DISSERTATION

MOLECULAR CHARACTERIZATION OF NOVEL TRANSCRIPTION OF ANTISENSE TOXIN-ANTITOXIN RNA IN REGULATING MYCOBACTERIUM TUBERCULOSIS SURVIVAL

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ABSTRACT

MOLECULAR CHARACTERIZATION OF NOVEL TRANSCRIPTION OF ANTISENSE TOXIN-ANTITOXIN RNA IN REGULATING MYCOBACTERIUM TUBERCULOSIS SURVIVAL

Despite more than seventy years of readily available anti-tuberculosis (or TB) treatments, *Mycobacterium tuberculosis* (or *Mtb*) remains the deadliest human pathogen. Novel short-course therapies are needed that effectively treat latent TB infection (or LTBI), which is a major source for new infections. However, the molecular determinants of LTBI, including a large repertoire of regulators encoded by *Mtb* that mediate survival, are largely uncharacterized. Gene expression studies have implicated numerous regulators and particularly toxin-antitoxin (or TA) systems in *Mtb* pathogenesis. Whole genome sequencing (*i.e.* WGS) studies have linked the massive genomic expansion of TA systems along with other pathogen-specific gene families to the emergence of TB-causing mycobacteria. In addition, a multitude of TA systems show genotypic differences that distinguish between ancient and modern lineages of *Mtb*. These predominantly include lineage-specific changes in amino acids, altering antitoxin DNA-binding, and nucleotides, generating new promoters. These mutations have led to an overrepresentation of differentially expressed *Mtb* TA genes responsible for mediating epigenetic changes that are associated with gains in virulence of modern lineages. Thus, the work presented in this dissertation begins to define the novel co-regulation of TA systems that underlie *Mtb* pathogenesis. Unraveling of more complex regulation of *Mtb* TA systems will provide keen insights into the phenotypic changes responsible for *Mtb* survival and persistence *in vivo*. This will ultimately help to streamline research and development of novel antibiotics as well as host directed immunotherapies against hard-to-treat tubercle bacilli, effectively shortening the duration of TB treatment.

TA systems are ubiquitous among bacteria, especially pathogens, and increasingly found to be essential for adaptation to host immune defenses and *in vivo* drug pressures, resulting in

the development of persistent or chronic infections. Phylogenomics comparisons have revealed that *Mtb* encodes a significantly expanded repertoire of TA systems that are solely conserved by tubercle bacilli, including homologous ParDE/RelBE systems like RelBE2 (*i.e.* Rv2865-Rv2866). Herein, we report a novel antisense (as)RNA, we call asRelE2, which is uniquely encoded by *Mtb* and involved in differentially post-transcriptionally regulating *relE*2 mRNA levels as part of the response to host-associated stress such as low pH in a cAMP-dependent manner. This dynamic regulation of the tripartite *relBE2/asrelE2* TA locus appear to be essential for long-term survival under acidic stress *in vitro*. In addition, the overexpression of *relE2* is found to mediate phenotypic development of a persistent state in *Mtb* associated with increasing tolerance towards frontline anti-TB drugs isoniazid (or Inh) and rifampicin (or Rif). In mice, asRelE2 acts in differentially regulating bi-cistronic *relB2* and *relE2* mRNA levels in a host tissue-specific manner dependent upon the downstream effector functions of the cytokine interferon gamma (*i.e.* IFN-γ) in murine TB. Specifically, *relE2* and *relB2* mRNA levels are found to steadily increase in lungs and in spleens, respectively, in the development of the chronic phase of *Mtb* infection. To our knowledge, this is the first time a *Mtb* TA system has been shown to be co-regulated by an asRNA antitoxin. Furthermore, this is linked with the development of the adaptive host immune response to *Mtb*, demonstrating that the post-transcriptional regulation of TA systems is an important mechanism, coordinating the epigenetic changes that are a hallmark of *Mtb* persistence and pathogenesis.

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CHAPTER 1: LITERATURE REVIEW PART I

1.1 Introduction.

Tuberculosis (or TB) has plagued humankind throughout history, accounting for as many as one-in-four deaths in the nineteenth century. This changed in the mid-1940s and 1950s with the discovery and widespread use of antibiotics that successfully treated TB [5]. Despite more than seven decades of effective chemotherapy though, TB is still a leading infectious killer worldwide today. The World Health Organization (or WHO) estimates that TB claimed 1.7 million lives in 2016, with another ten million new cases of active TB (*i.e.* ATB) [6]. Currently the WHO recommends two months of antibiotics, isoniazid (or Inh), rifampin (or Rif), ethambutol (or Etb), and pyrazinamide (or Pza), followed by four months of Inh and Rif to adequately treat ATB disease [7]. However, the duration of TB treatments challenge patient adherence thus limiting their effectiveness, contributing to the spread of multi and extensively drug resistant strains of *Mycobacterium tuberculosis* (*i.e. Mtb*).

Soon after the introduction of Inh in 1952, it was proposed that lengthy treatment regimens were necessary to eliminate persistent mycobacteria, refractory to bactericidal antibiotics that kill actively growing bacilli *in vivo* [8]. These cells are now thought to represent a sub-population that emerges during infection that is susceptible to sterilizing drugs such as Rif and Pza, thought to target non-replicating bacilli [9]. TB patients who have successfully completed treatment are still found to harbor dormant bacilli that are able to resume growth years, even decades later, causing relapse and ATB [10, 11]. Therefore, there is a recurrent need to understand the cellular and the molecular basis of *Mtb* dormancy and persistence to develop new effective sterilizing treatments.

1.2 Stepwise evolution of *Mycobacterium tuberculosis* from *Mycobacterium*.

TB is caused by a unique group of mycobacteria that form the *Mtb* complex (or MTBC), including *M. canettii* (*i.e. Mcn*), *M. africanum* (*i.e. Maf*), *M. bovis* (*i.e. Mbv*), and *Mtb* [12]. MTBC species and strains are acid-fast non-motile bacilli that are transmitted *via* aerosols generated

from patients with pulmonary ATB. The outcome of TB infection is highly variable, however, and can be rapidly cleared by the host, progress to ATB, or establish a latent infection (or LTBI) that may or may not develop into ATB long after initial infection. ATB is often a pulmonary disease, but *Mtb* dissemination to other parts of the body, including the lymph nodes, meninges, and bones occurs, producing extrapulmonary ATB disease as well. These extrapulmonary forms of disease (*i.e.* milary TB, TB meningitis, and Pott's disease) in part represent diverse host responses to *Mtb* [13]. Traditionally, the outcomes of *Mtb* infection and TB disease are attributed to various host and environmental factors [1]. However, an increasing number of phylogenomics studies indicate that mycobacterial determinants contribute to TB pathogenesis.

Today *Mtb* is the principal cause of TB in humans, but much of its high guanosine-cytosine (or GC) chromosome is shared by an ecologically diverse group of more than one hundred and fifty species belonging to the *Mycobacterium* genus. Phylogenetic trees reconstructed from 16S rRNA nucleotide (or NT)-sequences form two major branches: rapidly growing mycobacteria (or RGM), which grow to stationary phase in liquid media in one week; slow growing mycobacteria (or SGM), which grow to stationary phase in liquid media in approximately two-to-three weeks [14, 15]. Most RGM are harmless soil and aquatic species (*i.e.* over one hundred), while SGM, which split from RGM, comprise all the major human bacterial pathogens. Within the SGM, the MTBC clade emerges from early branching of *Mcn* and the smooth tubercle bacilli (or STB), and the more opportunistic pulmonary pathogen, *M. kansasii* (*i.e. Mkn*), which causes TB-like disease in immunocompromised individuals [16, 17]. Prior to this split, the MTBC shared a pathogenic ancestor with *M. marinum* (*i.e. Mmr*) and *M. leprae* (*i.e. Mlp*) [14, 18]. *Mmr* is an aquatic more opportunistic pathogenic SGM that causes granulomatous disease like TB in a wide host range [19], while *Mlp* is an obligate intracellular pathogen that causes leprosy [20].

Similarly, whole genome sequencing (or WGS) analyses indicate that *Mtb* evolved from an opportunistic environmental mycobacterium from extensive gains and losses in genome coding capacity. Genome-wide comparisons find that three thousand or seventy-seven percent

of *Mtb*'s open reading frames (*i.e.* ORFs) are encoded by *Mmr*, with at least eighty-five percent amino acid (or AA) sequence identity [19]. It appears that many of these orthologs were acquired by *Mmr* from horizontal gene transfer (or HGT) events, as they are not commonly encoded by other more distantly related SGM. Accordingly, whole genome alignments find that two thousand four hundred and ten (or eighty-nine percent) of *Mlp*'s genes are encoded by *Mtb*. One thousand four hundred and thirty-nine of these have been identified as ORFs, while the other nine hundred and seventy-one have been annotated as non-functional pseudogenes [21-23]. Many of these ORFs appear to be parental copies of genes families that have expanded in *Mtb* [24]. More recent phylogenomic comparisons find that *Mkn* is the most closely related SGM to *Mtb* outside of the MTBC [16]. Accordingly, *Mkn* encodes many *Mtb* virulence determinants, including two and thirteen PE/PPE proteins involved in antigen (or Ag) variation, thirty-six mammalian cell entry (or MCE) proteins, and fully functional early secreted Ag export (or ESX) proteins. *Mkn* is still missing a significant number of *Mtb* ORFs that are involved in two-component signaling (*i.e.* TCS) and regulation, however, and consequently, appears to be much less pathogenic than *Mtb*, with limited transmissibility [16].

Mcn and certain STB show similar levels of pathogenicity in murine *Mtb* infection models as well, and WGS analyses find that these STB have acquired most *Mtb* genes *via* extensive HGT and duplication events such as homologous recombination (HR) [25]. Comparing the genomes of STB patient strains in East Africa reveals that older STB strains are much more genetically diverse than *Mtb* [25]. In particular, the larger genomes of older and less pathogenic STB harbor many more mutations (*i.e.* single nucleotide polymorphism(s) (or SNP(s)) and insertions/deletions (or InDels)) in *Mtb*-specific genes [25]. More ancestral STB strains also encode numerous genes, which are not encoded in *Mtb*, including phage elements and recombinases, which are likely involved in increasing genomic diversity, and consequently, decreasing TB pathogenesis.

Mtb evolved from a combination of genome reduction, HGT, chromosomal expansion of gene families and functions, and their subsequent maintenance in the genome. *Mtb* strains from

around the world show little-to-no genetic variation compared to most other epidemic bacteria. Still, genome comparisons of MTBC species and ecotypes reveal phylogenetic differences, with clear implications in *Mtb*'s unique physiology [26]. It seems that while the overall genetic diversity of the MTBC is low, SNPs and InDels are fixed in genes in MTBC lineages and clades, with more limited abilities to cause TB (Figure 1.1) [27, 28].

Figure 1.1: Evolutionary history of the *Mycobacterium tuberculosis* complex taken from J.E. Galagan (2014) [2].

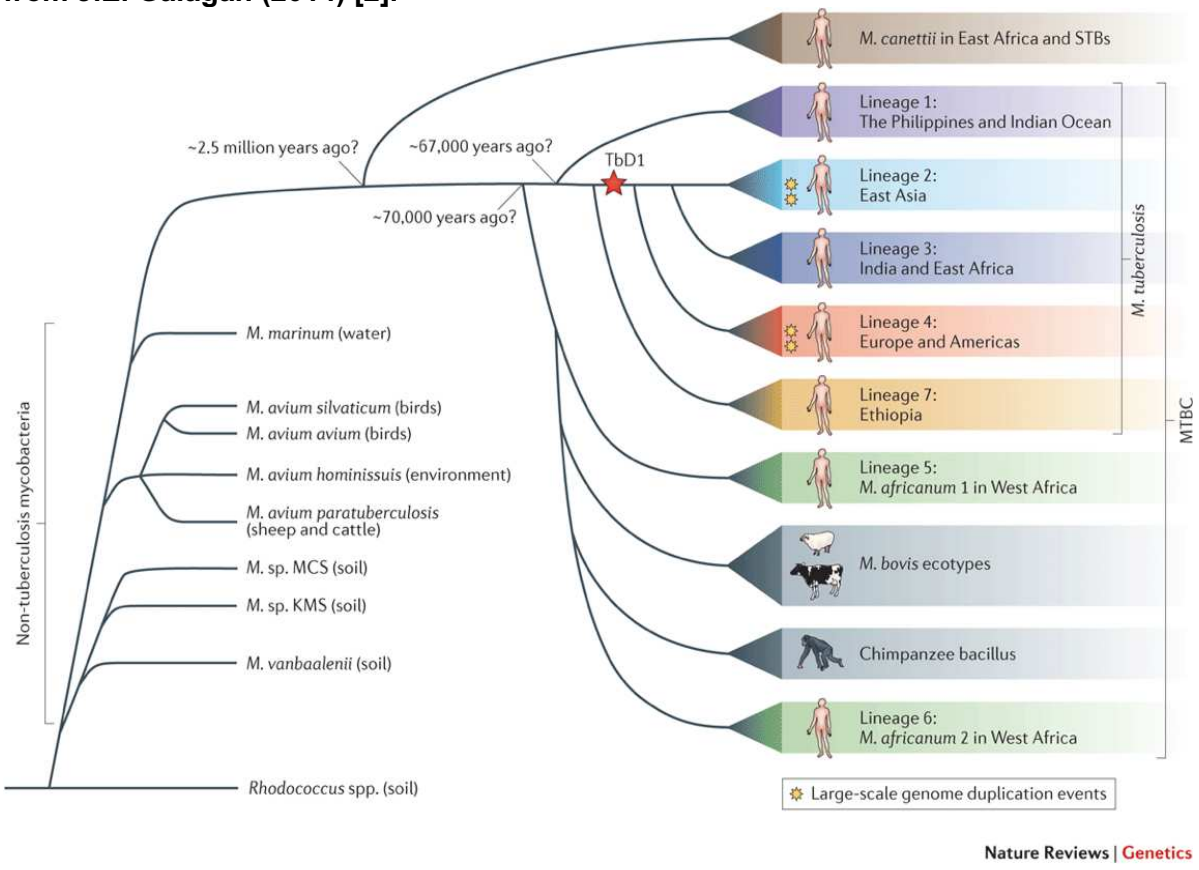

Figure 1.1: Evolutionary history of *Mycobacterium tuberculosis* complex taken from J. E. Galagan (2014) [2]. Evolution of the MTBC shown above, including the expansion of the genotypically diverse *M. avium* complex and the MTBC. From WGS comparisons, the MTBC likely arose from clonal expansion of a STB-like progenitor, with animal-adapted *Mbv* ecotypes subsequently evolving from a *Maf*-like progenitor.

1.3 Genetic diversity in the *Mycobacterium tuberculosis* complex.

MTBC species and strains with varying host specificities and pathogenicities are found to be ninety-nine percent identical at the NT-level [29]. One of the first complete MTBC phylogenetic trees built from the distribution of twenty variable regions identified a group of strains that were

missing a two thousand one hundred base pair (or bp) long region (*i.e.* TbD1) [30]. TbD1-deleted strains form lineages two, three, and four, which are widespread globally today [31]. Accordingly, WGS analyses have identified seven human lineages, wherein TbD1-deleted strains form the largely monophyletic modern clade [1]. More ancient MTBC lineages one, five, six, and seven in contrast appear to be much more genetically diverse [32] and are still by enlarge restricted phylogeographically [33]. *Maf* and lineage seven strains in particular are exclusively isolated from TB patients in Africa [34], similar to STB [35]. In addition, WGS comparisons find that *Mbv* strains, including Bacillus Calmette-Guerin (or BCG), are much more closely related to *Maf* than *Mtb* [30].

Figure 1.2: Phylogeography of *Mycobacterium tuberculosis* complex taken from M. Coscolla and S. Gagneux (2014) [1].

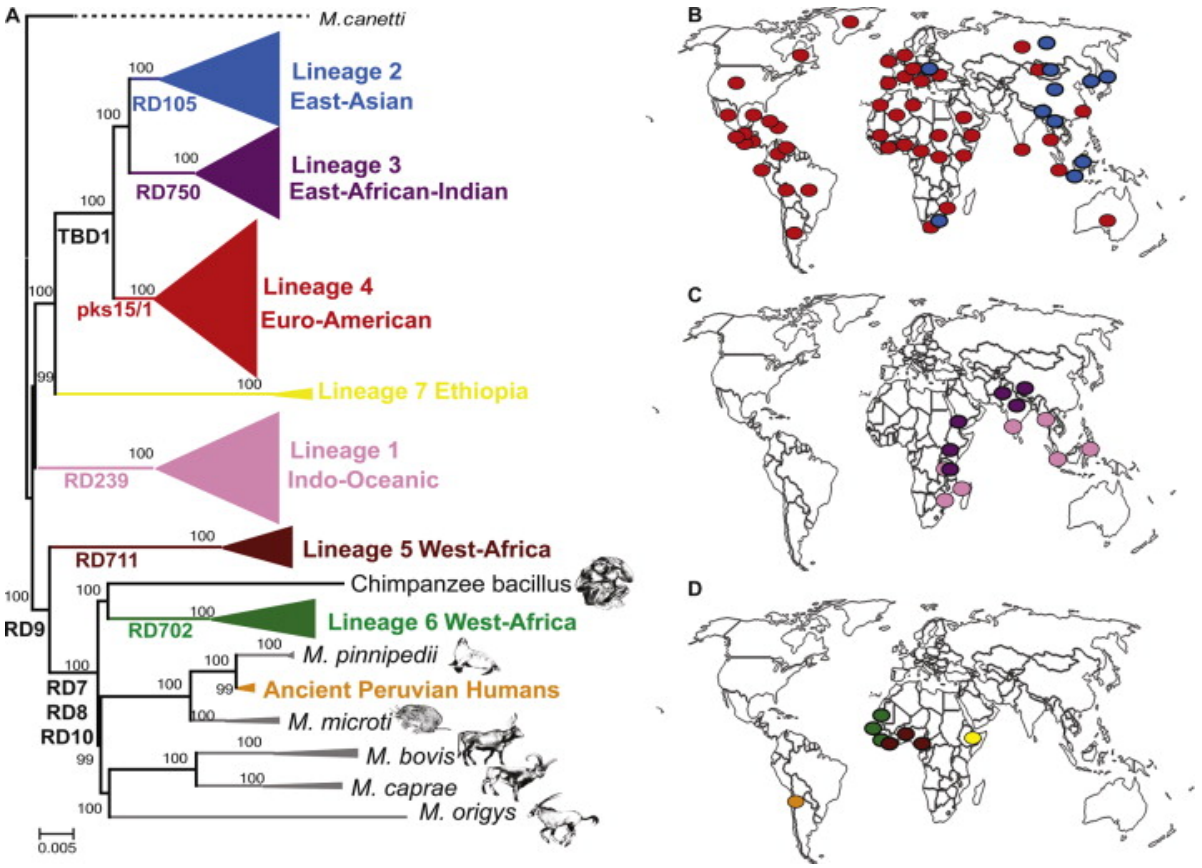

Figure 1.2: Phylogeography of *Mycobacterium tuberculosis* complex taken from M. Coscolla and S. Gagneux (2014). [1]. (A) Maximum likelihood tree inferred from SNPs and node support after 1000 bootstrap replications, with *Mcn* as outgroup. Corresponding regions of difference are shown at overlapping branch points and scale indicates SNPs per site. (B-D) Dominant MTBC lineages per country: Each dot corresponds to one-of-eighty countries of origin for eight hundred and seventy-five sequenced and annotated MTBC genomes; (B) shows most globally widespread lineages two and four; (C) shows more limited expanded lineages one and three; (D) shows most geographically restricted lineages five, six, and seven to Africa.

Human-adapted MTBC lineages differ on average by twelve hundred SNPs [1]. However, modern lineages only vary by nine hundred and seventy SNPs on average, while *Maf* and Indo-Oceanic lineages differ by approximately fifteen hundred SNPs. Remarkably, two-thirds of these SNPs in Maf and lineage one appear to be nonsynonymous (ns), altering AAs [26, 29], with over half affecting function [36]. Multiple reports of WGS analyses of globally diverse *Mtb* strains also show that many of the nearly one thousand experimentally validated human MTBC peptide T cell epitopes are hyperconserved in globally widespread East-Asian and Euro-American strains, and much more genetically diverse across *Maf*, Indo-Oceanic, and Ethiopian lineages [37-42]. These findings taken together ultimately implicate the genetic differences underlying dominant lineages two and four today in the successful establishment and maintenance of TB infection.

1.3.1 Genetic determinants.

Nonsynonymous, silent (s), and intergenic mutations in specific MTBC lineages are known to contribute to significant differences in MTBC pathogenesis. The PhoPR genes (*i.e. rv0757-rv0758*) encode a TCS transduction system essential for *Mtb* virulence in multiple infection models [43], and both ns and intergenic SNPs accumulating in and around orthologous *Maf* and *Mbv* PhoPR genes confer more limited virulence in humans [34, 44]. Similarly, *Mtb* cAMP receptor protein (Crp or Rv3676) is found to be indispensable for *Mtb* survival during mouse infections [45-47], and silent and ns mutations fixed in *Maf* and *Mbv* have likely resulted in defective Crp function [34, 36]. These mutations appear to be accumulating in globally diverse BCG vaccine strains moreover, and missing from virulent human-adapted *Mbv* strains (*e.g.* AF2122/97) [48]. More importantly, different lineage two- and four-specific mutations are linked to similar phenotypic differences marking the modern clade. A nsSNP in lineage two inactivates methyltransferase MamA (or Rv3263), while a nsSNP in lineage four inactivates the MamA homolog HsdM (or Rv2756c), and these mutations have been implicated in increased methylation and hemi-methylation of genomes of both modern lineages [49].

Silent mutations and mutations in intergenic regions are considered evolutionarily neutral

because phenotypic effects are often largely indiscernible. In *Mtb* though, silent mutations and mutations in intergenic regions can have important phenotypic consequences. A lineage two sSNP for instance creates a new internal transcriptional start site (*i.e.* TSS) in the *dosRS* locus (*i.e. rv3133c-rv3132c*) that leads to the constitutive upregulation of the dormancy regulon (or DosR) in lineage two [36]. Another sSNP puts a new internal TSS upstream of *mabA* (or *rv1483*), inducing high basal level production of MabA conferring Inh resistance in lineages two and four [50]. SNPs in intergenic regions (or IGRs) likewise create novel TSSs and promoters likely underpinning significant epigenetic differences between more phylogeographically restricted and globally widespread MTBC lineages [36]. WGS analyses of one hundred and sixty-one global isolates find that lineage two specific intergenic mutations are associated with acquired drug resistance and increased virulence [36, 51]. SNPs and smaller InDels in IGRs appear to be particularly important to the marked differences in gene expression underpinning phenotypic differences between MTBC lineages [52]. Many of these lineage-specific mutations are found in IGRs throughout the genome, which create novel promoters and TSSs contributing to pervasive transcription of antisense (or as)RNAs uniquely conserved by *Mtb* and the MTBC [36, 53-55]. *Mtb* antisense transcription appears to complement around seventy-five percent of all mRNAs, especially messages encoding for lipoproteins, secreted protein Ags, TCS transduction systems, serine / threonine kinases, and toxin-antitoxin (or TA) systems [55-60]. Moreover, a number of these intergenic SNPs and InDels appear to be fixed in globally diverse and more virulent modern sub-lineages [61, 62].

Gene duplications and the expansion of various gene families are other hallmarks of the *Mtb* genome. Mtb PE/PPE proteins are considered to play a role in Ag variation and arose from multiple duplication events involving secreted Ag ESAT-6 [63]. Accordingly, many expanded gene families, including TA systems, are missing copies in more ancient lineages [64], which are essential to TB pathogenesis in various animal infection models [65, 66]. A large duplication of approximately three hundred and fifty kilobases (or kb) seems to be responsible for elevated basal

expression of *dosR* in lineage two and four strains isolated from distinct geographic regions [27, 67]. This is an example of convergent evolution and suggests an evolutionary advantage to acquire and maintain this large duplication. On the other hand, various duplications of a thirty kb region encoding fifty-eight *Mbv* genes are phylogeographically distributed in BCG strains around the world [68], which may help to explain more variable protection afforded by BCG vaccine strains [69].

Repetitive elements such as insertion sequences (or IS(s)) have been used to successfully genotype MTBC lineages and strains, and their genomic locale can have important phenotypic consequences [1]. IS*6110* is a one thousand and four hundred kb fragment that acts as a strong promoter of expression of *Mtb* virulence factors when inserted in various regions of the genome [70-72]. Hyper-virulent strains often have IS*6110* inserted immediately upstream of *phoPR*, which constitutively upregulates the TCS transduction system, contributing to increased pathogenicity in *Mbv* [44]. Insertion events of IS*6110* and other repetitive elements followed by HR have also led to the deletion of TbD1 and other larger regions of difference (or RDs) [73, 74]. Successive loss of twenty RDs maintained by *Mtb* led to the attenuation of BCG [30, 75]. On the other hand, the deletion of *katG* from illegitimate HR of ISs confers Inh resistance in lineage two strains [76]. IS-dependent deletion of *rv1519* moreover, is associated with increased virulence in lineage two Beijing strains [77]. Patients with extra-pulmonary TB in particular are found to harbor *Mtb* strains missing the phospholipase C gene *rv1755c* [78].

1.3.2 Phenotypic implications of genetic variation.

Unlike many human pathogens, which are shed asymptomatically [79, 80], *Mtb* appears to require host lung-tissue damage to successfully transmit [81]. Accordingly, genetic variation in most wide-spread lineages two and four appear to increase pathogenicity [82]. In 1960, Mitchison *et al* found that *Mtb* strains from southern India were significantly less virulent than UK isolates in guinea pigs [83]. Subsequent genotyping (*e.g*. IS*6110* and RDs) found phylogenetic differences associated with different outcomes of infection and disease [84, 85]. Exposure to *Mtb* outbreak

strain CDC1551 is found linked with high tuberculin skin test (TST) conversion rates in household contacts of ATB patients [86] and more rapid and vigorous immune responses [87], resulting in LTBI [88, 89]. Lineage two strains in contrast are hyper-virulent in mice [90], rabbits [91], guinea pigs [92], and humans [93]. *Mtb* Beijing strain HN878, which was responsible for a TB outbreak in the US in the nineteen nineties is able to suppress early inflammatory responses, increasing pathology in multiple animal infection models [92, 94].

1.4 Host-pathogen dynamics of *Mycobacterium tuberculosis* infection.

Mtb is transmitted through aerosolized droplets and inhaled into the lungs, where it is able to establishes an infection. This may progress to ATB with clinical characteristics of a persistent cough and weight loss, radiographic evidence of lung involvement, markers of immune activation and inflammation, and sputa culture positivity [7, 95]. Infected individuals may control but not eliminate *Mtb* infection, without any obvious signs of ATB, and are diagnosed with LTBI. LTBI represents a spectrum of sub-clinical states of varying immunity, inflammation, and *Mtb* pathogen activity [96]. Immune activation and inflammation are essential for protection (or control of *Mtb* infection), preventing ATB [97], but their dysregulation is detrimental and exacerbate active disease [98].

Following airborne transmission to a naïve individual, *Mtb* reaches the lower lungs, where it is recognized by pattern recognition receptors (PRRs) and taken up by resident macrophages (Mφs). *Mtb* then enters the lung parenchyma and continues infecting local Mφs and dendritic cells (DCs). Recognition and uptake of *Mtb* by PRRs like toll-like receptor (TLR)2 induces production of pro-inflammatory mediators, including TNF-α, IL-1β, and IL-12 [3], that recruit additional innate immune cells to control infection. DCs in the lungs eventually engulf *Mtb* in a TLR-dependent manner and traffic live bacilli to the pulmonary lymph nodes (PLNs) to initiate adaptive immune responses [99]. Mannose receptor (MR) is PRR expressed on Mφs and DCs that recognizes mannosylated lipoarabinomannan (ManLAM) from the cell wall of *Mtb* [100]. This stimulation of

MR induces anti-inflammatory cytokines, IL-4 and IL-13, and inhibits IL-12, dampening protective pro-inflammatory responses [101, 102]. Thus, differential regulation of *Mtb* cell wall components can augment host immune responses to benefit *Mtb*.

Mtb is engulfed (phagocytosed) by host Mφs to isolate the threat and inform the adaptive immune responses [103, 104]. At this point, bacilli are eliminated, grow and expand exponentially, or adopt a persistent state to survive intracellularly. Phagocytosis of *Mtb* leads to recruitment of neutrophils, inflammatory monocytes, Mφs, and DCs [13, 105]. These cells become infected at the too, expanding the mycobacterial population. Accumulating innate immune cells form early granulomas in attempts to surround and eliminate infection. *Mtb* evolved multiple mechanisms though, to take advantage of intracellular niches during innate stage of infection [106]. First, *Mtb* manipulates trafficking and maturation of host phagosomes to avoid fusion with lysosomes and proteases that kill *Mtb* and alter presentation of Ags to naïve T cells [107]. Second, *Mtb* accesses the host cytosol to promote necrosis and attract other cells to infect [108, 109]. Finally, *Mtb* suppresses Mφ apoptosis, prolonging survival to maximize bacillary load before being released from necrosis. Mycobacteria continue to expand exponentially, spreading cell-to-cell, and eventually infect DCs, which migrate to PLNs to initiate adaptive immune responses [110].

There is a considerable delay in the onset of adaptive immunity in TB, which is a hallmark of chronic inflammatory diseases [111]. Tuberculin skin tests (TSTs) first show *Mtb* Ag-specific T cell responses from persons five-to-six weeks following exposure [95, 112]. This is similar in mice, which begin developing *Mtb* specific T cells one-to-two weeks following infection [113]. Accelerating this process through adoptive transfer does not affect *Mtb* growth or survival in mouse lungs [114], indicating that bacilli are unrecognizable and/or resistant to effector functions of Ag-specific $CD4^+$ T helper (T_H) cells [107]. Accordingly, the earliest activation of T_H cells occurs in the PLNs of mice, and requires live bacilli trafficked by DCs [115, 116]. After infected DCs make it to PLNs to present Ags, naïve $CD4^+$ T cells activate, proliferate, and differentiate into T_H

cell subsets prototypically, and home back to the site of infection [107].

The influx of $CD4^+$ T_H cells and some $CD8^+$ T cells halts mycobacterial growth and holds colony forming units (CFU) to approximately one million until mice succumb one-to-two years later [117]. This is attributed in part to local T cell production of IFN-γ and TNF-α, enhancing anti-mycobacterial functions of infected Mφs and other cells [118, 119]. The continued recruitment of T lymphocytes culminates in formation of mature granulomas [120, 121], the hallmark immune response to *Mtb* [122]. Immunohistochemistry reveals that human TB granulomas can be highly organized, with infected and uninfected Mφs, foamy Mφs, epithelioid cells, and multinucleated giant cells surrounded by a fibrotic cuff of mostly T and a few B cells [119, 123]. Granulomas are dynamic structures in various stages of differentiation throughout infection and can resolve and even disorganize over time [124-126]. Long-term control of *Mtb* infection requires maintenance of granulomas, with host defects increasing the likelihood of developing ATB [127-130], and *Mtb* has mechanisms to avoid host elimination and persist extensively. These mechanisms are tied into *Mtb* metabolism and growth and ultimately contribute in determining the outcome of infection.

1.4.1. Innate recognition and immune responses.

Although Mφs are a major intracellular niche for *Mtb* growth and survival during infection, they are also essential for protective immune responses that control or eliminate infection [131]. Mφs are found throughout the body, but their antimicrobial capacity depends on more local tissue environments that are shaped by associated cell types, growth factors, and cytokines. From an array of PRRs, lung Mφs recognize, bind to, and internalize *Mtb*. These processes initiate intracellular cascades that activate innate defenses and release cytokines and chemokines, recruiting inflammatory cells [131]. Consequently, Mφ responses to *Mtb* may vary significantly, from eradication to unrestricted growth of bacilli [132].

Mφs express multiple PRRs and other phagocytic receptors, including TLRs, C-type lectin receptors (or CLRs), and complement receptors (or CRs), which recognize *Mtb* products, and

especially cell wall components [3], and differentially regulate Mφ phagocytosis [133]. TLR2 for instance is expressed on alveolar Mφs, recognizing *Mtb* 19-kDA lipoprotein, LpqH, lipomannans, and phosphatidylinositol mannosides highlighted in Figure 1.3 [134-136]. TLR2 dimerizes with additional TLRs, including TLR1 and TLR6, when sensing various *Mtb* lipoproteins [3]. Following ligand binding, the cytoplasmic domains of TLR2, TLR1/TLR2, and TLR2/TLR6 recruit MYD88 [137], and in turn kinases (*e.g.* IRAKs, TAK1, and MAPKs) and ubiquitin ligases (*e.g*. TRAFs). These signaling cascades lead to activation of nuclear transcription factor NF-κB [138]. This prompts the transcription of genes involved in activating innate defenses, particularly TNF-α, IL-1β, IL-12, and nitric oxide (or NO) production [139].

Figure 1.3: Bioactive components of *Mycobacterium tuberculosis* cell wall taken from J. Kleinnijenhuis, M. Oosting, L. A. B. Joosten, M. G. Netea, & R. Van Crevel (2011) [3].

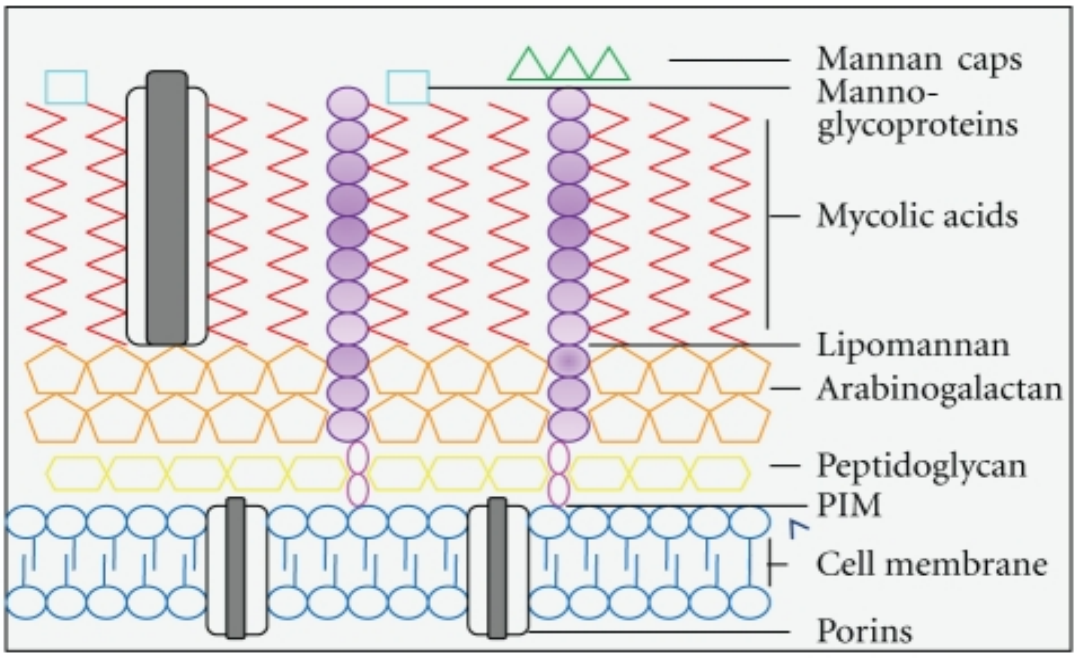

Figure 1.3: Bioactive components of *Mycobacterium tuberculosis* cell wall taken from J. Kleinnijenhuis, M. Oosting, L. A. B. Joosten, M. G. Netea, & R. Van Crevel (2011) [3]. The inner layer comprises peptidoglycan (or PG) covalently linked to arabinogalactan and the outer layer consists primarily of mycolic acids, lipomannan (or LM), mannosylated lipoarabinomannan (or ManLAM), and mannosylated glycoproteins.

TLR2 is particularly important for induction of TNF-α to activate Mφs [137, 140], which produce NO [141] and release antimicrobial peptides that kill intracellular bacilli [142]. TLR2/TLR6

activation leads to increased secretion of IL-1β, which induces antibacterial effector mechanisms such as autophagy and apoptosis in activated Mφs [143]. IL-12 is produced by Mφs in a TLR2-dependent manner, which leads to interferon gamma (or IFN-γ) production by T_H cells [144]. TLR2-deficient mice show severe deficiencies in granuloma formation and cannot control *Mtb*-infection long-term [145, 146]. In contrast, TLR4 activation on Mφs has been shown to induce a type I IFN response, which suppresses TNF-α, IL-1β, and IL-12 production and enhances pathology in *Mtb*-infected mice [147-149]. TLR4 recognizes secreted *Mtb* heat shock proteins [150, 151] and *Tlr4*-/- deficient mice show no differences in TB susceptibility compared to wild type (*i.e.* WT) mice [146]. This agrees with another report that a *Mtb* mutant strain overexpressing heat shock protein Rv0251c is attenuated in the chronic phase of murine infection [152]. These results taken together suggest that TLR2-dependent recognition favors the host, while TLR4-dependent recognition favors *Mtb*. Still, TLR2-dependent *Mtb* uptake may also lead to impaired Ag-presentation by major histocompatibility complex (or MHC) class II [153, 154] and diminished NO production by activated Mφs [155].

CLRs contribute to differential host recognition and innate responses in *Mtb* infection [3, 156]. MR is predominantly expressed on non-activated human Mφs and binds mannose caps on the *Mtb* cell wall [157-159]. MR-dependent *Mtb* recognition varies by strains though [160, 161], and MR is able to discriminate between variations in mannose caps on ManLAM [162] and higher-order phosphatidylinositol mannosides abundant on pathogenic bacilli [163]. This recognition of *Mtb* stimulates IL-4 and IL-13 production, which suppress Mφ production of IL-12 and reactive oxygen species (or ROS) [164-166], and results in the production of a unique pathogen vacuole with little-to-no lysosomal fusion [102].

Dectin-1 is a CLR expressed on Mφs and DCs that recognizes β-glucan, a fungal cell wall component [131, 167]. Dectin-1 is found to be required for TLR2-dependent uptake of pathogenic mycobacteria and induction of TNF-α and IL-6 by Mφs [168, 169]. Dectin-1 recognition appears

to enhance TLR4-mediated uptake of *Mtb* as well and subsequent IL-17 production by infected Mφs [170]. In contrast, Mφ inducible calcium-dependent CLR (Mincle) is expressed on Mφs following TLR activation and its stimulation leads to the induction of TNF-α, MIP-2, and IL-6 [171]. Mincle recognizes trehalose dimycolate, one of the most abundant lipids released by *Mtb*, [172, 173], and its activation is linked with increased mycobacterial resistance to host immune killing and necrosis [174, 175]. However, Mincle-deficient mice still produce effective granulomatous responses to *Mtb* infection, with T_H1 cells activating Mφs that effectively control *Mtb* [176].

In addition to CLRs, scavenger receptors are expressed on Mφs and recognize *Mtb* cell wall components facilitating its uptake [177]. Class A scavenger Mφ receptor with a collagenase structure specifically appears to tether trehalose dimycolate to murine Mφs, which is required for TLR2-dependent *Mtb* phagocytosis and activating NF-κβ [178]. This requires co-receptor CD14 that recognizes mycobacterial various lipoproteins and LAM [131, 179], which ultimately leads to enhanced induction of pro-inflammatory cytokines [180].

CR1, CR3, and CR4 are important for Mφ uptake of bacteria coated (or opsonized) with complement proteins. *In vitro* studies show that CR3 mediates eighty percent of phagocytosis of opsonized *Mtb* by human Mφs [181], but does not lead to any significant production of ROS or inflammatory cytokines by these infected Mφs [182]. CR3 recognizes and binds various glucans [183, 184], phosphatidylinositol mannosides, and glycosylated lipoproteins of *Mtb* [185], and is found to be highly expressed on alveolar Mφs [186].

CR-binding prompts actin-dependent movements by Mφs to engulf mycobacteria into a phagosome that recruits GTPases and v-ATPases to the membrane, which induce acidification of pathogen-containing vacuoles [187]. Acidified phagosomes fuse with lysosomes, which deliver hydrolases and ROS that effectively destroy bacteria to process Ags for activating T cells [188]. *Mtb* though, is able to subvert phagosomal trafficking and reside in immature phagosomes with a pH of approximately 6.20 [189-192] and high levels of ammonia [193]. *Mtb* phagosomes contain

iron and glycosphingolipids, indicating that they are fused with early endosomes [194, 195]. *Mtb* ManLAM perturbs calcium influxes in the Mφ cytoplasm, thereby inhibiting the recruitment of phosphatidyl-inositol-3-phosphate kinase to *Mtb* phagosomes [196-199], which is essential for acidifying phagosomes [200]. In addition, *Mtb* inhibits sphingosine kinase which further impedes Mφ calcium signaling [201]. Consequently, *Mtb* phagosomes are found to contain high levels of transferrin receptor [202] without v-ATPases [203] and late endosomal markers [204, 205]. The inhibition of phagosomal maturation likewise seems to stem from the lack of recruitment of effector proteins such as EEA1, which helps to deliver v-ATPases, hydrolases, and antimicrobial peptides to phagosomes [103, 196]. This is because *Mtb* phagosomes lacking phosphatidyl-inositol-3-phosphate kinase are unable to retain EEA1 found on endosomes [206], which inhibits GTPases acidifying *Mtb*-containing vacuoles [204, 205].

