

# *Mycobacterium tuberculosis* WhiB3: A Novel Iron–Sulfur Cluster Protein That Regulates Redox Homeostasis and Virulence

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## Abstract

**Significance:** *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis (TB), can persist in a latent state for decades without causing overt disease. Since latent *Mtb* is refractory to current antimycobacterial drugs, the discovery and characterization of the biological mechanisms controlling the entry, maintenance, and emergence from latent infection is critical to the development of novel clinical therapies. **Recent Advances:** Recently, *Mtb* WhiB3, a member of the family of intracellular iron–sulfur (Fe–S) cluster proteins has emerged as a redox sensor and effector molecule controlling several aspects of *Mtb* virulence. WhiB3 was shown to contain a 4Fe–4S cluster that specifically reacts with important host gases (O<sub>2</sub> and NO), and exogenous and endogenous metabolic signals to maintain redox balance. Notably, the concept of reductive stress emerged from studies on WhiB3. **Critical Issues:** The detailed mechanism of how WhiB3 functions as an intracellular redox sensor is unknown. Sustaining *Mtb* redox balance is particularly important since the bacilli encounter a large number of redox stressors during infection, and because several antimycobacterial prodrugs are effective only upon bioreductive activation in the mycobacterial cytoplasm. **Future Directions:** How *Mtb* WhiB3 monitors its internal and external surroundings and modulates endogenous oxido-reductive pathways which in turn alter *Mtb* signal transduction, nucleic acid and protein synthesis, and enzymatic activation, is mostly unexplored. Modern expression, metabolomic and proteomic technologies should provide fresh insights into these yet unanswered questions. *Antioxid. Redox Signal.* 16, 687–697.

## Introduction

THE ABILITY OF *Mtb* to maintain a state of latent TB infection is responsible for its remarkable success as a pathogen. Approximately 2.3 million people die each year of this disease and the devastation caused by this pathogen is exacerbated by the coexistent HIV epidemic and the emergence of multidrug resistant, extensively drug-resistant, and totally drug-resistant *Mtb* strains (19).

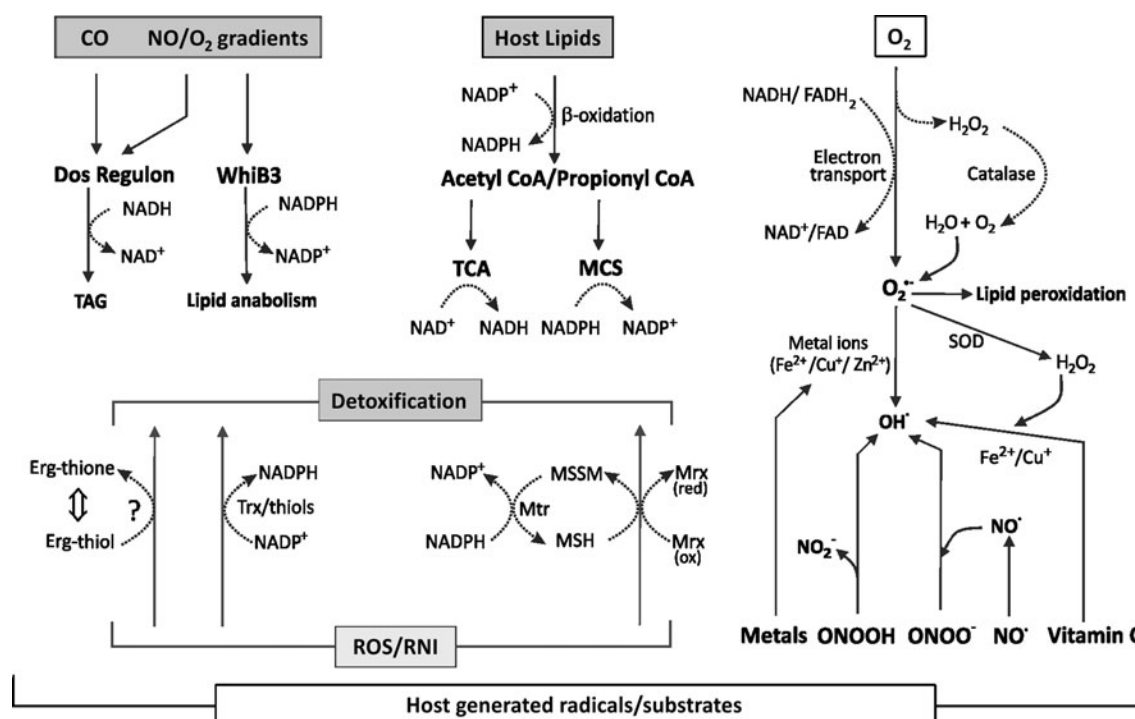
A major obstacle to the development of successful therapeutic strategies is the lack of a mechanistic understanding of how *Mtb* maintains a persistent, nonreplicating state in human tissues for decades, unresponsive to antimycobacterial drugs, to then abruptly resume growth and cause disease (24). This requires a detailed understanding of the metabolic flexibility of the bacilli that survive in the varied environments within the human host, ranging from high oxygen tension in the lung alveolus to hypoxic conditions within the tuberculous granuloma (6, 10). An important challenge is to under-

