUSION

The survival of Mtb depends on its ability to adapt to changes in its environment rapidly. It is becoming increasingly clear that riboregulation is an essential co-regulatory mechanism in adaptive responses (Schwenk & Arnvig, 2018). Our findings demonstrate that the type II TA loci relBE2 is co-regulated by an antisense mechanism in addition to cognate antitoxin-interaction. The elucidation of co-regulation by asRelE2 further highlights the importance of fine-tuning relBE2 in mediating the survival and persistent tolerant state in Mtb. This finding has significant implications regarding differential regulation of cognate TA genes and the coordination of type II TA loci in Mtb, and other adaptive metabolic processes necessary for infection and
survival in the host throughout infection. This notion is further supported by studies linking the genome-wide expansion of TA loci and other virulence genes to the evolution of Mtb (Dinan et al., 2014; Gupta et al., 2017; Sapriel & Brosch, 2019; Schwenk & Arnvig, 2018). These findings of a novel type IIb RelBE toxin-antitoxin system in Mtb defined by antisense RNA co-regulation ultimately indicate that antisense regulation of type II TA loci represents a key regulatory mechanism.

5 | EXPERIMENTAL PROCEDURES

5.1 | Bacteria and culture conditions

All bacterial strains used in these studies are listed in Table S1. Z-competent E. coli strain DHS Alpha (Zymo Research) cells were used for all cloning and propagation. One Shot® chemically competent E. coli strain BL21(DE3)pLysS (Invitrogen™) cells were used for the ectopic induction of recombinant Mtb proteins with 10 mM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG). E. coli strains were grown in Luria-Bertani (LB) broth at 37°C, shaking at 200 rpm, or on LB agar plates at 37°C. Mycobacteria were cultured in Middlebrook 7H9 broth (Difco™) supplemented with OADC (0.05 mg/ml oleic acid, 5 mg/ml BSA Fraction V, 2 mg/ml dextrose, 0.004 mg/ml catalase, and 0.85 mg/ml NaCl). 0.20% (v/v) glycerol, and 0.05% (v/v) Tween-80 (7H9-Tw) at 150 rpm, or on Middlebrook 7H10 agar (Difco™) plates supplemented with OADC and 0.20% glycerol at 37°C, as per standardized methods (Singh et al., 2013). For these studies, virulent Mtb strain H37Rv (ATCC 27294) was used as the wild-type (WT) parental strain for constructing overexpression and mutant strains. Unless otherwise noted, optical densities at 600 nm (OD₆₀₀) for growing Mtb in 7H9-Tw of 0.10–0.30, 0.40–0.60 (or ~6 days), 0.70–0.90, 1.00–1.20 (or ~10 days), and 1.30–1.50 (or ≥16 days) were considered to be early-log, mid-log, late-log, early-stationary, and late-stationary phase, respectively, as described before (Keren et al., 2011; Singh et al., 2013). Unless otherwise stated, antibiotics purchased from Sigma-Aldrich were used at the following concentrations: 50 μg/ml ampicillin (Amp) for E. coli; 34 μg/ml chloramphenicol (Chlor) for E. coli; 5 μg/ml gentamycin (Gm) for E. coli; hygromycin (Hyg) at 200 and 50 μg/ml for E. coli and Mtb, respectively; kanamycin (Kan) at 50 and 25 μg/ml for E. coli and Mtb, respectively.