Mtb also exports multiple proteins that impede phagosomal trafficking. *Mtb* releases lipid phosphatase SapM (or Rv3310), which dephosphorylates phosphatidyl-inositol-3-phosphate [197, 199], and eukaryotic-like protein kinase G (*i.e.* PknG or Rv0410c), which is essential to block phagosomal-lysosomal fusion and *Mtb* survival [207]. The *Mtb* blockade on phagosomal maturation in turn provides access to Mφ nutrients, including fatty acids, triacylglycerols [132, 208-210], iron, and zinc [211-213], which enable growth and expansion of tubercle bacilli during early infection [214-216]. The secretion of ESAT-6 by *Mtb* not only further impedes phagosomal maturation, it also enables *Mtb* to access the host Mφ cytosol [108, 217, 218]. More virulent *Mtb* strains appear to stimulate cytosolic PRRs in an ESAT-6-dependent manner [219-221] and move into the cytosol following phagosomal rupture [222, 223]. ESX-1-dependent secretion of ESAT-6 appears to perforate *Mtb* phagosomes, resulting in the mixing of phagosome contents with the Mφ cytosol [224, 225].

(NOD)-like receptors (or NLRs) are important cytosolic sensors in phagocytes, and pivotal to innate immunity, inducing antimicrobial responses to intracellular bacteria and regulating host

cell death [226, 227]. Of the four NLR protein families, NOD1 and NOD2 have a N-terminal caspase activation and recognition domain involved in a variety of cell signaling cascades, including apoptosis [227]. This domain is structurally similar to the N-terminal pyrin domain of NLRP3, an important sensor of cell integrity during infection [227]. Potassium ions efflux from innate cells with damaged membranes and activate NLRP3 [228], which recruits apoptotic protein PYCARD and in turn tyrosine kinase Syk, resulting in necrosis from increased mitochondria destabilization, organelle swelling, and membrane permeabilization, all of which are concomitant with caspase-I activation and IL-1β secretion [219, 223, 229, 230]. NOD1, NOD2, and NLRP3 are expressed in lung Mφs [226] and differentially recognize *Mtb* peptidoglycan and ESAT-6 [220, 231]. NOD1 and NOD2 bind discrete peptidoglycan components [232], activating NF-κβ-dependent transcription of pro-inflammatory and host defense genes [233]. NOD2 likewise interacts with NRLP3, activating capase-1 and IL-1β secretion [234], resulting in apoptosis of *Mtb*-infected Mφs [235]. NLRP3 also recognizes ESAT-6 though, which induces necrosis of *Mtb*-infected Mφs, enhancing its spread [219, 220, 229, 236].

To contain *Mtb*, Mφs undergo apoptosis, a cell death pathway that leads to reduced *Mtb* survival and enhanced Ag presentation to prime adaptive immunity [237-239]. Apoptotic Mφs are rapidly engulfed and degraded (or efferocytosed) by neighboring phagocytes [240] that ultimately kill bacilli with limited inflammation and immunopathology [241]. Accordingly, *Mtb* can exacerbate inflammatory responses of pro-apoptotic cells through various secreted effectors [242-244], while promoting necrosis, increasing *Mtb* replication, dissemination, and ATB disease progression [223, 245]. Intracellular *Mtb* replication rates help determine host cell fates, as Mφs infected with over twenty-five bacilli appear to become necrotic from increased damage to mitochondria and other cell membranes [246]. Mφs with low bacillary burdens in contrast readily undergo apoptosis [247]. Mφs can repair damaged cell membranes too, stopping necrosis and initiating apoptosis, but *Mtb* also blocks this repair pathway to infect new cells [248]. Virulent *Mtb* strains are known to cause

significant disruptions in mitochondrial membranes in Mφs [249] *via* secreting a necrotizing toxin that depletes NAD^+ for ATP synthesis [250]. Moreover, release of lysosomal protease cathepsin-B into the cytosol induces necrosis [251] and *Mtb* ESX-1-mediated escape into the host cytosol prompts this release [252]. Along with this, *Mtb* secretes protein Rv3654c, which decreases caspase eight levels in the cytosol and in turn apoptosis of infected Mφs [253]. *Mt*b also exports Rv3364c, which binds to and inhibits Mφ cathepsin G, blocking activation of caspase one [254]. Concomitantly, *Mtb* is able to induce the survival pathway in host cells. *Mtb* LAM in particular activates phosphatidyl-inositol-3-phosphate kinase-dependent phosphorylation of pro-apoptotic protein Bad, which activates growth factors that enhance Mφ survival [255]. Torrelles *et al.,* (2009) have reported that the deletion of the mannosyltransferase gene *pimB* (or *rv2188c*) reduces LAM in the *Mtb* cell wall, and *Mtb*Δ*pimB*-infected Mφs die much quicker than those infected with WT *Mtb* [256]. Phosphatidyl-inositol-3-phosphate kinase is important for the induction of MAP kinase pathways that are activated by the *Mtb* TA toxin Rv0475 [257]. These data show that *Mtb* manipulates host cell necrosis and apoptosis simultaneously, creating a protective niche for unrestricted growth and survival during innate stages of infection.

1.4.2 Initiating adaptive immune responses.

Extra-pulmonary TB manifests throughout much of the human body, with severe morbidity and mortality, including the lymph nodes, the central nervous system, and liver involvement [258-260]. Autopsy studies of LTBI people who died of non-TB-related causes found viable tubercle bacilli in lungs and LNs [261-263]. Lymphatic spread of *Mtb* during pulmonary infection induces adaptive immune responses that can drive transmissible host pathology [264]. *In situ* polymerase chain reaction (PCR) techniques identified viable bacilli in sixty-seven percent of lungs, spleens, kidneys, and livers from forty-nine LTBI individuals post-mortem [258]. Neyrolles *et al.,* (2006) similarly detected *Mtb* rRNA in adipose tissues surrounding LNs in almost one-third of forty-two *Mtb*-infected individuals [265]. They showed that *Mtb* can invade adipocytes and resides in a

non-replicating state, tolerant to Inh [265], which indicates that fat cells may serve as a potential reservoir for *Mtb*.

TSTs are first measurable in humans around six weeks post-exposure [95, 112]. This is similar in mice, with *Mtb* Ag-specific T cell responses beginning to emerge one-to-two weeks post-infection [113, 115, 266]. Activation of naïve $CD4^+$ T cells starts in PLNs from live *Mtb* trafficked by DCs from lungs to present Ags [113, 115, 266]. The subsequent proliferation, differentiation, and homing of effector $CD4^+$ T cells occurs normally, but DC transport of *Mtb* to the PLNs takes eight-to-ten days following infection [115]. This is in part because *Mtb* is able to impair chemokine-dependent migration of DCs to PLNs [115, 231, 267]. DCs express receptor DC-SIGN and its activation by ManLAM and phosphatidylinositol mannosides blocks maturation of infected DCs needed to traffic *Mtb* to the PLNs [101, 163, 268]. In addition, *Mtb* secretes proteins that impair the expression of MHC class II and co-receptor molecules on DCs, reducing development Ag-specific $CD4^+$ T cells [269, 270].

Neutrophils are also involved with trafficking *Mtb* to the PLNs in infection. In guinea pigs, neutrophils with large numbers of bacilli are found in pulmonary lymphatic lesions early in infection [271, 272]. In mice, the inhibition of neutrophil apoptosis by *Mtb* hinders DC trafficking too, as infection with *Mtb*Δ*nuoG* (or *Mtb*Δ*rv3151*) is found to result in accelerated DC-*Mtb* trafficking and subsequent development of effector $CD4^+$ T cells in PLNs, and this is reversed upon depletion of neutrophils [273]. Neutrophils also to transport *Mbv* BCG to PLNs post-vaccination [274].

Mtb disseminates extracellularly from pulmonary site of infection as well. *Mtb* appears to move across respiratory epithelium by triggering necrosis in the lining epithelial cells [275-277]. *Mtb*Δ*hbha* (or *Mtb*Δ*rv0475*) moreover cannot bind and invade respiratory epithelial cells, and is impaired for colonization of spleens but not lungs of infected mice [278]. The deletion of *Mtb* fatty acyl-AMP ligase Rv1345 attenuates bacilli growth in livers, but not in lungs or in spleens of mice following intravenous inoculation of Mtb [279], showing that this gene is specifically required for

Mtb dissemination to the liver. Similarly, *Mtb* protein kinase PknD (or Rv0931c) is found to be essential for causing central nervous system TB in mice and in guinea pigs, enabling invasion of endothelial cells but not pulmonary Mφs or epithelial cells [280, 281]. Importantly, these findings show that *Mtb* has acquired specific mechanisms to disseminate to more peripheral organs and tissues throughout the body.

1.4.3. Adaptive immune responses.

Many bacterial pathogens evade innate host defenses and cause acute infections, which are quickly cleared with the onset of adaptive immunity. TB represents a chronic infection though, due to the unique abilities of *Mtb* to avoid and subvert innate and adaptive immune responses. Host protection against TB relies on the development of T cell responses [97, 282], which requires tight regulation, and dysregulated T cell responses can be detrimental and drive ATB [283-285].

$CD4^+$ T cells support inflammation, contribute to protection, and regulate host immune responses to *Mtb*. In mice, the development of Ag-specific $CD4^+$ T cells leads to control of *Mtb* infection [113]. CD4-deficient transgenic mice are rendered highly susceptible to *Mtb* infection and succumb to severe lung pathology from unrestricted *Mtb* growth [117, 286]. The depletion of $CD4^+$ T cells following *Mtb* infection moreover leads to rapid reactivation marked by increasing bacterial growth and lung tissue damage, and reduced mouse survival [287]. Similarly, decreased $CD4^+$ T cell counts is found to lead to reactivation of LTBI in non-human primates (NHPs) [288], implicating the importance of $CD4^+$ T cells in controlling *Mtb* infection, preventing ATB, and reactivation disease in humans.

The development of Ag-specific T cell responses in *Mtb* infection is delayed considerably compared to other pathogens [231]. Activation of naïve $CD4^+$ T cells requires DC transport of live bacilli from lungs to PLNs in mice. Studies show that earlier transport of bacilli to PLNs leads to accelerated development of Ag-specific $CD4^+$ T cells and better host control of *Mtb* infection [112, 113]. However, adoptively transferred Ag-specific $CD4^+$ effector T cells in the lungs of infected mice still cannot recognize *Mtb* until around a week post-infection [114]. This is in line with others

reporting that *Mtb* inhibition of apoptosis delays and impairs development $CD4^+$ T_H cells [112, 273, 289, 290]. The inhibition of apoptosis seems to slow and limit DC acquisition of *Mtb*, which impairs its transport to PLNs and T cell priming [112, 273]. This likely limits the Ag-presentation of infected cells, enabling *Mtb* to influence $CD4^+$ T cells development.

$CD4^+$ T cells develop into different populations of T_H cells based on local cytokines in *Mtb* infection [114, 231]. DCs and Mφs produce IL-12, which binds to the IL-12 receptor on $CD4^+$ T cells, inducing transcription factors STAT4 and T-bet. STAT4 and T-bet activate transcription of numerous pro-inflammatory and defense genes such as IFN-γ by T_H1 cells [291, 292]. The advent of T_H1 cells is important for TB protection in various animal infection models in part because IFN-γ activates antibacterial functions of Mφs that control *Mtb* [293]. Reports likewise find that IL-12 is essential for the development and maintenance of T_H1 responses that control *Mtb* infection long-term [116, 294-296]. *IFNG*$^{-/-}$*, IFNGR*$^{-/-}$*, and IFNGR1*$^{-/-}$ mice are found to experience widespread tissue damage and accelerated mortality following *Mtb* infection [297-299]. Similar observations are reported in mice defective for downstream IFN-γ inducible effector functions, including phagosomal acidification (*LRG47*$^{-/-}$), NO production (*iNOS*$^{-/-}$), and the release of antimicrobial peptides (*GBP*$^{-/-}$) [299-301]. Humans with mutations in T_H1 immunity (*i.e.* IL-12p40, IL-12Rβ1, IFN-γR, and iNOS) moreover are highly susceptible to developing severe infections from BCG vaccination and exposure to non-pathogenic mycobacteria [302-307]. Moreover, long-term survivors of untreated TB (*i.e.* more than fifty years) appear to maintain circulating *Mtb* Ag-specific IFN-γ- and IL-2-producing T_H1 cells [308].

Naïve $CD4^+$ T cells are primed by MHC class II peptide Ag complexes on DCs [97]. These Ags are derived from proteases in *Mtb* phagolysosomes and loaded onto MHC class II molecules *via* the endocytic pathway [97]. Following binding of MHC class II-Ag, T cell receptor and CD4 co-stimulatory molecules interact, activating T cells to produce IFN-γ and IL-2. IFN-γ secreted by T_H1 cells enhances Mϕ phagocytosis and digestion of *Mtb*. T_H1 cells also activate Mφs *via* direct

contact and secretion of IFN-γ, converting them into potent antimicrobial cells. Activated Mφs are able to fully acidify *Mtb* phagosomes, promoting fusion with lysosomes and exposure of bacilli to lethal NO, ROS, and antimicrobial peptides, thereby enhancing Ag-presentation [208, 309, 310]. However, *Mtb* can inhibit the transcription of IFN-γ-stimulated genes, diminishing the antibacterial capacity of Mφs [311, 312]. *Mtb* activates STAT3 in human Mφs as well, which suppresses the production of pro-inflammatory cytokines and NO [313]. On the other hand, IFN-γ-dependent phagosomal acidification and release of antimicrobial peptides such as cathelicidin and cathepsin, has been shown to kill *Mtb* in human Mφs [314, 315]. Cathepsins specifically process *Mtb* proteins for MHC class II-Ag presentation [316]. In *Mtb*-infected immunocompetent mice, *Mtb* growth is arrested with the accumulation of IFN-γ-producing $CD4^+$ T cells in the lungs, which maintain a plateau population of approximately one million bacilli until mice succumb one-to-two years later [117, 317, 318]. These data collectively demonstrate that while T_H1 responses are sufficient to control *Mtb*, their ability to kill *Mtb* is limited in animal infection models. Studies show that *Mtb* significantly impairs surface expression of MHC class II-Ags on Mφs [153, 319] and alters its expression of Ag genes during the course of infection [320, 321]. In the case of Ag85, which is in several TB vaccine candidates, the downregulation of these genes in *Mtb* leads to a marked reduction in Ag85-specific $CD4^+$ T cells during chronic infection, likely contributing to *Mtb* survival during murine infection [322].

T_H1 cells are thought to be important for protection against TB in animal infection models because of the Mφ-activating effects of IFN-γ. However, the depletion of $CD4^+$ T cells from mice chronically infected with *Mtb* appears to result in reactivation without affecting IFN-γ activity in the lungs [287]. Adoptive transfer studies in WT, *IFN-*$\gamma^{-/-}$, and *iNOS*$^{-/-}$ deficient mice moreover show that *Mtb* infection is effectively controlled by the development of T_H1 cells independent of any IFN-γ production [323]. Numerous *Mtb* Ag-specific $CD4^+$ T cells are found during murine infection expressing cell surface marker PD-1 and producing little-to-no IFN-γ [324]. These cells are a

precursor population that maintain IFN-γ-producing T_H1 cells [231]. Accordingly, *PD-1*$^{-/-}$ mice are found to rapidly succumb to *Mtb* infection from neutrophil-dominated pathology and significantly increased production of IFN-γ, TNF-α, and IL-17 in the lungs [325, 326], and depleting $CD4^+$ T cells rescues these mice from early mortality [326]. It thus seems that PD-1 acts to regulate differentiation of $CD4^+$ T cells to avoid immunopathology, while maintaining T cell populations to effectively control *Mtb*.

Naïve $CD4^+$ T cells differentiate into T_H17 cells as well, contributing to adaptive immune responses in *Mtb* infection. T_H17 cells produce IL-17, which is involved in neutrophil recruitment and activation, and sustained inflammation. IL-17 induces high-level production of inflammatory molecules by phagocytes and epithelial cells that also underly chronic inflammatory diseases [327, 328]. Studies in IL-17-deficient mice infected with *Mtb* strain H37Rv reveal that IL-17 is dispensable for host control of *Mtb* [329]. However, Gopal *et al,.* (2014) showed that *IL-17*$^{-/-}$ mice are much more susceptible to hypervirulent *Mtb* strains, with increased organ burdens and the impaired localization of IFN-γ-producing $CD4^+$ effector T cells and activated Mφs in lungs [330]. Others reports likewise show that IL-17 contributes to protective T_H1 responses in lungs of *Mtb*-infected mice [331, 332]. On the other hand, T_H17 responses are implicated in immunopathology from excess recruitment and activation of neutrophils in lungs of Mtb-infected mice [333, 334]. In cases of ATB, IL-17 production is directly correlated with *Mtb* Ag load [335] and clinical signs of disease severity [336]. Others have shown an association between increased circulating IL-17-producing T cells and ATB [337, 338]. Thus, it's thought that while T_H17 cells contribute to protection early on in *Mtb* infection, their tight regulation is required to avoid pathogenic T_H17 responses with the potential to exacerbate ATB [339].

$CD8^+$ of cytotoxic T cells (CTLs) are also involved in adaptive immunity to *Mtb*. Anti-CD8 antibody-depleted mice and *TAP*$^{-/-}$ mice, which are unable to develop Ag-specific CTLs, are found to succumb early in *Mtb* infection, with increased lung burdens and tissue pathologies, relative to

WT mice [340, 341]. TAP is essential to pump peptide Ags from the host cytosol across the endoplasmic reticulum to load MHC class I molecules for Ag-presentation to CTLs [341]. MHC class I restricted CTLs are found to accumulate in mouse lungs throughout *Mtb* infection that are important for producing IFN-γ and TNF-α [342, 343] and delivering granzymes proteases with perforin and/or granulysin to kill bacilli in infected Mφs [344]. CTLs in mice and in humans produce perforin [345, 346], which acts as a pore to deliver granzymes that effectively lyse *Mtb*-infected Mφs, killing intracellular bacilli [347]. Human CTLs make granulysin too, which deliver granzymes proteases into Mφs and directly kills *Mtb* [348, 349]. Accordingly, the dysregulation of granulysin and perforin production by CTLs appears to contribute to increased TB pathology [350-352]. Lung tissue samples from ATB patients show an influx of CTLs producing granzymes with little-to-no perforin or granulysin in a majority of TB lesions [353]. Anti-TNF therapy moreover is found to lead to the depletion of perforin- and granulysin-producing CTLs in LTBI individuals, contributing to reactivation ATB disease [125].

Most Ag-specific CTLs isolated from *Mtb*-infected individuals appear to be MHC class I restricted [344] and murine infection with the pro-apoptotic mutant, *Mtb*Δ*secA2* (or *Mtb*Δ*rv1821*), accelerates Ag cross-presentation and development of classically restricted CTLs, improving host control [290]. Accordingly, *Mtb* is found to suppress MHC class I Ag presentation on infected Mφs [354] and secrete 'decoy' Ags [355] limiting protective CTL responses. *Mtb* cell wall lipids promote involvement of non-classically restricted $CD8^+$ T cell responses. Human CD1-restricted $CD8^+$ T cells recognize multiple immunomodulatory *Mtb* lipids presented by CD1 surface molecules (*i.e.* a-d) on Ag presenting cells [356]. CD1c-restricted T cells for instance recognize *Mtb* polyketides, whereas CD1b-restricted T cells become activated by ManLAMs [357] and mycolic acids [358]. Notably, CD1 molecules show little-to-no genetic variability and NT polymorphisms are associated with increased susceptibility to ATB [359]. These published observations ultimately implicate the development of CD1-restricted T cells in TB protection.

Increased susceptibilities of individuals with mutations in T_H1 responses to mycobacterial infections highlight the importance of adaptive immunity in human TB. It is also evident though, that TB is a chronic inflammatory disease. Earlier studies in rabbits show that lung tissue necrosis and destruction occurs concomitantly with the onset of T cell responses [360], indicating that host immunopathology contributes to the outcomes of *Mtb* infection. A more recent systemic review of human immunodeficiency virus positive (or HIV^+) individuals co-infected with *Mtb* found that those persons with $CD4^+$ T cell counts below two hundred are less likely to develop cavitary TB and transmit to non-HIV-infected individuals [361]. In addition, studies of genetic diversity found that unlike most pathogens, five hundred experimentally confirmed human T cell epitopes are strictly conserved at the sequence level in over two hundred *Mtb* strains isolated from patients worldwide [37, 362]. In fact, there appear to be a larger number of fixed mutations in essential housekeeping genes, suggesting that *Mtb* does not rely on Ag variation. This is in line with others reporting that differential regulation of Ags by *Mtb* helps to undermine protective immune responses, potentially increasing host pathology and transmission [310, 363, 364].

1.5 Determinants of *Mycobacterium tuberculosis* persistence and pathogenesis.

Mtb significantly and differentially regulates the expression of its Ags, including ESAT-6 (or Rv3875) and Ag85, throughout *Mtb* infection [320], rendering many $CD4^+$ T cells largely non-functional [322, 365]. The *Mtb* DosR is induced by environmental cues associated with adaptive immunity, including hypoxia [74,75] and NO [73], and a number of DosR genes encode peptide Ags recognized by T cells from humans with LTBI but not ATB [366, 367]. Moreover, NHP lungs infected with *Mtb*Δ*dosR* (or *Mtb*Δ*rv3133c*) or *Mtb*Δ*dosS* (or *Mtb*Δ*rv3132c*) attenuated strains show transcriptional signatures of accelerated and greater T cell responses compared to those infected with WT *Mtb* [368], implying that *Mtb* mediates establishment and maintenance of LTBI, and that latency does not only reflect the inhibitory effects of T cell responses on *Mtb* growth and survival *in vivo*. Accordingly, *Mtb* encodes five resuscitation promotion factors (Rpfs), of which RpfA (or Rv0867c) and RpfB (or Rv1009) are recognized by T cells of LTBI but not ATB individuals [369,

370], and their successive deletion significantly impairs reactivation ATB in mice [371, 372]. Supplementing liquid media with only twenty nanomolar concentrations of various Rpfs is found to trigger the outgrowth of over eighty percent of tubercle bacilli recovered from patient sputa pre-chemotherapy, and the relative proportions of these Rpf-dependent and multi-drug tolerant bacilli appear to increase markedly during treatment [373]. Moreover, although T_H1 responses enhance host killing of *Mtb* [310, 312], they also induce multiple TA systems [374], which are shown to be essential for successful adaptation and survival of mycobacteria exposed to drugs in anerobic low pH host environments during infection [65, 66, 375, 376]. Many of these TA genes appear to mediate epigenetic changes essential for maintaining respiration, ATP synthesis, and *Mtb* survival during extended periods of stress *in vitro* [65, 377, 378], and in turn virulence in mice [363, 379] in guinea pigs [65, 375].

Mtb recognizes environmental pressures throughout infection, eliciting changes in growth and metabolism that promote mycobacterial survival. *Mtb* counters hypoxia in the granuloma [380, 381] for instance through induction DosR [378, 382]. DosR is essential for *Mtb* pathogenesis in multiple animal models of *Mtb* infection [383, 384] and influences the delay and dampening of T cell responses [368]. Besides Ag production, DosR regulates a number of genes involved in protein recycling, alternative electron transport, and DNA repair that augment *Mtb* growth and metabolism *in vivo* [366, 385, 386]. Hypoxic tubercle bacilli in particular do not inhibit phagosomal acidification, and this enhances T cell recognition of Ags not expressed by bacilli during chronic infection [310]. And although this certainly enhances the anti-mycobacterial effector mechanisms of T cells and infected Mφs [387, 388], this persistent state misdirects host responses during later stages of the infection. Adjunctive therapies in particular appear to reduce tissue hypoxia, thereby increasing efficacy of anti-TB drug regiments [389, 390], indicating that mycobacterial persistence is acquired *in vivo*.

Similar to hypoxia, the host limits *Mtb* access to nutrients such as iron, phosphate, and carbon, which induces bacterial persistence [391-393]. *Mtb*-infected phagocytes in particular are

known to sequester metals, AAs, and carbon [394, 395]. Limiting nutrients and especially carbon renders *Mtb* insensitive to frontline anti-TB drugs such as Inh and Rif, with little-to-no replication, modified RNA polymerase activity, and altered metabolism [377, 396-398]. This is the stringent response, which is regulated by hyper-phosphorylated guanosine NTs pppGpp and ppGpp (*i.e.* (p)ppGpp), which are made by dual functioning *Mtb* enzyme RelA (or Rv2583c) [399]. RelA is found to be essential for long-term survival of *Mtb* in nutrient starved environments *in vitro*, as well as pathogenesis in mice, in guinea pigs, and in NHPs [66, 400, 401]. The stringent response directly affects lung pathology [363, 401], as its induction leads to diminished T_H1 cell responses, resulting in increased lung burdens and pathology of murine *Mtb*-infected lungs [363]. There is a striking absence of necrotic granulomas in the lungs of guinea pigs infected with *Mtb*Δ*relA* (or *Mtb*Δ*rv2583c*) as well [401]. These findings suggest that the *Mtb* stringent response is an evolved adaptive mechanism that functions dually to enhance survival and virulence.

*Mt*b stringency appears to have evolved as an integrated response to numerous pressures linked to the host immune responses to infection. For example, (p)ppGpp metabolism is regulated by phosphate availability, which is put into linear chains by polyphosphate kinase (or Rv2984) and removed by exopoloyphosphatase (or Rv1026) [402]. Both of these enzymes are essential for *Mtb* survival in Mφs and in guinea pig lungs [402, 403]. Accordingly, *Mtb* transcription factor PhoY2 (or Rv0821c), which regulates phosphate transport, is found to be essential for developing Rif and Pza tolerance *in vitro* and long-term survival in murine lungs as well [404]. Phosphate starvation activates TCS systems SenX3-RegX3 (*i.e.* Rv0490-Rv0491) and PhoPR (or Rv0757-Rv0758) as well [405, 406], which in addition to upregulating phosphate transport, induce the transcription of numerous genes encoding immunomodulatory proteins [407-411]. SenX3-RegX3 and PhoPR are essential for full virulence during multiple animal infection models [412-415], and this is because they induce epigenetic changes that ultimately affect the lipid composition of the *Mtb* cell wall [413, 416]. Central carbon metabolism is known to generate toxic byproducts, which

Mtb incorporates into various bioactive cell wall lipids [209, 417].

Mtb encodes seven WhiB proteins that are responsive to multiple endogenous (*e.g*. lipids) and exogenous (*e.g*. pH, ROS, and NO) factors, which modulate the redox potential of *Mtb* [418-420]. WhiB3 (or Rv3416) in particular responds to low pH and is essential for bacillary survival in activated Mϕs, decreasing their pro-inflammatory responses and contributing to increased lung pathology [420]. Similarly, WhiB5 (or Rv0022c) is another redox sensor involved in stringent control and is essential for *in vivo* persistence [421]. Notably, the lungs of mice infected with *Mtb*Δ*whiB5* are found to have smaller bacterial burdens with larger granulomas with high amounts of IFN-γ [421]. Furthermore, *Mtb*Δ*whiB5* is found to be unable to cause reactivation TB in mice receiving corticosteroids [421]. Global analyses ultimately show that this is likely because WhiB5 directly regulates almost forty genes directly implicated in *Mtb* virulence, including the secretion of ESAT-6 [421].

Like DosR, MprAB (or Rv0981-Rv0982) encodes another TCS regulatory module that has been integrated with the *Mtb* stringent response. As a result, *Mtb*Δ*mprAB* is unable to regulate replication in Mϕs, and consequently, cannot persist in mouse lungs long-term [422, 423]. This is because MprAB regulates numerous genes, including *pks4* (or *rv1181*), *mbtB* (or *rv2383c*), and *mbtD* (or *rv2381c*) for mycobactin synthesis, as well as *fdxA* (or *rv2007c*), and *ppe37* (or *rv2123*) [424], which also form to the *Mtb* SigE (or Rv1221) regulon [422]. Alternative sigma factors such as SigE are important transcription factors implicated in *Mtb*-infection [425-427]. Eubacteria often encode only a few sigma factors, but *Mtb* harbors thirteen that appear to be uniquely involved in persistence and virulence [428]. Indeed, SigE is found to be essential for intracellular survival, and increased virulence in mice [427]. Transcriptomic analyses indicate that SigE regulates an array of genes involved in protein recycling, mycolic acid biosynthesis, transcription, as well as ATP biosynthesis [422, 429].

Like alternative sigma factors, *Mtb* encodes seventeen adenylyl cyclases that synthesize

3',5'-cyclic adenosine monophosphate (*i.e.* cAMP) in response to various discrete environmental cues [66, 430, 431]. Accumulating cAMP intracellularly in turn activates cAMP-dependent transcription factors, Crp (or Rv3676) and Cmr (or Rv1675c) [45, 432]. Crp and Cmr induce a variety of genes of genes encoding polyketide synthesis, ESAT-6 secretion, and TA proteins [433, 434].

Figure 1.4 The *Mycobacterium tuberculosis* stringent response: An integrated network regulating persistence and pathogenesis adapted from N.K. Dutta and P.C. Karakousis (2014) [4].

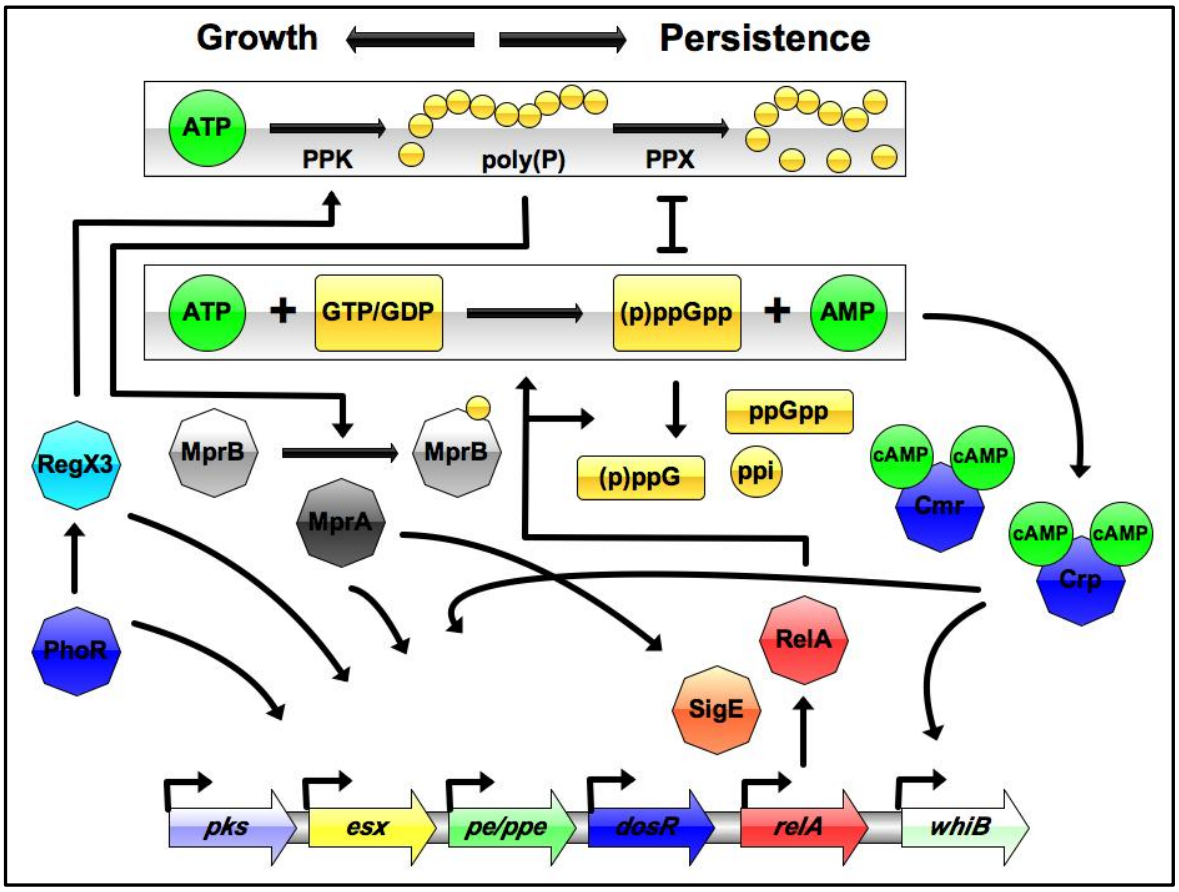

Figure 1.4: The *Mycobacterium tuberculosis* stringent response, integrating persistence and pathogenesis adapted from N.K. Dutta and P.C. Karakousis 2014 [4].

TA modules in particular are thought to facilitate epigenetic changes augmenting *Mtb* persistence and virulence [435]. They are expressed by *Mtb* in limiting environments *in vitro* and *in vivo* [374, 436], and their actions are ascribed to increased pathology in several animal models of infection

[65, 66, 437]. And yet, the sheer number of *Mtb* TA genes (*i.e.* over one hundred) has made it difficult to discern their more individual contributions to TB pathogenesis.

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CHAPTER 2: LITERATURE REVIEW PART II

2.1 Latent tuberculosis infection.

Since the WHO declared TB a global health threat nearly ~25 years ago, significant efforts have reduced global TB incidence by nearly 40% [1]. However, *Mtb* is still the leading infectious cause of morbidity and mortality worldwide today, with ten million new cases of ATB and one-and-a-half million deaths reported each year [2]. In nearly 95% of *Mtb* infected individuals, the host immune response effectively controls *Mtb*, resulting in an asymptomatic (or persistent) LTBI [3, 4]. Mφs, monocytes, T_H cells, are recruited along with other immune cells to the lungs to control LTBI, effectively walling off the infection and ensuring maintenance of persistent tubercle bacilli [4, 5]. Consequently, individuals with LTBI may develop ATB at any time, often years or even decades later, accounting for a majority of newly reported ATB cases [6, 7]. Current diagnostics cannot accurately discriminate between LTBI and ATB, and successful treatment requires lengthy drug regimens. The duration of treatment challenge patient adherence [8], and this poor patient compliance has led to the rise and spread of multi-and extensively-drug resistant TB [9-11]. Drug resistant TB treatment requires at least nine-to-twenty months of multi-drug regimens that are still less than 50% effective [12]. Accordingly, the development of shorter more effective therapies is paramount to increase the rate of successful treatment and prevent the ongoing spread of multi- and extensively-drug resistant TB now threatening the utility of frontline anti-TB drugs [13].

LTBI is a clinical diagnosis that is made after excluding ATB (*e.g.* from chest x rays and sputum cultures) and prior exposure to *Mtb*. The tuberculin skin test is still the primary diagnostic test for LTBI and assesses the T cell responses of a person to an intradermal injection of purified *Mtb* proteins [8]. Memory T cells from LTBI recognize *Mtb* Ags and the injection site becomes inflamed, indicating prior infection and/or clearance [14, 15]. While it is estimated that only 10% of persons with LTBI ever relapse (or reactivate) [16, 17], the risk of developing clinically severe

ATB that is associated with increased mortality is significantly increased in people with LTBI who are co-infected with HIV, receiving immunosuppressive therapies, including anti-TNF-α agents, steroids, and cancer treatment, or have other chronic systemic diseases such as diabetes mellitus [18]. People with LTBI are found to harbor viable tubercle bacilli in the lungs and extra-pulmonary sites [19, 20]. In the pre-antibiotic era, Opie and Aronson found that nearly 25% of tissue samples recovered from lesions in lungs and in PLNs of individuals with LTBI who succumbed to non-TB related causes are infectious [21]. Notably, a quarter of these lesions appeared to be fibrotic and calcified, indicating that they were healed [21]. Almost a third of lung tissue samples without gross or histopathological evidence of TB from people who died of unrelated causes are found to cause ATB in experimental animals during this time as well [22]. Even with effective treatments today, more advanced molecular techniques commonly find viable tubercle bacilli in LTBI. *In situ* PCR amplifies *Mtb* genomic (g)DNA, ribosomal (r)RNA, and messenger (m)RNA from almost one-third of lung and extra-pulmonary tissues in individuals who succumbed to non-TB related causes [23, 24]. Similar *in situ* methods even detect viable bacilli in patients who have successfully completed treatment [25-27]. It thus seems that persistent mycobacteria represent a critical component of *Mtb* pathogenesis, ensuring long-term survival in the host, with a potential for reactivation of LTBI-to-ATB [28].

Currently, successful preventive LTBI treatment requires six-to-nine months of Inh, which inhibits *Mtb* mycolic acid synthesis and subsequently cell wall formation [29], three-to-four months of Rif, which interferes with *Mtb* RNA polymerase (RNAP) subunit RpoB (or Rv0667) [30], or two-months of Rif and Pza, targeting bacilli in acidified intracellular host environments [31], although this regimen is no longer recommended because of increased toxicity [32]. More recently it was shown that three-to-four months of Rif and Inh is just as effective as nine-months of Inh alone [33, 34]. This is because bactericidal drugs such as Inh target actively replicating tubercle bacilli, while sterilizing drugs, including Rif and Pza, specifically kill slow growing, non-replicating mycobacteria [35]. This reduced susceptibility to Inh and other antibiotics, which target processes essential to

actively growing tubercle bacilli, including cell wall synthesis, DNA replication, and translation, is known as drug tolerance [36], and this is a hallmark of *Mtb* persistence in LTBI [19]. Killing by Inh and other bactericidal frontline antibiotics (*e.g*. Etb) during TB treatment is directly proportional with tubercle bacilli replication and metabolism [37-39], underlying increasing drug tolerance [28, 40, 41]. Novel short-course therapies that successfully clear LTBI thus need to eliminate dormant (or persistent) tubercle bacilli. However, little is known about the cellular and molecular basis of *Mtb* persistence. More specifically, how does *Mtb* enter into and exit from dormancy? Are there any vulnerable points in *Mtb* dormancy that may be exploited for new potential drug targets?

2.2 *Mycobacterium tuberculosis* persistence in latent tuberculosis infection.

Emergent multidrug tolerant tubercle bacilli recovered from TB patient sputum prior to the start of treatment shows that *Mtb* persistence is induced by the host immune environment during infection, prompting epigenetic development of a reversible, alternate physiological state enabling bacilli to evade and survive innate and adaptive host defenses, and exposure to drugs [4]. This phenomenon was originally described by Joseph Bigger in 1944 soon after the discovery of the first anti-tubercular drug streptomycin, noting that a small fraction of drug sensitive staphylococci survive long-term exposure to penicillin *in vivo* [42]. Then in 1958 Walsh McDermott took Bigger's observations along with his own and first proposed that an alternative physiological state *in vivo* confers tolerance or the "capacity for drug susceptible microorganisms to survive extensive drug attack when subsisting in the host" [43]. McDermott and others later showed that two-thirds of *Mtb* infected mice that complete chemotherapy relapse with drug sensitive bacilli, spontaneously or from immunosuppression, and develop ATB with increased mortality [44]. Their work revealed that tubercle bacilli underlying disease relapse in mice are in a non-replicating or slowly growing state, relatively unaffected by drugs. Assessing genetic variation through ISs indicates that *Mtb* replicates minimally over years of human LTBI [6]. This agrees with several WGS studies, finding that few SNPs accumulate in the *Mtb* genome over lengthy human LTBI [45-48]. Subpopulations of dormant, drug tolerant bacilli appear to be especially important to establish and maintain *Mtb*

infection in humans [49-51]. For instance, proportions of pre-existing Inh tolerant bacilli increase significantly in TB patient sputa over time during treatment [12, 52], with an initial and rapid decline in *Mtb* colony forming units (CFU) slowing early in treatment [12]. This bi-phasic killing of *Mtb* by Inh and other bactericidal antibiotics is shown in Figure 2.1 below from A. Harms, E. Maisonneuve, and K. Gerdes (2016) [53]. Notably, the outgrowth of these tolerant (or persistent) bacilli produces a brand new population of drug sensitive bacilli, showing that drug tolerance, which is mediated epigenetically, is genetically distinguishable from drug resistance [12, 49, 54].

Figure 2.1: The development and enrichment of *Mycobacterium tuberculosis* persister cells during infection and subsequent treatment taken from A. Harms, E. Maisonneuve, & K. Gerdes (2016) [53].