stand how *in vivo* gases [*e.g.*, oxygen (O<sub>2</sub>), nitric oxide (NO), carbon monoxide (CO), carbon dioxide (CO<sub>2</sub>), hydrogen (H<sub>2</sub>), and hydrogen sulfide (H<sub>2</sub>S)] and nutrients (*e.g.*, host fatty acids), several of which are thought to play a crucial role in persistence, affect *Mtb* physiology and consequently redox homeostasis (Fig. 1). Furthermore, frontline anti-*Mtb* drugs such as isoniazid (34), ethionamide (7), and PA-824 (32) require bioreductive activation to exert antimycobacterial effects, which magnifies the importance of understanding how *Mtb* maintains redox balance during treatment. It is this lack of drug efficacy against dormant *Mtb* that represents a fundamental challenge to investigators worldwide. A better understanding of how environmental host factors affect *Mtb* redox homeostasis (Fig. 1) may shed light on the biology of *Mtb* persistence and lead to the development of new and improved antimycobacterial drugs.

Exciting progress has been made in understanding how members of the WhiB family sense and respond to host gases NO and O<sub>2</sub> through their iron–sulfur (Fe–S) clusters, and how

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**FIG. 1. Redox machinery of *Mtb*.** During infection, *Mtb* encounters redox active compounds that have the capacity to alter or skew intracellular redox balance. *Mtb* has evolved several mechanisms to maintain redox homeostasis. *Mtb* utilizes host lipids as a source of carbon *in vivo*, which are metabolized via the  $\beta$ -oxidation pathway. This leads to the generation of high concentrations of NAD(P)H during the conversion of fatty acids to acetate and propionate. The accumulation of high levels of NAD(P)H causes reductive stress in *Mtb* (18, 40). Furthermore, host-generated free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are also capable of disturbing *Mtb* redox balance. However, *Mtb* counterbalances the stress generated by these radicals primarily via the abundant intracellular redox buffer, mycothiol (MSH). In addition, protein thiols and mycobacterial thioredoxins (Trx) are also involved in detoxifying the adverse effect of free radicals. Another yet uncharacterized redox active molecule, ergothioneine (Erg), is also expected to play a role in detoxification of free radicals and maintaining redox balance. Endogenous byproducts of mycobacterial respiration such as  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\bullet-}$  may react with mycobacterial lipids to generate lipid peroxides. In the presence of iron or copper, these ROS can generate the highly redox active  $\text{OH}^{\bullet}$ . ROS or RNS such as peroxide, peroxyntrous acid (ONOOH), and peroxyntrite (ONOO $^{\bullet}$ ), and compounds such as vitamin C (in the presence of metal ions) may also lead to the production of intramycobacterial  $\text{OH}^{\bullet}$  that can cause the oxidation of intracellular substrates. Host-generated gases also play a role in inducing mycobacterial redox changes. *Mtb* has well-defined sensor systems such as the DosS/T/R and WhiB3 signaling pathways, which specifically sense NO, CO, and changes in  $\text{pO}_2$ . CO, NO, and  $\text{O}_2$  interact with the Dos signaling pathway, leading to the induction of the 47-member Dos regulon. It also leads to the production of the storage lipid TAG, which requires large quantities of NAD(P)H for synthesis. Similarly, WhiB3 also senses and responds to alterations in cytoplasmic NO and  $\text{O}_2$ . Mrx; mycoredoxin, Mtr; mycothiol disulfide reductase.