5.2 | Construction of Mtb mutant, overexpression, and reporter strains

The Mtb ΔrelE2 and ΔasrelE2 deletion mutant strains were generated using a two-step allelic exchange with a temperature-sensitive replicative vector, pPR27-xylE, as initially described by (Pellicic et al., 1997). In brief, to create mutant strains, ~1000 base-pairs (bp) upstream and downstream flanking the 264-bp relE2 gene and the 372-bp intergenic region (IGR) intervening in between relE2 and rv2867c were PCR amplified from Mtb str. H37Rv genomic (g)DNA with gene-specific primers (GSPs) in Table S2 using GoTaq® Green (2X) Master Mix (Promega Corporation) enriched with 5% (final concentration) PCR-grade DMSO (Sigma-Aldrich) per the manufacturer’s notes (Promega Corporation). For the generation of MtbΔrelE2, the upstream and downstream regions were cloned into a mycobacterial shuttle vector, pVV16, flanking the hygromycin resistance (HygR) gene hph. The resulting 4049-bp dsDNA fragment, rv2864c-relB2-hph-rv2867c, was excised and then cloned into pPR27-xylE at NotI and Spel restriction digest (RD) sites for sucrose (Suc) counter selection. To develop MtbΔasrelE2, upstream and downstream regions were cloned into a mycobacterial shuttle vector, pMIND, flanking the Kan-resistance (KanR) gene aphA. The resulting 3800 bp fragment, rv2864c-relBE2-aphA-rv2867c, was then cloned into pPR27-xylE at NotI and XbaI RD sites, creating pPR27-asrelE2KO. freshly prepared electroporative WT Mtb H37Rv was electroporated with various allelic exchange vectors. Following the outgrowth of HygR and SucR colonies in 7H9-Tw with 50-Hyg (MtbΔrelE2) and KanR and SucR in 7H9-Tw with 25-Kan (MtbΔasrelE2) for 4 weeks at 37°C, successful deletions from genomes of mutant Mtb strains were confirmed by PCR analyses using GSPs listed in Table S2.

For overexpression studies in Mtb, relE toxins were PCR amplified from gDNA using forward GSPs, producing N-terminal tetra-cysteine tags for in situ protein detection. Controlled overexpression was achieved using anhydrotetracycline (ATc)-inducible overexpression vector, pST-KT, essentially as first reported by (Park et al., 2013). RelE2ΔR65L and RelE2ΔR31LΔY95F toxin genes were constructed by changing G-182 and G-242 and A-254 to T based on prior reporting (Neubauer et al., 2009), using reverse GSPs with single nucleotide polymorphisms (SNPs) in PCRs Table S2. For co-overexpression of antitoxin genes, P₅₉₅₇tetO1 was PCR amplified from pST-KT and re-cloned into ATc-inducible pE2 derivatives, thereby creating a duplicate promoter P₅₉₅₇tetO2. Mtb RelB2 and asRelE2 antitoxin genes were cloned in NotI, and HindIII RD sites in 7H9-Tw with 25-Kan (MtbΔasrelE2) for 4 weeks at 37°C, successful deletions from genomes of mutant Mtb strains were confirmed by PCR analyses using GSPs listed in Table S2.

For overexpression studies in Mtb, relE toxins were PCR amplified from gDNA using forward GSPs, producing N-terminal tetra-cysteine tags for in situ protein detection. Controlled overexpression was achieved using anhydrotetracycline (ATc)-inducible overexpression vector, pST-KT, essentially as first reported by (Park et al., 2013). RelE2ΔR65L and RelE2ΔR31LΔY95F toxin genes were constructed by changing G-182 and G-242 and A-254 to T based on prior reporting (Neubauer et al., 2009), using reverse GSPs with single nucleotide polymorphisms (SNPs) in PCRs Table S2. For co-overexpression of antitoxin genes, P₅₉₅₇tetO1 was PCR amplified from pST-KT and re-cloned into ATc-inducible pE2 derivatives, thereby creating a duplicate promoter P₅₉₅₇tetO2. Mtb RelB2 and asRelE2 antitoxin genes were cloned in NotI, and HindIII RD sites were engineered immediately downstream of P₅₉₅₇tetO2. Mtb was electroporated with relBE2 and asrelE2 overexpression vectors listed in Table S1, as reported before (Parish & Stoker, 1998), and incubated at 37°C on 7H10 agar with 25-Kan for 3–4 weeks or until colonies became visible.