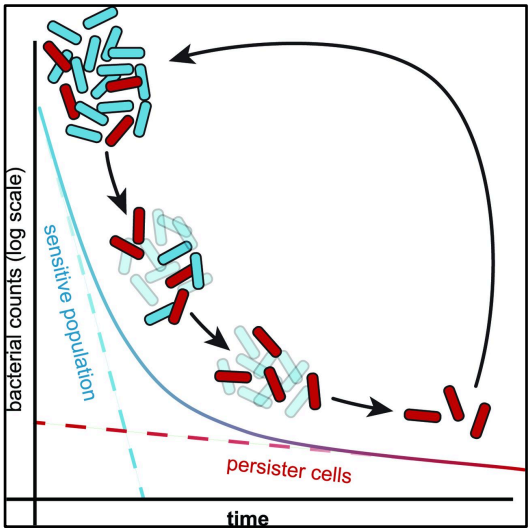

Figure 2.1: Development and enrichment of *Mycobacterium tuberculosis* persister cells during infection and treatment taken from A. Harms, E. Maisonneuve, & K. Gerdes (2016). A schematic representation of the development and enrichment of *Mtb* persister cells underlying LTBI and subsequent relapse following early treatment withdrawal and/or treatment failure. Blue tubercle bacilli represent an actively growing, Inh susceptible subpopulation; red tubercle bacilli represent a slow growing or nonreplicating, Inh tolerant subpopulation. An initial rapid decline in *Mtb* CFUs in TB patient sputa very early on in treatment is followed by steadily increasing pre-existing subpopulations of mycobacteria that are tolerant to Inh and other frontline anti-tubercular drugs.

Mtb persistence appears to be largely induced by host immune responses during infection. Studies in humans and in mice find that neutralization of TNF-α, which is essential for host control of *Mtb* infection, greatly enhances the efficacy of Inh and Rif treatment regimens, with little-to-no development of drug tolerant bacilli [55-57]. Another more recent report reveals that slow growing but metabolically active Inh tolerant bacilli emerge in WT but not GKO *Mtb* infected mice [58]. A

significant decrease in *Mtb* replication over time *in vivo* is known to be driven by T cell responses [59], and contributes to long-term persistence of *Mtb* in Mφs, neutrophils, and other phagocytes [60]. Intracellular survival is a key component of the *Mtb* life cycle, enriching for drug tolerant (or persistent) bacilli [61, 62]. Persistent bacilli show phenotypic heterogeneity [39, 58], ensuring *Mtb* survival to an array of environmental stresses [53, 63]. For example, while one phenotype may be well suited to survive nutrient starvation (NS), another phenotype may better ensure survival to ROS [53]. This arises from stochastic (or noisy) gene expression in *Mycobacteria* [64]. Noisy gene expression produces differences in mRNA and protein levels between individual bacilli [65], which are significantly amplified by positive and negative transcriptional regulatory feedback loops [66] that integrate the stochastic and deterministic factors of *Mtb* persistence [67].

Figure 2.2: The stochastic and deterministic nature of *Mycobacterium tuberculosis* persister cells taken from J.E. Michiels, B. Van den Berg, N. Verstraeten, & J. Michiels (2016) [49].

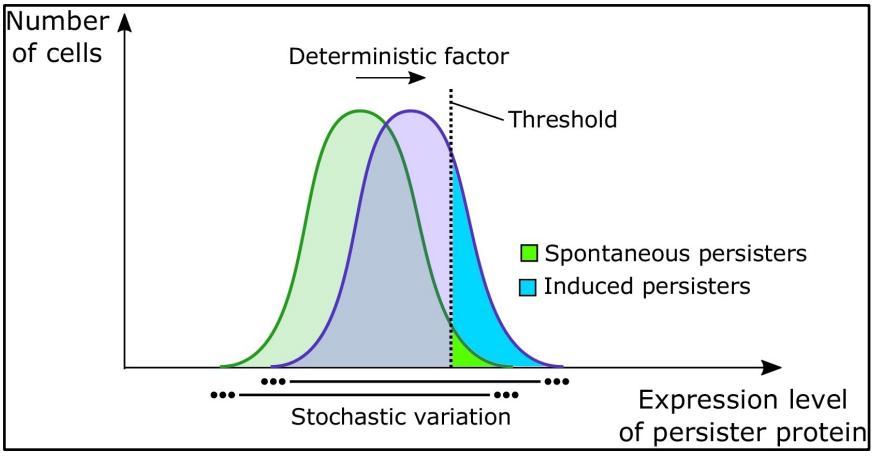

Figure 2.2: Stochastic and deterministic nature of bacterial persisters taken from J.E. Michiels, B. Van den Berg, N. Verstraeten, & J. Michiels (2016) [49]. Schematic representation of the dual stochastic and deterministic nature underlying the development and maintenance of bacterial persistence. Green and blue bell represent stochastic and induced persisters, respectively, whereas purple represents the dual nature of *Mtb* persister cells.

The dual nature of *Mtb* persistence is best exemplified from the molecular characterization of type II toxin protein kinase HipA (*i.e*. Rv3744). HipA-levels appear to naturally fluctuate within a population, producing a mix of actively growing and quiescent drug tolerant cells [68]. However, HipA activity also increases kinase levels to a threshold within a population that leads to marked

induction of drug tolerant persister cells [69, 70]. In other words, while drug tolerant *Mtb* persister cells are in any given population, the frequency of their formation is inducible, which is illustrated in Figure 2.2 above from J.E. Michiels, B. Van den Berg, N. Verstraeten, and J. Michiels (2016) [49]. Proportions of multidrug tolerant tubercle bacilli appear to increase considerably following exposure to NS [71, 72], low pH [61], ROS [73], hypoxia [74], and antibiotics [75] *in vitro*. Others report that pre-existing subpopulations of drug tolerant tubercle bacilli steadily increase during treatment in humans [28, 76]. This is in line with more recent transcriptomics studies in mice and in humans, finding that *Mtb* genes involved in the phenotypic development of a dormant multidrug tolerant state are specifically induced by the development of T_H1 cell immune responses [62, 77].

2.3 Phenotypic development of *Mycobacterium tuberculosis* persistence.

Numerous *Mtb* genes appear to be specifically involved in the development of a persistent state [78-81]. Many of these genes encode TA systems and other two-component regulators that are induced as part of two essential *Mtb* stress responses: the stringent and SOS responses. These adaptive responses are known to be induced by an array of host-associated environmental cues through two-component signaling and NT messengers, including cAMP [82, 83], eliciting global changes to transcription, translation, and metabolism that produce an altered physiological state, phenotypically resistant to drugs and essential to *Mtb* pathogenesis [84].

2.3.1 The stringent response.

Under stressful conditions like NS, kinase HipA phosphorylates elongation factor (EF)-Tu and tRNA synthetases [85, 86] to induce the stringent response, which significantly reduces rates of transcription, translation and DNA replication [87]. Accumulating uncharged tRNAs in the ribosome activate RelA (*i.e.* Rv2583c) to make hyper-phosphorylated guanosines pppGpp and ppGpp (or (p)ppGpp) [88-90]. Carbon, iron, magnesium, and phosphate starvation, as well as exposure to low pH all lead to increased intracellular (p)ppGpp levels, and this is essential for mediating *Mtb* persistence *in vitro* [91] and *in vivo* [92-95]. (p)ppGpp blocks translation by directly binding to initiation factor two (or Rv2839c), blocking ribosomal assembly [96], and to elongation

factor two (*i.e.* Rv0685), increasing the rate of mistranslation [97]. Error prone translation appears to be important for developing Rif tolerance [98] and Ag variation [99] in *Mtb*. However, ppGpp-bound EF-Tu is found to enhance the fidelity of stringently translated *Mtb* proteins [97, 100-103], indicating that alternative pathways of protein synthesis are activated in stressful environments. Along with this (p)ppGpp halts and preserves ongoing DNA replication for more favorable growth conditions by directly binding to the origin of replication [104, 105], DNA polymerase G (*i.e.* DnaG or Rv2343c) [106, 107], and GTPases like Obg (or Rv2440c) that are also found to be essential to *Mtb* pathogenesis [108, 109]. (p)ppGpp also binds to RNAP, stalling transcription, further inhibiting ongoing DNA replication [110, 111]. Additionally, (p)ppGpp-RNAP shifts its focus from rRNA, tRNA, and σ^A (*i.e.* Rv2703) promoters to DNA motifs recognized by alternative transcription factors and regulators [112-117], which have been shown to be critical to regulate *Mtb* genes that are essential for virulence and complex lipid production [118-125]. WhiB3 (or Rv3416) for instance is known to function as a redox sensor that activates the transcription of *Mtb* genes needed to recycle potentially toxic lipid intermediates into immunomodulatory polyketides *in vivo* [126, 127]. Moreover, the regulon of stringently-induced *Mtb* transcription factor cAMP receptor protein Crp (*i.e.* Rv3676) is much larger than that of BCG TB vaccine strains, with the significant upregulation of multiple TA genes and other two-component regulators [128-130] that are known to augment discrete aspects of DNA replication, transcription, and translation needed to mediate *Mtb* persistence *in vitro* [131, 132] and *in vivo* [61, 133, 134].

2.3.2 The SOS response.

The SOS response was discovered by Miroslav Radman and Evelyn Witkin in the 1970s, finding that DNA damage induces repair mechanisms [135, 136]. The SOS regulon has expanded significantly in *Mtb* to include coding and non-coding (nc)RNA genes involved in an array of physiological processes in addition to DNA repair [137-141]. Consequently, while single- and double-stranded breaks in genomic DNA trigger the SOS response, the downstream effects may vary widely, and depending on the amount of damage, may even lead to enhanced genetic

diversity [142, 143]. In the absence of DNA damage, LexA (or Rv2720) binds to operator sequences or SOS boxes and represses the transcription of ~50 *Mtb* genes [138, 141, 144]. DNA damage caused by stresses like ROS results in auto-decay of LexA [145]. The amount of LexA decay is dependent on binding affinities of SOS boxes though, thereby enabling the serial activation of DNA repair mechanisms, including NT excision repair, HR repair, and non-homologous end joining repair [146]. *Mtb* NT excision repair appears to be constitutively induced because of relatively weak SOS boxes [138, 147], and this is essential to counter DNA damage from intracellular exposure to host-derived ROS and RNI [148, 149]. Increasing ROS and RNI levels in an activated immune environment produce double stranded breaks in *Mtb* gDNA, thereby activating principal regulator RecA (*i.e.* Rv2737c) and HR, which is especially important for *Mtb* growth and survival in human Mϕs [145, 150]. The inhibition of host-derived ROS and RNI in particular is found to restore WT intracellular kinetics to *Mtb*Δ*recA* [150]. Intriguingly though, many SOS-inducible *Mtb* genes are regulated independently of RecA and LexA, especially TA loci that are known to be uniquely conserved among pathogenic SGM and the MTBC [138, 139, 151-155]. HigBA1 (*i.e.* Rv1955-Rv1956) and HicAB1 (*i.e.* Rv2016-Rv2017), which are both unique to the MTBC, are induced by DNA damage in a LexA-/RecA-independent manner despite SOS boxes present in upstream promoters [151, 152, 156]. These and other SOS-inducible *Mtb* TA loci are transcriptionally activated by ClgR (*i.e.* Rv2745c) and sigma factors such as σ^B (or Rv2710) [154, 157, 158]. In contrast, several small (s)RNAs are shown to be transcriptionally activated in LexA-/RecA-dependent manner, as a key part of the *Mtb* SOS response [138]. This likely enables the induction of numerous *Mtb* TA genes that are encoded bicistronically as part of a much broader and integrated response DNA damage and stress in general. This would ultimately indicate that SOS-inducible TA systems are key mediators of *Mtb* pathogenesis.

2.4 Toxin-antitoxin systems.

TA loci encode small two-component systems that were first discovered as selfish genetic

elements, stabilizing plasmids by killing progeny that loose the antidote antitoxin-encoding gene [159]. However, WGS has since revealed that TA systems are ubiquitously encoded by archaeal and bacterial genomes, especially pathogens, indicating added functions [160]. TA loci typically encode a toxin protein that inhibits growth, or even causes death, and a more labile antagonistic antitoxin that is a ncRNA or protein [161]. Under certain circumstances, including environmental stress or bacteriophage infection, the antitoxin is selectively degraded, freeing the toxin to target vital processes to active growth such as cell division, transcription, and protein synthesis, thereby inhibiting growth or causing death [162]. Growth inhibition by toxins is often reversible when new antitoxins are made, thus, it has been thought that the activation of TA systems facilitate bacterial survival in times of stress [163].

To date, six TA families have been described, which are shown in Figure 2.3 below, based on the mechanisms of toxin inhibition by cognate antitoxins. While all toxins are proteins, antitoxins function as RNAs (*i.e*. in type I and III TA systems) and proteins (*i.e*. in type II, IV, V, and VI TA systems) [160, 164-167]. Type I and III antitoxins are small (s)RNAs that are known to function in *cis* (*i.e.* complementary base-pairing) and/or in *trans* (*i.e.* secondary structure interactions). Type I antitoxins in particular bind to toxin mRNAs, blocking their translation while mediating their decay *via* RNase III (*i.e.* Rv2925c in *Mtb*) [168]. In times of stress, sRNA antitoxins are degraded, thereby enabling the translation of small and potentially lethal type I toxins [162]. Type III sRNA antitoxins in contrast form pseudoknots that directly bind to cognate toxins, thus inhibiting their activity [169]. In the archetype III TA locus, ToxN is a RNase that is encoded just downstream of five tandem repeats in the primary *toxIN* message [170, 171]. ToxN processes *toxIN*, producing five 36-NT antitoxin sRNAs able to bind to ToxN [162]. Intriguingly, certain type I and III TA systems are demonstrated to be much more commonly encoded in high GC-content actinomycetes, including *Mycobacteria* [165]. Type II, IV, V, and VI antitoxins regulate cognate toxins through protein-protein interactions. Type II antitoxins in particular inhibit cognate toxins by assembling into complexes that auto-regulate transcription [172]. Type II antitoxins are

selectively degraded by Lon and Clp proteases under stress like NS, thereby freeing toxins [173, 174]. Type IV toxins and antitoxins in contrast do not directly interact and have the same target [160]. Type IV TA systems have more specifically been demonstrated to protect against lytic phage infection and many of these are more commonly encoded by *Mycobacteria* and other more

Figure 2.3: The regulation and activity of six major widespread types of bacterial toxin-antitoxin systems.

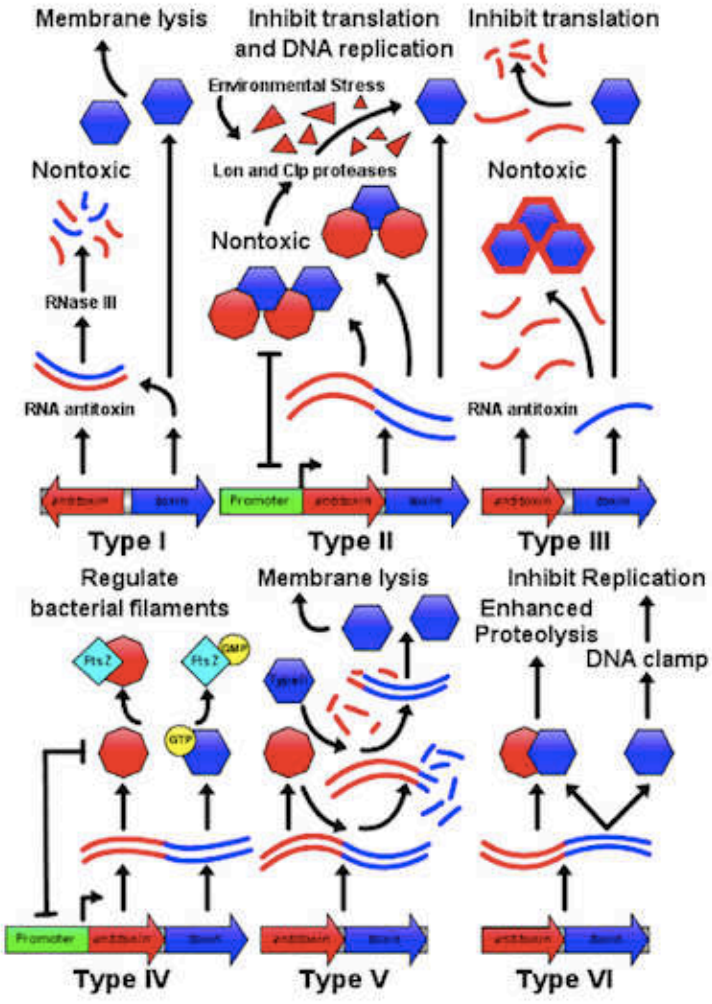

Figure 2.3: Types of bacterial TA systems. Toxins, antitoxins, and promoters are shown in blue, red, and green, respectively. (1) Type I: the sRNA antitoxin interacts with toxin mRNA, inhibiting translation of small toxic proteins that function to perturb cell membrane permeability, disrupt ATP biosynthesis, and/or alter NT biosynthesis. (2) Type II: TA proteins form protein complexes during active growth, inhibiting the toxin activity. Type II antitoxins and TA complexes commonly auto-regulate transcription. Lon and Clp degrade selectively degrade antitoxins under stress, freeing toxins to target macromolecular processes such as translation and DNA replication. (3) Type III: sRNA antitoxins are processed by their cognate RNase toxin, resulting in RNA pseudoknot-toxin complexes inhibiting activity. (4) Type IV: the antitoxin binds to and thus stabilizes bacterial cell filaments and the toxin destabilizes them, thereby block cell division. (5) Type V: the RNase antitoxin GhoS degrades *ghoT* mRNA under optimal growth conditions; *ghoS* antitoxin mRNA is degraded by type II toxin MqsR in response to stress, especially phage infection, leading to the translation of GhoT, which induces cell death. (6) Type VI: the SocA antitoxin acts as an adaptor, binding to SocB toxin, thereby enhancing Clp-mediated decay. Upon activation, SocA functions to suspend ongoing DNA replication.

closely related *Actinomycetales*, including Rv0837c-Rv0836c, Rv1044-Rv1045, and Rv2827c-Rv2826c [160, 175]. In the only known type V TA system, the antitoxin GhoS is a RNase that, in ideal growth conditions, selectively degrades *ghoT* toxin mRNA [176]. In response to stress, *ghoS* is rapidly degraded, resulting in the translation of its small phage-like hydrophobic toxin, GhoT, which affects cell membrane potential [177, 178]. Finally, the type VI TA system comprises a protein toxin, SocB, which suspends DNA replication by directly binding to DnaN (*i.e.* Rv0002 in *Mtb*) [179]. Unlike other protein antitoxins though, SocA functions as an proteolytic adaptor, thereby enhancing Clp-dependent decay of SocB [179].

2.4.1 Mechanisms of type I toxin-antitoxin regulation.

Small type I toxins are regulated by *cis*- and/or *trans*-acting sRNAs [180]. They are the most functionally diverse TA family but only *hok*/*sok* [181], *ldr*/*rdl* [182], *symE* /*symR* [183], and *tisB*/*istR* [163] appear to be widespread. The antitoxins from these four systems are all small antisense (as)RNAs that function to silence translation of cognate toxins by direct binding to toxin mRNA [184]. This allows for their tight regulation indicative of their lethality [185]. The first type I TA system, *hok/sok*, was discovered as a plasmid-stabilization system [181], encoding a 52-AA toxin, Hok, along with a 64-NT asRNA antitoxin, Sok [186]. Sok binds to the untranslated region (UTR) of *hok* in *cis* [187], effectively hiding the shine-dalgarno (SD) sequence and marking it for decay *via* RNase III [188]. And although the half-life of Sok is significantly less than that of *hok* [189, 190], it is processed before being translated [191]. Even still, cleavage events hinder Sok-binding-to-*hok* [192, 193], and without Sok (*e.g.* plasmid-free progeny), low-level accumulation of processed *hok* rapidly kills cells [168]. Plasmids encoding *hok*/*sok* are commonly maintained in bacteria, phenomenon known as post-segregational killing (PSK). Plasmid-encoded Hok toxins enact PSK, which led to the early idea that TA loci merely serve selfish genetic roles. However, numerous *hok*/*sok* loci have been found throughout bacterial genomes suggesting additional roles [194]. The chromosome of *E. coli* strain K12 for instance encodes six *hok*/*sok* TA loci named A-E and X. Notably, HokA and HokC homologs encoded by more pathogenic *E. coli* strains appear

have resisted accumulating mutations that have rendered them non-functional in *E. coli* strains such as K12 [195]. This suggests that chromosomally encoded Hok toxins evolved functionally from PSK to mediate virulence. Like *hok/sok*, there are four *ldr*/*rdl* systems (*i.e*. A-D) encoded in *E*. *coli* strain K12 genome [196, 197]. This type I TA system encodes Ldr, which is a small toxin that is translated from processed *ldr* mRNA, along with an asRNA antitoxin Rdl, which acts in *cis* mediating *ldr* decay via RNase III [193]. However, *ldr*/*rdl* are not found to be plasmid-encoded [182], which further suggests uniquely evolved function from PSK. Screens for ncRNA identified *symE*/*symR* system, which encodes a 77-NT asRNA SymR that complements the UTR of *symE* mRNA [183]. SymR-binding effectively hides the *symE* start-codon from the ribosome and marks it for decay by RNase III. Additionally, *symE* is regulated in a LexA-/RecA-dependent manner in response to DNA damage [183, 193]. Another SOS-induced type I TA system, *tisB*/*istR*, is found to be required for developing ciprofloxacin tolerance [198]. The *tisB*/*istR* locus encodes IstR1 and IstR2 asRNA antitoxins upstream of toxin gene *tisB* [199-202]. Although *istR1* and *istR2* are co-regulated from the same SOS box, an internal promoter constitutively upregulates IstR1 [202], which inhibits *tisB* translation in *trans* [200]. The primary *tisB* transcript is cut 42-NT downstream of its transcriptional start site (TSS) though, enhancing interactions between the *tisB* SD sequence and the ribosome that interfere with IstR1 binding, maintaining low-level translation of TisB [201, 202]. This ultimately shows that while TisB is essential for SOS-inducible persistence, it is tightly regulated on multiple levels to limit any potentially lethal effects from runaway activity.

2.4.2 Mechanisms of type I toxin inhibition.

Many type I toxins are small proteins, usually less than 60-AAs in length, which contain an α-helical transmembrane domain similar to that in bacteriophage holin proteins [203]. These toxins form pores in cell membranes as well, but their downstream effects may vary widely, from inhibiting growth-to-cell lysis [169]. For instance, plasmid-encoded Hok toxins function in PSK [180], while chromosomally-encoded homologs appear to be largely non-functional in this regard [161]. The overexpression of the *hok* gene encoded on the widespread pR1 plasmid collapses

the cell membrane potential, rapidly killing *E*. *coli* [189]. The ectopic induction of chromosomally-encoded *hokD* (or *relF*) gene also quickly collapses the cell membrane potential, killing *E. coli*, but it fails to stabilize pR1 [186, 204]. HokD along with other homologs encoded in the *E. coli* genome are known to function like certain holins [205], producing ghost cells indicative of an apoptotic-like death [186, 206]. HokB collapses the cell membrane potential too and does not kill cells, and this seems to be an important component of the response to NS [131] and DNA damage [207]. Ldr toxins been shown to be functionally distinct as well [194], causing nucleoid condensation, arrest of translation, and cell elongation [193] that is linked with the upregulation of genes involved in cAMP metabolism [182]. LdrA localizes to the cell membrane and collapses its potential, thereby inhibiting DNA replication [208], which indirectly affects transcription and protein synthesis [209]. Ldr toxins thus likely function in coordinating concurrent DNA replication with cell growth and division. SymE in contrast is much larger (i.e. 113-AAs in length), shares little-to-no sequence identity, and does not target cell membrane potential similar to many other type I toxins [169]. Bioinformatics have revealed that SymE belongs to the AbrB superfamily of transcriptional regulators comprising type II MazE antitoxins [183], while the overexpression of SymE is found to function like type II toxin MazF [193]. Given that other similar *mazE*-like genes are encoded next to *symE*, it seems that SymE evolved into a RNase from an antitoxin-like progenitor [183]. SymE has been shown to localize with ribosomes [183], and this seems to be involved in SOS-induced persistence [210]. In contrast, SOS-inducible type I toxin TisB is 29-AA and localizes to the cell membrane [163], wherein it forms pores that disrupt cell membrane potential, indicating a role in bacterial survival [210-212]. The ectopic induction of TisB fatally collapses the cell membrane potential but its SOS-dependent induction dissipates the PMF in a protective manner [163]. TisB seems to mediate persistence by inhibiting ATP synthesis, which in turn halts DNA replication, transcription, and translation [198]. This is in line with another report that IstR1 serially regulates TisB based on the level DNA damage, thereby limiting any potential TisB cytotoxicity [213].

2.4.3 Mechanisms of type II toxin-antitoxin regulation.

Type II TA loci are ubiquitously encoded in *Archaea* and *Bacteria*, especially CcdAB [214], ParDE [215], MazEF [216], VapBC [217], HigBA [218], RelBE [219], and Phd-Doc [220]. Many homologs of these particular type II TA systems share several important features: TA loci encode polycistronic operons [221]; TA proteins assemble into complexes inhibiting toxin activity [222]; Antitoxins alone and/or TA complexes auto-regulate expression [223]; Antitoxins are much more susceptible to Lon and Clp proteases than toxins [216], and thus selectively and rapidly degraded to activate toxins [173]. The first type II TA locus was first discovered nearly 40 years ago, as an operon "coupling cell division" (or *ccd*) to plasmid stabilization [224]. It was revealed soon after that *ccd* encodes a cytotoxic protein, CcdB, which enacts PSK, along with its regulator, CcdA [225, 226]. CcdAB and other similar systems were largely ignored until chromosomally-encoded homologs were identified [227]. This led to the discovery of another module ParDE and thus their apt naming TA systems [228-231]. CcdB and ParE target DNA gyrase [232] but share little-to-no sequence identity [233], revealing distinct mechanisms of action [215]. CcdA and ParD evolved independently as well, yet they both maintain a N-terminal DNA-binding domain and a C-terminal toxin-binding domain [234]. C-terminal antitoxin domains are largely disordered, which is key for binding to cognate toxins [235-237]. And while this makes antitoxins much more susceptible to decay [238], it enables them to interfere with toxic interactions, restoring processes such as DNA replication [239]. Unstructured C-terminal antitoxin domains also allow the formation of multimeric TA complexes that inhibit activity [239] and auto-repress transcription [240]. TA ratios over one lead to de-repression though, as increasing toxins in TA complexes significantly weakens DNA-binding of N-terminal antitoxin domains [241]. This is conditional cooperativity and is employed to limit runaway activity of toxins [242]. Bioinformatics analyses find that antitoxins such as CcdA and ParD are often identified from "guilt-by-association" and instead comprise various antitoxins, including PhD and RelB [234]. This would explain why most type II antitoxins have functional N-terminal DNA-binding domains and C-terminal toxin-binding domains although they share little-to-no sequence homology [243]. Many chromosomally-encoded antitoxins are also able to auto-

regulate expression without toxins [244, 245], supporting the notion that chromosomally-encoded TA systems are functionally distinct. CcdB shares homology with the common chromosomally-encoded type II toxin MazF [246] that is a sequence-specific RNase [247] required for stringent control [216, 227, 248]. Studies have determined that MazEF proteins form heterotetramers to inhibit toxin activity [249, 250]. In times of NS, MazE is quickly degraded by proteases [216, 247], thereby activating MazF-dependent programmed cell death (*i.e.* PCD) to ensure survival in replete environments [251]. This is because MazE decay leads to rapid de-repression of *mazEF* through conditional cooperativity [252] and replenishment of MazE to tightly regulate PCD [251, 253].

RelBE is another important type II TA system induced by stress. Mutations in *E. coli relA* produce a "relaxed" (*i.e.* for *rel*) response to NS marked by continued tRNA and rRNA expression [248]. Accordingly, the first chromosome-encoded *relBE* TA locus was discovered from mutations in *relB* creating a "delayed-relaxed" response to NS marked by a brief halt in rRNA and tRNA transcription [204, 254]. Mutations in *relB* have are known to significantly enhance RelB decay, thereby dysregulating RelE, which significantly decreases bacterial survival to stationary phase growth and NS [132]. Unlike other antitoxins such as MazE, RelB exists as a monomer [255, 256] that binds to and in turn collapses the RNase site of RelE [257, 258]. RelBE proteins form hetero-trimers and -tetramers though, with C-termini of RelB dimers effectively "wrapping around" RelE toxins [236, 258, 259], and this enables RelB binding to both operators in the *relBE* promoter [223, 242]. RelBE heterotrimers appear to bind to operators significantly better though, indicating transcriptional regulation *via* conditional cooperativity [259]. Nonetheless, basal *relBE* expression is known to be considerably higher than most type II TA genes even when RelBE ratios are greater than one [172, 260]. This is likely because *relBE* mRNA is also found to be post-transcriptionally regulated under stringent conditions, resulting in accumulating *relE* mRNA that in turn promotes the transcriptional cross-activation of *relB* and *mazF* [261]. This suggests that post-transcriptional events represent an important but underappreciated level of type II TA regulation, supporting the idea that chromosome-encoded type II TA systems evolved as a broad regulatory network. Post-

transcriptional regulation of bi-cistronic type II TA operons would ultimately help to contribute to the expansion of function roles encoded in the genome.

2.4.4 Mechanisms of type II toxin inhibition.

Type II toxins target two major processes: DNA replication and translation [262, 263]. And while most share sequence homology with each other, as well as viral and eukaryotic RNases, they have evolved to affect more discrete aspects of DNA replication and protein synthesis [217, 264]. CcdB and ParE are the only type II toxins known to directly target DNA replication. They both bind to DNA gyrase *via* distinct mechanisms of action [215, 265], effectively converting it into a constitutive endonuclease [266, 267]. This leads to an accumulation of breaks in gDNA and in turn a lethal production of ROS causing PSK [230, 268, 269]. Chromosome-encoded CcdB and ParE appear to be functionally similar, enacting PCD [245, 270, 271], and it seems that inherent differences in their binding reflect distinct PCD pathways [272, 273]. CcdB and MazF toxins form one-of-two major type II toxin superfamilies based on structural homology [246]. However, MazF toxins are RNases targeting discrete aspects of translation [234, 251]. *E. coli* MazF is shown to cut at ACA-NTs, which rapidly depletes total RNA, while modifying rRNA and mRNA for effective translation of leaderless messages [251, 274, 275]. MazF cleavage occurs independently of the ribosome, but translating mRNAs appear to be much more efficiently cut [251, 276]. Functional characterization of other MazF homologs encoded by high-GC content bacteria moreover, has revealed multiple recognition sequences, three-to-seven-NTs long [251, 277, 278], that reflect an evolving sequence specificity in a range of MazF activities [279].

Similar to CcdB/MazF, ParE and RelE form to the other major superfamily of type II toxins [280]. And because of clear differences between their catalytic sites [272], RelE toxins function as RNases, cleaving translating mRNAs engaged at the ribosome A-site [219, 281]. Structural studies have found that RelE strongly interacts with 16S rRNA and mRNA in actively translating 70S ribosomes, thereby blocking the subsequent access of elongation factors and tRNAs [219, 282]. These interactions with RelE seem to position mRNA to be cut between the second and

third NT of the codon in the ribosomal A-site [282]. This is in line with earlier reporting that RelE selectively cleaves codons in the A-site with guanosines at the third NT position; *e.g.* stop codons [219]. RelE toxins are found to share homology with extracellular microbial RNases that cut RNAs strictly before guanosine-NTs, but because these homologs are missing several key AA-residues, they are unable to cleave RNA without the ribosome [282, 283]. RelE-dependent mRNA cleavage commonly produces non-stop mRNA (*i.e*. without a stop codon), effectively halting translation [284]. Stalled ribosomes are rescuable by transfer-messenger (tm)RNA (*i.e.* SsrA or MTB000042 in *Mtb*) though [285], which provides a stop codon to finish translation, while tagging mRNA and aberrant proteins for rapid decay [286], directly implicating SsrA in augmenting the effects of RelE.

Additional members of the ParE/RelE toxin superfamily are found to target translation in other discrete ways, even in a ribosomal-independent manner [287]. Rel-family toxins YoeB (*i.e.* Rv3358 in *Mtb*) and MqsR share a high degree of sequence homology with extracellular RNases, and are thus found to cleave non-translating RNAs as well [263, 288]. YoeB and MqsR still cleave actively translating mRNAs but by distinct mechanisms, preferentially cleaving start codons and GCU-NT-sequences, respectively [288, 289]. Moreover, MqsR appears to directly bind to DNA in its upstream promoter [290], which is involved in the transcriptional activation of this toxin during stress [173, 290]. HigB (*i.e.* Rv1955, Rv2022c, and Rv3182 in *Mtb*) is another RelE-like toxin that instead suppresses translation initiation by cutting alanine-rich codons held by 50S sub-units of 70S ribosomes [291, 292]. Other HigB homologs are still found to interact with 16S rRNA in 30S sub-units of 70S ribosomes, which is important for ribosomal-dependent RNase activity *in vitro* [293] and *in situ* [294]. This is even more significant considering that HigB ribosomal-dependent RNase activity is known to be induced as part of the stringed response, following the select decay of HigA antitoxins (*i.e.* Rv1956, Rv2021c, and Rv3183 in *Mtb*) [287]. These observations show that Rel-family toxins provide a spectrum of functional activities.

While they do not form to either type II toxin superfamily, virulence associated protein toxins (*i.e.* VapC) likewise function as RNases, with a wide range of target and NT-sequence

specificities. VapBC systems represent the most widely-distributed type II family, as VapC toxins are identified based on the presence of a PIN-domain [295]. PIN-domain proteins are found in all three domains of life, but they are primarily associated with VapC toxins [296]. PIN-domains are especially common among RNases that process rRNA and preferentially degrade ncRNA [297, 298], comprising around one-hundred-thirty-AAs that dimerize to bind to and position target RNAs in the active site of VapC [295, 299]. VapC homologs with little-to-no sequence similarity outside of their PIN-domains are found to recognize and cut at similar RNA NT-recognition sequences [300]. Even still, other VapC toxins recognize and cleave target RNA sequences based on their secondary structures. The VapC toxin encoded by *Shigella flexneri* for example is demonstrated to recognize the structure and cleave the anti-codon stem loop of initiator tRNA, thereby altering translation initiation [301]. Similarly, other VapC toxins are found to selectively cleave degenerate tRNA anti-codon stem loops that specifically encode serine and tryptophan [302].

Despite little-to-no sequence homology, other type II toxins are also found to discretely regulate translation. The ectopic induction of the Doc toxin for instance has been shown to block the incorporation of ^{3}H-leucine [303] concomitant with the accumulation of RelE-dependent cut transcripts [304]. This is because Doc phosphorylates and thus inhibits EF-Tu-binding of charged tRNAs, blocking translation elongation [305, 306]. Another type II toxin, HipA, also functions as a kinase, phosphorylating glutamyl tRNA synthetase, subsequently blocking translational initiation [85]. Ultimately, HipA and Doc activity leads to induction of MazF and RelE toxins form generating “hungry” codons at the A-site, which also triggers the stringent response [85, 304, 307, 308]. This further implicates a large TA regulatory network in augmenting more discrete contributions of type II toxins to bacterial adaptation.

2.4.5 Toxin-antitoxin systems in mediating bacterial persistence.

Type II TA systems have important roles in mediating bacterial persistence in response to stress [262]. Their individual contributions often remain largely unknown because most bacterial genomes encode multiple homologous TA systems. Nonetheless, it has become clear that they

are involved in mediating persistence. Transcriptomics studies reveal that type II TA systems are markedly upregulated in subpopulations of drug tolerant bacteria [71, 309], while their induction is known to be essential for mediating persistence [281, 310]. In *E. coli*, global translation rates of persistent bacteria are relatively low, which favors the de-repression and transcription of type II TA systems [309]. This replenishes antitoxins to quench toxins and thereby exit the persistent state [311]. In *E. coli*, the development and maintenance of a persisters seems to be dependent on intracellular type II toxin levels [68], as their induction produces significantly more drug-tolerant persister cells without affecting growth *per se* [310]. Thus, regulation of TA gene expression likely plays a critical role in the entrance into and exit from a persistent state [312]. Mutations in *hipA* resulting in its constitutive induction increase persister cells in a population by as much as 1,000-fold [313, 314]. MqsR is also found to selectively degrade antitoxin mRNA, which is essential for developing ampicillin tolerance *in vitro* [177]. This leads to transcriptional cross-activation of RelE from the accumulation of *relE* messages [261], which regulates PCD during persistence in a cell density-dependent manner [315, 316]. RelE-mediated PCD is dependent on differential induction of MazF in response to stress, which rapidly degrades total RNA [317, 318], arresting cell growth and eventually causing cell death [216, 261, 319, 320]. MazF-dependent PCD likely functions in part to block the integration of phage elements into the bacterial genome [307, 321]. However, it has also been found that MazF functions to kill most of the population, while promoting the survival of a small persistent sub-population [322, 323], which is an essential component of the life cycle of *Myxococcus xanthus* [324]. This pathway suppresses the SOS-induced PCD, which does not seem to be involved with mediating bacterial persistence [323, 325]. More importantly, MazF-induced persisters still translate MazF-resistant mRNA and remain metabolically active [326]. The RNase activities of RelE-family toxins also contribute to transcriptomic and proteomic remodeling underlying this drug tolerant state [174, 327, 328]. For instance, RelE homolog YafQ degrades tryptophanase mRNA, which inhibits indole production, thereby dramatically increasing the level of persister cells [329]. In addition, MqsR selectively targets outer membrane protein (*i.e. omp*)

mRNA, thereby enriching for toxin mRNA, which dramatically increases persistence levels [177, 330]. Finally, RelE-like toxin HhA activates a number of type II TA genes along with lytic prophage genes limit PCD in mediating persistence [331, 332]. These findings taken together illustrate that cognate and non-cognate toxins and antitoxins integrate cell growth and cell death in mediating bacterial persistence.

2.4.6 Toxin-antitoxin systems in mediating bacterial pathogenesis.

TA systems are found to be much more abundantly encoded in many pathogenic bacterial genomes, especially *Mtb* [333]. Pathogenic *E. coli* strains that cause sepsis and urinary tract infections in particular are found to maintain a number of TA systems, which are non-functional or missing from the common *E. coli* strain K-12 [334]. *In vivo* assessments of knock out mutant strains have revealed that three of these TA loci (*i.e. yefM-yoeB*, *ybaJ-hhA*, and *pasTI*) are each individually essential for colonization of the bladder or kidneys [334]. YoeB and HhA are found to regulate fimbriae production in *E. coli*, promoting the dispersal of persisters *in vitro* [331, 332], which likely explains the impaired ability of respective mutant strains to colonize the bladder. In contrast, PasT appears to be critical for mediating the pathogen response to NS, ROS, and RNI, *via* altering translation [334, 335], prompting subsequent dissemination and colonization of the kidneys during *E. coli* infection. *Salmonella enterica* serovar *typhimurium* is another major human pathogen, which causes an infection that can rapidly produces fully disseminated disease. This is because *S. typhi* and other more pathogenic salmonella harbor plasmids that are maintained by TA loci [336-338]. In addition, the genome of *S. typhi* uniquely encodes TA loci acquired from HGT [333] that are important to virulence [339-342]. *S. typhi* encodes multiple stringently-induced RelE toxins in particular that have been shown to be essential for mediating growth and survival in Mφs [61], but not fibroblasts and epithelial cells [339], which demonstrates that RelE homologs act in discrete aspects of the *S. typhi* pathogenesis. The *S. typhi* RelBE-like TA system SehAB for example is revealed to be significantly induced in Mφs, which activates genes that regulate

intracellular replication, and the deletion *sehB* attenuates salmonella replication in host Mφs [341]. This Δ*sehB* mutant is also attenuated in mice inoculated perorally and intraperitoneally, unable to colonize mesenteric lymph nodes early on in infection [341]. This is similar with another *S. typhi* RelBE-like TA system, ShpAB, wherein its deletion significantly impairs the development of drug tolerance *in vitro* [343] and in turn the colonization of mesenteric lymph nodes [61]. These findings taken together demonstrate that TA genes contribute to distinct aspects of the *S*. *typhi* pathogen life cycle, and that persistence and virulence are one in the same phenomenon, with TA systems ensuring pathogen survival in detrimental environments. *Vibrio cholerae* is an epidemic pathogen that persists extensively in aquatic environments and human intestines, causing Cholera [344, 345]. The acquired ability of *V*. *cholerae* to adapt to diverse ecological niches is attributed to two chromosomes. The second chromosome of *V*. *cholerae* has accumulated eighteen TA loci, which is significantly more than any other *Vibrio* species [346]. Several of these TA systems have been shown to be collectively important in maintaining the integrity of virulence factors encoded on the smaller chromosome [245, 347], which is in line with others reporting that their individual deletions impair parts of *V*. *cholerae* infection; i.e. dissemination and transmission [344, 348]. TA systems thus contribute to pathogenesis by stabilizing horizontally acquired virulence genes. *Haemophilus influenzae* is an opportunistic pathogen that can cause systemic diseases such as bacteremia and meningitis, and more virulent strains that encode certain homologous VapBC and RelBE TA systems [349, 350]. Accordingly, individual and serially deleted Δ*vapBC* mutant strains appear to be severely attenuated for long-term intracellular survival and virulence [349]. Similar defects arise from deletion of *H*. *influenzae* RelBE-like TA system HigBA as well [350]. Transcriptomics show that this HigBA homolog in particular regulates tryptophan metabolism, which is an essential component of *H*. *influenzae* virulence. Other HigBA TA homologs have also been implicated in recurrent *Pseudomonas aeruginosa* infections, complicating cystic fibrosis, severe burn wounds, and urinary tract infections [351]. HigBA TA loci uniquely encoded by more virulent *P*. *aeruginosa*

strains [352] are demonstrated to constitutively upregulate type III secretion, thereby increasing Mφ necrosis [352, 353]. This ultimately shows that TA systems are directly involved in mediating virulence. A number of *Rickettsia* species cause chronic human infections that may vary widely in severity depending on the treatment regimen [354]. Fluoroquinolone antibiotics are prescribed because of their broad spectrum activity, but their use to treat rickettsia infections leads to poorer patient outcomes [355]. Ciprofloxacin in particular is found to increase apoptosis of Mϕs that are infected with rickettsia, which encode considerably more TA systems [354, 356]. This is because ciprofloxacin cues intracellular rickettsia to release these toxins into the host cytoplasm, wherein they are functional RNases, inducing host cell death [354]. This is in line with additional reports that TA homologs unique to pathogenic bacteria such as *Mtb* RelBE2 (*i.e.* Rv2865-Rv2866) are particularly potent inducers of host cell death [357, 358]. These findings further substantiate that TA systems have evolved as important mediators of bacterial pathogenesis. Similar to Rickettsia, *Leptospira* species cause asymptomatic infections that develop into fatally disseminated diseases [359]. *L. interrogans* selectively releases MazF toxins into host Mϕs, thereby enhancing necrosis, and subsequent ectopic induction of cognate MazE antitoxins is found to inhibit decay of host Mϕ mRNA, thus suppressing host cell death [360]. Epidemic bacteria such as *Mtb* uniquely function in suppressing host cell apoptosis, while promoting necrosis [361]. Apoptosis is considered to be an immunologically silent death [362], which taken along with findings in *L. interrogans*, ultimately implicate TA systems in both host subversion and manipulation during infection.

2.5 *Mycobacterium tuberculosis* toxin-antitoxin systems.

Mtb harbors an inordinately large number of TA systems, especially when compared to related *Mycobacteria* [34], and WGS links this genomic expansion to the most recent emergence of *Mtb* [164, 166, 363]. *In silico* searches and functional studies identify up to ninety-four TA loci throughout the genome of *Mtb* reference strain H37Rv, encoding for type I, II, and IV TA systems [160, 167, 364-369]. Phylogenomics studies find that post-transcriptional regulation of *Mtb* TA

systems is a hallmark of the MTBC [363]. Only IGRs in the *Mtb* genome appear to be shaped by positive selective forces [370], producing new promoters and TSSs that drive sense and antisense transcription [363]. This has led to an overrepresentation of differentially expressed *Mtb* TA genes that has been directly implicated in mediating *Mtb* pathogenesis [371]. *In silico* searches for regulatory DNA motifs in particular fail to identify any major σ^A (*i.e.* Rv2703) binding sites, instead finding that an unusually high frequency of *Mtb* TA systems are regulated by σ^B (*i.e.* Rv2710) [158]. Others report that a number of *Mtb* TA genes are differentially expressed by σ^B [372, 373], which functions as a master regulator of persistence *in vitro* and *in vivo* [374, 375].

Table 2.1: The distribution of *Mycobacterium tuberculosis* toxin-antitoxin systems among *Mycobacterial* species and ecotypes:

Mycobacterium	Classification; Isolation source	ORFs	VapBC	MazEF	ParDE/RelBE
Mtb H37Rv	MTBC; Pulmonary tissue of patient with ATB	3906	52	11	14
Maf GM041182	MTBC; Pulmonary tissue of patient with ATB	4045	51*	08*	14
Mcn STB-D	STB; Pulmonary tissue of patient with ATB	4040	49*	07*	13*
Mcn STB-K	STB; Cutaneous tissue of patient with extra-pulmonary ATB	3957	45*	08*	11*
Mdc TBL 12000985	MTBCAP; Cutaneous tissue lesion of patient with NTM infection inducing a granulomatous host immune response.	4676	00	00	00
Msh CCUG 53584	MTBCAP; Sputum of patient with a pulmonary NTM infection similar to that of TB	3981	00	00	00
Mlc DSM 44577	MTBCAP; Synovial tissue of patient with NTM infection similar to that of	4203	00	00	00
Mkn ATCC 12478	SGM; Pulmonary tissue and lesions of patient with fatal NTM infection	5497	03*,**	00*,**	04*,**
Mmr M	SGM; Cutaneous tissue and lesion of fish with *Mmr* infection	5426	00	00*,**	01*,**
Mgo DSM 44160	SGM; Gastric lavage of patient with NTM infection	6496	00	00	00

Notes: Mycobacteria include *M. decipiens* (*i.e. Mdc*), *M. shinjukuense* (*i.e. Msh*), *M. lacus* (*i.e. Mlc*), *M. gordonae* (*i.e. Mgo*), MTBC associated-phylotype (*i.e.* MTBCAP), and non-tuberculosis mycobacteria (*i.e.* NTM). Orthologs of *Mtb* H37Rv (N=52) VapBC, (N=11) MazEF, and (N=14) ParDE/RelBE TA systems identified in *Mycobacterial* genomes using tBLASTn *in silico* searches, as bidirectional best hits (*i.e.* at least 50% identical over at least 80% of the query sequence), and assessed for synteny, similarly as recently described [376]. Upstream and downstream IGRs, as well as adjacently oriented genes considered in synteny analysis to identify true orthologs of most significantly expanded *Mtb* TA systems. (*) denotes orthologous orphaned toxin and antitoxin genes found not counted, while ** denotes orthologous TA loci lacking synteny counted.

As many as ninety-four TA systems have been found in the genome of *Mtb*, including two type I TA systems (*i.e.* Rv0024-Rv0025 and Rv0078B-Rv0078A); eighty-eight type II TA systems (*i.e.* fifty-two VapBC, eleven MazEF, fourteen ParDE/RelBE, one HipBA, one SirR, and nine

unclassified), and four type IV systems (*i.e. rv0078A*, *rv1044-rv1045*, *rv1051c*, and *rv2827c-rv2826c*). WGS finds that *Mkn* is the closest non-tuberculosis mycobacteria (NTM) relative to *Mtb* [377, 378], but only two of the seven *Mtb* TA loci encoded by *Mkn* are truly orthologous, showing synteny (*i.e. vapBC39* (*i.e. rv2530a-rv2530c*) and *vapBC43* (*i.e. rv2871-rv2872*)). Both of these TA loci have accrued multiple mutations though, rendering them nonfunctional. At least nine *Mtb* ParDE/RelBE TA systems are absent from the genomes of *Mmr* and *Mkn* [164, 378]. *Mkn* harbors RelBE3 and ParDE1 (*i.e.* Rv1960c-Rv1959c) on a plasmid without synteny, further implicating their genomic expansion in the emergence *Mtb*. STB chromosomes are known to be larger and more diverse from extensive HR, and thus form the deepest seeded branch of the MTBC [379]. Studies show a significant accumulation of mutations likely affecting STB TA orthologs [379, 380]. WGS reveals that strain K is oldest STB, with the fewest orthologs, while strain D, which is the closest relative to *Mtb*, harbors nearly all ninety-four *Mtb* TA systems [379]. Moreover, clear genotypic differences in the distribution and genetic variation of *Mtb* TA loci arise, underlying the phenotypic differences between ancient and modern MTBC clades [367]. Accordingly, AA-substitutions altering antitoxin DNA-binding and SNP-driven promoters have been found to be a key discerning feature of MTBC lineages [166, 363, 367, 371, 379].

2.5.1 Regulators of translation and DNA replication.

VapBC is the most abundant type of *Mtb* TA system [381]. Bioinformatic analyses of PIN-domains identify up to fifty-two VapC toxins in the *Mtb* genome [167, 217, 382]. VapB antitoxins in contrast make up several families of transcriptional regulators with RHH, Phd, ArbR, MerR, and other novel N-terminal DNA-binding domains [218, 383]. Thus, *Mtb* VapBC protein complexes are stoichiometrically diverse. VapBC4 (or Rv0596c-Rv0595c) and VapBC5 (or Rv0626-Rv0627) appear to bind at a ratio of one-to-one [384, 385], while VapBC15 (or Rv2009-Rv2010) is found to bind at a ratio of one-to-one or two-to-one [386]. VapBC2 (or Rv0309-Rv0310), VapBC26 (or Rv0581-Rv0582), and VapBC30 (or Rv0623-Rv0624) bind at a ratio of two-to-two [387-389], and a majority of *Mtb* VapC toxins appear to be exclusively regulated in a cognate manner [364, 365,

390]. Accordingly, *Mtb* VapC toxins are known to share little-to-no AA-sequence similarity outside of their PIN-domains with more discrete functional roles. *Mtb* VapC toxin genes encode RNases [295, 365, 391] that alter distinct aspects of translation [390, 392, 393]. VapC1 (or Rv0065) and VapC19 (or Rv2548) cut at GC-rich sequences, revealing that most of the *Mtb* transcriptome is susceptible [300]. The ectopic induction of both toxins quickly depletes total RNA, thus inhibiting mycobacterial growth [364, 390]. The overexpression of VapC45 (or Rv2019) halts growth too from select decay of 16S and 23S rRNAs [365]. VapC20 (or Rv2549c) and VapC26 target 23S rRNA too, cutting the sarcin-ricin loop needed for recycling ribosomes [302, 392, 394]. VapC4, VapC28 (or Rv0609), and VapC30 in contrast all target *Mtb* serine tRNAs [392, 393]. Intriguingly, VapC25 (or Rv0277c), VapC29 (or Rv0617), VapC33 (or Rv1242), VapC37 (or Rv2103c), and VapC39 (Rv2530c) all selectively cleave the only *Mtb* tryptophan tRNA [392]. It thus seems that *Mtb* VapC toxins have a range of effects on translation, from its complete shutdown to the select protein synthesis of leaderless codon biased mRNA [395]. These findings ultimately suggest that *Mtb* VapC toxins act to sync translation with dynamic fluctuations in environmental conditions.

Mtb has eleven MazF toxins with unique RNase activities [364, 365]. MazF3 (or Rv1102c) for instance cuts RNAs at 5'-UU↓CCU-3', including 23S rRNA [396, 397]. Specifically, MazF3 is able to cleave helix seventy of 23S rRNAs in 50S ribosomal subunits, but not in complete 70S ribosomes [397, 398], demonstrating that MazF3 functions at least in part to block ribosome assembly. MazF4 (or Rv1495) on the other hand not only cleaves RNA at 5'-U↓CGCU-3' [399], it directly binds to and inhibits DNA topoisomerase I [400]. These published results demonstrate that MazF4 is able to differentially affect *Mtb* growth *via* two distinct mechanisms. WGS recently showed that MazF6 (or Rv1991c) cleaves mRNAs, as well as 23S and 16S rRNAs at 5'-U↓CCUU-3' sequences [401]. MazF6 also cuts 16S rRNAs in ribosomes, effectively removing their anti-SD sequences [401], enabling the translation of leaderless mRNAs that make up much of the *Mtb* transcriptome [402]. MazF7 (or Rv2063A) cuts numerous mRNAs at 5'-U↓UU-3', as well as

proline and lysine tRNAs, thereby making it a potent inhibitor of translation [403, 404]. Similarly, MazF9 (or Rv2801c) recognizes ~70% of *Mtb* transcripts at 5'-U↓AC-3' [405]. Ultimately, these differences in sequence-specificity suggest that *Mtb* MazF toxins differentially affect translation and thus growth, from a complete shutdown to more selective translation of leaderless messages.

Mtb also harbors twelve TA systems that belong to the ParDE/RelBE superfamily. ParE1 and ParE2 (or Rv2142c) share 30% and 40% AA-sequence similarity with the prototypic plasmid-encoded toxin, respectively [364]. Only ParE2 appears to maintain key catalytic residues, making its overexpression cytotoxic to mycobacteria [366]. However, ParE2 is also found to be involved in developing highly drug tolerant viable but non-culturable bacilli [366]. This is likely because ParD2 is able to disrupt ParE2-binding to DNA Gyrase I [366]. *Mtb* likewise encodes two *bona fide* RelBE TA systems, RelBE1 and RelBE2, and one RelBE-like YefM-YoeB TA system, RelBE3 [160, 364]. Because RelE3 is missing AA-residues, which are essential for ribosomal-dependent mRNA cleavage (*i.e*. arginine-81 (R81) and tyrosine-85 (Y85)), is functionally distinct from RelE1 and RelE2 [160, 406]. Consequently, the ectopic induction of RelE3 only has limited effects on bacterial growth compared to that of RelE1 and RelE2 [132, 406, 407]. The RNase activities of RelE1 and RelE2 in contrast potently inhibit mycobacterial growth, producing elongated cells [406, 408, 409]. Intriguingly, RelBE1 and RelBE2 are found to be uniquely conserved by closely related NTM. *M. avium* RelE1 functions canonically, cleaving actively translating mRNAs [410]. In *Mtb* though, the induction of RelE1 and RelE2 leads to the differential expression of numerous genes involved in cell-wall processes and alternative metabolic pathways, indicating that they evolved functionally [409]. This would explain why the inhibitory effects *relE2* overexpression are not fully neutralized by RelB2 [364, 409, 411]. And because RelB2 activates transcription without RelE2, which leads to constitutive upregulation of *relBE2* [411, 412], post-transcriptional events are likely key to control RelE2 activity [409, 411, 413]. *Mtb* regulates *relBE2* in particular similarly to that of a type I system [414]. This also seems to be the case for another three *Mtb* RelBE-like systems, HigBA1 (or Rv1955-Rv1956-Rv1957), HigBA2 (or Rv2022c-Rv2021c), and HigBA3 (or Rv3182-

Rv3183) [160]. The ectopic induction of HigB1 is especially cytotoxic to *Msm*, *Mmr*, and *Mtb* [415, 416] and uniquely requires HigA1 along with Sec-like chaperone Rv1957, which is co-transcribed, to be neutralized [152, 415]. Rv1957 refolds HigA1, making it more resistant to proteolysis, which is essential to inhibit HigB1 [415]. Accordingly, *higA1* cannot be deleted from the genome of *Mtb* and the deletion of *rv1957* severely attenuates mycobacterial growth [152]. This indicates that HigB1 is exclusively regulated in a cognate manner at the protein-level. Even still, *higB1* mRNA levels often appear to be constitutively upregulated compared to those of *higA1* and *rv1957* [364, 414, 417, 418], indicating post-transcriptional regulation of potentially lethal activity of HigB1.

2.5.2 Regulators of persistence.

Under stressful conditions *Mtb* antitoxins are selectively degraded [103, 419, 420], freeing their toxins to target DNA replication and translation. *Mtb* TA genes are differentially expressed as part of a broad response to stress underlying the epigenetic development of persistence [160, 409, 413]. Accordingly, many *Mtb* TA genes are determined to be essential for developing tolerance to fluoroquinolones, Inh, and Rif [133, 409]. Multiple *Mtb vapBC* genes for example seem to be individually essential for developing drug tolerance *in vitro* [375, 421, 422], which are transcriptionally induced by hypoxia [364, 423], NS [102], and drug exposure [138]. However, many of these VapC toxins accumulate extracellularly, while their cognate antitoxins are retained intracellularly [103, 420, 424]. VapC toxins are shown to be among the most abundantly exported proteins by *Mtb in vitro* [425, 426]. Because VapC toxins are predominantly regulated in a cognate manner, toxin protein export likely represents a critical point of regulation, which limits their lethal effects. MazF toxins have also been heavily implicated in mediating *Mtb* persistence in vitro [103, 133, 427, 428]. MazF3, MazF6, and MazF9 in particular are collectively essential for *Mtb* survival to NS, ROS, and drug exposure [133]. Moreover, these toxins degrade cognate *mazE* mRNAs [133], indicating that post-transcriptional *Mtb* TA regulation is important for developing a persistent state. Increased MazF-dependent cleavage of rRNA is known to be a hallmark of Inh and Rif tolerant *Mtb* [429], with MazE being replenished in a limited manner [103, 420, 430, 431]. The

ectopic induction of *Mtb* ParE2 is lethal [432, 433] but also produces viable but non-culturable bacilli [366] that are recovered from TB patient sputa [429, 434]. ParDE2 genes are differentially regulated by stress, wherein post-transcriptional events that change *parD* and *parE* mRNA levels to regulate ParE2 activity [102, 366, 435]. This is also observed in *Mtb* RelBE TA homologs, which have drug-specific effects on tolerance [409]. Like *parDE2*, significant changes in *relB2* and *relE2* mRNA levels, resulting in marked induction of RelE2 [80, 130, 413], appear to be additionally regulated similarly to type I toxins by asRNA antitoxins [371, 414, 436], ultimately indicating the importance of type I TA regulation of *Mtb* persistence.

2.5.3 Regulators of pathogenesis.

Numerous *Mtb* TA genes are found to be differentially expressed in INH- and RIF-tolerant bacilli recovered from patient sputa [28, 437]. Correlating patient indicators of TB disease severity with *Mtb* gene expression reveals that transcriptomic changes associated with the development persistent tubercle bacilli are induced by the host immune response [77, 81, 437]. This includes the marked upregulation of numerous *Mtb* TA loci. An array of VapBC genes are transcriptionally activated *in vivo* [62, 418, 423, 438, 439], and transposon screens find that many are required for *Mtb* survival in Mφs and persistence in mice and nonhuman primates [440-443]. The deletion of VapBC36 (or Rv1982A-Rv1982c) in particular leads to the attenuation of *Mtb* in mouse models of infection, and this TA system is missing from the genome of vaccine strain BCG [444]. Increased basal expression of VapBC36 genes that are essential for intracellular *Mtb* replication and survival [445, 446] leads to the production of antigenic PE/PPE proteins [447], and secreted immunogenic proteins [448]. Antigenic VapC toxins are also secreted by *Mtb* [425, 426] that are functional in host cells [357, 358], subverting responses. *Mtb* VapC toxins, which have accrued mutations in BCG and STB orthologs are recognized by T cells of ATB patients [449-451] or LTBI individuals [452, 453]. The increased basal expression of *vapB47* (or *rv3407*) in a recombinant BCG strains is in turn found to significantly enhances the vaccine efficacy against TB [454, 455].

MazEF TA systems are also directly implicated in *Mtb* pathogenesis [133, 418, 456]. While *mazE* and *mazF* mRNA levels are similar in the lungs, only *mazF6* is transcribed in the spleens of infected mice [457]. *Mtb*Δ*mazE5* (*or rv1943c*) and *Mtb*Δ*mazE7* mutant strains moreover show persistence defects in the chronic but not acute phase of mouse infection [441]. Because non-cognate antitoxins neutralize MazF toxins [458], it is seems likely that complex *Mtb* TA interactions

Figure 2.4: *Mycobacterium tuberculosis* toxin-antitoxin systems in the regulation of persistence and tuberculosis pathogenesis.

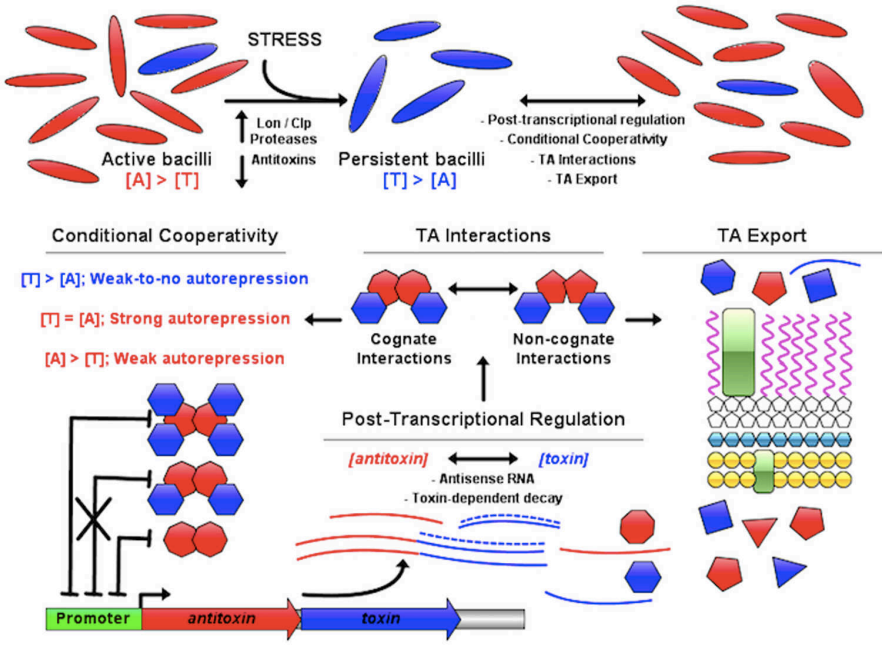

Figure 2.4: *Mycobacterium tuberculosis* toxin-antitoxin regulation of persistence and pathogenesis. During exponential growth such as the acute (or the pre-immune) phase of *Mtb* infection, TA genes are co-transcribed and/or translated to thereby suppress and/or limit any toxic effects on processes that are essential to actively replicating tubercle bacilli, especially DNA replication and translation. This is achieved *via* multiple levels of TA regulation in *Mtb*: (1) Transcription is auto-regulated by antitoxins and/or TA complexes *via* conditional cooperativity, as well as activated by novel promoters accumulating in *Mtb* and the MTBC; (2) TA mRNAs are post-transcriptionally processed (or degraded), thereby enabling rapid and global changes to intracellular toxin and antitoxin stoichiometries; (3) Cognate and non-cognate TA protein-protein interactions, thus help to integrate the regulation of a massively expanded repertoire of chromosome-encoded functions; (4) TA protein and mRNA export, which further aids in limiting any potentially toxic effects from high-level basal expression of many *Mtb* toxin and antitoxin genes. In stressful times such as the chronic (or immune-mediated) phase of *Mtb* infection, these multiple levels of regulation ultimately lead to the striking overrepresentation of differentially induced toxin and antitoxin genes that is principally involved in regulating *Mtb* pathogenesis.

regulate MazF toxins in an infection phase-dependent manner. For example, MazF6 and MazE10 (or Rv0298) are each predicted to be important for mycobacterial survival in IFN-γ activated Mφs [442]. And while *Mtb*Δ*mazF3* and *Mtb*Δ*mazF9* mutant strains are not shown to be attenuated *in vivo* [409], *Mtb*Δ*mazF3*Δ*mazF6*Δ*mazF9* appears to be considerably less virulent than WT *Mtb* in

guinea pigs in acute and chronic phases of infection [133]. Strikingly, the lung granulomas of *Mtb*Δ*maz-F3,-F6,-F9*-infected guinea pigs do not appear to be necrotic and are comprised mostly of T_H cells, with relatively few Mφs and other innate cells [133]. All three RNase toxins accumulate in *Mtb* culture filtrate [103, 431] that are functional in host cells [280, 459], and thus likely target host transcripts to increase tissue damage. The exosomes of *Mtb*-infected Mφs likewise contain *mazEF* mRNAs [460], further suggesting that they serve an immunomodulatory role(s) [461, 462]. *Mtb* MazEF TA genes are differentially upregulated by the TCS system SenX3-RegX3, which is essential for regulating iron scavenging and protein transport inside host Mφs [463].

Homologous ParDE/RelBE superfamily TA systems appear to be directly involved in Mtb pathogenesis too. ParE1 is important for *Mtb* survival in IFN-γ-activated Mφs that likely subverts host cell responses [440]. This is supported by others finding that ParE1 is uniquely required for *Mtb* dissemination to the spleens during infection [441]. A majority of *Mtb parDE*/*relBE* TA genes are predominantly upregulated *in vivo* as part of the broad adaptive response to the host immune response to infection [439]. Among these, RelE1 induces strong T cell responses in *Mtb* infected mice at four-weeks post-infection, whereas RelE2 elicits similar T cell responses at eight-weeks post-infection [464]. RelE2 and not RelE1 moreover is determined to induce comparable T cell responses in otherwise healthy individuals with LTBI [453], and be among the most seroreactive Ag proteins in human TB as well [465, 466]. These findings are in further support of transposon screens that reveal Sec-like antitoxin chaperone Rv1957 is key for *Mtb* survival in IFN-γ-activated Mϕs [440] and during the chronic phase of infection in mice [441]. This likely explains why HigA1 is known to specifically activate the transcription of multiple *Mtb* genes involved with intracellular survival [152, 440, 467]. But because HigA2, HigA4 (or Rv0023), and HigB2 also appear to be uniquely essential for *Mtb* persistence following development of T_H1 mediated immune responses [375, 440, 442, 468], complex non-cognate TA interactions likely play important functional roles in mediating more discrete aspects of TB pathogenesis. Rv1957, HigA2, HigB3, and HigA4 for

instance are recognized by T cells in patients with ATB or LTBI, while HigA1 only produces robust T cell responses in latent TB [439, 469-473]. The presence of infection phase specific T cell Ags ultimately indicates that differential regulation of *Mtb* TA genes serves a principle role in mediating *Mtb* pathogenesis.

2.6 A look back.

Mtb is the principal cause of human TB worldwide today, but much of its high-GC content genome is shared by an ecologically diverse group of species and strains belonging to the deeply seeded *Mycobacterium* genus. Phylogenomic analyses with closely related mycobacteria such as *Mmr*, *Mkn*, and *Mcn* shed insights into ancestry and evolution of *Mtb*. WGS comparisons to *Mkn* and STB in particular link downsizing of metabolic capacity and massive genomic expansion of TA loci and other pathogen-specific gene families to the emergence of *Mtb* and the MTBC. In addition, a multitude of *Mtb* TA systems exhibit genotypic differences among ancient and modern MTBC lineages. These predominantly include lineage-specific changes in AAs, affecting antitoxin DNA-binding, and NTs, generating new promoters in IGRs. These mutations have resulted in an overrepresentation of differentially expressed *Mtb* TA genes responsible for mediating epigenetic changes distinguishing more virulent modern MTBC lineages. And yet, while post-transcriptional regulation (or mRNA cleavage) is essential for the differential expression of *Mtb* TA genes, the molecular mechanisms governing changes in TA mRNA remain largely unknown. Many *Mtb* TA genes seem to be differentially expressed in a manner similar to that of type I TA systems *via* an asRNA antitoxin, especially ParDE/RelBE superfamily TA homologs that are uniquely conserved in the MTBC. Type I regulation of *Mtb* homologs is likely important for coordinating TA expression in successful pathogen adaptation. *Mtb relBE2* for instance is commonly differentially regulated in response to stress, wherein *relE2* toxin mRNA levels increase significantly compared to those of *relB2*, resulting in development of persistent tubercle bacilli, which are tolerant to frontline anti-tubercular drugs such as Rif and Inh, as well as the host immune environment. Increased *relE2* mRNA levels likely enable the rapid shutdown of the translation of canonical mRNAs concomitant

with new protein synthesis from leaderless messages, including *relB2* and *relE2*. This suggests that post-transcriptional regulation leads to the differential expression of numerous *Mtb* TA genes in turn providing control over downstream effects, from growth inhibition-to-PCD. *Therefore, the working hypothesis is that post-transcriptional control of TA systems is an important but undefined point of regulation that is unique to Mtb pathogenesis.* A comprehensive understanding of TA mRNA cleavage and/or decay will provide keen insights into sub-cellular processes that are vital for *Mtb* persistence. This would ultimately help streamline the research and development of novel antibiotics as well as host directed immunotherapies targeting dormant multidrug tolerant tubercle bacilli, effectively shortening TB treatment.

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CHAPTER 3: ANTISENSE RNA REGULATION OF THE MYCOBACTERIUM TUBERCULOSIS TYPE II TOXIN-ANTITOXIN SYSTEM RELBE2

3.1 Introduction.

Despite more than 60 years of available treatments, *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), remains the deadliest human pathogen [6]. It is thought that nearly one-third of the world's population has been exposed to or infected with *Mtb*, and while most individuals either do not become infected or control infection long-term [7], up to ten percent will develop active TB and thus, represent a major source for new infections [8, 9]. The ability of *Mtb* to establish and maintain a latent or chronic infection is attributed to its unique ability to resist or avoid killing by the host immune response. The decreased mycobacterial growth associated with transition to latent TB infection is linked in part with the cytokine IFN-γ that activates Mφs to control *Mtb* [10]. While *Mtb* appears to subvert IFN-γ-dependent immune responses [11], its adoption of a persistent (or phenotypically resistant) state in an activated host environment appears to be critical to establishing [12] and maintaining latent TB infection [13].

There have been considerable efforts to identify the molecular basis of *Mtb* persistence. Numerous regulatory elements, including sigma factors and transcription factors, are associated with the adaptation, regulation, and alternative metabolism of *Mtb* to *in vitro* and *in vivo* host-associated stresses [14]. TA systems are increasingly implicated to play essential roles in the biological processes underlying persistent and chronic *Mtb* infections [15-17]. TA systems are classified into six major types based on the antitoxin and the mechanism by which it regulates the cognate toxin [17]. In general, TA systems are comprised of a protein toxin and a cognate protein or asRNA antitoxin [17]. During periods of exponential growth such as the acute (or pre-immune) phase of *Mtb* infection, transcription and translation of toxins and antitoxins maintains near equivalent intracellular stoichiometries [18]. Under stressful conditions such as the chronic (or immune-mediated) phase of *Mtb* infection, antitoxins are degraded, thereby freeing toxins to

target aspects of DNA replication and translation [19].

In silico analyses and functional studies identify up to 94 TA systems encoded in the *Mtb* strain H37Rv genome, including 52 VapBC, 11 MazEF, and 14 ParDE/RelBE type II homologs [20-22]. Ours along with others published works show that *Mtb* TA systems, especially from the ParDE/RelBE TA superfamily, are differentially regulated in response to host-associated stresses during infection, highlighting the importance of these systems in mediating a persistent state [20, 23-26], contributing to characteristic long-term treatment regimens and latent TB [14, 27]. RelE toxins induce bacteriostasis by cleaving mRNAs codons engaged with the ribosome, resulting in ribosomal stalling on truncated messages [28]. The expression of the *relBE* operon in *E. coli* is minimal under physiological growth conditions, but stresses, such as NS, lead to rapid depletion of RelB and arrested growth and altered metabolism mediated by RelE [29], coinciding with select accumulation of *relE2* mRNA from bi-cistrons [30]. Still, hyperactivation of RelE is shown to be detrimental to long-term bacterial growth and survival [31].

A growing body of evidence has indicated that post-transcriptional regulation is a hallmark of bacterial pathogens [32], including antisense (as) transcription, which appears to be especially abundant in *Mtb* [33]. *Mtb* expresses asRNA to two-thirds of its ORFs in exponential phase and over ninety percent throughout stationary phase [34] that likely modulate gene expression levels by dsRNA-mediated decay *via* Rnc [35]. Antisense transcription appears to lead to the differential expression of virulence genes as well. The *Mtb ino1* gene (*i.e. rv0046c*) for instance is essential for virulence [36] and has a complementary RNA, asIno1, which is differentially regulated along with *ino1* during *in vitro* growth [34]. *Mtb pks12* (*i.e. rv2048c*) encodes a polyketide synthase involved in immunomodulatory lipid biosynthesis [37], which likewise has a complimentary RNA, asPks, that is inversely regulated in response to host acidification and ROS [38, 39]. Accordingly, *Mtb* TA genes that are found to be essential for long-term persistence *in vivo* appear to be post-transcriptionally regulated similarly [12, 15, 16, 40].

Based on findings that many *Mtb* TA genes, which are differentially post-transcriptionally regulated as a broad stress response *in vivo*, are also implicated in latent human TB, has led us to hypothesize that *Mtb* TA systems are tightly regulate growth and survival in an infection-phase dependent manner [41, 42]. To begin to address this possibility, we focused on characterizing the regulatory mechanism(s) of the *relBE2* TA locus. Our investigation revealed a novel asRNA antitoxin, asRelE2, which is involved in differentially regulating *relE2* mRNA-levels, compared to those of co-transcribed *relB2*. Our studies determined that *relBE2* and asRelE2 are co-regulated by promoters recognized by essential *Mtb* transcription factor Crp and accumulates inversely with *relE2* mRNAs in a growth phase-dependent manner. Gene expression analyses found that *relE2* mRNA levels are decreased relative to those of *relB2* and asRelE2 in early-to-mid-log phase. In stationary phase and in response to stress though, *relE* mRNA levels increase, contributing to long-term *Mtb* survival *in vitro*. Notably, inverse expression profiles from murine infection show that *relB2* and *relE2* mRNA levels are differentially regulated *via* asRelE2 in response to the host immune status during infection. To our knowledge, this is the first report that identifies the involvement of antisense asRNA in the post-transcriptional regulation of a type II TA locus that is uniquely encoded by *Mtb* and the MTBC, and links post-transcriptional antisense regulation of an endogenous translational inhibitor to the host immune response, which implicates asRNA in regulating and mediating *Mtb* pathogenesis.

3.2 Materials and methods.

3.2.1 Bacteria and culture conditions.

All bacterial strains used in these studies are listed in Table 3.1. Z-competent *E. coli* strain DH5 Alpha (Zymo Research) cells were used for all cloning and propagation. One Shot® chemically competent *E. coli* str. BL21(DE3)pLysS (Invitrogen™) cells were used for the ectopic induction of recombinant *Mtb* proteins with 10 mM (final concentration) of isopropyl β-D-1-thiogalactopyranoside (IPTG). *E. coli* strains were grown in Luria-Bertani (LB) broth at 200 rpm or on LB agar plates at 37°C. Mycobacteria were cultured in Middlebrook 7H9 broth (Difco™)

supplemented with OADC *(i.e.* 0.05 mg mL^{-1} oleic acid, 5 mg mL^{-1} BSA Fraction V, 2 mg mL^{-1} dextrose, 0.004 mg mL^{-1} catalase, and 0.85 mg mL^{-1} NaCl), 0.2% (v/v) glycerol, and 0.05% (v/v) Tween-80 (*i.e.* 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 [43]. For these studies, virulent *Mtb* strain H37Rv (ATCC ® 27294™) was used as a WT parental strain in the construction of various overexpression and mutant (or Mut) strains. Unless otherwise noted, optical densities at 600 nm (*i.e.* OD_{600}) for growing *Mtb* in 7H9-Tw of 0.10-0.30, 0.40-0.60 (or 6 d), 0.70-0.90, 1.00-1.20 (or 10 d), and 1.30-1.50 (or ≥16 d) were considered to be in early-log, mid-log, and late-log, early-stationary, and late-stationary phase, respectively, as described earlier [25, 43]. Antibiotics purchased from Sigma-Aldrich were used at the following concentrations: 50 μg mL^{-1} ampicillin for *E. coli*; 34 μg mL^{-1} chloramphenicol (34-Chlor) for *E. coli*; 5 μg mL^{-1} gentamycin for *E. coli*; hygromycin (Hyg) at 200 and 50 μg mL^{-1} (50-Hyg) for *E. coli* and *Mtb*, respectively; kanamycin (Kan) at 50 (50-Kan) and 25 (25-Kan) μg mL^{-1} for *E. coli* and *Mtb*, respectively.

Table 3.1: Bacterial strains and plasmids used in these studies.

Strains and Plasmids	Additional Information	Source and Reference
Strains		
E. coli strain DH5α	F^- φ80*lacZ*ΔM15 Δ(*lacZYA-arg*F)U169 *deoR*, recA1 *end*A1 *hsd*R17(rK^-, mK^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Zymo Research
E. coli strain BL21(DE3)pLysS	F^- *omp*T *hsd*S_B (r_B^-, s_B^-) *gal dcm* (DE3) pLysS(Cam^R)	Invitrogen™
Mtb strain H37Rv	Virulent *Mtb* strain ATCC ® 27294™	[44]
*Mtb*Δ*relE2*	*Mtb* H37Rv *relE2* mutant strain with Hyg^R	This study
*Mtb*Δ*asrelE2*	*Mtb* H37Rv *asrelE2* mutant strain with Kan^R	This study
Plasmids		

pVV16	Constitutive overexpression mycobacterial shuttle vector encoding Hyg^R	Kindly provided by Karen Dobos (TBVRM, Colorado State University)
pMIND	Tetracycline-inducible overexpression mycobacterial shuttle vector encoding Kan^R	Made available by Brian Robertson at Addgene; [45]
pPR27-*xylE*	Thermosensitive replicative *Mtb* vector with sucrose counterselection and Gm^R	Kindly provided by Mary Jackson from [46]
pPR27-*relE2*KO	pPR27-*xylE* with pVV16 Hyg^R gene *hph* flanked by *rv2864c-relB2* and *rv2867c*	This study
pPR27-*asrelE2*KO	pPR27-*xylE* with pMIND Kan^R gene *aphA* flanked by *rv2864c-relBE2* and *rv2867c*	This study
pST-KT	Mycobacterial non-toxic ATc-inducible overexpression vector encoding Kan^R	Made available by Vinay Nandicoori at Addgene; [47]
$pE2^{WT}$	pST-KT encoding ATc-inducible $RelE2^{WT}$	This study
$pE2^{\Delta R61L}$	pST-KT encoding ATc-inducible $RelE2^{\Delta R61L}$	This study
$pE2^{\Delta R81L,Y85F}$	pST-KT encoding ATc-inducible $RelE2^{\Delta R81L,Y85F}$	This study
$pBE2^{WT}$	$pE2^{WT}$ encoding $RelB2^{WT}$ controlled by P_{myc2}*tetO2*	This study
$pASE2^{WT}$	$pERRY2^{WT}$ encoding $asRelE2^{WT}$ controlled by P_{myc2}*tetO2*	This study
pET28a	N- and C-terminal His_6 tag IPTG-inducible overexpression vector with Kan^R	Novagen
pET28a-*his-relB2-his*	pET28a encoding IPTG inducible N- and C-terminal His^6-tagged Mtb RelB2 and Kan^R	This study
pETcoco2	N-terminal His_6 tag and C-terminal HSV tag IPTG-inducible overexpression vector with Amp^R	Novagen

pETcoco2-*relE2-hsv*	pETcoco2 encoding IPTG-inducible C-terminal HSV-tagged *Mtb* RelE2 and Amp^R	This study
pETcoco2-*his-rnaseIII*	pETcoco2 encoding IPTG-inducible N-terminal His_6-tagged *Mtb* RNase III and Amp^R	This study
pETcoco2-*his-crp*	pETcoco2 encoding IPTG-inducible N-terminal His_6-tagged *Mtb* Crp and Amp^R	This study
pCHERRY3	*Mtb* reporter plasmid encoding codon-optimized mCHERRY and Hyg^R	Made available by Tanya Parish at Addgene; [48]
pUV15-*pHGFP*	*Mtb* reporter plasmid encoding codon-optimized pH-sensitive GFP and Hyg^R	Made available by Sabine Ehrt at Addgene; [49]
WT pGREENCHERRY	pCHERRY3 encoding mCHERRY-SsrA and pHGFP-SsrA controlled by WT $P_{asrelE2}$CBS and P_{relBE2}CBS	This study
Mut pGREENCHERRY	pCHERRY3 encoding mCHERRY and GFP controlled by Mut $P_{asrelE2}$CBS and P_{relBE2}CBS	This study

3.2.2 Construction of *Mycobacterium tuberculosis* mutant, overexpression, and reporter strains.

The *Mtb* Δ*relE2* and Δ*asrelE2* deletion mutant strains were generated using the two-step allelic exchange with temperature sensitive replicative vector, pPR27-*xylE,* as originally reported [46]. Briefly, to generate mutant strains, ≥1000 bp upstream and downstream flanking the 264-bp *relE2* gene and the 372-bp IGR intervening between *relE2* and *rv2867c* were PCR amplified from *Mtb* H37Rv gDNA with gene-specific primers (GSPs) listed in Table 3.2 using GoTaq® Green (2X) Master Mix (Promega Corporation) enriched with five percent PCR-quality DMSO (Sigma-Aldrich) per the manufacturer's instructions (Promega Corporation). For the generation of Δ*relE2*, the upstream and downstream regions were cloned into the mycobacterial shuttle vector, pVV16, flanking hygromycin resistance (Hyg^R) gene *hph*. The resulting 4049-bp fragment, *rv2864c-relB2-hph-rv2867c*, was excised and cloned into pPR27-*xylE* at NotI and SpeI restriction

digestion (RD) sites for sucrose (Suc) counter selection. For the development of *Mtb*Δ*asrelE2*, the upstream and downstream regions were cloned into mycobacterial shuttle vector, pMIND, flanking the kanamycin resistance (Kan^R) gene *aphA*. The resulting ~3,800 bp fragment, *rv2864c-relBE2-aphA-rv2867c*, was cloned into pPR27-*xylE* at NotI and XbaI RD sites, creating pPR27-*asrelE2*KO. Freshly prepared electrocompetent WT *Mtb* strain H37Rv was electroporated with the allelic exchange vectors. Following the outgrowth of Hyg^R and Suc^R colonies in 7H9-Tw with 50-Hyg (*i.e. Mtb*Δ*relE2*), and Kan^R and Suc^R in 7H9-Tw with 25-Kan (*i.e. Mtb*Δ*asrelE2*) for three-to-four weeks at 37°C, successful deletions from the genomes of mutant strains was confirmed by PCR analyses using GSPs listed in Table 3.2.