For dual transcriptional reporter assays, pGREENCHERRY plasmids were constructed, encoding the pH-sensitive green fluorescent protein (GFP) (Vandal et al., 2008) regulated by the Mtb relBE2 promoter, containing a Crp binding site (CBS), P₅₉₅₇CBS, and mCHERRY (Carroll et al., 2010), which is controlled by the convergent Mtb asrelE2 promoter, containing another CBS, P₅₉₅₇CBS. Initially, a constitutive promoter P₅₉₅₇ was excised from pCHERRY3 and replaced with the ~120-bp P₅₉₅₇CBS intervening between asrelE2 and rv2867c. The mCHERRY gene was PCR amplified using GSPs in Table S2 and re-cloned into pCHERRY3 to add a C-terminal tag (ADSHQRDYALAA) encoded by SerA (MTB0000042). This fusion tag enhances the mCHERRY decay (Andersen et al., 1998; Personne & Parish, 2014). The 105-bp P₅₉₅₇CBS encoded between rv2864c and relBE2 was subsequently cloned into the pCHERRY derivative.
Then, the GFP gene from pUV15-pHGFP made available by (Vandal et al., 2008), was PCR amplified, producing an additional fusion C-terminal SsrA decay tag, and re-cloned downstream of PrelBE2-CBS at Clal and Spel RD sites, creating a WT transcriptional reporter pGREENCHERRYWT (Table S1). For the construction of nonfunctional mutant dual transcriptional reporter, pGREENCHERRYWT-Mut, PrelBE2-CBS and PaseRE2-CBS were PCR amplified using forward GSPs, making their CBSs non-functional. Specifically, the left arm of PrelBE2-CBS (tGAGa) was mutated to tGGAa, while the left arm of PaseRE2-CBS (cGACg) was mutated to cGCAg, abating Crp-DNA-binding, as shown before (Agarwal et al., 2006; Rickman et al., 2005). Mut PaseRE2-CBS and PrelBE2-CBS PCR amplicons were then cloned into pGREENCHERRY similarly, and transcriptional reporters were electroporated into WT Mtb H37Rv.

5.3 | Functional interaction analysis of Mtb relBE2/asrelE2 TA genes

Mtb relBE2/asrelE2 merodiploid strains in Table S2 were incubated shaking at 150rpm and 37°C for at least 16 days to the late-stationary phase. These cultures were then diluted in 150ml fresh 7H9-Tw with 25-Kan to an OD600 of ~0.10, and 150μl of 2mg/ml ATP (Takara™) was added to induce the expression of relBE2/asrelE2 TA genes. Ectopic inductions were carried out at 37°C and 150rpm for up to 20 days in the dark, and for every 2 days, OD600, CFU/ml, and N-tetracysteine-RelE2 fluorescence (RFU or excitation/emission = 508nm/528nm) were assessed. To measure in situ RFUs, up to 10 ml of ectopically induced cultures were washed three times in TBST (pH 6.50), resuspended in 500μl of 10% formalin (Sigma-Aldrich), containing 20μM FlAsH-EDT2 biersacinal labeling reagent (Invitrogen™), and fixed in the dark at 4°C for 2 d. Formalin-fixed tubercle bacilli were rinsed twice in BAL wash buffer per the manufacturer’s instructions (Invitrogen™) and resuspended in TBST (pH 6.50). The whole-cell RFUs were measured with an EnSpire Multimode microplate reader (PerkinElmer) and normalized to OD600.