For overexpression studies in *Mtb*, *relE* toxin genes were PCR amplified from gDNA with forward primers producing N-terminal tetra-cysteine tags for *in situ* protein detection. Controlled expression was achieved using anhydrotetracycline (ATc)-inducible overexpression vector, pST-KT, as previously reported [47]. Non-functional $RelE2^{\Delta R61L}$ and $RelE2^{\Delta R81L,Y85F}$ toxin genes were constructed by exchanging G-182 and G-242 with A-254 to T based on earlier published findings [28], using reverse GSPs with SNPs in PCRs (Table 3.2). For co-overexpression of antitoxin genes, P_{myc1}*tetO1* was PCR amplified from pST-KT and then re-cloned into anhydrotetracycline (ATc)-inducible pE2 derivatives, thereby creating duplicate promoter P_{myc2}*tetO2*. *Mtb* RelB and asRelE2 antitoxin genes were then cloned in NotI and HindIII RD sites immediately downstream of P_{myc2}*tetO2*. *Mtb* was next electrotransformed with *relBE2* and *asrelE2* overexpression vectors (Table 3.1), as described before [50] and incubated 37°C on 7H10 with 25-Kan for three-to-four weeks or until colonies became visible.

For dual transcriptional reporter assays, pGREENCHERRY plasmids were constructed, encoding GFP [49], which is regulated by the WT *Mtb relBE2* promoter containing a *Mtb* Crp DNA-binding site (or CBS), (*i.e.* P_{relBE2}CBS), and mCHERRY [48], which is controlled by the convergent *Mtb asrelE2* promoter containing another CBS (*i.e.* $P_{asrelE2}$CBS). Initially, the constitutive promoter

P_{smyc} was excised from pCHERRY3 and replaced with 120-bp $P_{asrelE2}$CBS intervening in between *asrelE2* and *rv2867c*. The mCHERRY gene was PCR amplified using GSPs in Table 3.2 below and re-cloned into pCHERRY3 to add the C-terminal tag (*i.e.* ADSHQRDYALAA) encoded by *Mtb* SsrA (*i.e.* MTB000042). This fusion tag enhances mCHERRY degradation [51, 52]. The 105-bp P_{relBE2}CBS 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) [49], was PCR amplified, producing another fusion C-terminal SsrA decay tag, and cloned downstream of P_{relBE2}CBS at ClaI and SpeI RD sites to make dual transcriptional reporter pGREENCHERRY^{WT} listed in Table 3.1. For the construction of non-functional mutant pGREENCHERRY, P_{relBE2}CBS and $P_{asrelE2}$CBS were PCR amplified, making Mut CBSs. Specifically, the left arm of P_{relBE2}CBS (*i.e.* tGAGa) was mutated to tGGAa, and the left arm of $P_{asrelE2}$CBS (*i.e.* cGACg) was mutated to cGCAg, ablating Crp-binding [5, 53]. Mut $P_{asrelE2}$CBS and P_{relBE2}CBS PCR amplicons were cloned into pGREENCHERRY, and reporters were electroporated into WT *Mtb* H37Rv.

Table 3.2: Oligonucleotides used in this studies.

Oligo function and gene(s) or region of interest	**Forward Primer (5'-to-3')**	**Reverse Primer (5'-to-3')**
Allelic Exchange		
relE2-up	CCCAAAGCGGCCGCCGGTGT GATCACCACGCC	TTTGGGGGTACCTCAGTGGGG GCGTCGCG
relE2-down	CCCAAATCTAGAAACTCACCG ACGGGCGCT	TTTGGGCATATGTTACTAGTTG TACGGGGCGAAGTGATC
relE2-KO	GTGCCTTACACCGTGCGG	CTATCGGCGGTAGATGTCC
asRelE2-up	CCCAAACATATGGCGGCCGC CGGTGTGATCACCACGCC	TTTGGGGCTAGCCTATCGGCG GTAGATGTCC
asrelE2-down	CCCAAATTCGAATCAGTCCAG CAACACCGTC	TTTGGGTCCCGGATTTCTAGAT GTACGGGGCGAAGTGATC
asrelE2-KO	AACTCACCGACGGGCGCT	AGCTGCGGGCATATCAGC
Ectopic Induction		

P_{myc2}*tetO2*	CCCAAATCTAGAGGATCGTCG GCACCGTCA	TTTGGGAAGCTTTTAATTAAGC GGCCGCATCGATGGATCGTGC TC ATTTCGG
relE2$_{WT}$	CCCAAAGGATCCGGCGGCTG TTGTCCCGGCTGTTGTGGCGG CGTGCCTTACACCGTGCGGT	TTTGGGGATATCCTATCGGCG GTAGATGTC
relE2$_{\Delta R61L}$	CCCAAAGGATCCGGCGGCTG TTGTCCCGGCTGTTGTGGCGG CGTGCCTTACACCGTGCGGT	TTTGGGGATATCCTATCGGCG GTAGATGTCCGCGCGGTGATC GACGCGCAGGATCACTACCGT TGTGTGCTCGTCGTCAATCCG GTACAGCAGGAGGTACGTTCC GCGACGCG
relE2$_{\Delta R81L,\Delta Y85F}$	CCCAAAGGATCCGGCGGCTG TTGTCCCGGCTGTTGTGGCGG CGTGCCTTACACCGTGCGGT	TTTGGGGATATCCTATCGGCG GAAGATGTCCGCGAGGTGATC GACGCGCAGGAT
relB2$_{WT}$	CCCAAAGCGGCCGCAATGCG GATACTGCCGATTTC	TTTGGGAAGCTTTCAGTGGGG GCGTCGC
asrelE2-1$_{WT}$	CCCAAAGCGGCCGCAGCAAT ACCGTCCACGCCA	TTTGGGAAGCTTGTGCCTTACA CCGTGCGG
asrelE2-2$_{WT}$	CCCAAAGCGGCCGCAGCAAT ACCGTCCACGCCA	TTTGGGAAGCTTCAACTCACCG ACGGGCGCT
asrelE2-3$_{WT}$	CCCAAAGCGGCCGCCTATCG GCGGTAGATGTCCG	TTTGGGAAGCTTGTGCCTTACA CCGTGCGG
his-relB2-his	CCCAAAGCTAGCATGCGGATA CTGCCGATTTC	TTTGGGCTCGAGGTGGGGGCG TCGCGGGA
relE2-hsv	CCCAAAGCATGCTCATGCCTT ACACCGTGCGG	TTTGGGGCGGCCGCTCGGCG GTAGATGTCCG
his-rnaseIII	CCCAAAGCTAGCATGATCCGG TCACGACAAC	TTTGGGTTAATTAATTAGGCGG AGGTTTTGCC
his-crp	CCCAAAGCTAGCGTGGACGA GATCCTGGCC	TTTGGGTTAATTAATTACCTCG CTCGGCGGG
5'-,3'-DIG Labeled Riboprobes		
relB2	CAGUACAGCGUCUCCUGCAACGAUUCCCACUCGUCGGCGCCGA CCAGAAC	
relE2	UUGCCCACCCGCAGGGGCUCGCGCGACAGAUCGCCGAACGCG AAUUCGAC	

asRelE2	GUCGAAUUCGCGUUCGGCGAUCUGUCGCGCGAGCCCCUGCGGGUGGGCAA	
5S rRNA	AUCGGCGCUGGCAGGCUUAGCUUCCGGGUUCGGAAUGGGACCGGGCGUUU	
T7 *In Vitro* Transcribed RNA		
T7 *relE2*	CGAAATTAATACGACTCACTATAGGGAGAGTGCCTTACACCGTGCGG	CTATCGGCGGTAGATGTC
T7 asRelE2-3	CGAAATTAATACGACTCACTATAGGGAGAGCAATACCGTCCACGCCA	GTGCCTTACACCGTGCGGTT
EMSA DNA probe primers and oligos		
WT P_{serC}CBS	AGGGCTTGCATGTGAGCTT	GCCATCAGGGTAGTGAGG
WT P_{relBE2}CBS	CCAGCAAACCTGAGACGC	ACCGCCATACTAGGCCGT
WT $P_{asrelE2}$CBS	GCTGACGACGTCCTGTGC	TAACCCATTCTTTCAAGACC
Mut P_{serC}CBS	AGGGCTTGCATGGTAGCTTGTTCACACTACGCGCCTGTGCCGGCAGGCTTTCGTTCCGCTCGAGTCGCCGCGGTTCGGTACCCCTCACTACCCTGATGGC	
Mut P_{relBE2}CBS	CCAGCAAACCTGGAACGCCGCGCACAAAGTGCGAAACCACTGGAAGGTGAGCCCTAATTTAGGGCTGAGCAGGACCTGTATAACGGCCTAGTATGGCGGT	
Mut $P_{asrelE2}$CBS	GCTGACGCAGTCCTGTGCACGGCGATGCGGTCTGGCAAGTGTCGGCGCTGCCAGTACCTCTCGATGCCAACAGGATCGTCGGTCTTGAAAGAATGGGTTA	
Transcriptional Reporter		
WT $P_{asrelE2}$CBS	CCCAAATCTAGACGAGCTGCGGGCATATCA	TTTGGGGGATCCAACCCATTCTTTCAAGACCG
Mut $P_{asrelE2}$CBS	CCCAAATCTAGACGAGCTGCGGGCATATCAGCGCTGACGCAGTCCTGTGCACGGCGATGCG	
mcherry-ssrA	CCCAAAGGATCCGGAGGAATCACTTCGCAATGGTCTCGAAGGGCGAG	TTTGGGAAGCTTTCAGGCGGCCAGGGCGTAGTCGCGCTGGTGCGAGTCGGCCTTGTACAGCTCGTCCATG
WT P_{relBE2}CBS	CCCAAAAAGCTTAAGCACCAGCAAACCTGAG	TTTGGGACTAGTATCGATACCGCCATACTAGGCCGT

Mut P_{relBE2}CBS	CCCAAAAAGCTTAAGCACCAG CAAACCTGGAACGCCGCGCA CAAAGTGCG	
phgfp-ssrA	CCCAAAATCGATGGAGGAATC ACTTCGCAATGAGTAAAGGAG AAGAACTT	TTTGGGACTAGTTCAGGCGGC CAGGGCGTAGTCGCGCTGGTG CGAGTCGGCTTTGTATAGTTCA TCCATGCC
5'/3' RACE		
5' RNA Adaptor	GCUGAUGGCGAUGAAUGAAC ACUGCGUUUGCUGGCUUUGA UGAAA	
3' Oligo$(dT)_{20}$		TGGCGATGAATGAACACTGCG TTTGCTGGCTTTGATGAAA$(T)_{20}$
5' RNA Adaptor F1	CCCAAAGGATCCGCGTTTGCT GGCTTTGATGAAA	
3' Oligo $(dT)_{20}$ R1		TTTGGGGATATCGCGTTTGCTG GCTTTGATGAA
3' *relE2* F1	CCCAAAGGATCCGTGCCTTAC ACCGTGCGG	
5' *relE2* R1		TTTGGGGATATCTATCGGCGGT AGATGTCCG
3' *relB2* F1	CCCAAAGGATCCATGCGGATA CTGCCGATTT	
5' *relB2* R1		TTTGGGGATATCTCAGTGGGG GCGTCGCG
3' asRelE2 F1	CCCAAAGGATCCGCAATACCG TCCACGCCA	
5' asRelE2 R1		TTTGGGGATATCGTGCCTTACA CCGTGCGG
5' asRelE2 R2		TTTGGGGATATCATGGCGGTAT GCGGATAC
Sequencing Primer	TTTGCTGGCTTTGATGAAA	
RT-qPCR		
cya (*rv1264*)	CGTATCCGCAAGGGAGATTAG	CGTCAGGATCATCCACTCTG

crp	GTCCCGAAATCTCCGAACAG	GGCACATCGGTGAAGATGA
relB2	CACAGGACCAGATCACCATC	GATCGACTCCCTGATTCCG
relE2	TTACACCGTGCGGTTCAC	AACGCGAATTCGACCACT
asRelE2-3	AACGCGAATTCGACCACT	TTACACCGTGCGGTTCAC
16S rRNA	GGGTTAAGTCCCGCAACGAG	GCATGTGTGAAGCCCTGGAC
Lrg-47	AAAGGTCCACAGACAGCGTCACTCG	CAGGGGAGCATAATGGGTCTCTGC
Igtp	CCCATGGATTTAGTCACAAAGTTGC	GCTGGTGAGTCACTTTATTCCAGCC
Cftr	ACGCCCCTATGTCGACCAT	ACGACTATTATAGCTCCAATCACAATGA
Atp6v1h	AGCACTGCCTGGCCATACTTCCTG	TTCAACAGCAACACCGCTGCCAC
Nramp1	GCCACTGTGCTAGGTTTGCT	AATGGTGATCAGTACACCGC
β-actin	AGAGGGAAATCGTCGGTGAC	CAATAGTGATGACCTGGCCGT

3.2.3 Functional interaction analyses of *Mycobacterium tuberculosis* RelBE2/asRelE2 toxin-antitoxin genes.

Mtb relBE2/asrelE2 merodiploid strains shown in Table 3.1 were incubated at 37°C to late-stationary phase (or sixteen days). These cultures were then diluted in 150 mL fresh 7H9-Tw containing 25-Kan to an OD_{600} = 0.10, and 150 μL of 2,000 ng mL^{-1} ATc (Takara™) was added to induce expression of *relBE2/asrelE2* TA genes. Ectopic inductions were carried out at 37°C and ~150 rpm for 20 days in the dark, and every two days, OD_{600}, CFU mL^{-1}, and N-tetracysteine-RelE2 relative fluoresence units (or RFUs) (*i.e.* Excitation / Emission = 508 nm / 528 nm) were assessed. To measure *in situ* fluoresence, up to ten mL ectopically induced cultures were washed three times in TBST (pH 6.50), resuspended in 500 μL of 10% neutral buffered formalin (Sigma-Aldrich) containing 20 μM FlAsH-EDT2 biarsenical labeling reagent (Invitrogen™), and fixed in the dark at 4°C for at least two days. Formalin fixed bacilli were washed twice in BAL wash buffer

per the manufacturer's notes (Invitrogen™) and resuspended in (1X) TBST, pH 6.50. Whole cell fluorescence was measured using Enspire Multimode microplate reader and normalized to OD_{600}.

3.2.4 Physical interaction analyses of *Mycobacterium tuberculosis* RelBE2 toxin-antitoxin proteins.

RelBE2 protein-protein interaction studies were performed essentially, as described before by [26], with several modifications. In brief, *relB2* and *relE2* gene fragments were amplified from WT *Mtb* strain H37Rv gDNA using GoTaq® Green (2X) Master Mix (Promega Corporation) enriched with five percent PCR-grade DMSO (Sigma-Aldrich) per the manufacturer's instructions (Promega Corporation), and cloned into pET28a and pETcoco2, respectively (Table 3.2). DNA constructs were transformed into *E. coli* strain DH5 Alpha (Zymo Research) and transformants were selected from growth on LB agar containing 50-Kan for pET28a and 50-Amp for pETcoco2. Sequenced constructs were transformed into *E. coli* strain BL21(DE3)pLysS (Invitrogen™) cells and selection was carried out overnight by growth in LB broth supplemented with 34-Chlor, and 50-Kan for pET28-*relB2* selection or 50-Amp for pETcoco2-*relE2* selection, or both for co-transformation. Overnight cultures were then diluted one-to-fifty in fresh LB media containing the necessary antibiotics. When pETcoco2-relB2 construct was used, LB media contained 0.01% (v:v) L-arabinose to amplify the plasmid copy number prior to protein induction. Once cultures reached an OD_{600} of ~0.50, protein production was induced by the addition of IPTG to achieve a final concentration of 10 mM. Cultures were incubated for an additional five hour at 150 rpm and 37°C, bacterial cell pellets were collected by brief centrifugation. Crude bacterial cell lysates were obtained using BugBuster® including Benzoase® (Novagen) according to the manufacturer's protocol. Crude whole cell lysates were clarified by centrifugation at 12,500 X *g* for twenty minutes at 4°C and then passed through a 0.20 μM filter. Each milliliter of clarified whole cell lysate was combined with 250 μL pre-washed Ni-NTA His-Bind® Resin (Qiagen) and rocked gently at 4ºC for one hour before packing into a column with five-to-ten mL of bind buffer (100 mM Tris-HCl, 250

mM NaCl, and 5 mM imidazole, pH 7.80). The column was rinsed three times with wash buffer one (*i.e.* 100 mM Tris-HCl and 250 mM NaCl, 10 mM imidazole, pH 7.80), and then three more times with wash buffer two (*i.e.* 100 mM Tris-HCl and 250 mM NaCl, 25 mM imidazole, pH 7.80). Recombinant protein and/or protein complexes were eluted in a stepwise fashion in buffer (*i.e.* 100 mM Tris-HCl, 500 mM NaCl, pH 7.80) containing 50 mM, 125 mM, and 250 mM imidazole. All wash and elution fractions were separated on NuPAGE® 12% Bis-Tris gels (Invitrogen™) in (1X) MES running buffer (Invitrogen™) at 200 V, followed by transfer to 0.2-micron nitrocellulose membranes (BioRad) at 50 V for western blotting. Membranes were then blocked in 4% BSA in TBST (pH 7.60), incubated with primary Penta-His antibody (Qiagen) or anti-HSV-Tag® antibody (Novagen), both diluted 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).

3.2.5 Extraction and purification of *Mycobacterium tuberculosis* total RNA.

Total RNA was isolated from fifty milliliter culture aliquots of *Mtb* were centrifuged at 3,500 X *g* for ten minutes at 4°C, washed twice in TBST (pH 6.50), and resuspended in one milliliter of TRIzol® Reagent (Invitrogen™). Mycobacterial cells were lysed by physical disruption in 1.50 mL screw-cap tubes (USA Scientific) filled with ~200 µL of 0.10 mm zirconia glass beads (BioSpec Products), and subjected to 2400 oscillations for 30 s six times using the Mini-BeadBeater-1 (BioSpec Products), with cooling on ice for at least two minutes in between each round of beadbeating. Following disruption, 200 µL chloroform was mixed by vigorous vortexing for fifteen seconds and then whole cell lysates were centrifuged at 12,500 X *g* for fifteen minutes at 4°C. 500 µL aqueous layer of clarified lysates were transferred to new 1.50 mL microcentrifuge tubes containing 500 µL of cold molecular biology grade isopropanol (Sigma-Aldrich), vortexed briefly, incubated at -20°C for at least overnight, and centrifuged at 12,500 X *g* for fifteen minutes at 4°C to pellet RNA precipitate. RNA pellets were washed once in 80% v/v molecular biology grade

200 proof ethanol (Sigma-Aldrich) in DEPC-treated water (Sigma-Aldrich), briefly dried at room temperature, and treated with 10 Units (U) DNase I (Thermo Scientific™) at 37ºC for one hour. Equivalent volumes of phenol:chloroform (5:1, pH 4.30-4.70) (Sigma-Aldrich) were mixed with DNase I reactions with vigorous vortexing for fifteen seconds and centrifuged at 12,500 X *g* for three minutes at 4ºC. Top aqueous layers were transferred to new 1.5 mL microcentrifuge tubes with ten volumes 80% (v/v) ethanol, 10% v/v 3 M sodium acetate (Sigma-Aldrich), and 0.50 μg mL^{-1} glycogen (ThermoFisher Scientific™) in DEPC-treated water and incubated at -20ºC for at least one day to precipitate total RNA. Following three rounds of DNase I treatment, total *Mtb* RNA was then quantified and qualified using the NanoDrop (ND-1000) UV/VIS spectrophotometer (ThermoFisher Scientific™), and only total RNA samples with absorbance ratios at 260-to-280 nm of 1.90-2.00 were used for downstream gene expression analyses.

3.2.6 Northern blotting analyses of *Mycobacterium tuberculosis* total RNA.

Northern blotting of *Mtb* RNA was performed as recently described [54], using 5'- and 3'-digoxigenin (DIG)-labeled riboprobes listed in Table 3.2 synthesized by IDT DNA Technologies (Coralville, IA). Five micrograms of total RNA samples were heated at 75ºC for five-to-ten minutes in (2X) TBE-Urea Sample Buffer (Invitrogen™), resolved on 6% TBE-Urea gels in (1X) TBE Buffer (Invitrogen™) at 180 volts for thirty-to-forty-five minutes, then transferred to Ambion® BrightStar® positively charged nylon membranes at thirty volts for fifty-to-sixty minutes using the XCell II™ Blot Module (Thermo Fisher Scientific), and crosslinked using the UV Stratalinker® 1800 per the manufacturer's instructions (Stratagene). UV crosslinked transferred membranes were then pre-hybridized with ULTRAhyb™ Ultrasensitive Hybridization Buffer (Thermo Fisher Scientific) for at least one hour at 68ºC before adding riboprobes and incubating overnight at 68ºC with gentle movement. Membranes were rinsed twice with (0.5X) SSC NorthernMax™ Low Stringency Wash Buffer (Invitrogen™) at 68ºC. Membranes were washed and blocked for thirty minutes at room temperature with (1X) DIG Wash and Block Buffers (Roche), and then incubated with 1:2,500 (final concentration) of anti-DIG-AP-conjugate in (1X) DIG Block Buffer (Roche). Northern blots

were developed using DIG Nucleic Acid Detection Kit per the manufacturer's instructions (Roche) and imaged with ChemiDoc^{TM} XRS^{+} (Bio-Rad).

3.2.7 5' and 3' rapid amplification of complementary ends of *Mycobacterium tuberculosis* total RNA:

5'/3' RACE was performed essentially as reported before [55]. 5'/3' RACE was subjected to three pools of RNA: 5' $3PO_4$ primary RNA; 5' PO_4 processed RNA; 5' OH cleaved RNA. For primary transcripts, two microgram of total RNA was incubated with two units of 5' PO_4-dependent riboexonuclease (Lucigen) for sixty min at 30^{o}C to degrade 5' PO_4 RNAs, followed by 100 U of RNA Pyrophosphhydrolase (RppH – NEB) to remove pyrophosphate from 5' $3PO_4$ ends, and then with ten units of T4 RNA Ligase I (ThermoFisher Scientific) and 0.10 mg mL-1 BSA for three hours at 37^{o}C and overnight at 16^{o}C to attach 5' RNA oligo adaptor (Table 3.2) to 5' PO_4 ends. To select for 5' PO_4 processed RNA, equal amounts (μg) total RNA and RNA adaptor were directly incubated with T4 RNA Ligase I and BSA similarly. For selection of 5' OH RNA, two micrograms of total RNA was treated with four units of RppH, then two units of 5' PO_4-dependent riboexonuclease (Lucigen), followed by two units of T4 polynucleotide kinase (ThermoFisher Scientific) at 37^{o}C for thirty minutes to phosphorylate 5' OH ends, and then T4 RNA Ligase I and BSA. After extracting RNA in acid-phenol:chloroform and precipitating overnight in 0.50 μg mL^{-1} glycogen at -20^{o}C, two micrograms of each 5' ligated RNA pool was subsequently incubated with two units of *E. coli* poly(A) polymerase (NEB) at 37^{o}C for thirty minutes to polyadenylate their 3' ends, and reverse transcribed with Oligo$(dT)_{20}$ using Transcriptor First Strand cDNA Synthesis Kit (Roche) at 50^{o}C for one hour. Single-stranded cDNA was column purified (Zymo Research) and PCR amplified with 0.40 μM forward adaptor-specific primer and reverse GSPs (Table 3.2) in GoTaq® Green Master Mix (Promega) and 0.16 mg mL^{-1} PCR grade DMSO added for 5' RACE. 3' RACE was performed essentially the same but with nested forward GSPs and the reverse

Oligo(dT)$_{20}$-specific primer (Table 3.2). 5'/3' RACE PCRs were run on 1.5% agarose gels at ninety-five volts for seventy minutes in (1X) TAE gel purified, cloned into pMIND, and sequenced.

3.2.8 Double stranded RNA degradation assay with *Mycobacterium tuberculosis* RNase III.

N-terminal hexa-histidine tagged *Mtb* RNase III (*i.e.* His-RNase III) was overproduced and purified essentially as described before [56]. *Mtb* strain H37Rv *rnaseIII* was amplified from gDNA, cloned into pETcoco2 and overproduced in *E. coli* str. BL21(DE3)pLysS (Invitrogen^{TM}) at an OD_{600} of 0.50 and 37^{o}C with one millimolar IPTG for five hours. Harvested bacterial were resuspended in BugBuster^{TM} Reagent (Millipore) with EDTA-Free Protease Inhibitor (Roche) and 250 U of Benzoase Nuclease (Novagen), lysed at room temperature, rocking gently for thirty minutes, and centrifuged at 12,500 X *g* for twenty minutes at 4^{o}C. Clarified whole cell lysate was incubated with Ni-NTA His•Bind® resin (Millipore) for one hour at 4^{o}C, rocking gently, and loaded onto a column preequilibrated with ice-cold buffer (*i.e.* Tris-HCl (pH 7.90) and 500 mM NaCl) with 10 mM imidazole. The column was washed with six volumes of ice-cold buffer containing fifty millimolar imidazole. His-RNase III was eluted in three volumes of ice-cold buffer with two-hundred and fifty millimolar imidazole. Elution fractions were pooled into a three kilodalton MWCO Amicon Ultra-15 Centrifugal Filter Unit (Millipore) and dialyzed against at 4^{o}C in Tris-HCl (pH 7.90) and 5% (v:v) glycerol with 500, 250, and then 150 mM NaCl. His-RNase III was resolved on a 12% Bis-Tris Gel (Invitrogen^{TM}), stained with SimplyBlue SafeStain (ThermoFisher Scientific), and estimated to be at least eighty percent pure.

In vitro His-RNase III dsRNA cleavage assays were performed similarly as previously described [57]. Full length *relE2* and *asrelE2-3* dsDNA templates were PCR amplified from *Mtb* strain H37Rv gDNA using GoTaq® Green Master Mix (Promega) with GSPs with additional 5' TAATACGACTCACTATAGGG 3' T7 promoters (Table 3.2), and gel purified. RNA was *in vitro* transcribed using the T7 RiboMAX Express large-scale RNA Production System (Promega) and purified *via* Acid-Phenol:Chloroform (pH 4.50) and overnight ethanol precipitation at -20^{o}C. Four-

hundred ng μL^{-1} (final concentration) of *in vitro* transcribed RNA was mixed with DEPC water and (5X) dsRNA decay buffer (150 mM Tris-HCl (pH 7.60), 250 mM NaCl, 0.50 mM EDTA, and 0.50 mM DTT to create fifty microliter (final volume) reactions, heat denatured at 70°C for ten minutes, and immediately cooled to 4°C. One microgram of His-RNase III and five microliters of 0.1-to-50 millimolar $MgCl_2$ were added on ice. RNase III dsRNA decay reactions, including negative control reactions with one microgram of His-RNase III, 50 millimolar $MgCl_2$, and one microgram of *relE2* or asRelE2-3, were incubated at 37°C for thirty minutes, quenched by adding five microliters of 440 millimolar EDTA, and RNA was extracted with acid-phenol-chloroform and overnight ethanol precipitation as described above. Five microliters of one microgram per microliter dsRNA isolated from RNase III dsRNA degradation reactions was mixed with five microliters of (2X) TBE-Urea Sample Buffer (Invitrogen™), heated to 75°C for five minutes, centrifuged at 6,000 X *g* for three minutes at 4°C, and separated on 6% TBE-Urea gels in 1X TBE Buffer (Invitrogen™) at 180 volts for fifty minutes. Resolved gels were stained in SYBR® Gold (Invitrogen™) for forty-five minutes and then imaged using ChemiDoc™ XRS+ (Bio-Rad).

3.2.9 Electrophoretic mobility shift assay with *Mycobacterium tuberculosis* transcription factor cAMP receptor protein.

The *Mtb* Crp gene was PCR amplified from gDNA and cloned into pETcoco2 at NheI and PacI restriction digest sites, encoding a N-terminal hexa-histidine tag. His-Crp was overproduced in *E. coli* BL21(DE3)pLysS at an OD_{600} = ~0.50 and 37°C with one millimolar of IPTG for four-to-five hours. Cells were pelleted at 3,500 X *g* for ten minutes at 4°C, resuspended in BugBuster™ Reagent (Millipore) with EDTA-Free Protease Inhibitor (Roche) and 250 U Benzoase Nuclease (Novagen), lysed at room temperature, rocking gently for thirty minutes, and centrifuged at 12,500 X *g* for twenty minutes at 4°C. Ni-NTA purification of His-Crp was done essentially as described before [58]. His-Crp elution fractions were then combined into a three kilodalton MWCO Amicon Ultra-15 Centrifugal Filter Unit (Millipore) and dialyzed at 4°C for one hour at 4,500 X *g* in buffer (or 10 mM Na_2HPO4, 2 mM KH_2PO4, 2.7 mM KCl, and 10% (v:v) glycerol, pH 7.40) with 250 mM

NaCL, 125 mM NaCL, then 62.50 mM NaCl to remove excess salt. His-Crp purity was assessed *via* SDS-PAGE on a 12% Bis-Tris Gel in (1X) MES running buffer, quantified using the BCA Assay (ThermoFisher Scientific), and then stored at -20°C until further use.

Electrophoretic mobility shift assays (or EMSAs) with *Mtb* His-Crp were carried out using EMSA Kit with SYBR™ Green (Invitrogen™). dsDNA probes were made from PCR amplification of 100-bp P_{serC}CBS, WT 100-bp $P_{asrelE2}$CBS, and WT 100-bp P_{relBE2}CBS (Table 3.2), while corresponding Qblocks® gene fragments were used for mutant dsDNA probes. In 40 μL, 100 ng of gel purified DNA, one millimolar cAMP (Sigma-Aldrich), and zero-to-five microgram of His-Crp were incubated in EMSA reaction buffer (*i.e.* 150 mM KCl, 0.10 mM DTT, 0.10 mM EDTA, 10 mM Tris, pH 7.60) for thirty minutes at room temperature. Eight microliters of (6X) EMSA Gel Loading Solution was added to each reaction and dsDNA-protein complexes were separated on 6% DNA retardation gels (Novex™) at one-hundred volts for ninety minutes in (0.5X) TBE buffer. Resolved gels were then stained in SYBR™ Green in (1X) TBE buffer, rocking gently for forty-five minutes at room temperature, and imaged using ChemiDoc™ XRS+ (Bio-Rad). EMSAs were repeated (N=3) times and densitometry was used for relative quantification of Crp-DNA-binding affinities of P_{relBE2}CBS and $P_{asrelE2}$CBS similarly as previously shown [59].

3.2.10 *Mycobacterium tuberculosis in vitro* stress and persistence assays.

Mid-to-late stationary phase WT and mutant *Mtb* cultures were diluted to an OD_{600} of 0.05-0.10 in seventy-five milliliters of fresh 7H9-Tw containing 50-Hyg or 25-Kan, and sub-cultured at 37°C and 150 rpm for up to twenty days. In this time, outgrowth was assessed *via* measuring the OD_{600} and enumerating CFU mL^{-1} from plating of ten-fold serial dilutions onto 7H10 agar of culture aliquots every day for up to twenty days. Results represent the means ± 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 as before [43, 60, 61]. In brief, *Mtb* strains were grown in 7H9-Tw plus

50-Hyg or 25-Kan until mid-to-late-log phase, and (N=3) fifty milliliter culture aliquots were briefly centrifuged at 4,500 X *g* for ten minutes at 4°C, washed twice, and resuspended to an OD_{600} of 0.20-0.25 (or 6.50-7.00 Log_{10} CFU mL^{-1}) in (1X) TBST (*i.e.* 20 mM Tris, 150 mM NaCl, and 0.05% (v:v) non-metabolizable tyloxapol). For cAMP studies, fifty milliliters of cell aliquots resuspended in TBST (pH 6.50) were incubated with zero-to-ten millimolar of cell soluble analog dibutyryl cyclic adenosine monophosphate (db-cAMP), rocking gently, at 37°C for up to two days, as published before [5]. At zero hours, four hours, one day, and two days post-db-cAMP-exposure, bacilli were centrifuged at 4,500 X *g* for ten minutes at 4°C, washed twice, and resuspended in TBST, pH 6.50. For assessing intracellular cAMP levels, one milliliter sample aliquots were immediately boiled in 0.1 molar HCl and stored at -80°C until assayed. One milliliter was fixed in 10% neutral buffered formalin (Sigma-Aldrich) for at least two days at 4°C prior to reading GFP (Excitation / Emission = 395 nm / 510 nm) and mCHERRY (Excitation / Emission = 587 nm / 610 nm) RFUs using Enspire Multimode microplate reader (PerkinElmer). One milliliter was serially diluted and plated onto 7H10 agar containing 50-Hyg or 25-Kan when necessary for enumerating CFUs. For low pH stress, fifty milliliter culture sample aliquots were washed and resuspended in TBST (pH 6.50, 5.50, or 4.50) to an OD_{600} 0.20-0.25 (or 6.50-7.00 Log_{10} CFU mL^{-1}) and incubated, rocking gently for up to eight days at 37°C. After zero, one, two, four, and eight days of NS under low pH, bacilli were recovered as described above: one milliliter was prepped for estimating intracellular cAMP levels; one milliliter was fixed in 10% neutral buffered formalin for assessing GFP and mCHERRY RFUs; one milliliter was serially diluted and plated onto 7H10 agar with 50-Hyg or 25-Kan when for determining Log_{10} CFU mL^{-1}. Significant differences in survival of WT and mutant *Mtb* strains were determined through comparing means ± the standard deviation of at least three independent experiments using regular two-way ANOVA with Tukey's post-tests (*i.e.* * = $p<0.05$, ** = $p<0.01$, and *** = $p<0.001$), as previously reported [62].

3.2.11 Measurement of intracellular cAMP in *Mycobacterium tuberculosis*.

Intracellular cAMP levels in *Mtb* were measured using Direct cAMP Enzyme Immunoassay Kit according to the acetylated version of the manufacturer's instructions (Sigma-Aldrich). Sample culture aliquots were recovered and resuspended to approximately $1x10^8$ CFU mL^{-1} in (1X) TBST, pH 6.50, centrifuged at 4,500 X *g* for ten minutes at 4°C, resuspended in 0.10 M HCl, and boiled for ten minutes at 100°C [63]. Whole cell lysates were transferred to 1.50 mL screw-cap tubes (USA Scientific) containing 200 µL 0.10 mm diameter zirconia glass beads (BioSpec Products), and exposed to three rounds of bead beating (*i.e.* 2400 oscillations over thirty seconds) using the Mini-BeadBeater-1 (BioSpec Products), cooling on ice for at least two minutes in between each round. Bacterial debris was removed from centrifugation at 12,500 X *g* for fifteen minutes at 4°C and clarified lysates were kept at -20°C until further use. Intracellular cAMP levels were measured by reading the optical density at 405 nm of one-hundred microliters of immunoassayed whole cell lysates using the Enspire Multimode microplate reader (PerkinElmer). Intracellular cAMP levels were estimated from standard curves generated from reading optical densities at 405 nm of zero-to-twenty pmol mL^{-1} cAMP in 0.10 M HCl, and then cAMP per 10^8 CFU was calculated by dividing pmol cAMP mL^{-1} by CFU mL^{-1} samples, like VanderVen *et al.*, (2015) [64].

3.2.12 Reverse transcription quantitative PCR of *Mycobacterium tuberculosis* and murine total RNA.

For reverse transcription quantitative PCR (or RT-qPCR) gene expression analyses, one microgram of total RNA was heated to 65°C for ten minutes with 2.5 µM reverse GSPs, cooled to 4°C, mixed with twenty units of Transcriptor RT reverse transcriptase and eighty units of RNase inhibitor, and reverse transcribed at 58°C for one hour. No RT (NRT) and no template control (NTC) control RT-qPCR reactions were included. Four microliters of 1:25 and 1:50 dilutions of cDNA were used in 25.00 µL qPCR reactions containing 12.50 µL (2X) SYBR Green I Master Mix (Roche), two microliters of five micromolar forward and reverse GSPs shown in Table 3.2, two microliters of DMSO, and 4.50 µL DEPC-treated water carried out on LightCycler® 480 System

per the manufacturer's details (Roche). GSP sets were optimized through generating standard curves from the amplification of cDNA (*i.e.* Cp values) reverse transcribed from serially diluted early-to-mid-exponential phase total RNA.

Figure 3.1: Amplification efficiency of *Mtb* and host murine RT-qPCR GSPs.

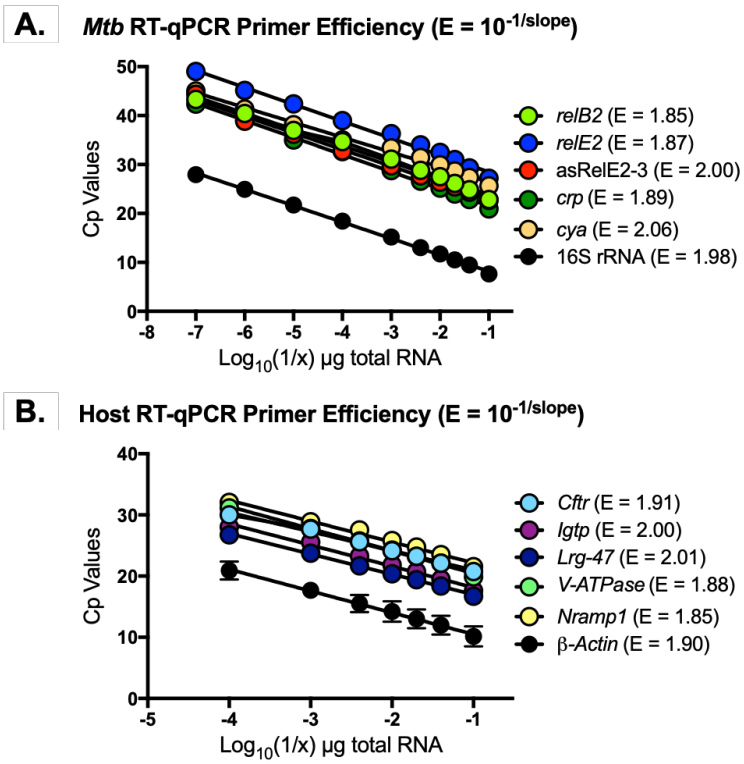

Figure 3.1: RT-qPCR GSP amplification efficiency (E=10^{-1}/slope) of cDNA reverse transcribed from 10-fold serially titrated total RNA isolated from (A.) mid-exponential phase *Mtb* growing in 7H9-Tw and (B.) uninfected WT C57/BL6J host murine lungs. Plotted data represent the means ± the standard deviations of (N=3) independent experiments; slopes determined using linear regression analyses in GraphPad Prism. (A.) Cp values of qPCR reactions of cDNA reverse transcribed from Log_{10} 10.00-to-1.00E-05 µg total RNA was subjected to qPCR reactions. (B.) Cp values of qPCR reactions of cDNA reverse transcribed from Log_{10} 10.00-to-1.00E-02 µg total RNA isolated from uninfected WT C57/BL6J mouse lungs.

Amplification efficiency (*i.e.* E) was measured using a linear regression model (E=$10^{-1/slope}$), and only GSPs with at least eighty-five percent amplification efficiency were used for relative quantification. Genes of interest (GOIs) were normalized to 16S rRNA (or MTB000019) and approximate fold induction was calculated using the delta-delta Cp method (*i.e.* $E^{\Delta GOI} / E^{\Delta 16S}$ for *Mtb* and $E^{\Delta GOI} / E^{\Delta \beta\text{-}Actin}$ for mice), compared to zero hour controls [65]. Melt curve analyses were likewise performed to confirm qPCR specificity and the mean fold inductions ± standard deviation calculated from (N=3) independent experiments.

3.2.13 Mouse infection and gene expression studies with *Mycobacterium tuberculosis.*

All mouse infection experiments were performed in accordance with NIH guidelines and approval of the Institutional Animal Care and Use Committee at Colorado State University. Six-to-eight week-old female C57BL/6J (WT) mice and IFN-$\gamma^{-/-}$ (or GKO) transgenic mice purchased from Jackson Laboratories were challenged intranasally with approximately five-hundred CFU of *Mtb* strain H37Rv [66, 67]. This was achieved by anesthetizing mice with a ketamine-xylazine solution and delivering around 250 CFU of mid-to-late exponential phase *Mtb* strain H37Rv in 25 μL TBST (pH 6.50) into each nare. *Mtb* infected mice were housed in sterile micro-isolator cages in the ABSL-3 facility at the Infectious Disease Research Cluster at Colorado State University, provided water and food *ad libitum*, and monitored daily for morbidity and mortality. At seven, fourteen, twenty-one, and twenty-eight days post-infection, four-to-five WT and GKO mice were sacrificed by CO_2 inhalation, and their lungs and spleens were immediately harvested. In addition, four WT mice were sacrificed at forty-two days post-infection to assess Log_{10} CFU mL^{-1}. On these days, organs were quickly weighed and halved; one half was immersed in RNAlater® (Ambion®) and stored at -80°C until total RNA was extracted; one half was homogenized using TissueLyser with bead milling and ten-fold serial dilutions of lung and spleen homogenates were plated onto 7H11 agar containing fifty μg mL^{-1} carbenicillin and ten μg mL^{-1} cyclohexamide, and incubated at 37°C for four-to-five weeks or until CFUs became visible.

For *in vivo* gene expression analyses, total RNA was extracted from lungs and spleens of four-to-five WT and GKO infected mice following seven, fourteen, and twenty-eight days of *Mtb* infection. Frozen lung and spleen tissues stored in RNAlater® were thawed and transferred to two milliliter screw-capped tubes containing one milliliter of TRIzol (Ambion™) and half a milliliter of 1.00 and 0.10 mm glass beads, and homogenized through six rounds of bead beating using the Mini-BeadBeater-1 (BioSpec Products), with cooling on ice for at least two minutes in between each cycle. Organ homogenates were mixed with equivalent volumes of Acid-Phenol:Chloroform (pH 4.50) (ThermoFisher Scientific) by vigorous vortexing for fifteen seconds and then centrifuged

at 12,000 X *g* for twenty minutes at 4°C. The aqueous phase was added to ten volumes of eighty percent 200 proof ethanol, ten percent three molar sodium acetate, and 0.50 μg μL^{-1} glycogen, vortexed, and then incubated at -20°C overnight. Total RNA from infected lungs and spleens was purified with three-to-six rounds of DNase I treatment and then quantified and qualified using the NanoDrop (ND-1000) UV/VIS Spectrophotometer (ThermoFisher Scientific). Relative differences in expression of mouse and *Mtb* genes were calculated by comparing qPCR Cp values of GOIs from time-matched WT and GKO infected lungs and spleens, normalized to β-actin for mice and 16S rRNA for *Mtb*.

3.3 Results.

3.3.1 *Mycobacterium tuberculosis* toxin RelE2 is co-regulated through cognate protein-protein interactions and a novel asRNA, asRelE2.

Type II TA systems are defined by regulation of the toxin *via* neutralization interaction by the antitoxin. To begin to understand the mechanisms involved in co-regulating RelBE2, we first sought to confirm the functional and physical interactions of the RelB2 antitoxin and RelE2 toxin. *In situ* overexpression of *relE2* inhibits *Mtb* growth, resulting in the expected bacteriostatic phenotype. Co-overexpression of *relB2* under similar ectopic induction conditions did not produce a discernable change in growth in Figure 3.2A. Notably, the bacteriostatic phenotype resulting from ectopic induction of *relE2* is rescued by the co-induction of *relB2*, demonstrating a functional interaction between this cognate TA protein pair (Figure 3.2).

Overproduction of RelE2 in *Mtb* was detected by monitoring the N-tetracysteine-tagged toxin protein detected throughout the ectopic induction (Figure 3.2B). To assess a physical interaction between RelB2 and RelE2 proteins, these homologs were co-purified and identified by Western blotting shown in Figure 3.2C. When His-RelB2 was produced and subjected to Ni^{2+} affinity column chromatography, it was only found in the elution fractions, with at least 50 mM imidazole. In contrast, when RelE2-HSV was overproduced alone and subjected to Ni^{2+} affinity column chromatography, it was only detected in the unbound wash fractions, revealing that it is

not retained on the column. However, when His-RelB2 and RelE2-HSV were co-overproduced, His-RelB2 and RelE2-HSV were co-eluted, further substantiating that they physically interact *in situ.* These results demonstrated that *Mtb* RelBE2 acts as a *bona fide* type II TA homolog.

Figure 3.2 *Mtb relBE2* functions as a *bona fide* type II TA system.

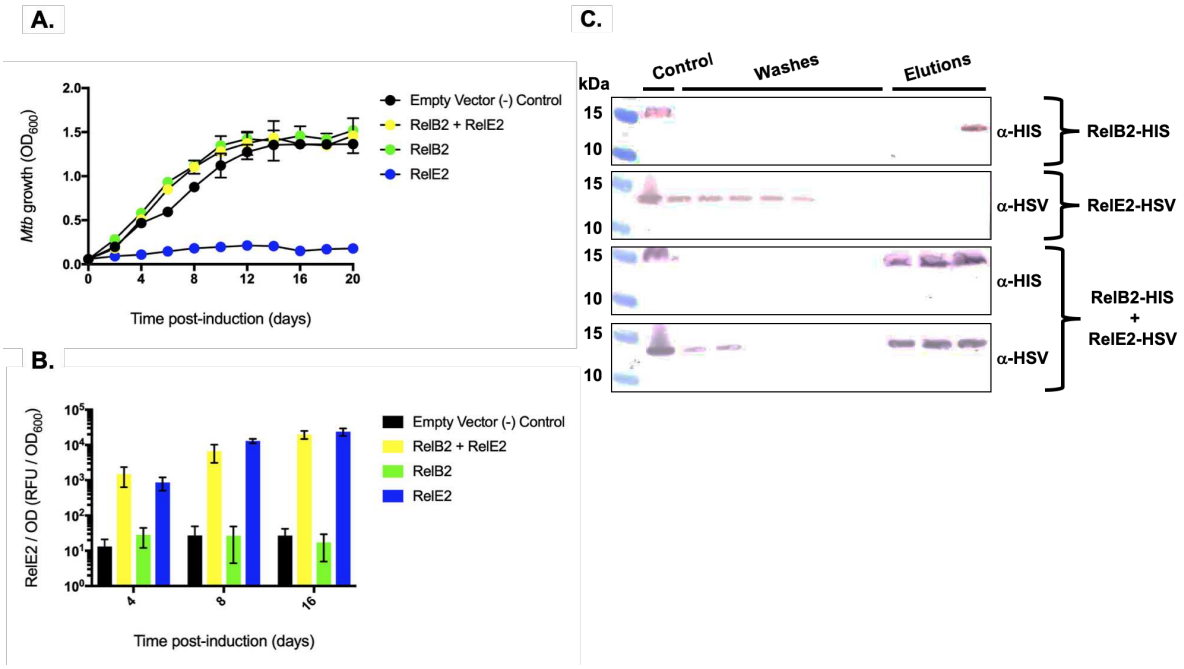

Figure 3.2: *Mtb* RelB2 and RelE2 functionally and physically interact *in situ*. Plotted data are the means ± the standard deviations of (N=3) independent experiments. (A.) Growth of WT *Mtb* in 7H9-Tw containing 2,000 ng mL^{-1} ATc overexpressing empty vector negative control pST-KT, N-tetracysteine-RelE2, RelB2, or N-tetracysteine RelE2 with RelB2 find that arrested growth from the overproduction of WT N-tetracysteine RelE2 is rescued by the co-overproduction of RelB2. (B.) Corresponding N-tetracysteine-RelE2 RFU (Ex / Em = 508 / 528 nm) per OD_{600} reveal that toxin RelE2 is overproduced during arrested and rescued growth. (C.) In *E. coli* BL21(DE3)pLysS clarified whole cell lysates, His-RelB2 is uniquely found in the elution fractions (≥50 mM imidazole); RelE2-HSV in contrast is solely detected in the wash fractions when ectopically overproduced alone with IPTG. Upon co-overproduction, RelE2-HSV is shown to co-elute with RelB2-HIS, indicating that they physically interact *in situ*. These data represent N=3 separate experiments.

Normally only type I TA systems are regulated by an antisense RNA mechanism. Based on our previously published observations though [26], which indicated that *Mtb* TA systems are post-transcriptionally regulated, we investigated asRNA as a co-regulatory mechanism controlling *relE2* mRNA levels. Accordingly, Northern blot analysis was employed in combination with 5'/3' RACE on total RNA isolated from early-exponential phase *Mtb* (*i.e.* OD_{600} = 0.10-0.30) using 5'/3' dual DIG-labeled riboprobes detecting sense and antisense *relBE2* expression. Two mRNAs that are estimated to be 282-NT and 549-NT in size, were identified using the *relB2*-specific riboprobe,

with the smaller *relB2* mRNA appearing much more abundant (Figure 3.3). Similarly, two low abundant mRNAs, approximately 264-NT and 549-NT in length, that represent *relE2* and *relBE2*, respectively, were identified with the *relE2*-specific riboprobe in Figure 3.3B & Figure 3.4D. Moreover, two novel asRNAs to *relE2* were discovered, found to be 264-NT and 512-NT in length, that were shown to be expressed at similar levels to *relB2* and *relBE2*, respectively.

Figure 3.3: *Mtb relBE2*/*asrelE2* forms a novel tripartite TA locus.

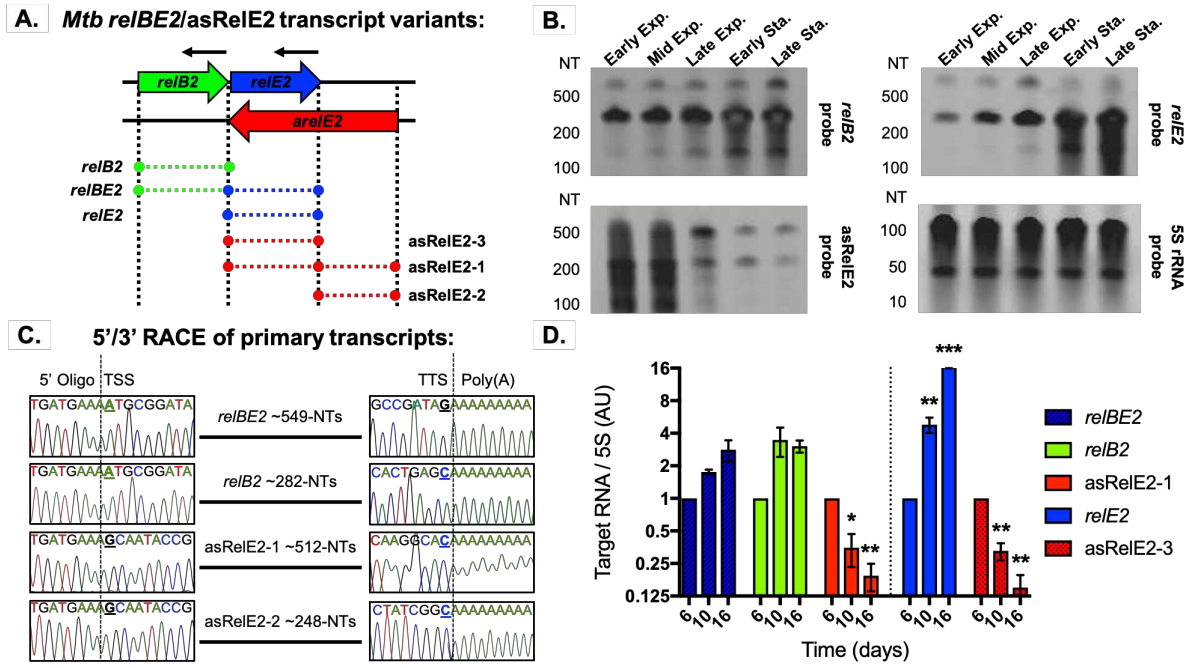

Figure 3.3: Expression profiles of *relBE2*/asRelE2 in growth of Mtb in 7H9-Tw. (A.) Schematic of the chromosomal context of *relBE2/asrelE2* tri-partite TA cassette encoded in WT *Mtb* H37Rv. (B.) Representative northern blots of 2.00 μg total RNA isolated from WT *Mtb* growing in 7H9-Tw at early-exponential phase (OD_{600} = 0.10-0.30), mid-exponential phase (OD_{600} = 0.40-0.60 or 6 d), late-exponential phase (OD_{600} = 0.70-0.90), early-stationary phase (OD_{600} = 1.00-1.20 or 10 d), and late-stationary phase (OD_{600} = 1.30-1.50 or ≥16 d) probing against *relE2*, *relB2*, asRelE2, and 5S rRNA. (C.) Consensus mapping of 5' (N=10) and 3' (N=20-30) ends of endogenous early-exponential *relBE2*/asRelE2 primary transcripts: (1) 549-NT *relBE2* mRNA 5' end in *relB2* start codon and 3' end in *relE2* stop codon; (2) 282-NT *relB2* mRNA 5' end in *relB2* start codon and 3' end near *relE2* start codon; (3) 512-NT asRelE2-1 transcript 5' end antisense 248-NT downstream of *relE2* stop codon and 3' end complementary to *relE2* stop codon; (4) ~248-NT asRelE2-2 represents the non-complementary downstream truncated variant of full-length asRelE2-1 transcript. (D.) Relative quantification and differences of the mean of ± the standard deviation of (N=3) independent experiments profiling *relBE2*/asRelE2 transcript variants in mid-exponential phase (6 d), early-stationary phase (10 d), and late-stationary phase (16 d). WT *Mtb relBE2, relB2*, *relE2*, asRelE2-1, and asRelE2-3 transcript levels were quantified by densitometry, normalizing target transcripts to corresponding 5S rRNA blots (AU), similarly as previously reported [4]. Relative to d 6, significant differences were assessed using a two-way ANOVA with Tukey's multiple comparisons post-tests (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001).

5'/3 RACE was applied for mapping the ends of sense and antisense RNAs identified with *Mtb relBE2*/*asrelE2* riboprobes. This led to the identification of *relBE2* mRNA variants and novel asRNAs to *relE2* from total RNA isolated from early-log phase *Mtb*. Sanger sequencing of 5'/3'

RACE of primary (*i.e.* 5' $3PO_4$) RNA pools mapped TSSs of a 549-NT amplicon corresponding to *relBE2* and a 282-NT product corresponding to *relB2* to the start codon of *relB2*, and the 3' ends to G-3178085 and C-3177820 in the stop codons of *relE2* and *relB2*, respectively (Table 3.3 and Figure 3.2). 5'/3' RACE of the corresponding processed (*i.e.* 5' PO_4) mRNA pools produced amplicons of *relE2* variants, while concomitant 5'/3' RACE of complementary *relBE2* expression amplified PCR products from a full-length primary transcript, asRelE2-1 (*i.e.* 512-NT), and a processed asRNA, asRelE2-3 (*i.e.* 264-NT in length). Sanger sequencing of ten-to-thirty RACE clones identified the TSS and the 3' end of asRelE2-1 as G-3178333 and C-3177822, respectively.

Table 3.3: 5'/3' RACE sequencing of *relBE2*/asRelE2 RNAs from early-log phase *Mtb*.

Primary (5' $3PO_4$) Transcripts

		Consensus 3' ends of (N=10-20) RACE Clones					
	TSS	C-3178080	G-3178081	A-3178082	T-3178083	A-3178084	G-3178085
relBE2	A-3177537	1	0	0	1	5	13
	TSS	C-3177815	T-3177816	G-3177817	A-3177818	G-3177819	C-3177820
relB2	A-3177537	1	3	0	11	3	2
	TSS	C-3177817	T-3177818	C-3177819	G-3177820	C-3177821	C-3177822
asRelE2-1	G-3178333	3	0	4	1	0	12

Processed (5' PO_4) Transcripts

	Consensus 5' ends of (N=10-20) clones				**Consensus 3' ends of (N=10-20) clones**			
	G-3177819	C-3177820	G-3177821	G-3177822	A-3178082	T-3178083	A-3178084	G-3178085
relE2	4	2	0	14	5	3	0	12
	T-3178084	C-3178085	G-3178086	T-3178087	G-3177819	C-3177820	G-3177821	G-3177822
asRelE2-3	1	3	4	12	4	1	0	15

Mapping the 5' and 3' ends of processed asRNA found that asRelE2-3 complements *relE2* and is cleaved from asRelE2-1. Sequencing of ten-to-twenty RACE PCR products of 5' PO_4 RNAs in particular identified six NTs upstream (*i.e.* 5' UGAGCG 3') as the 5' end of *relE2*, and

corresponding complementary NTs as the 3' end of asRelE2. Antisense dsRNA-mediated decay of toxin mRNA is found to be essential for logarithmic growth and survival of tubercle bacilli [68], and our Northern blot analyses demonstrated that *relE2* and asRelE2 transcript levels are inversely regulated with respect to growth phase (Figure 3.3B). Profiling the expression of the novel tripartite *Mtb relBE2*/asRelE2 TA locus from early-exponential-to-late-stationary phase (*i.e.* OD_{600} of 1.30-1.50 or at sixteen days), *relE2* mRNA levels were determined to increase significantly more than twenty-fold over time in *Mtb*, relative to 5S rRNA (*i.e.* Rrf or MTB000021) expression. In contrast, asRelE2 expression levels were found to start to decline sharply during late-exponential phase (OD_{600} = 0.80-1.00 or six days) and decreased ten-to-twenty-fold in late-stationary phase. This was concomitant with high basal *relB2* mRNA levels increasing two-fold in stationary phase *Mtb* growth, which shows that asRelE2 mediates *relE*2 mRNA decay.

3.3.2 *Mycobacterium tuberculosis* asRelE2 silences *relE2* translation *in situ*.

To experimentally determine if asRelE2 functions to target and silence *relE2* expression *in situ*, we assessed co-expression of *relE2* and asRelE2 from separate ATc-inducible P_{myc}tetO promoters in *Mtb*. Non-functional mutant RelE2 proteins harboring AA-substitutions at arginine-(R)61, and R81 and tyrosine-(Y)85, corresponding to the critical catalytic residues in the prototypic *E. coli* RelE toxin [28], were engineered and included to serve as RelE2 non-functional negative controls. Accordingly, ectopic induction of $RelE2^{WT}$, $RelE2^{\Delta R61L}$, or $RelE2^{\Delta R81L,Y85F}$ alone resulted in significantly increased RelE2 RFUs *in situ* (*i.e.* N-tetracysteine RelE2 per OD_{600}), allowing direct monitoring of RelE2 overproduction. In contrast, co-overexpression of WT *relE2* with asRelE2-1 or asRelE2-3 inhibited the production of RelE2, demonstrating that complementary *relE2* asRNAs inhibited translation of *relE2*. This is supported by the co-overexpression of asRelE2-2, wherein the non-complementary fragment of asRelE2 did not seem to affect RelE2 overproduction. The ability of asRelE2 to rescue the inhibited growth phenotype of *Mtb* as a result of RelE2 production was assessed by co-overexpression of *relE2* and asRelE2. As expected, the ectopic production

of WT RelE2 potently inhibited *Mtb* growth, while the production of mutant RelE2$^{\Delta R61L}$ and RelE2$^{\Delta R81L,Y85F}$ did not affect mycobacterial growth over twenty days (Figure 3.4).

Figure 3.4: *Mtb* asRelE2 functions as a type I asRNA antitoxin *in situ*.

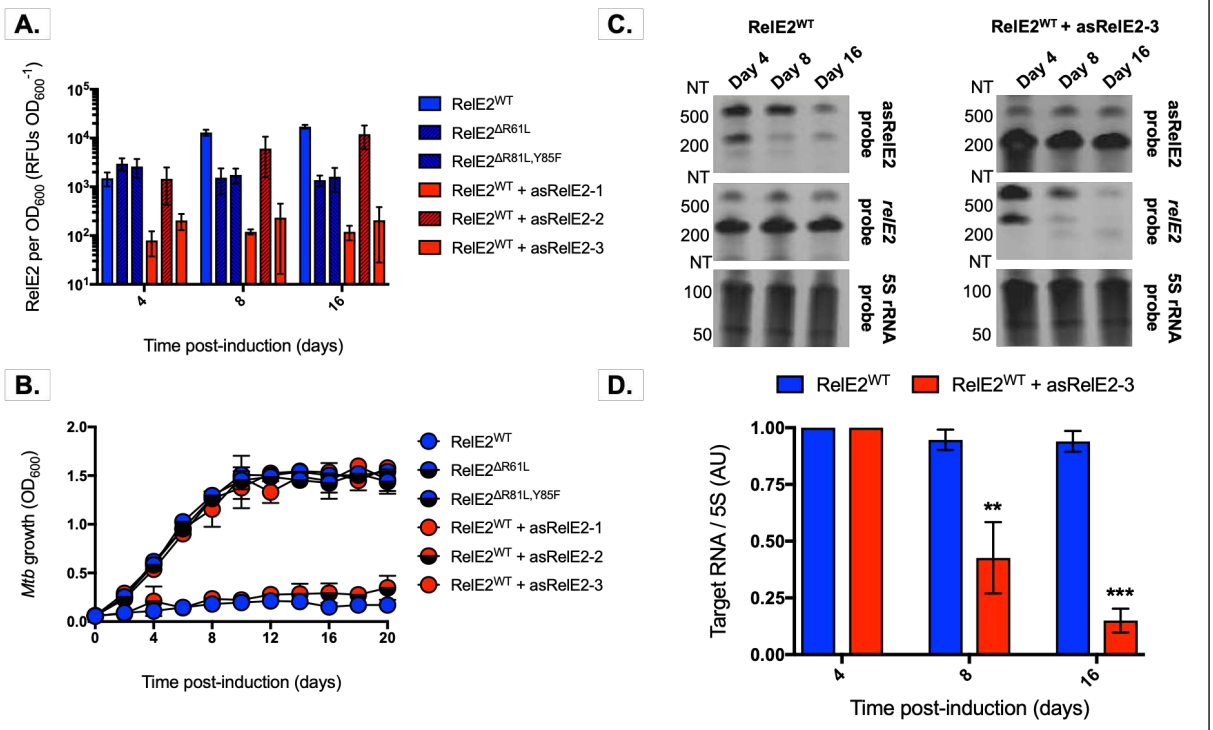

Figure 3.4: Ectopic induction of *relE2*/*asrelE2* TA genes in *Mtb*. Electrotransformants harboring *relE2* and *asrelE2* overexpression vectors in late-stationary phase were sub-cultured for 20 d in 7H9-Tw medium containing 2,000 ng mL^{-1} ATc (Day 0 OD_{600} = 0.05-0.10). Plotted data represent the mean ± the standard deviation of (N=3) independent experiments. (A.) *In situ* RFU (Ex / Em = 508 / 528) of N-tetracysteine-tagged (*i.e.* GGCCPGCCGG)-RelE2 proteins in formalin fixed mycobacteria stained with biarsenical detection reagent normalized to OD_{600} 4, 8, and 16 d post-induction. (B.) Growth (or OD_{600} at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 d) of *Mtb* transformants overexpressing WT *relE2* and *asrelE2* genes over 20 d. (C.) Representative northern blots of total RNA isolated from *Mtb* strains overexpressing WT *relE2* and WT *relE2* with asRelE2 at 4, 8, and 16 d post-ectopic-induction. (D.) Relative quantification and differences using densitometry, normalizing WT *relE2* and asRelE2 target RNA to corresponding 5S rRNA blots (AU) over time. Relative to d 4, significant differences were assessed using a two-way ANOVA with Tukey's multiple comparisons post-tests (**, p-value < 0.01; ***, p-value < 0.001).

Co-overexpression of asRelE2-1 or asRelE2-3 with WT *relE2* restored normal exponential phase growth. In contrast, co-overexpression of WT *relE2* with non-complementary asRelE2-2 did not reverse growth inhibition demonstrated with *relE2* alone. These observations were supported by Northern blotting of *relE2* and asRelE2 transcripts, revealing that *relE2* was downregulated ten-to-one-hundred-fold with the co-overexpression of asRelE2-3 in Figure 3.4C and Figure 3.4D below. These findings collectively indicate that asRelE2 acts to silence *relE2* translation *in situ*, and that inhibition of RelE2 production requires the complementary *relE2*

portion for activity. This is consistent with *in silico* analyses, which predict that the complementary portion of asRelE2 (*i.e.* 1-150-NT) interact most favorably with *relE2* (ΔG = -226.525 kcal mole^{-1}; p-value < 0.0001) [69].

3.3.3 *Mycobacterium tuberculosis* asRelE2 mediates RNase III *relE2* mRNA degradation *in vitro*.

Figure 3.5: *Mtb* asRelE2 targets *relE2* for decay by *Mtb* RNase III *in vitro*.

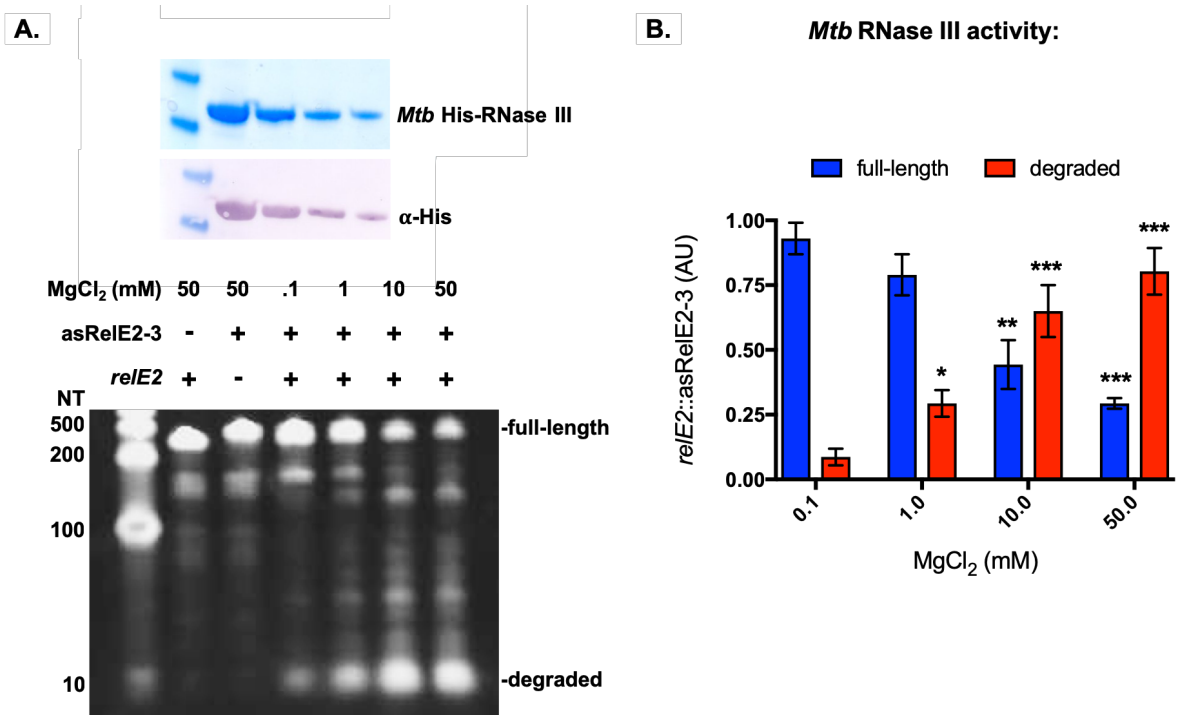

Figure 3.5: *Mtb relE2*::asRelE2-3 dsRNA decay by *Mtb* RNase III. (A.) Degradation of *in vitro* transcribed *relE2*::asRelE2-3 dsRNA hybrids by dialyzed recombinant (6X) Histidine-tagged *Mtb* RNase III (His-RNase III) >80% pure from Coomassie stained 12% SDS-PAGE gels. His-RNase III activity against *relE2*::asRelE2-3 was assessed at 37ºC for 30 min, as described in methods and materials, supplying 0.10-to-50.00 mM Mg^{2+} as a co-factor. Control reactions, with 1 μg His-RNase III, 50.00 mM $MgCl_2$, and 1 μg *relE2* or asRelE2-3, were included to normalize amounts (AU) of full-length *relE2* and asRelE2-3 substrates, and ~10-15-NT dsRNA decay products resolved on 6% TBE-Urea gels, as reported before [2]. (B.) *Mtb* RNase III activity plotted data represent the mean of ± the standard deviation of (N=3) separate experiments. Relative amounts (AU) of full-length substrates and degradation products were normalized to 0.10 mM $MgCl_2$ dsRNA decay reactions and significant differences were assessed using a two-way ANOVA with Tukey's multiple comparisons post-tests (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001).

To assess if *relE2* is degraded by *Mtb* RNase III or Rnc in an asRelE2-dependent manner, *relE2* and *asRelE2*-3 RNAs that were *in vitro* transcribed from gel purified PCR products using T7 phage promoters and incubated in the presence of purified recombinant *Mtb* His-Rnc. Double-stranded RNA decay reactions were activated with addition of zero-to-fifty millimolar magnesium

chloride, incubated at 37°C for thirty minutes prior to quenching with EDTA, and resolved on six percent TBE-Urea gels. Negative (-) control reactions were included with either full-length *relE2* or asRelE2-3, one microgram of His-Rnc, and fifty millimolar magnesium chloride, showing little-to-no degradation. In contrast, when *reE2* and asRelE2-3 were included in decay reactions, full-length transcripts seem to decrease concomitantly with the appearance and accumulation of Rnc dsRNA degradation products (*i.e.* 10-15-NTs in size) in Figure 3.5. These decay products were found to increase significantly in a magnesium chloride-dependent manner, degrading up to 75% of *relE2*::asRelE2-3 dsRNA hybrids in the presence of fifty millimolar magnesium chloride shown in Figure 3.5 above. This observed Rnc-dependent decay of *relE2*::asRelE2 dsRNA hybrids demonstrates that *relE2* is post-transcriptionally regulated by *Mtb* RNase III in an asRelE2-dependent manner *in vitro*.

3.3.4. *Mycobacterium tuberculosis* cAMP receptor protein regulates *relBE2* and *asrelE2* co-expression.

The expression of *relBE2* has been shown to be differentially regulated by alternative Mtb transcription factor Crp [63]. Therefore, an *in silico* search for CBSs flanking *relBE2* and *asrelE2* was performed, as reported before [70]. Bioinformatics analyses putatively identified two CBSs, one located 75-90-NT upstream of the *relBE2* TSS (*i.e.* 5'-t**GAG**acgccgcg**CAC**a-3'), and the other located 80-95-NT upstream of the *asrelE2* TSS (*i.e.* 5'-c**GAC**gtcctgtg**CAC**g-3'). These CBSs are centered at -82.50- and -87.50-NT, respectively, and their left and right arms share a high degree of sequence similarity with others' reported consensus CBSs [58, 71].

To determine if these CBSs regulate *relBE2* and asRelE2 expression, we next performed EMSAs using recombinant *Mtb* His-Crp and dsDNA probes representing the IGRs flanking the *relBE2*/*asrelE2* tripartite TA locus. To monitor Crp DNA-binding, the well characterized intergenic CBS that is known to regulate the expression of *Mtb serC* (or *rv0884c*) was included as a positive control [1]. Following thirty-minute incubations, binding reactions resolved on 6% DNA retardation gels revealed that His-Crp directly interacts with predicted CBSs based on the significantly altered

mobility of WT P_{relBE2}CBS and $P_{asrelE2}$CBS dsDNA fragments in a Crp-dependent manner in Figure 3.6A. Compared to the free DNA in control binding reactions without His-Crp, nearly all of the P_{serC}CBS dsDNA probe was bound in the presence of one microgram of His-Crp, and this was reversed in binding reactions with mutant P_{serC}CBS.

Figure 3.6: *Mtb* Crp regulates convergent *relBE2/asrelE2* expression *in vitro*.

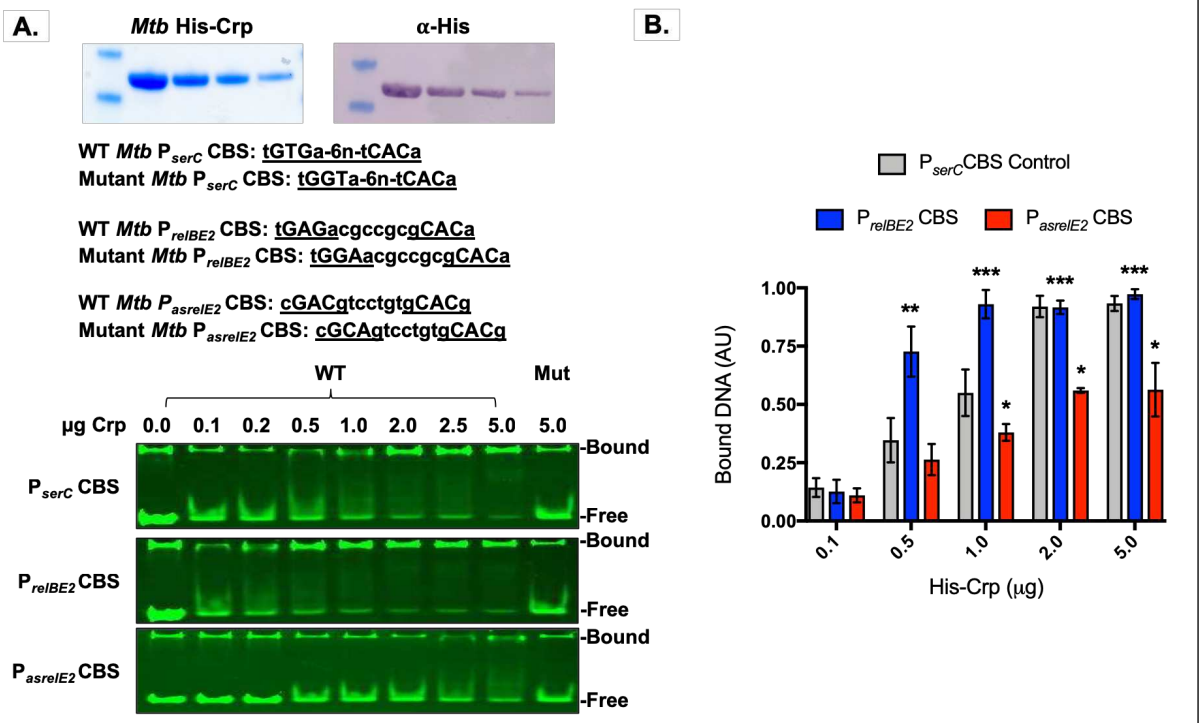

Figure 3.6: *Mtb* Crp binding *in vitro* to putative CBSs regulating convergent *relBE2/asrelE2* expression. (A.) Representative EMSAs of (N=3) independent experiments showing 0.00-5.00 µg recombinant (6X) Histidine-tagged *Mtb* Crp (His-Crp) interactions with 100 ng DNA probes and 1 mM cAMP: WT P_{serC} CBS, 100 bp intergenic region between *serC-rv0885c* containing well-characterized *Mtb* CBS [1] used to qualify strength of His-Crp interactions with putative CBSs flanking *relBE2/asrelE2*; WT P_{relBE2} CBS, 100 bp intergenic region between *rv2864c-relB2* containing putative CBS centered at -82.50; WT $P_{asrelE2}$ CBS, 110 bp intergenic region between *relE2-rv2867c* containing putative CBS centered at -87.50. Mutant dsDNA oligo probes harboring left arm mutations, ablating interactions with 5.00 µg His-Crp: Mut P_{serC} CBS, tG**GT**a; Mut P_{relBE2} CBS, tG**GA**a; Mut $P_{asrelE2}$ CBS, cG**CA**g. (B.) Plotted data are the mean ± the standard deviation of (N=3) independent experiments. Densitometric analyses of bound and free DNA in EMSAs were normalized to control binding reactions without His-Crp. Compared to 0.1 µg His-Crp, significant differences in DNA-binding interactions were determined using a two-way ANOVA with Tukey's multiple comparisons post-tests (*, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001).

His-Crp showed a similarly high affinity for P_{relBE2}CBS, with the majority of the WT dsDNA probe demonstrated to be bound in the presence of at least one-hundred nanograms of His-Crp. In contrast, the affinity of His-Crp for $P_{asrelE2}$CBS was determined to be much weaker in the

presence of one millimolar cAMP in Figure 3.6. These data demonstrate that *relBE2* and *asrelE2* are dynamically regulated in a Crp-dependent manner.

3.3.5. *Mycobacterium tuberculosis relBE2*/*asrelE2* is differentially expressed *via* changes in intracellular cAMP:

Figure 3.7: *Mtb* harboring pGREENCHERRY reveals cAMP-dependent regulation of *relBE2* and *asrelE2* expression.

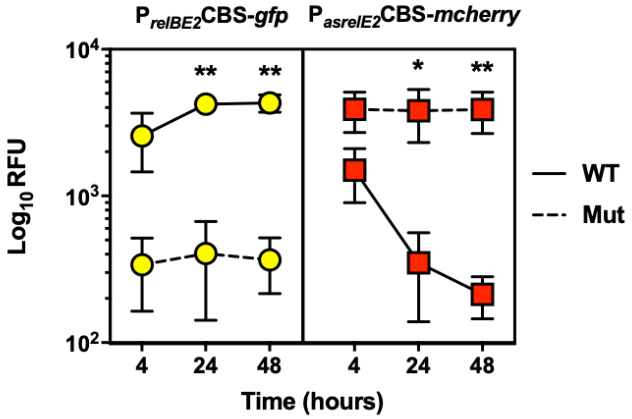

Figure 3.7: cAMP-dependent regulation of GFP and mCHERRY RFU in pGREENCHERRY. Data represent the means ± the standard deviations of (N=3) separate experiments; significant differences in GFP RFU between WT and Mut P_{relBE2}CBS (yellow circles) and in mCHERRY RFU between WT and Mut $P_{asrelE2}$CBS (red squares) in response to elevated intracellular db-cAMP determined using a regular two-way ANOVA with Tukey's post-tests (*, p-value < 0.05; **, p-value < 0.01). Straight lines and dashed lines profile WT and Mut P_{relBE2}CBS and $P_{asrelE2}$CBS activities in response to elevated intracellular db-cAMP, showing co-regulation of *relBE2* and *asrelE2*.

To examine the dynamics of *relBE2/asRelE2* co-expression, a dual transcriptional reporter was used to simultaneously probe the cAMP-dependent regulatory activities of P_{relBE2}CBS and $P_{asrelE2}$CBS. This reporter was constructed with unstable *gfp* and *mcherry* variants encoding ssrA-tags that are regulated by P_{relBE2}CBS and $P_{asrelE2}$CBS, respectively [51, 72]. Specifically, WT *Mtb* strain H37Rv was transformed with pGREENCHERRY, regulating *gfp* and *mcherry* expression with the 105-NTs and 120-NTs in the upstream IGRs of *relBE2* and *arelE2*, respectively. As a control, WT left arm NTs GAG and GAC in P_{relBE2}CBS and $P_{asrelE2}$CBS were altered to GGA and GCA, respectively. Mid-exponential phase *Mtb* harboring WT and mutant reporters were exposed to ten millimolar of non-toxic cell soluble analog, db-cAMP, in (1X) TBST, pH 6.50, for up to forty-eight hours. Compared to mutant GFP RFU, WT GFP fluoresence was increased significantly by

approximately ten-fold at twenty-four hours and forty-fold at forty-eight hours, demonstrating that *relBE2* transcription is activated in a cAMP-dependent manner in Figure 3.7. In contrast, WT mCHERRY RFUs were determined to be decreased around two-, ten-, and twenty-fold after four, twenty-four, and forty-eight-hour timepoints, respectively, compared to mutant mCHERRY RFUs, indicating that $P_{asrelE2}$CBS downregulates asRelE2 expression in a cAMP-dependent manner.

To further assess cAMP-dependent regulation of *Mt*b *relBE2/asrelE2* expression *in situ*, mid-to-late-exponential *Mtb* cultures were supplemented with db-cAMP, and relative changes in *relB2*, *relE2*, and asRelE2-3 expression were measured following four-, twenty-four-, and forty-eight-hours exposure using RT-qPCR [3, 73]. These analyses revealed that *relB2* and *relE2* of the *relBE2* bi-cistronic mRNA are differentially regulated compared to asRelE2-3 in a time- and dose-dependent manner relative to zero-hour controls shown in Figure 3.8.

Figure 3.8: *Mtb relBE2* and *asrelE2* expression is dynamically regulated in a cAMP-dependent manner.