5.4 | Physical interaction analysis of Mtb RelBE2 TA proteins

RelBE2 TA protein–protein interaction studies were performed as described by (Ramirez et al., 2013), with few modifications. In brief, RelB2 and RelE2 gene fragments were amplified from Mtb H37Rv gDNA using GoTaq® Green (2X) Master Mix (Promega Corporation) enriched with 5% PCR-grade DMSO (Sigma-Aldrich) per the manufacturer’s notes (Promega Corporation), and cloned into pET28a and pETcoco2, respectively (Table S1). DNA constructs were transformed into E. coli strain DH5 Alpha (Zymo Research) and transformants were selected from overnight growth at 37°C on LB agar with 50-Kan for pET28a and 50-Amp for pETcoco2. Sequenced vectors were transformed into chemically competent E. coli strain BL21(DE3) pLysS (Invitrogen™) cells. The selection was carried out overnight by growth in LB broth supplemented with 34-Chlor 50-Kan for pET28-relB2 selection or 50-Amp for pETcoco2-relE2 selection, or both for co-transformation. Overnight cultures were then diluted 1:50 into fresh LB media containing the necessary antibiotics. When the pETcoco2-relB2 construct was used, LB media had 0.01% (v/v) L-arabinose to amplify plasmid copy number before ectopic induction. Once subcultures reached an OD600 of ~0.50, protein production was induced by adding 10mM (final concentration) IPTG. Subcultures were incubated for another 5 h at 150rpm and 37°C, and bacterial cell pellets were collected via brief centrifugation. According to the manufacturer’s protocols, crude whole cell lysates were obtained using BugBuster® with Benzoase® (Novagen). Crude whole cell lysates were then clarified by centrifugation at 12,500 x g for 20 min at 4°C and passed through a 0.20μm filter. Each mL of clarified lysate was combined with 250μl of pre-washed Ni-NTA His-Bind® Resin (Qiagen) and rocked gently at 4°C for about 1 hour before packing into a column with 10ml of bind buffer (100mM Tris–HCl, 250mM NaCl, and 5mM imidazole, pH 7.80). The column was rinsed three times with wash buffer-one (100mM Tris–HCl and 250mM NaCl, 10mM imidazole, pH 7.80), and then three times with wash buffer-two (100mM Tris–HCl and 250mM NaCl, 25mM imidazole, pH 7.80). Recombinant TA proteins and/or protein complexes were eluted stepwise in elution buffer (100mM Tris–HCl, 500mM NaCl, pH 7.80) containing 50-, 125-, and 250-mM imidazole. All wash and elution fractions were separated on NuPAGE® 12 Bis-Tris Gels (Invitrogen™) in MES running buffer (Invitrogen™) at 200 V, followed by transfer to a 0.2micron nitrocellulose membrane (BioRad) at 5V for western blotting. Membranes were blocked in 4% BSA in TBST (pH 7.60), incubated with primary Penta-His antibody (Qiagen) or anti-HSV-Tag® antibody (Novagen), diluted at 1:10,000, followed by goat anti-mouse-alkaline phosphatase (Sigma Aldrich), diluted 1:10,000. Membranes were developed with the addition of NBT/BCIP substrate solution (Sigma-Aldrich).

5.5 | Extraction and purification of Mtb total RNA

Total RNA was isolated from 50ml culture aliquots of Mtb. Bacilli were collected by centrifugation at 3500 x g for 10 min at 4°C, washed two times in TBST (pH 6.50), and resuspended in 1 ml of TRizol® Reagent (Invitrogen™). Bacilli were lysed by physical disruption in 1.50ml screw-cap tubes (USA Scientific) with 250μl of 0.10mm zirconia glass beads (BioSpec Products) subjected to 2400 oscillations for 30seconds six times, using the Mini-BeadBeater-1 (BioSpec Products), with cooling on ice for 2 min in between each round of bead beating. Following the disruption, 200μl of chloroform was mixed by vigorous vortexing for 15 seconds, and whole-cell lysates were centrifuged at 12,500 x g for 15 min at 4°C. 500μl of the aqueous layers were transferred to new 1.50ml microcentrifuge tubes containing 500μl of ice-cold molecular biology grade isopropanol (Sigma-Aldrich), vortexed, incubated at -20°C overnight, and centrifuged at 12,500 x g for 15 min at 4°C to pellet RNA. RNA pellets were washed once in 80% molecular biology grade 200-proof
ethanol (Sigma-Aldrich) in DEPC-treated H2O (Sigma-Aldrich), dried at room temperature, and treated with 10 units (U) DNase I (Thermo Scientific™) at 37°C for 60 min. Equal volumes of phenol:chloroform (5:1) pH 4.30–4.70 (Sigma-Aldrich) were mixed with DNase I reactions with vigorous vortexing for 15 seconds and centrifuged at 12,500 × g for 3 min at 4°C. Top aqueous layers were transferred to new 1.5 ml microcentrifuge tubes with 10 volumes of 80% ethanol, 10% 3 M sodium acetate (Sigma-Aldrich), and 0.50 μg/ml glycogen (ThermoFisher Scientific™) in DEPC-treated H2O and incubated at -20°C overnight to precipitate RNA. Following three rounds of DNase I treatment, total RNA was quantified and qualified using the NanoDrop (ND-1000) UV/VIS Spectrophotometer (ThermoFisher Scientific™), and only samples with absorbance ratios at 260–280 nm of 1.90–2.00 were used in downstream gene expression analyses.