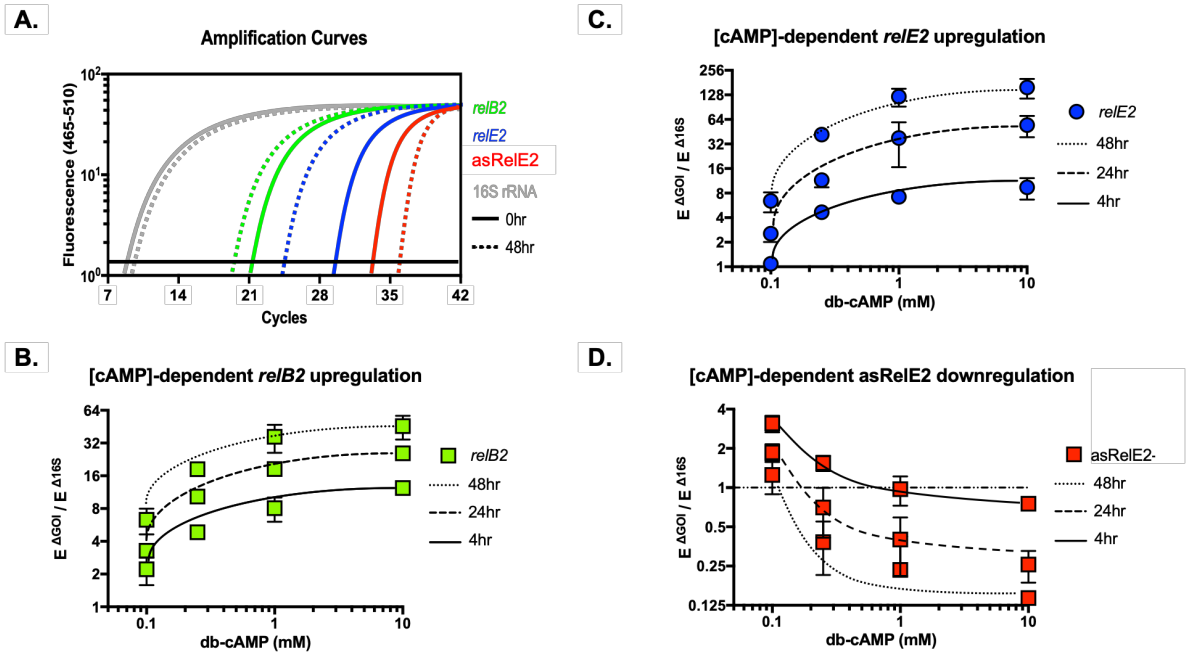

Figure 3.8: cAMP-dose-dependent regulation of *Mtb relBE2*/asrelE2 expression *in situ*. RT-qPCR gene expression analyses of total RNA isolated from mid-exponential phase *Mtb* washed, resuspended to an OD_{600} of 0.20-0.25, and incubated in (1X) TBST, pH 6.50, with up to 10 mM cell soluble analog, db-cAMP, for 4, 24, and 48 h at 37°C, similar to prior reports [5]. Relative changes in gene expression were calculated using the $E^{-\Delta\Delta Ct}$ method, normalized to 16S rRNA and compared to 0 h controls. (A.) Representative traces of *relB2*, *relE2*, asRelE2, and 16S rRNA qPCR amplifications from 0 h (solid lines) and 48 h (dashed lines) (B.-D.) Dose-response curves were estimated using a non-linear one phase association fit in GraphPad Prism. Data are the mean of (N=3) separate experiments ± the standard deviation.

Specifically, *relB2* was found to be induced two-to-five-fold from zero-to-ten millimolar db-cAMP supplementation over a forty-eight-hour period of time. These changes were accompanied with an approximately two-to-fifty-fold increase in *relE2* expression levels, which are five-to-ten-fold greater than that of *relB2* by forty-eight hours post-exposure in Figure 3.8B and Figure 3.8C below. In contrast, asRelE2-3 was markedly decreased from increasing the intracellular db-cAMP over time shown in Figure 3.8D, which taken together shows that increasing intracellular cAMP levels augments dynamic expression levels of the *relBE2*/*asrelE2* tripartite TA locus, leading to a steady and stable accumulation of *relE2* toxin mRNA *in situ*.

3.3.6 *Mycobacterium tuberculosis relE2* mRNA is upregulated in response to low pH host-associated stress *in vitro*.

It has been established that *Mtb* responds to low pH and other host-associated stresses by rapidly raising its intracellular cAMP levels to elicit necessary adaptive changes [3, 73, 74]. To characterize the relationship between acidification and cAMP, *Mtb* was subjected to (1X) TBST, pH 4.50, pH 5.50, and pH 6.50 and relative changes in *cya* (*i.e. rv1264*), *crp*, *relB2*, *relE2*, and asRelE2 expression were measured by RT-qPCR after four-, twenty-four-, and forty-eight-hours of exposure. RT-qPCR further revealed that *relE2* and asRelE2 are acid-inducible based on the differential expression patterns between pH 4.50 and pH 6.50, further linking *relE2* expression to cAMP-dependent asRelE2 repression. Both *cya* and *crp* were found to be upregulated by four- and twenty-four-hours at pH 4.50, confirming the conditions in Figure 3.9A. These findings are coincident with relative increases in intracellular cAMP, which was also at peak levels after four hours at pH 4.50 and pH 5.50 (*i.e.* eighty and twenty pmole per 10^8 CFU, respectively). Moreover, intracellular cAMP pools at pH 4.50 and pH 5.50 remained significantly elevated compared to pH 6.50 over twenty-four-hours (*i.e.* two-way ANOVA; *, p-value < 0.05; **, p-value < 0.01) in Figure 3.9B. Intracellular cAMP levels in *Mtb* at pH 6.50 in contrast decreased nearly four-fold by forty-eight hours compared to the time of initiation, which is consistent with the observed transcriptional responses of *cya*, *crp*, *relB2*, *relE2*, and asRelE2 expression compared to pH 4.50 and pH 5.50.

Figure 3.9: *Mtb* regulates *relBE2/asrelE2* expression in a cAMP-dependent manner under acidic stress *in vitro*.

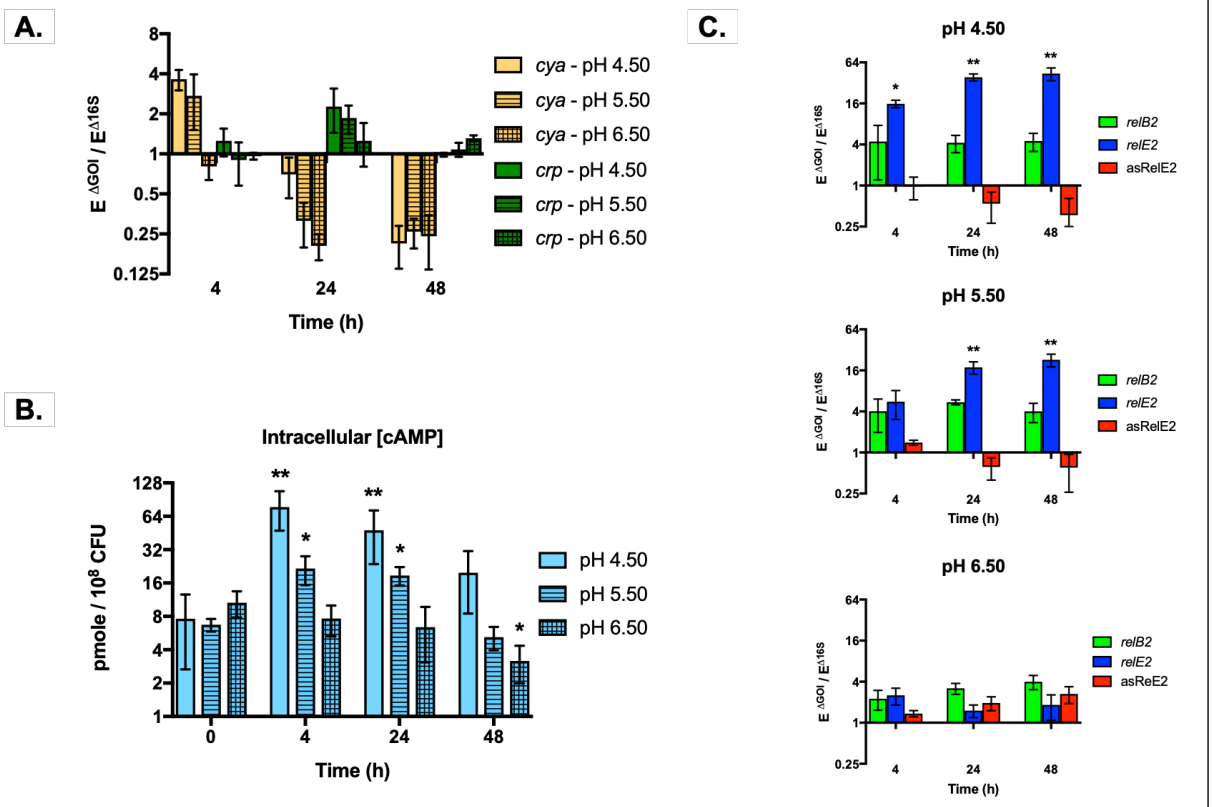

Figure 3.9: cAMP-dependent regulation of *Mtb relBE2*/asrelE2 expression under acidic stress *in situ*: RT-qPCR and cAMP ELISA analyses of mid-exponential phase *Mtb* washed, resuspended to an OD_{600} of 0.20-0.25, and incubated in (1X) TBST, pH 4.50, 5.50, or 6.50, up to 48 h at 37°C. Plotted data represent the mean ± the standard deviation of at least 3 independent experiments and significant differences were assessed using a two-way ANOVA with Tukey's multiple comparisons post-tests (*, p-value < 0.05; **, p-value < 0.01). (A.) & (C.) RT-qPCR gene expression analyses of pH-sensitive adenylyl cyclase *cya, crp*, *relB2*, *relE2*, and asRelE2 from total RNA isolated from *Mtb* after 0, 4, 24, and 48 h under acidic stress. (B.) pmoles intracellular cAMP levels measured from acetylated direct cAMP ELISA and then normalized to OD_{600}, as others have previously reported [3].

These observations indicate that *relB2*, *relE2*, and asRelE2 are responsive to low pH and that both relB2 and asRelE2 are mechanisms of post-transcriptional regulation.

3.3.7 *Mycobacterium tuberculosis* RelE2 mediates adaptation and survival to low pH host-associated stress *in vitro*.

To determine the importance of RelE2 in mediating adaptation and drug tolerance and the role of asRelE2 in its regulation, *Mtb*Δ*relE2* and *Mtb*Δ*asrelE2* knockout deletion mutant strains were constructed. Under normal *in vitro* culture conditions, no significant differences in growth were observed for *Mtb*Δ*relE2* and *Mtb*Δ*asrelE2* in comparison to WT until late-exponential phase,

when *Mtb*Δ*asrelE2* Log_{10} CFU mL^{-1} were observed to be significantly less (*i.e.* up to ten-fold) than the WT and *Mtb*Δ*relE2* strains (Figure 3.10). To further characterize the impact of *Mtb*Δ*asrelE2* dysregulation on long-term *Mtb* growth, *relE2* and asRelE2-3 transcript levels between WT and *Mtb*Δ*asrelE2* were compared from late-exponential (*i.e.* eight days) to stationary (*i.e.* sixteen and twenty days) phases of growth. Relative to WT expression, *relE2* was found to be constitutively

Figure 3.10: Generation and growth of WT *Mtb*, Δ*relE2*, and Δ*asrelE2* mutant strains *in vitro*.

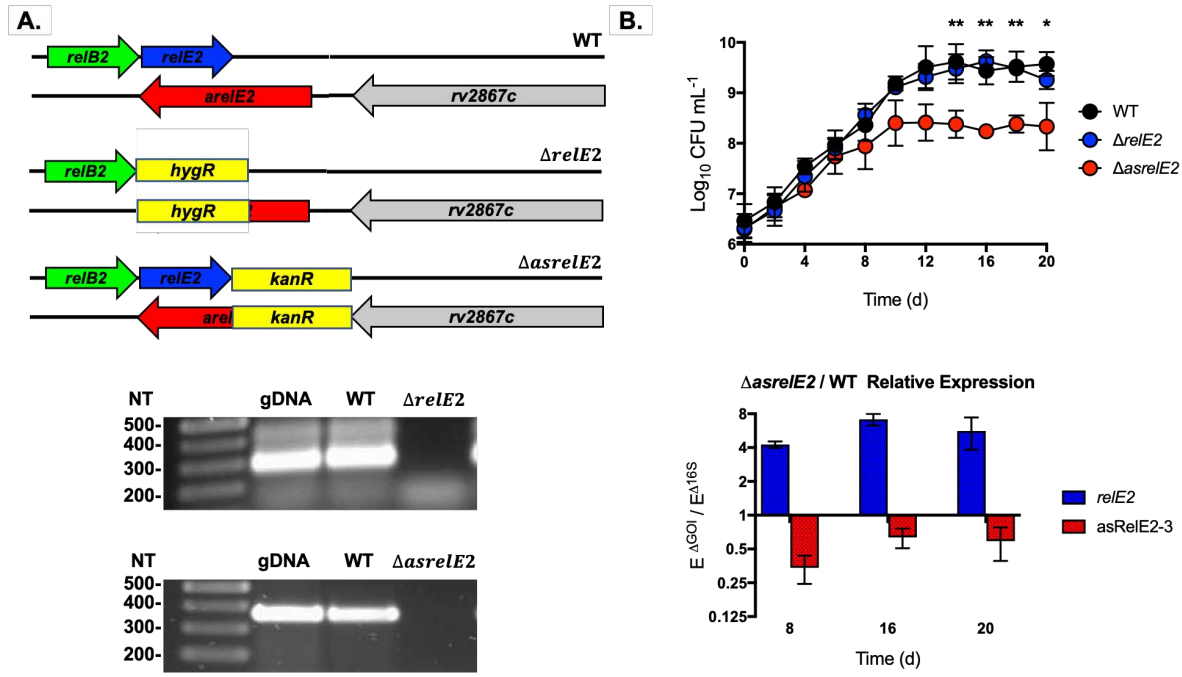

Figure 3.10: Generation and characterization of *Mtb* Δ*relE2 and* Δ*asrelE2* mutant strains. (A.) Schematic representation and PCR confirmation analyses of mutant KO strains generated from two-step allelic exchange using temperature-sensitive constructs encoding sucrose counter-selection: *Mtb*Δ*relE2*, 264-NT gene replaced with Hyg^R gene cassette; *Mtb*Δ*asrelE2*, 372-NT intergenic region between *asrelE2-rv2867c* replaced with Kan^R gene cassette. (B.) Log10 CFU mL^{-1} and RT-qPCR gene expression analyses of WT and *relE2/asrelE2* mutant KO *Mtb* strains sub-cultured (Day 0 OD_{600} = 0.05-0.10) in 7H9-Tw over 20 d at 37°C. Differential expression of *relE2* and asRelE2-3 in mutant *Mtb*Δ*asrelE2* was compared with WT gene expression levels after 4, 8, and 16 d outgrowth, normalized to 16S rRNA. Plotted data are the mean ± the standard deviation of (N=3) independent experiments. Significant differences were identified using a two-way ANOVA with Tukey's multiple comparisons post-tests (*, p-value < 0.05; **, p-value < 0.01).

upregulated nearly four-fold at eight, sixteen, and twenty days. In contrast, asRelE2-3 expression levels were determined to be reduced almost four-fold in late-log phase, compared to WT, and remained downregulated throughout stationary phase (*i.e.* in Figure 3.10B). These findings

demonstrate that the dysregulation of asRelE2 results in hyper-expression of *relE2*, underlying reduced CFU mL^{-1} throughout the stationary phase of growth in *Mtb*.

Figure 3.11: Essentiality of *relE2/asrelE2* expression in adaptation and survival of *Mtb* under low pH stress *in vitro*.

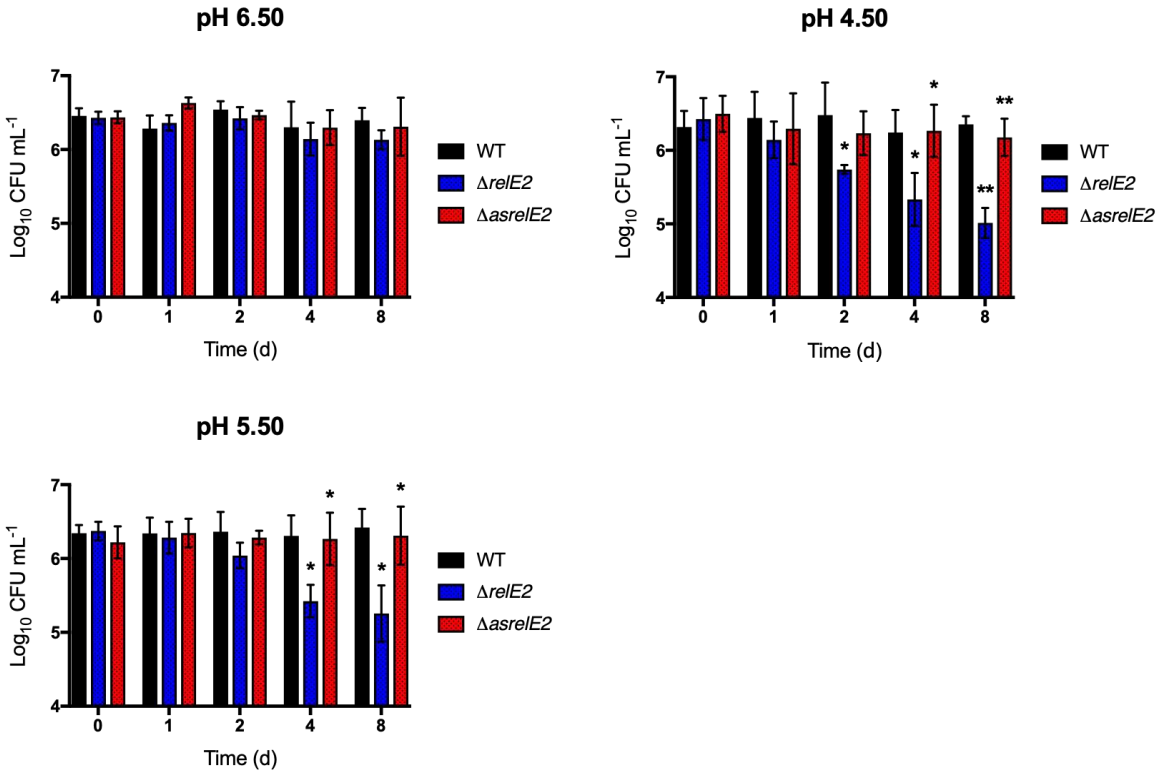

Figure 3.11: Essentiality of *relE2/asrelE2* expression in adaptation and survival of *Mtb* under acidic stress *in vitro*. WT and mutant strains sub-cultured to mid-exponential phase (OD_{600} = 0.40-0.60) in 7H9-Tw were washed, resuspended to an OD_{600} of 0.20-0.25 (or ~6.50 x 10^6 CFU mL^{-1}), and incubated in (1X) TBST, pH 4.50, 5.50, or 6.50, at 37°C. After 0, 1, 2, 4, and 8 d exposure to low pH stress *in vitro*, mycobacteria were enumerated on 7H10 agar at 37°C. Data depict the mean ± the standard deviation of independent experiments. Significant differences in log10 CFU mL^{-1} were found using a two-way ANOVA with Tukey's multiple comparisons post-tests (*, p-value < 0.05; **, p-value < 0.01).

WT, *Mtb*Δ*relE2*, and *Mtb*Δ*asrelE2* strains were evaluated similarly for long-term survival and persistence in response to stress such as NS at low pH. Relative to day zero, no differences in Log_{10} CFU mL^{-1} were observed between WT and mutant strains after one day in (1X) TBST, pH 4.50. However, *Mtb*Δ*relE2* Log_{10} CFUs were found to steadily decrease in TBST, pH 4.50, over time, resulting in a nearly twenty-fold reduction in survival by day eight, compared to WT and Δas*relE2* strains shown in Figure 3.11. An intermediate survival phenotype was observed at pH 5.50, with a more moderate reduction in *Mtb*Δ*relE2* Log_{10} CFUs after four and eight days. No

significant survival differences were observed following NS at pH 6.50. Accordingly, this survival defect appeared to be restored in *Mtb*Δas*relE2*, with no significant differences in Log_{10} CFU mL^{-1} at pH 4.50, pH 5.50, or pH 6.50, over eight days (*i.e.* in Figure 3.11). Taken together, these observations indicate that *relE2* overexpression is important for long-term survival of *Mtb* under acidic stress *in vitro*.

3.3.8 *Mycobacterium tuberculosis relBE2/asrelE2* is dynamically expressed in response to host immune activation during infection.

To investigate a regulatory connection between the host environment and the activity of TA loci *in vivo*, relative changes in *relBE2* and asRelE2 TA gene expression were examined in murine *Mtb* infection models. IFN-γ is known to be critical for TB control in mice in part because it activates infected Mφ and recruits other phagocytes to restrict *Mtb in vivo* [75, 76]. Accordingly, to assess the effects of host immune status on *relB2*, *relE2*, and asRelE2 RNA levels and *Mtb* persistence *in vivo*, we compared changes in gene expression between WT and GKO infected mice over time. At seven days post-infection, there were no significant differences between Log_{10} CFUs recovered from lungs and spleens of GKO and WT *Mtb* infected mice. Starting at fourteen days post-infection though, GKO lung and spleen bacillary burdens were observed to steadily increase throughout the duration of infection, with GKO lungs harboring 10^7-10^8 CFUs, while WT lungs contain 10^5-10^6 CFUs at twenty-eight days post-infection in Figure 3.12A. In addition, approximately twenty-fold more Log_{10} CFU mL^{-1} were recovered from the spleens of GKO infected mice, compared to those of WT infected mice at twenty-eight days post-infection shown in Figure 3.13A. Accordingly, *Mtb* infection ended after twenty-eight days for GKO mice because of significantly increased mortality, compared to that of WT infected mice. *Mtb* Log_{10} CFU mL^{-1} were cultured from WT infected lungs and spleens forty-two days post-infection, demonstrating the development of host immune control over infection. Compared to the lungs of GKO infected mice, IFN-γ-stimulated genes, including LRG-47, IGTP, V-ATPase, CFTR, and NRAMP1 were found to

be increasingly expressed following seven, fourteen, and twenty-eight days infection in WT *Mtb* mice, with the mounting of a normal T_H cell immune response (*i.e.* in Figure 3.12B).

Figure 3.12: Dynamics of *Mtb* growth and adaptation in infected murine lungs.

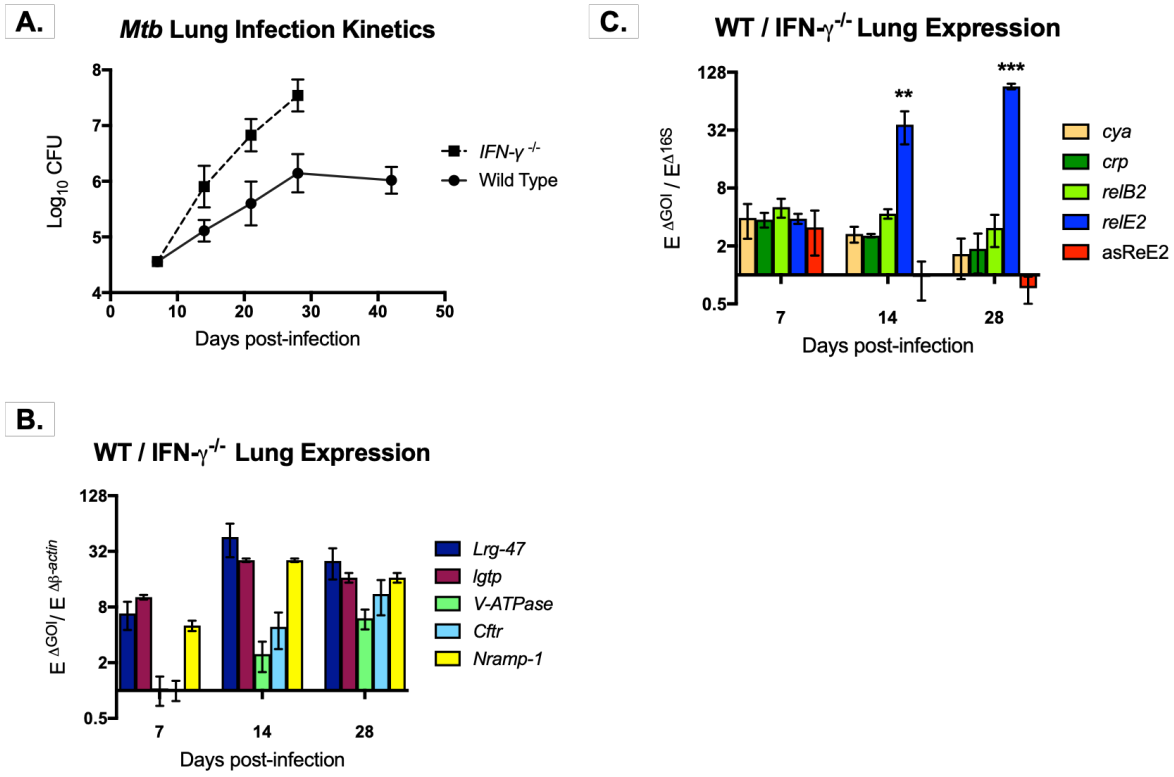

Figure 3.12: Differential expression of *relBE2*/asRelE2 in lungs of *Mtb*-infected WT and IFN-$\gamma^{-/-}$ GKO mice. WT and IFN-$\gamma^{-/-}$ (GKO) mice were infected with *Mtb* strain H37Rv for up to 42 days. Data represent the means ± the standard deviation of (N=4) WT and GKO infected mouse lungs at 7, 4, 21, 28, and 42 dpi. Significant differences in $Log_{10}CFU\ mL^{-1}$ were identified using a two-way ANOVA with Tukey's post-tests (*, p-value < 0.05; **, p-value < 0.01). (A.) Tubercle bacilli were enumerated from culturing of lung homogenates of WT and IFN-$\gamma^{-/-}$ mice on 7H11 with 50 µg mL^{-1} carbenicillin and 10 µg mL^{-1} cyclohexamide. (B.) RT-qPCR of IFN-γ-regulated genes, LRG-47, IGTP, V-ATPase, CFTR, and NRAMP1 in total RNA isolated from lungs of *Mtb*-infected mice, normalized to *β-actin*; changes in gene expression relative to IFN-$\gamma^{-/-}$ GKO mice. (C.) RT-qPCR of *Mtb cya*, *crp*, and *relBE2/*asRelE2 in total RNA isolated from *Mtb*-infected lungs using $E^{-\Delta\Delta Ct}$ method, normalized to 16S rRNA; changes in gene expression compared to IFN-$\gamma^{-/-}$ GKO mice.

All of these genes were also found to be upregulated in the spleens of infected mice at seven, fourteen, and twenty-one days post-infection in Figure 3.13B. Only *Lrg-47* and *Igtp* showed temporal expression profiles, however, similar to those measured in the lungs, which were still five-to-ten-fold lower. In contrast, there were no significant changes in *ATPase*, *Cftr*, and *Nramp1* expression levels measured in mouse spleens throughout the rest of infection. Accordingly, *Mtb cya* and *crp* were demonstrated to be maximally expressed two-to-four-fold at seven days post-infection and decreased thereafter in WT infected mice lungs. When *relE2* and asRelE2

transcriptional activity was assessed, it was found that their expression was inversely regulated in infected WT mice lungs over time, marked by the upregulation of *relE2* over twenty-fold at fourteen and twenty-eight days post-infection (*i.e.* in Figure 3.12C), whereas *relB2* expression levels were shown to be significantly increased in WT infected mice spleens, compared to that of *relE2*, in a temporal manner (*i.e.* in Figure 3.13C).

Figure 3.13 Dynamics of *Mtb* growth and adaptation in infected murine spleens.

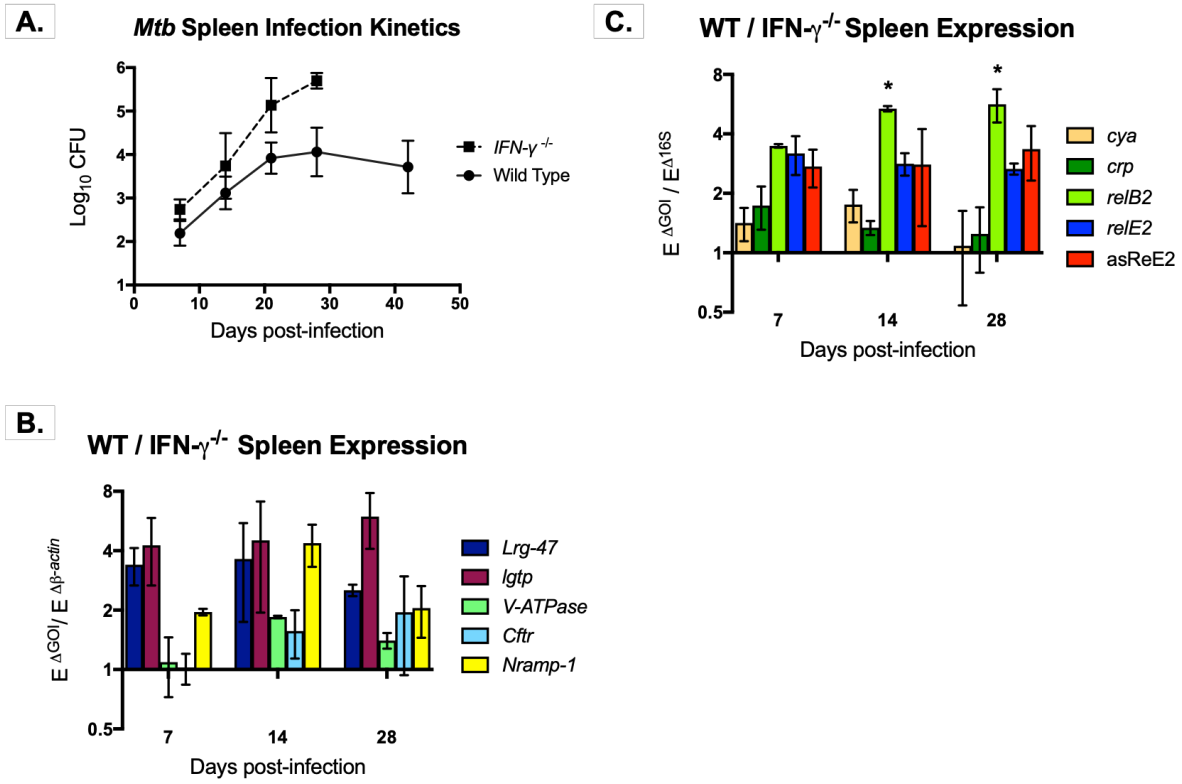

Figure 3.13: Differential expression of *relBE2*/asRelE2 in spleens of *Mtb*-infected WT and IFN-γ$^{-/-}$ GKO mice. WT and IFN-γ$^{-/-}$ (GKO) mice were infected with *Mtb* strain H37Rv for up to 42 days. Data represent the means ± the standard deviation of (N=4) WT and GKO infected mouse spleens at 7, 4, 21, 28, and 42 dpi. Significant differences in Log_{10}CFU mL^{-1} were identified using a two-way ANOVA with Tukey's post-tests (*, p-value < 0.05; **, p-value < 0.01). (A.) Tubercle bacilli were enumerated from culturing of spleen homogenates of WT and IFN-γ$^{-/-}$ mice on 7H11 with 50 µg mL^{-1} carbenicillin and 10 µg mL^{-1} cyclohexamide. (B.) RT-qPCR of IFN-γ-regulated genes, LRG-47, IGTP, V-ATPase, CFTR, and NRAMP1 in total RNA isolated from spleens of *Mtb*-infected mice, normalized to *β-actin*; changes in gene expression relative to IFN-γ$^{-/-}$ GKO mice. (C.) RT-qPCR of *Mtb cya*, *crp*, and *relBE2/*asRelE2 in total RNA isolated from *Mtb*-infected spleens using E$^{-\Delta\Delta Ct}$ method, normalized to 16S rRNA; changes in gene expression compared to IFN-γ$^{-/-}$ GKO mice.

The use of immune competent and deficient mice ultimately revealed that the dynamic regulation of *relBE2*/*asrelE2* coincides with development of a chronic or persistent TB infection in mice and depends on a fully constituted IFN-γ-dependent host response to *Mtb* in a tissue-specific manner.

3.4 Discussion.

The development of a persistent state of infection requires the coordination of multiple bacterial processes, including halting of cell cycle progression, induction of alternative metabolic pathways, in addition to adaptive regulatory systems, including TA loci. Ours and others reports show that during infection, as well as in response to stress associated with the host environment, TA genes are differentially regulated in a manner that is concomitant with the transition to a drug tolerant persistent state [23, 26, 77]. Although post-transcriptional regulation in *Mtb* is not well understood, we predicted that *relBE2* is differentially regulated similar to that of a type I system by an asRNA antitoxin. Northern blotting of sense and antisense *relBE2* expression *in vitro* initially identified that *relBE2* mRNA and novel antisense transcript, asRelE2, are differentially regulated in a growth phase-dependent manner, wherein *relE2* mRNA levels steadily increase throughout stationary phase. Sanger sequencing of 5'/3' RACE PCR products identified *relB2* and *relE2* as leaderless messages, which is in line with previous reporting that the majority of *Mtb* mRNAs are leaderless [78, 79]. This likely explains why *Mtb* MazF3 toxin is found to cleave the anti-Shine Dalgarno sequence on 16S rRNA [55]. A *relBE* homolog in post-transcriptionally regulated in a manner that results in the development of drug tolerant *E. coli* persister cells [30]. Accordingly, the overexpression of WT *relE2* strongly inhibited *Mtb* growth, while the co-ectopic induction with either *relB2* or *asrelE2* rescued this growth defect. Unlike others reporting of bactericidal activity of certain *Mtb* toxin proteins [80], the overexpression of *Mtb relE2* resulted in a reversible state of inhibited growth. Sub-culturing of late stationary phase (*i.e.* twenty day) *Mtb*Δ*asrelE2* reveals a considerably longer lag phase, compared to WT and Δ*asrelE2 Mtb*, by six-to-seven days in 7H9-Tw. Moreover, there are no significant differences in proportions of Rif- and Inh-tolerant tubercle bacilli that develop between WT and Δ*asrelE2 Mtb*, while *Mtb*Δ*relE2* is severely attenuated in the development of Rif- and Inh-tolerance. We have found that inducible expression of MazF6 in *Mtb* was also mycobacteriostatic [26], which indicate that RelE and MazF do not function in mediating

PCD. Still, the possibility remains that these and other toxins may act in concert with other TA proteins to different host environmental stresses such as low pH NS to enact PCD. This would explain why RelE2 is found to be co-regulated by cognate antitoxins RelBE and asRelE2 in *Mtb*. Co-overexpression of RelB2 downregulates RelE2 toxin activity *via* TA protein-protein interactions, while the co-overexpression of asRelE2 reduces *relE2* activity *via* increased *relE2* mRNA decay from more type I regulation in Figure 3.4. Similar to other published reports of RNase III decay of toxin mRNA in *B. subtilis* and in *E. coli* [57, 68], *Mtb* RNase III was determined to degrade *relE2*::asRelE2-3 double-stranded RNA hybrids in a magnesium-dependent manner *in vitro* (*i.e.* in Figure 3.5). This is line with additional reporting that *relB2* and *relE2* expression levels are differentially regulated by asRNA in a cAMP-dependent manner [63]. Intriguingly, Kahramanoglou *et al*., (2014) determined that Crp binds to an antisense CBS, approximately 15-NT downstream of the stop codon of *relE2* mRNA [63]. Recombinant *Mtb* Crp binds to P_{relBE2}CBS and $P_{asrelE2}$CBS, albeit it with significantly lower affinity, comparable to that of canonical P_{serC}CBS. This additional putative CBS likely contributes to differential regulation of *relBE2*/*asrelE2* expression levels. Mutations in left arms of P_{relBE2}CBS and $P_{asrelE2}$CBS were shown to ablate DNA-binding in EMSAs and dysregulate GFP and mCHERRY fluoresence *in situ*, compared to those of WT left arms, in the presence of up to ten millimolar cAMP in Figure 3.6 and Figure 3.7. We likewise employed RT-qPCR and determined that *relE2* mRNA levels are significantly upregulated in a cAMP-dependent manner, while asRelE2 is downregulated. This is similar with another ncRNA that is differentially regulated along with acid-inducible adenyl cyclase (or *cya*), leading to increasing intracellular cAMP levels in *Mtb* [81]. Exposure to low pH stress induced *cya* and *crp* expression in *Mtb* up to four-fold after four hours (*i.e.* in Figure 3.9). This leads to elevated intracellular levels of cAMP concomitantly with the overexpression of *relE2* in *Mtb*. Accordingly, the deletion of *relE2* resulted led to low pH-dependent decreases in Log_{10} CFUs over time *in vitro* (*i.e.* in Figure 3.11). *Mtb*Δ*asrelE2* on the other hand showed no significant differences in Log_{10} CFUs, compared to that of WT *Mtb*, over time in response to NS at acidic pH. These

findings agree with others overexpression and mutant studies of *relE2*, showing that this toxin is specifically essential for developing *Mtb* Rif-tolerance *in vitro* [24]. Compared to WT and Δ*relE2* *Mtb*, *Mtb*Δ*asrelE2* Log_{10} CFUs were five-to-ten-fold less in late exponential and stationary phase, corresponding with the constitutive upregulation of *relE2* mRNA. Thus, it appears that the hyperexpression of *relE2* rescues the survival defect of *Mtb*Δ*relE2* under low pH stress in Figure 3.11. The development of T_H cell mediated immune responses is important for long-term host control of *Mtb* infection [76, 82]. Host control arises in mice from IFN-γ-dependent induction of GTPase Lrg-47, thereby effectively acidifying *Mtb* phagosomes in infected Mφs [83]. Compared to GKO hypersusceptible mice, *V-ATPase*, *Cftr*, and *Nramp-1* encoding ion pumps were found to be increasingly expressed over time in the lungs of WT infected mice (*i.e.* in Figure 3.12). In contrast, their expression levels in *Mtb* infected spleens failed to show similar temporal increases. Consequently, while *relE2* was found to be upregulated, with asRelE2 downregulated over time in mycobacteria from the lungs, *relB2* mRNA levels in *Mtb* was determined to be markedly higher in the spleens. We have observed tissue-specific differences in *Mtb mazEF6* TA gene expression levels too [26]. The dynamic regulation of *relBE2* and *asrelE2* in the lungs and spleens of infected mice implicates post-transcriptional regulation of *Mtb* TA loci in mediating long-term survival (or persistence) during infection. This is in line with other published results that the expression levels of *relB2* and *relE2* are inversely regulated in *Mtb* recovered from TB patient sputa following the start of multi-drug treatment [27]. On the other hand, *relB2* is found to be significantly upregulated in the reactivation of *Mtb* from a hypoxia-inducible drug tolerant dormant state [84]. These finding taken together ultimately highlight the importance of post-transcriptional regulation of TA genes, particularly *relBE2*/*asrelE2*, mediating the entry into and exit from a persistent state in *Mtb in vivo*. WGS comparisons find that the genome-wide expansion of TA loci and other virulence genes are important drivers in the evolution of *Mtb* and the MTBC [32, 85]. These include lineage-specific mutations changing AAs, altering antitoxin DNA-binding, and NTs, generating novel promoters in

IGRs, including CBSs [63, 86, 87]. These mutations have resulted in an overrepresentation of differentially expressed *Mtb* TA genes, which is essential for mediating epigenetic changes that distinguish virulent modern *Mtb* lineages [40]. These findings ultimately indicate that co-regulation of RelE2 by asRelE2 represents an important mechanism, coordinating the differential expression of a large number of TA genes, which is unique to the physiology of *Mtb*.

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CHAPTER 4: FINAL DISCUSSION

4.1 Introduction.

The development of new drugs that are active against resistant strains and phenotypically diverse organisms continues to present the greatest challenge in the future. The presence of many chromosomally-encoded TA systems in *Mtb* raises important questions with regard to their expansion, regulation, and thus function. The cumulative results indicate that they are involved in regulating adaptive responses to stresses associated with the host environment and drug treatment. Here we review the TA-families encoded in *Mtb,* discuss the duplication of TA loci in *Mtb,* regulatory mechanism of TA loci, and phenotypic heterogeneity and pathogenesis.

The significant reductions in annual TB incidence have been attributed to better disease management programs, improving poverty conditions, and diagnostics and therapeutic regimens that successfully detect and treat ATB [1]. However, this decrease falls short of the estimated 20% reduction rate needed to enter into the elimination phase of the disease management [2]. The ongoing failure to achieve such reduction rates has been directly linked to the global burden of LTBI [3]. Indeed, individuals with LTBI can develop ATB years or even decades after initial infection. Reactivation (or relapse) of LTBI accounts for a significant proportion of newly reported active TB cases, which in turn drives the transmission and thus the maintenance of global TB burden [4].

The successful treatment of *Mtb* infections requires lengthy multi-drug therapeutic regimens to achieve a durable cure. The development of multidrug and extensively drug resistant TB has been attributed to poor adherence to the protracted treatment regimens because of intermittent drug exposures ultimately resulting in difficult to treat, highly transmissible clinical strains. Identifying LTBI individuals at increased risk for developing ATB, as well as developing new antibiotics that are active against both resistant strains and phenotypically diverse LTBI organisms that would make significant progress towards reducing the substantial reservoir of

difficult to treat *Mtb*. This strategy is key in reducing the overall global burden of TB yet continues to present the greatest challenge in the future.

The regulatory mechanisms that coordinate mycobacterial adaptation during infection and lead to the establishment and maintenance of LTBI capable of reactivation are largely unknown. It is well accepted that TB patients enter a state of "clinical latency", wherein any surviving tubercle bacilli tolerate host immunity and chemotherapy to maintain a persistent infection [5, 6]. Historically, the development of LTBI was thought to be a relatively passive event on the part of the bacilli, however, it is now clear that *Mtb* responds to the restricted and altered growth environment that occurs as a result of the host response to infection. This is reflected in the literature, which has mainly focused on *Mtb*'s adaptive metabolic response to host environmental cues such as hypoxia [7], low pH [8], and NS [9]. The adaptive metabolic responses identified in these studies provide a foundation but are incomplete because the large set of *Mtb* regulators underlying bacterial adaption in the development of LTBI, and reactivation to ATB remain undefined.

While long-term *Mtb* infections appear to be tolerant to treatment as a result of non-replicating persistent organisms, LTBI can reactive and progress to ATB at any time in a person's life [10]. Accordingly, long treatment regimens consisting of nine-months of Inh, three-months of Rif and Inh, or two-months of Pza and Rif, which are thought to target difficult to treat organisms and effectively reduce the risk of relapse and development of ATB years after the completion of treatment [11]. The rate of killing by Inh is found to be directly proportional to mycobacterial growth in patients, and likely only kills rapidly dividing tubercle bacilli [12]. This reduced susceptibility to drug treatment during infection is referred to as 'drug-tolerance', and is a hallmark of tubercle bacilli recovered from LTBI individuals [13, 14]. Moreover, the shortening of therapy by Pza and Rif is predicated on the idea that these drugs specifically target persistent sub-populations of *Mtb* dominating patient sputa before and after treatment [15]. Thus, although tolerance and LTBI are classically linked to *in vivo* drug pressures and host immune defenses,

respectively, both phenomena appear to reflect a similar regulatory mechanism, resulting in a persistent physiological state of *Mtb*.

TA systems are widely-distributed in prokaryotic genomes and have been increasingly implicated in mediating bacterial adaptive responses resulting in the development of latent infections [16]. TA systems are grouped into six major types (*i.e.* I-VI) based on the physical type of antitoxin and its regulation of cognate toxin [17]. Accordingly, type II TA systems are principally defined by a protein antitoxin, and quite often share several other key features: cognate toxin and antitoxin genes form small bi-cistronic operons [18]; toxin and antitoxin proteins assemble into protein complexes to neutralize toxin activity [19]; auto-regulate their expression [20]; antitoxins are much more susceptible to proteolysis than toxins [21], and are selectively degraded to activate toxins [22]. The presence of TA loci in numerous bacterial pathogens and *Mtb* raises important questions: (1) why do organisms encode so many functional TA loci; (2) how did this expansion occur; (3) how is the activity of TA loci regulated in an expanded complex system; (4) what is the functional redundancy and what advantage do they provide to the bacterium during the infection; (5) what instigates their activity?

4.2 Toxin-antitoxin systems of *Mycobacterium tuberculosis*.

Numerous bioinformatics analyses find that *Mtb* genome encodes a significantly expanded repertoire of TA loci that makes up the *Mtb* TA-system consisting of well-assigned Type II VapBC, MazEF, ParDE, RelBE, and HigBA TA families. Additional ORFs are annotated as putative toxin and antitoxin genes or TA loci [23-25]. Most of the *Mtb* TA systems, which are conserved by the MTBC and a number of more pathogenic NTM, are not encoded in the genomes of environmental mycobacteria, suggesting that TA loci play unique role(s) in *Mtb* pathogenesis [24].

The genome of *M. smegmatis* (or *Msm)* for instance, which is twice the size of *Mtb,* only encodes two *Mtb* TA loci [26], while *Mlp*, which has lost nearly sixty percent of its coding capacity comparatively, maintains five *Mtb* TA systems [27]. Thus, the functional roles of TA loci in *Mtb* pathogenesis have received considerable attention over the last decade. These results indicate

that they are involved in regulating adaptation to stresses associated with the host environment and drug treatment.

VapBC TA systems are the most abundant family encoded in the *Mtb* genome [28, 29]. The identification of *vapBC* TA loci has relied on the presence of *vapC* PIN-domains, which are a hallmark of prokaryotic and eukaryotic RNases. *Mtb vapB* homologs are defined by location to *vapC* and the presence of RHH, Phd, ArbR, MerR, and other novel DNA-binding domains. The significance of the heterogeneity in the *vapB* coding sequence is known, but could indicate functional diversity under a number of growth conditions [28].

Multiple VapBC TA loci are transcriptionally activated by environmental conditions associated with the host such as hypoxia (*e.g. vapBC20*, *vapBC22, and vapBC45*) and to IFN-γ stimulated Mφs (*e.g. vapBC4*, *vapBC5*, and *vapBC47*) [30-32]. Increased constitutive expression of *Mtb vapBC* genes have been shown to be essential for regulating intracellular mycobacterial replication [33, 34], thus enhancing PE/PPE protein production (*i.e*. *vapBC13* and *vapBC25*) [35], and secretion of immunogenic proteins Mpt70 and Mpt80 *via* transcriptional fusions (*i.e*. *vapBC43*) [36]. Transposon screens revealed that many members of the VapBC TA family including *vapC4*, *vapB5*, *vapC20*, *vapC26*, and *vapC45* are important for the long-term survival of *Mtb* in Mφs, in the lungs of mice and in nonhuman primates [37-40]. VapC4 is essential for *Mtb* survival in nonhuman primate lungs [40], while VapC45 is essential for inhibiting apoptosis of *Mtb*-infected Mφs [41], indicating that VapC toxins function in more discrete aspects of infection.

The role of VapBC proteins in pathophysiology of *Mtb* has been experimentally demonstrated in vaccine studies that revealed that increased recombinant production of VapB47 in *Mbv* strain BCG significantly enhances BCG vaccine efficacy in a murine model of TB [42, 43]. Transcriptomic comparisons between WT *Mtb* and *Mtb*Δ*RD1* reveal that *vapBC47* is among the most abundant transcripts in *Mtb*Δ*RD1* [44]. Studies have shown that a *Mtb* strain missing the region-of-difference-two (or RD2) encoding *vapBC36* is attenuated in a murine model of TB, which

is restored by complementation with the RD2 region. This is consistent with observations that *Mbv* BCG has lost RD2 from extensive *in vitro* propagation [45]. VapBC TA proteins, particularly VapB47 stimulate IFN-γ production by peripheral blood mononuclear cells (or PBMCs) recovered from ATB patients [46-48] and are also uniquely recognized by PBMCs from LTBI individuals [49, 50]. Given that *Mtb* VapC toxins are among the most abundantly extracellular proteins [51, 52], and many have demonstrated function in host cells [53-55], it's likely that VapBC TA loci mediate persistence and function on multiple levels at discrete stages of infection to directly influence both mycobacterial and host translation.

The *Mtb* genome encodes eleven MazEF TA loci. MazEF TA proteins share AA-sequence similarity and overexpression studies that have demonstrated MazF3, MazF6, and MazF9 arrest mycobacterial growth, while activity for the remaining MazF toxins has not been experimentally shown [56, 57]. MazEF toxins and antitoxins appear to be discretely induced during infection by host cues, including hypoxia, oxidative stress and low pH [31, 57, 58]. Although *mazE6* and *mazF6* are expressed similarly in the lungs of infected mice, only *mazF6* transcripts are increased significantly in spleens [56]. *Mtb*Δ*mazE5* and *Mtb*Δ*mazE7* are impaired for dissemination to the spleens of infected mice, which implicates non-cognate MazEF TA interactions and functions in *Mtb* dissemination and pathogenesis [38]. There is evidence that some MazEF components such as MazF6 and MazE10 appear to be essential for mycobacterial survival, and there is evidence that other MazEF components functionally interact because a *Mtb*Δ*mazF3*Δ*mazF6*Δ*mazF9* strain was considerably less virulent than the comparator wildtype (WT) *Mtb* strain in the guinea pig model of TB [39, 57]. Strikingly, lung granulomas of *Mtb*Δ*mazF3*Δ*mazF6*Δ*mazF9*-infected guinea pigs were not necrotic and comprised mostly T lymphocytes with relatively few Mφs and epithelioid cells [57].

In vitro functional studies have shown that the RNase activity of *Mtb* MazF toxins have target specificities, which is significant because this specificity impacts the extent of transcriptional

remodeling performed by each MazF toxin [59-61]. For example, only 20% of *Mtb*'s transcriptome is susceptible to MazF3 RNase activity, while approximately seventy percent of *Mtb* total RNA is susceptible to MazF6 RNase activity [28, 59]. In addition to degrading mRNAs, MazF3 and MazF6 have been shown to target helix loop seventy of 23S rRNA and the anti-SD sequence of 16S rRNA [62-64]. MazF9 regulates translation by selectively degrading proline and lysine tRNAs [65, 66]. And while MazF4 doesn't directly inhibit mycobacterial growth [57], it binds to DNA topoisomerase, which likely serves as a key regulatory checkpoint for *Mtb* [28, 67, 68]. This is consistent with our work, which has implicated MazEF6 in mediating the transition to a persistent state in *Mtb* [56].

MazEF proteins may likewise directly target host RNAs that results in an increased pro-inflammatory response and altered growth environment due to host tissue damage. MazF proteins have been reported to be secreted by *Mtb* and their RNase activities have been shown to be functional in host eukaryotic cells [69-72]. This is consistent with the observation that MazEF toxin proteins are differentially recognized by PBMCs from ATB and LTBI individuals [50, 73]. The exosomes of *Mtb*-infected Mφs containing *mazF* transcripts have also been shown to induce potent pro-inflammatory responses in recipient Mφs [74], which suggests that TA mRNAs are immunomodulatory too, like other *Mtb* transcripts [75-77]. Taken together, these studies indicate that MazEF toxins and antitoxins play a role in establishing *Mtb* infection and directly participate in the host-pathogen interactions on multiple levels.

Mtb encodes fourteen TA systems that belong to the functionally diverse ParDE/RelBE TA superfamily, including two ParDE systems, three RelBE systems, and three HigBA systems [24]. Members of ParDE/RelBE TA superfamily function by inhibiting translation through cleavage of RNAs or binding units of the ribosome. Of all the *Mt*b ParDE/RelBE TA proteins, ParE1 appears to be particularly important for *Mtb* survival in activated Mφs and in dissemination to spleens of mice [37, 38]. Unlike most toxins in this superfamily, ParE homologs bind DNA gyrase to block

replication, which results in phenotypic heterogeneity, with some tubercle bacilli becoming viable but non-culturable [78, 79].

This heterogeneity has also been observed with *Mtb* RelE toxins that are known to have differential effects on drug tolerance and host cell death [80, 81]. This is consistent with the demonstration that *Mtb relBE* TA genes are components of *in vivo* adaptive responses and upregulated *in vivo* at different times of infection suggesting that they participate at different stages of infection. This was demonstrated by immune stimulation by RelE1 at four-weeks post-infection and wanes at up to eight-weeks post-infection when RelE2 becomes more antigenically recognized [30, 82]. This is in line with the observation that RelE2 and not RelE1 elicits a much more robust IFN-γ responses from the PBMCs of LTBI individuals [50].

The *Mtb* ParDE/RelBE family includes three HigBA TA systems [83]. Hig toxins target translation by binding to 50S sub-units of 70S ribosomes and cleaving AAA-NT-sequences of translating mRNAs, a mechanism unlike RelE1, RelE2, or RelE3 [84]. Exogenous expression of HigB1 is particularly cytotoxic to *Mtb* [85, 86], and is neutralized by the co-expression of HigA1 and its Sec-like chaperone, Rv1957, which enhances the antitoxin's stability [87]. This enhanced stability of the HigA1 antitoxin is thought to contribute to its unique role as a global transcriptional regulator directly involved in regulating iron scavenging and metal transport genes as well as multiple other genes that are essential for intracellular survival [37, 85, 88]. HigA2 has been shown to have a more modest effect than HigA1 and is important for bacterial survival in acidified phagosomes and in guinea pig lungs [39, 89]. This is supported by the recognition of HigA2 by PBMCs from persons with ATB and LTBI [90], and recognition of *Mtb* HigA1 uniquely by LTBI persons [30, 91].

4.3 Expansion of the ParDE/RelBE superfamily toxin-antitoxin systems in *Mycobacterium tuberculosis*.

TA systems identified in *Mtb* are uniquely encoded by other pathogenic Actinomycetes such as Nocardia and Corynebacteria [92]. *Mtb* TA systems are encoded in more mobile regions

of the genome, which indicates that they were first acquired *via* HGT [24]. In NTMs such as *M. abscessus*, the MAC, and *Mkn* many TA loci are maintained on accessory plasmids [93]. Because *Mtb* TA systems are highly conserved in genetically diverse strains of STB, which form the oldest lineage of the MTBC, their integration and subsequent chromosomal expansion appear beneficial to the unique physiology of the MTBC [94].

Extensive gene duplication events have led to the accumulation of functional VapBC, MazEF, ParDE, RelBE and HigBA TA homologs. However, this duplication process appears to have been non-congruent, as antitoxins have evolved to function to suppress toxins that alter or regulate bacterial growth. Accordingly, there are several evolutionary scenarios post-duplication that would lead to such functional gains: (1) TA genes accumulate mutations rendering their proteins non-functional. Once duplicated inactive TA genes continue to evolve and their proteins ultimately regain function, but with discrete activities compared to ancestral proteins; (2) TA genes co-evolve to maintain functionality, but under an alternative regulatory context; (3) TA proteins retain functionality, while evolving through more promiscuous non-cognate TA interactions, which ultimately enable the non-congruent evolution and co-existence of various functional TA systems.

Figure 4.1: Phylogeny of *Mycobacterium tuberculosis* RelBE toxin-antitoxin systems in *Mycobacterium*.

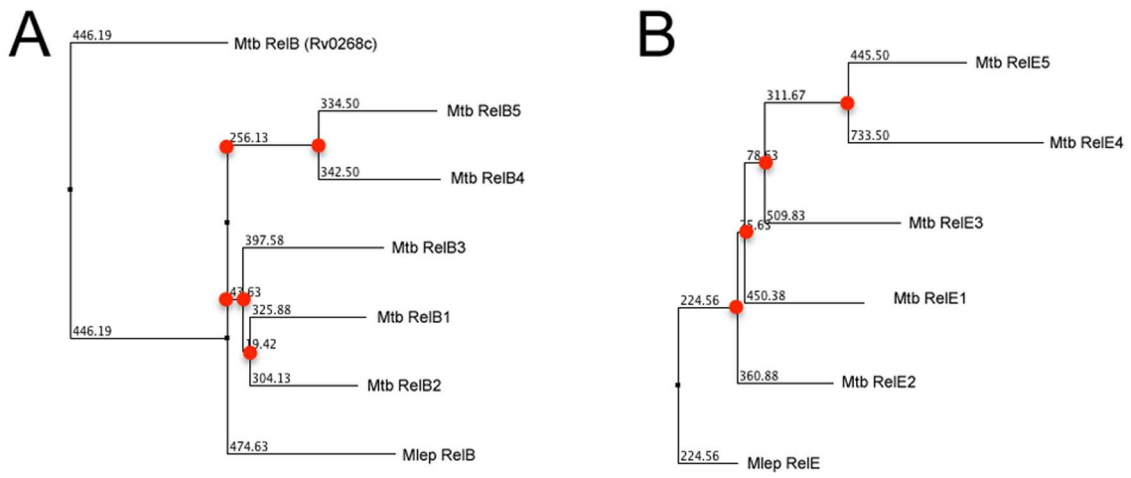

Figure 4.1: Phylogeny of *Mycobacterium tuberculosis* RelBE toxin-antitoxin systems. Phylogenetic patterns between (A) *Mtb* RelB and *Mlp* RelB and (B) *Mtb* RelE proteins and *Mlp* RelE. Red dots indicate duplication events in the lineages. Labels refer to bootstrap support for the node and the scale for expected number of mutational steps is indicated.

Mtb ParDE/RelBE TA proteins in particular appear to have undergone non-congruent evolution based on phylogenetic comparisons. Phylogenetic analysis of experimentally validated ParDE/RelBE TA homologs of *E. coli*, *S. pneumoniae*, and *S. typhimurium* discern evolutionary relationships of ParDE/RelBE TA proteins in *Mtb*. This analysis initially revealed that all *Mtb* TA proteins cluster with non-*Mtb* ParD, ParE, RelB, RelE, HigB, or HigA proteins. Topologies of non-*Mtb* TA proteins also demonstrate different branching orders of cognate *Mtb* TA components, which indicates that a number of mutational/evolutionary steps are necessary to diverge the *Mtb* ParDE/RelBE proteins. Phylogenetic analysis of RelBE proteins encoded by *Mtb* and the RelBE proteins encoded by *Mlp,* revealed different topologies for RelB proteins and RelE proteins. The *Mtb* RelB1-through-3 homologs tightly cluster, RelB4 and RelB5 cluster and the orphan RelB encoded by *rv0268c* groups alone in Figure 4.1. Cognate RelE toxin proteins map sequentially. *Mtb* RelE2, RelE1 and RelE3 map in order one branch point from the *Mlp* RelE toxin, and RelE4 and RelE5 co-cluster (*i.e.* in Figure 4.1). As observed from the topology of the phylogeny, the evolutionary paths of cognate *Mtb* RelBE1 and RelBE2 proteins are different, and therefore lack phylogenetic congruence (*i.e*. non-congruent coevolution).

4.4 Complex regulation of *Mycobacterium tuberculosis* toxin-antitoxin systems.

Expression profiling and transcriptomic studies in *Mtb* find that many toxin and antitoxin genes are transcribed under any given condition. This presents the situation that multiple TA proteins cooperatively function in response to changing environmental conditions, which is consistent with numerous studies discussed above. Several mechanisms of bacterial regulation seem to be uniquely evolved in regulating the functions of *Mtb* TA components and include cognate and non-cognate TA protein interactions, autoregulation of TA transcription, differential asRNA regulation of TA transcripts, and transcriptional coupling of TA loci to metabolic processes responsive to the growth environment and adaption. Studies have found that some *Mtb* toxins are able to interact with both non-cognate and cognate antitoxins to regulate function (*i.e.* in Figure 4.2) [56, 95, 96]. In the cases where non-cognate interactions between toxin and antitoxin pair

have been demonstrated there is a specific pattern of neutralizing activity or interaction of non-cognate proteins indicating that there was specificity in the mutual function between non-cognate TA components. In addition to the identification of physical non-cognate interactions between TA loci components, studies provided evidence that there are differences in the interaction strength of non-cognate TA protein complexes (*i.e.* Slayden & Dawson, *unpublished*). It is believed that structural difference between RelE proteins impacts the full extent of functional inhibition by cognate and non-cognate RelB partners [24, 80, 81, 97]. In certain cases, there is evidence that RelBE heterodimers consist of combinations of cognate and non-cognate TA protein components to regulate uncontrolled toxin activity [98]. TA proteins are able to interact in cognate and non-cognate toxin-antitoxin heterodimers. An example of multiple non-cognate interactions is $MazE_2$ that binds to MazF dimers, until selectively degraded by proteases during the stringent response to activate MazF-dependent function [21, 99, 100].

Figure 4.2: Complex regulation underlying the overrepresentation of differentially expressed toxin and antitoxin genes in *Mycobacterium tuberculosis*.

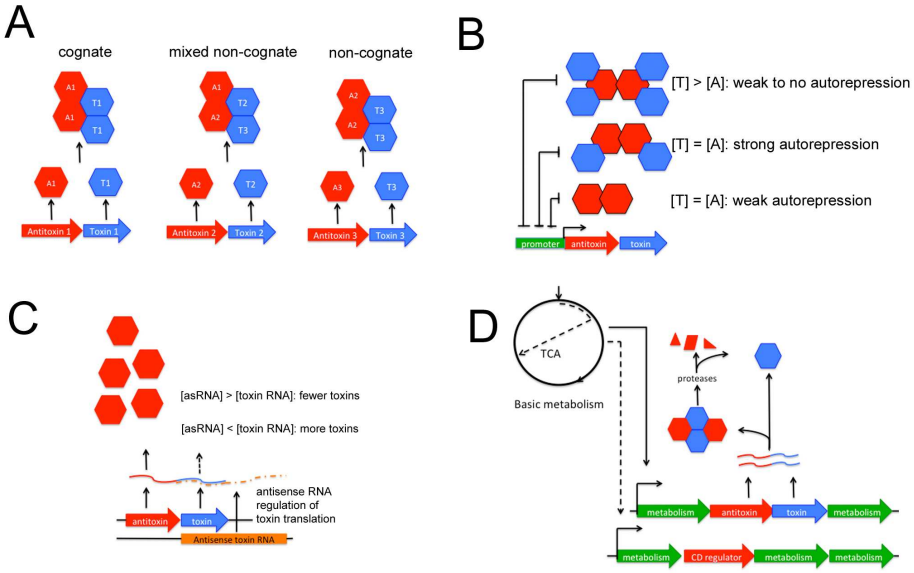

Figure 4.2: Complex regulation underlying the overrepresentation of differentially expressed toxin and antitoxin genes in Mycobacterium tuberculosis. (A) Cognate, mixed non-cognate and non-cognate toxin antitoxin interactions resulting in regulation of toxin activity. (B) Autoregulation of toxin antitoxin loci by toxin:antitoxin protein complexes. (C) *Cis*-antisense regulation of toxin transcription in a Type II TA loci. (D) Transcriptional coupling of a TA loci with basic metabolism and alternative regulators involved in adaptive responses to nutrient changes in the environment.

While sequence conservation exists in TA families, it is also known that there is a

degree of non-conservation, and it is generally considered that non-conservation and protein structure underlie interactions of TA proteins [97]. Protein sequence alignments of *Mtb* RelBE proteins highlighted regions of conservation among *Mtb* Rel-family toxins and antitoxins, providing insights into protein characteristics that may be critical for TA protein-protein interactions and potential cognate and non-cognate partner specificity (*i.e.* in Figure 4.2). *Mtb* RelBE TA proteins provide an example of how protein sequences can be used to understand protein interaction specificity. These RelB and RelE alignments found conserved amino acid residues characteristic of RelB and RelE *Mtb* proteins and also residues of lower conservation. Interestingly, the single *relBE2* TA locus encoded by *Mlp,* which has been annotated as a non-functional pseudogene, is missing several critical residues known to be important for RelE activity further substantiating that the *Mlp* RelE has been inactivated consistent with observed extensive gene decay throughput the genome [101]. Protein alignments were combined with protein structures to map the physical orientation of the conserved regions of *Mtb* RelE proteins. The combination of using conserved amino acids identified in RelE3 and mapping to these to the RelE3 crystal structure revealed location of conserved and non-conserved regions in *Mtb* RelE toxin proteins. This was extended to include co-crystal structures of RelB and RelE TA proteins to identify potential interacting amino acid residues involved in TA interactions (*i.e.* in Figure 4.3). It was found that AA-E48 in RelB3 is in close proximity to RelE residue AA-R60, and AA-E53 and AA-E57 located on the third alpha helix of RelB2 and are in close proximity to the binding site residues AA-R71 and AA-R76 of the RelE2. The conserved AA-residues among all RelE family proteins is thought to provide function and interaction with the antitoxin and ribosome, whereas AA-residues not conserved among RelE members are thought to be involved in more unique non-cognate TA protein-protein interaction patterns observed experimentally because the secondary structure, particularly among antitoxins is important for interaction specificity. TA expression is auto-regulated at the transcriptional level by TA components binding at the promoter resulting in induction or repression of the bi-cistron (*i.e.* in Figure 4.2). This type of TA regulation is considered to occur in multiple TA families, and

is tightly controlled by the toxin that rapidly de-represses *mazEF* transcription [102]. This is coupled with the MazF-dependent decay of *mazF*-only mRNA to replenish MazE, and this is likely an important feature of regulating TA loci function [21]. RelB proteins are also known to function as transcriptional activators alone and in complex with RelE proteins, so post-transcriptional regulation of TA loci by TA proteins appears to be a more widespread and important regulatory mechanism to control toxin activity [97]. RelB-RelE dimers assemble into heterotetramers through the N-terminus of RelB2 [103]. These complexes only weakly bind to DNA operator sequences (*i.e. relO1* and *relO2*) in the upstream *relBE* promoter (*i.e.* P_{relBE}) [98].

Figure 4.3: RelBE sequence alignments and modeling to guide understanding of *Mycobacterium tuberculosis* toxin-antitoxin interactions.

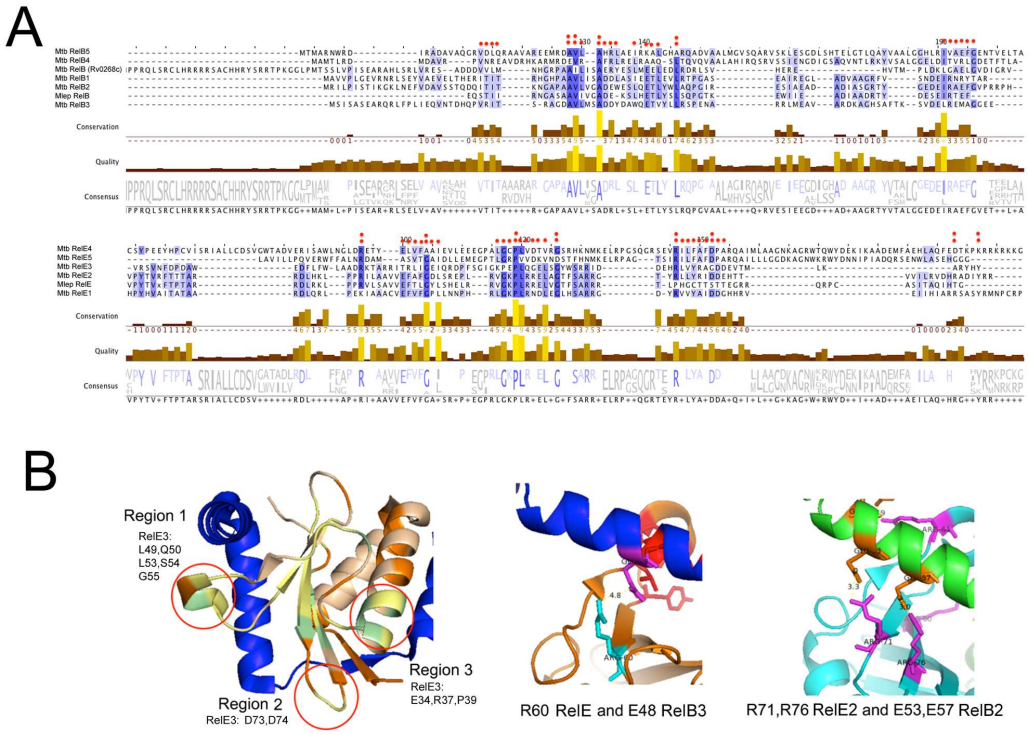

Figure 4.3: RelE and RelB protein sequence alignments and modeling to guide understanding of TA protein-protein interactions. A) *Mtb* RelB and *Mlp* RelB sequence alignment. *Mtb* RelB and *Mlp* RelB sequence alignment. Conserved amino acids residues in (A) and (B) alignments are indicated by double red dots and regions of conservation are indicated by single red dots. Shading indicates % similarity in the alignment for that amino acid residue, the darker the shade the great the % identity. (B) Mapping RelE of regions of conservations and conserved amino acids and mapping conserved interacting residues of RelE and RelB.

Instead, RelB-to-RelE ratios that are greater than one permit DNA-binding (*e.g.* $RelB_2$-RelE and $RelB_2$-RelE-$RelB_2$), which ensures basal RelE production [20, 98]. During adaptive processes to

stress, RelB can be rapidly degraded resulting in induction of RelE, yet *relBE* is still significantly upregulated even after RelB is replenished [104]. Considering that P_{relBE} resembles a very strong phage promoter, it is considered that *relBE* is post-transcriptionally regulated in a manner to prevent RelE hyper-activation [105]. Taken together, these observations further substantiate that chromosomally-encoded TA loci have evolved functionally as part of a larger TA system.

It is generally thought that type II toxins are regulated through protein-protein interactions and type I systems are regulated by asRNA, inhibiting the translation of the toxins. However, there are numerous *in vivo* observations (*i.e.* RelBE2 and MazEF6) of imbalances between type II cognate toxin and antitoxin mRNAs that are encoded in, and apparently co-expressed as part of a bi-cistronic message [56]. This widespread observation of TA transcript imbalance suggests that there are other mechanisms of regulation, including post-transcriptional processing mediated *via* asRNA, whereby the binding of complementary RNAs either alter the transcription-translation of mRNA and/or protects against RNase-mediated decay that is shown in Figure 4.2. There is an example of antisense regulation of the type II RelBE2 system where the toxin is regulated by a *cis*-acting asRNA that maps to the *relE2* portion of the *relBE2* transcript to suppress *relE2* (*i.e.* Slayden and Dawson, *unpublished*). While this is an example of *cis*-asRNA regulation, *in silico* analyses based on sequence homology of toxin genes also raises the possibility that *trans*-asRNA regulation may occur and therefore antisense regulation maybe a general mechanism at the TA-system level. These observations indicate that asRNA may be a general regulatory mechanism of type I and II TA systems. The finding of an antitoxin asRNA involved with the regulation of a type II toxin represents a novel level of complexity to co-evolution and regulation of TA systems.

It is becoming increasingly evident that the timing of TA loci may be associated with their location in the genome. Many of the TA loci encoded in the *Mtb* genome are co-localized or encoded in bi-cistronic operons with genes encoding proteins involved in cell cycle regulation and basic metabolism (*i.e.* in Figure 4.2). This co-localization in operons results in transcriptional coupling of TA loci with *Mtb* cell cycle and metabolism responsive to availability of nutrients and

the growth environment. RelBE1 encoded by *rv1246c-rv1247c* is located in a five-gene operon, which includes *rv1248c* (or *kgd*), which encodes a multifunctional protein capable of catalyzing alpha-ketoglutarate decarboxylase, alpha-ketoglutarate dehydrogenase, and hydroxyoxoadipate synthase activities [106]. The co-localization of a gene encoding a protein involved in basic metabolism and the TCA cycle, and the *relBE1* provides a mechanism to transcriptionally coupling TA loci regulation and function to the TCA cycle and carbohydrate and fatty acid carbon source availability. Furthermore, a known and conserved cell cycle regulator MadR1 encoded by *rv2216* is co-localized with *rv2215*, which encodes a component of the pyruvate dehydrogenase complex [107-109]. Therefore, the co-localization and transcriptional coupling of RelBE1 with proteins essential to basic metabolism and responsive to carbon source availabilities with a cell cycle regulator exemplifies coupling adaptive responses and cell cycle with growth environment.

4.5 Toxin-antitoxin systems in mediating *Mycobacterium tuberculosis* persistence.

The vast majority of encoded TA loci are induced as part of various adaptive responses to host-associated environmental stress such as hypoxia, NS, and low pH [7, 9, 70, 110], the activation status of infected Mφs [7, 9, 70, 110] and the development of T cell-mediated immunity to infection [32]. Importantly, these findings in *Mtb* are consistent with other studies in model and pathogenic organisms demonstrating that TA proteins are involved with adaptation to stress and bacterial survival [97]. It is now accepted that during a natural infection an array of phenotypic alterations, allow for sub-populations of bacilli to become refractory to natural immunological mechanisms and/or chemotherapy. Observations that bacterial populations exhibit a high degree of phenotypic heterogeneity is thought to ensure survival, as at least a small proportion of cells is pre-disposed to adequately respond to potential threats (*e.g*. antibiotics). While one phenotype provides an advantage in a certain environment, other phenotypes may improve survival under entirely different circumstances [111]. This occurs in *Mtb* [112], and is referred to as “bet-hedging” [111]. It has been reported that these emerge spontaneously as the result of stochastic or “noisy” gene expression that leads to significant differences in mRNA and proteins between individual

cells in bacterial populations [113, 114].

However, there is emerging evidence that the phenotypic diversity observed in bacterial populations is the result of specific regulatory systems that couple cell cycle progression and adaptive bacterial metabolism with changing growth environments rather than only by stochastic gene expression [23, 56, 109, 115]. Inclusion of adaptive regulatory elements to the stochastic noise mechanism for the generation of phenotypically diverse bacteria within a population, has led to a multicellular concept we refer to as the phenomic potential. Phenomic potential is an idea that any single bacterial chromosome encodes more functions than can be accommodated in any one bacterial cell, but through intricate regulatory control mechanisms beyond stochastic noise, individual cells within the total population are specifically adapted in response to changing environments, leading to a selective advantage for the whole population. This is consistent with the identification of cell cycle regulators in *Mtb* that share significant homology and molecular action, but regulate different repertoire of adaptive responses [109, 115]. For one of these cell cycle regulators, the response includes MazEF TA loci [56]. This concept is also supported by the analysis of mutation libraries in bacteria that define the minimal coding capacity to sustain growth at twenty percent of the total genome [68]. This low “nominal coding capacity” supports the idea of phenomic potential and reflects that one genome has the ability to encode multiple phenotypes enabling diversification of the bacterial population.

Phenotypically diverse bacteria within a population become different from one another because they synthesize and accumulate different sets of mRNAs and proteins. Stochastic noise alone though, is unlikely able to generate specific metabolically diverse groups of microorganisms without further amplification from feedback loops [116]. Eldar and Elowitz found that both positive- and negative-feedback loops amplify noise to produce a stable, phenotypically diverse population of cells [117]. They are formed through cell-signaling cascades, which underlie the so-called “threshold-based” persistence mechanism [118]. The importance of phenotypic heterogeneity to LTBI and treatment is still unknown, but it is clear that persistent mycobacteria are in an altered

metabolic and persistent state [119, 120].

4.6 Toxin-antitoxin systems in mediating *Mycobacterium tuberculosis* pathogenesis.

TA systems are overly abundant in the genomes of pathogenic SGM and particularly *Mtb*, implicating them in adaptive metabolism, pathogenesis, persistence and tolerance to treatment during infection. The sheer number of *Mtb* TA loci makes it difficult to discern their more individual contributions of TA components to pathogenesis. TA loci are induced as part of the *Mtb* response to stress such as hypoxia [24], NS [9], and DNA damage [121], yet singular deletions lead to few, if any phenotypic defects, as homologous TA systems often function in a compensatory manner [57, 80, 122, 123]. *In vitro* studies have found that levels of multidrug-tolerant persisters decrease significantly only after the serial deletion of at least five type II toxin homologs, which indicates that they are functionally redundant [123, 124]. In contrast, Helaine *et al* have shown that all ten RelE/ParE superfamily toxins are all essential for developing persistence in IFN-γ-stimulated Mφs, which shows that they are functionally unique [122]. Thus, more discrete contributions of TA loci to *Mtb* pathogenesis are largely inferred from 'genome-wide' screens using transposon mutant libraries. *In vitro* characterization has further shown that they function to alter the transcriptome and , the proteome, culminating in the emergence of drug-tolerant sub-populations of *Mtb* [24, 80, 125]. There is mounting evidence though, that *Mtb* TA systems are much more involved in virulence. *Mtb*'s TA systems are found to be basally expressed at significantly higher levels in more virulent *Mtb* strains [126]. The post-transcriptional regulation of TA loci is a dynamic process though [125, 127], which is differentially reflected *in vivo* [32, 128]. TA systems function as global transcriptional regulators and are likewise important for long-term survival *in vivo* [39, 89]. Now given that TA loci appear to be under positive selection in most bacterial pathogen genomes, it is likely that they uniquely contribute to gains in virulence [25]. This phenomenon was first described by Joseph Bigger in 1944, noting that a small proportion of drug-susceptible bacteria survived lengthy chemotherapy [129]. Then in 1958, D.W. McDermott applied Bigger's terminology and defined *Mtb* persistence as the "capacity for drug-susceptible organisms to survive extensive drug

attack upon subsisting in the host" [130]. By enlarge this phenomenon was ignored until only more recently, when persisters have been implicated in a numerous chronic bacterial infections [116]. Moreover, it is understood that persister cells underlie the recurrent need for long-term antibiotic therapy for infections caused by medically important but difficult to treat pathogens and particularly *Mtb* [111, 116].

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LIST OF ABBREVIATIONS

1. Tuberculosis ..... TB
2. Active tuberculosis disease ..... ATB
3. World Health Organization ..... WHO
4. Isoniazid ..... Inh
5. Rifampin ..... Rif
6. Ethambutol ..... Eth
7. Pyrazinamide ..... Pza
8. *Mycobacterium tuberculosis* ..... *Mtb*
9. *Mycobacterium tuberculosis* complex ..... MTBC
10. *Mycobacterium canettii* ..... *Mcn*
11. *Mycobacterium africanum* ..... *Maf*
12. *Mycobacterium bovis* ..... *Mbv*
13. Latent tuberculosis infection ..... LTBI
14. Guanosine-cytosine ..... GC
15. Nucleotide ..... NT
16. Rapid growing mycobacteria ..... RGM
17. Slow growing mycobacteria ..... SGM
18. Smooth tubercle bacilli ..... STB
19. *Mycobacterium kansasii* ..... *Mkn*
20. *Mycobacterium marinum* ..... *Mmr*
21. *Mycobacterium leprae* ..... *Mlp*
22. Whole genome sequencing ..... WGS
23. Open reading frame ..... ORF
24. Amino acid ..... AA

25. Horizontal gene transfer ..... HGT

26. Antigen ..... Ag

27. Mammalian cell entry ..... MCE

28. Early secreted antigen export ..... ESX

29. Homologous recombination ..... HR

30. Two-component signaling ..... TCS

31. Single nucleotide polymorphism ..... SNP

32. Insertion and/or deletion ..... InDel

33. *Mycobacterium avium* complex ..... MAC

34. Non-synonymous single nucleotide polymorphism ..... nsSNP

35. Silent single nucleotide polymorphism ..... sSNP

36. Transcriptional start site ..... TSS

37. Dormancy regulon ..... DosR

38. Intergenic region ..... IGR

39. Antisense RNA ..... asRNA

40. Toxin-antitoxin ..... TA

41. Kilobase ..... kb

42. Insertion sequence ..... IS

43. Pattern recognition receptor ..... PRR

44. Macrophage ..... Mφ

45. Dendritic cell ..... DC

46. Pulmonary lymph nodes ..... PLNs

47. Mannose receptor ..... MR

48. Mannosylated lipoarabinomannan ..... ManLAM

49. T Helper ..... T_H

50. Nitric oxide ..... NO

51. Interferon ..... IFN

52. Wild type ..... WT

53. Major histocompatibility complex ..... MHC

54. Reactive oxygen species ..... ROS

55. Nucleotide-binding oligomerization domain-like receptor ..... NLR

56. Polymerase chain reaction ..... PCR

57. Non-human primate ..... NHP

58. Cytotoxic T lymphocyte ..... CTL

59. Human immunodeficiency virus ..... HIV

60. Resuscitation promotion factor ..... Rpf

61. Hyper-phosphorylated guanosine ..... (p)ppGpp

62. 3'-,5'-cyclic adenosine monophosphate ..... cAMP

63. Genomic DNA ..... gDNA

64. Ribosomal RNA ..... rRNA

65. Messenger RNA ..... mRNA

66. Tumor necrosis factor alpha ..... TNF-α

67. RNA polymerase ..... RNAP

68. Nutrient starvation ..... NS

69. Non-coding RNA ..... ncRNA

70. Small RNA ..... sRNA

71. Untranslated region ..... UTR

72. Post segregational killing ..... PSK

73. Programmed cell death ..... PCD

74. Isopropyl β- d-1-thiogalactopyranoside ..... IPTG

75. Luria broth ..... LB

76. Mutant ..... Mut

77. Base-pair ..... bp

78. Restriction digestion ..... RD

79. Relative fluoresence unit ..... RFU

80. Rapid amplification of complementary ends ..... RACE

81. Electrophoretic mobility shift assay ..... EMSA

82. Reverse transcription quantitative polymerase chain reaction ..... RT-qPCR

83. Arginine ..... R

84. Tyrosine ..... Y

85. Peripheral blood mononuclear cells ..... PBMCs