5.6 Northern blotting analysis of Mtb total RNA

Northern blotting of Mtb total RNA was performed as reported before (Gerrick et al., 2018), using 5′- 3′-digoxigenin (DIG)-labeled riboprobes listed in Table S2, which were synthesized by IDT DNA Technologies (Coralville, IA). Approximately 5 μg of total RNA samples were heated to 75°C for 5-10 min in (2X) TBE-urea sample buffer (Invitrogen™), run on 6% TBE-urea gels in (1X) TBE + Nucleic Acid Detection Kit per the manufacturer's protocol (Roche) and transferred to BrightStar® positively charged nylon membranes at 30 V for 60 min. Northern blots were washed twice with (0.5X) SSC NorthernMax™ Low stringency Wash and Block Buffers (Roche), respectively, and then in (1X) DIG Wash and Block Buffer (Roche). Membranes were washed, dried, and incubated for 3 h at 37°C and overnight at 16°C to attach the 5′ RNA oligo adaptor (Table S2) to 5′ PO4 ends. To select 5′ PO4 processed RNA, single-stranded cDNA was column purified (Zymo Research) and PCR-amplified using 0.40 μM forward adapter-specific primer and reverse GSPs in Table S2 in GoTaq® Green Master Mix (Promega) and 0.16 mg/ml PCR grade DMSO added for 5′ RACE. 3′ RACE was performed essentially the same but with nested forward GSPs and a reverse Oligo(dT)20-specific primer listed in Table S2. 5′/3′ RACE PCR products were run on 1.5% agarose gels at 95 V for ~70 min in (1X) TAE, gel purified, cloned into pMIND, and Sanger sequenced.

5.8 Double-stranded (ds)RNA cleavage assay with Mtb RNase III

N-terminal hexahistidine tagged Mtb RNase III (His-RNase III) was overproduced and purified essentially as reported before (Akey & Berger, 2005). Mtb H37Rv RNase III was PCR amplified from gDNA, cloned into pETcoco2, and overproduced in E. coli BL21(DE3)pLysS (Invitrogen™) at an OD600 of ~0.50 and 37°C with 1 mM of IPTG for 5 h. Harvested cells were resuspended in BugBuster™ Reagent (Millipore) with EDTA-free protease inhibitor (Roche) and 250 μg of Benzoase Nuclease (Novagen), lysed at room temperature, rocking gently for 30 min, and centrifuged at 12,500 × g for 20 min at 4°C. The clarified whole cell lysate was incubated with Ni-NTA His-Tag® resin (Millipore) for 60 min at 4°C, rocking gently, and loaded onto a column pre-equilibrated with ice-cold buffer (Tris-HCl [pH 7.90] and 500 mM NaCl) with 10 mM imidazole. The column was washed with six volumes of ice-cold buffer with 50 mM imidazole. His-RNase III was eluted in three volumes of ice-cold buffer with 250 mM imidazole. Elution fractions were pooled into a 3 kDa MWCO Amicon Ultra-15 Centrifugal Filter Unit (Millipore) and dialyzed against at 4°C in Tris–HCl (pH 7.90) and 5% glycerol with 500, 250, and 150 mM NaCl. His-RNase III was resolved on 12% Bis-Tris gels (Invitrogen™), stained with SimplyBlue SafeStain (ThermoFisher Scientific), estimated to be at least 80% pure quantified using BCA assay (ThermoFisher Scientific), and stored at -20°C until further use.

In vitro, His-RNase III dsRNA cleavage assays were performed as recently published by Gordon et al. (2017). Full-length reIE2 and asreIE2-3 DNA templates were PCR amplified from Mtb H37Rv gDNA using GoTaq® Green Master Mix (Promega) with GSPs adding
5′-TAATACGACTACTAGGG-3′ upstream of T7 promoters (Table S2), and gel purified. RNA was in vitro transcribed using T7 RiboMAX Express large-scale RNA Production System (Promega) and then purified by acid-phenol: chloroform (pH 4.50) with overnight ethanol precipitation at -20°C. Approximately 400.0ng/ml (final concentration) of in vitro transcribed RNA was mixed with DEPC-treated H2O and (5X) dsRNA cleavage buffer (150mM Tris–HCl (pH 7.60), 250mM NaCl, 0.50mM EDTA, and 0.50mM DTT) to create 50μl reactions, heated to 70°C for 10min, and immediately cooled on ice. One μg of His-RNase III and 5μl of 0.10–50.00mM MgCl2 were added on ice. RNase III dsRNA cleavage reactions, including negative control reactions with one μg of His-RNase III, 50mM MgCl2, and 1 μg of relE2 or asRelE2-3, were incubated at 37°C for 30min, quenched with the addition of 5μl of 440mM EDTA, and RNA was extracted with acid-phenol-chloroform and precipitated overnight as described above. Five microliters of 1 μg/μl RNA isolated from RNase III dsRNA cleavage reactions were mixed with (2X) TBE-urea sample buffer (Invitrogen™), heated to 75°C for 5min, centrifuged at 6000×g for 3min at 4°C, and separated on 6% TBE-urea gels in (1X) TBE buffer (Invitrogen™) at 180V for 50min. Resolved gels were stained in SYBR® Gold (Invitrogen™) for 45min and imaged using ChemiDocTM XRS+ (Bio-Rad).

5.9 | Mtb in vitro stress and persistence assays

Mid-to-late stationary phase cultures of WT and mutant Mtb strains were diluted to an OD600 of ~0.10 in 75ml of 7H9-Tw with 50-Hyg or 25-Kan, and subcultured at 37°C and 150rpm for 20days. During this time, outgrowth was assessed by measuring OD600 and enumerating CFU/ml from plating 10-fold serial dilutions of culture aliquots onto 7H10 agar with antibiotics every 2days. Results represent the means ± of the standard deviation of at least three independent experiments. To assess the effects of in vitro stress conditions associated with the host, WT and mutant Mtb cultures were treated similarly as reported before (Bettis et al., 2002; Early et al., 2019; Singh et al., 2013). In brief, Mtb strains were grown in 7H9-Tw with 50-Hyg or 25-Kan until the mid-to-late log phase. Three 50ml culture sample aliquots were briefly centrifuged at ~4500×g for 10 min at 4°C, washed twice, and then resuspended to an OD600 of 0.20–0.25 (or 6.50-to-7.00 Log10 CFU/ml) in (1X) TBST (i.e., 20mM Tris, 150mM NaCl, and 0.05% nonmetabolizable tyloxapol). For cAMP studies, 50ml cell aliquots were recovered and resuspended in TBST at a pH of 6.50, 5.50, or 4.50 to an OD600 of ~0.25 (or 6.50–7.00 Log10 CFU/ml) and incubated, rocking gently for 8days at 37°C. Following 0, 1, 2, 4, and 8days of acid stress, bacilli were enumerated as described above: (1) 1 ml was prepped to estimate intracellular cAMP levels; (2) up to 10 ml was fixed in 10% neutral buffered formalin to assess GFP and mCHERRY RFUs. Significant differences in survival of various WT and mutant strains were made by comparing means ± of the standard deviations of three independent experiments using a two-way ANOVA with Tukey’s post-tests (*p<0.05, **p<0.01, and ***p<0.001), as recently reported (Gallant et al., 2016).

5.10 | Measurement of intracellular Mtb cAMP levels

Intracellular Mtb cAMP levels were measured using the Direct cAMP Enzyme Immunoassay Kit according to the acetylated version of the manufacturer’s protocol (Sigma-Aldrich). Sample culture aliquots were recovered and resuspended to ~1x108 CFU/ml in TBST, pH 5.50, centrifuged at 4500×g for 10 min at 4°C, resuspended in 0.10 M HCl, and boiled for 10 min at 100°C (Kahramanoglu et al., 2014). Whole-cell lysates were transferred to 1.50ml screw-cap microcentrifuge tubes (USA Scientific) filled with 200μl 0.10mm diameter zirconia glass beads (BioSpec Products) and exposed to three rounds of bead beating (2400 oscillations in 30s), using the Mini-BeadBeater-1 (BioSpec Products), with cooling on ice for at least 2 min in between each round. Bacterial cell debris was removed via centrifugation at 12,500×g for 15 min at 4°C, and clarified lysates were stored at ~20°C until further use. Intracellular cAMP levels were measured by reading the optical density at 405nm (OD405) of 100μl of immunoassay whole cell lysates using an EnSpire Multimode microplate reader (PerkinElmer). Intracellular cAMP levels were estimated from standard curves generated from reading the OD405 of 0–20 pmol/ml of cAMP in 0.10 M HCl, and cAMP per 106CFU was calculated by dividing pmol cAMP/ml by CFU/ml, similarly to prior reporting (VanderVen et al., 2015).

5.11 | Reverse transcription-quantitative PCR (RT-qPCR) of Mtb and murine total RNA

For RT-qPCR gene expression analyses, 1 μg of total RNA was heated to 65°C for 10 min with 2.50μM reverse GSPs, cooled to 4°C,
and mixed with 20U of transcriptor RT reverse transcriptase and 80U of RNase inhibitor and reverse transcribed at 58°C for 60min. No RT (NRTs) and no template controls (NTCs) were included with every reaction. Four microliters of 1:25 and 1:50 dilutions of cDNA were used in 25.00μl qPCR reactions containing 12.50μl (2X) SYBR Green I Master Mix (Roche), 2 μl of 5μM of forward and reverse GSPs (Table S2), 2μl of DMSO, and 4.50μl of DEPC-treated H2O carried out on the LightCycler® 480 System per the manufacturer’s instructions (Roche). GSPs were optimized by generating standard curves of qPCRs of cDNA (or Cp values) reverse transcribed from serially diluted early-to-mid-log phase total RNA (0h). Amplification efficiencies (E) were determined using linear regression analyses (E = 10^{-1/slope}), and GSPs with at least 85% qPCR E was used for relative quantification (Figure S1). Genes of interest (GOIs) were normalized to 16S rRNA (MTB000019), and fold inductions were calculated using E ΔΔCt for Mtb and EΔΔCt/ΔΔActin in comparison to 0 controls (Livak & Schmittgen, 2001). Melt curve analyses were run in tandem to confirm qPCR amplicon specificity, and mean fold inductions ± standard deviations were calculated from at least three independent experiments.

5.12 | Mtb infection of THP-1 cells

Human monocytic THP-1 cells (ATCC TIB-202) were maintained in RPMI-1640 (ATCC 30–2001) culture medium supplemented with 10% fetal bovine serum (FBS, ATCC 30–2020) and 0.05mM betamercaptoethanol (Sigma) at 37°C, 5% CO2. THP-1 cells were seeded in flat-bottom 24-well plates at 5×10^5 cells/well and treated overnight with 100nM phorbol 12-myristate 13-acetate (PMA, Sigma). The resulting differentiated cells were incubated in supplemented RPMI without PMA for 24h. Activated macrophages were established by incubating differentiated cells with 20ng/ml IFN-gamma (R&D Systems) and 20pg/ml LPS (Sigma) for 16h. Both activated, and nonactivated macrophages were infected with log-phase Mtb H37Rv WT and KO cultures at an MOI of 10 for 4h. The remaining inoculum was serially diluted and plated in duplicate on 7H11 agar plates for CFU enumeration. Cells were washed twice with sterile PBS following the incubation period to remove extracellular bacilli. At each desired time post-infection, infected cells were lysed with 0.05% SDS in 7H9 broth. Replicate cell lysates were pooled and centrifuged at 3500 rpm to remove extracellular bacilli. At each desired time post-infection, the remaining inoculum was serially diluted, and plated in duplicate on 7H11 agar plates for CFU enumeration.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

CCD and RAS conceived original project design. CCD, JEC and JMS performed experimentation. CCD, JEC, JMS and RAS performed data analysis. All authors contributed to original draft, CCD and RAS were responsible for final draft. RAS obtained funding for this research, provided resources and project supervision. All authors contributed to the article and approved the submitted version.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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