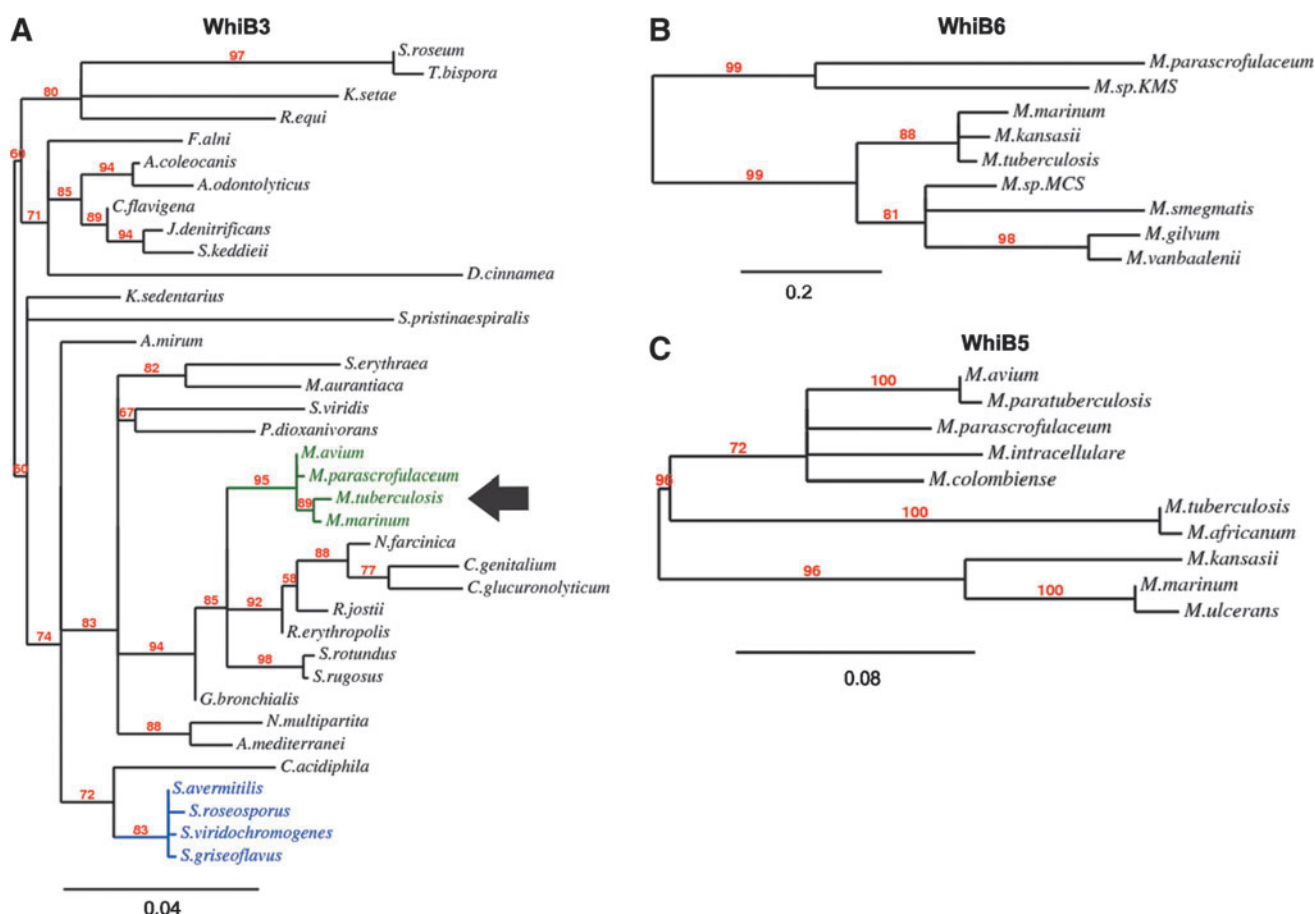
they may modulate *Mtb* virulence. Here we review the current knowledge of the *Mtb* WhiB-like family of proteins, focusing on the best characterized member, WhiB3.

### Phylogenetic Distribution of the Wbl Proteins

Comprehensive phylogenetic analysis showed that *Mtb* WhiB1, WhiB2, WhiB3, WhiB4, and WhiB7 are evolutionary closer to *Corynebacterium* and *Nocardia* WhiB-like (Wbl) proteins (Fig. 2A). Furthermore, using Interpro and Pfam databases, protein bioinformatic analyses have identified more than 1600 Wbl homologues (database ID: IPR003482/PF02467), which are exclusive to actinobacteria and the double-stranded DNA (dsDNA) containing siphoviridae family of phages. Notably, WhiB5 and WhiB6 are restricted to mycobacteria (Figs. 2B and 2C) with WhiB5 only present in virulent strains (Fig. 2C). All Wbl proteins with the exception of WhiB5

contain four invariant Cys residues arranged in a Cys- $X_{14-22}$ -Cys- $X_2$ -Cys- $X_5$ -Cys motif (Fig. 3A), which is typically associated with metal co-coordinating DNA-binding proteins. Secondary structure prediction of Wbl proteins indicates an acidic amphipathic  $\alpha$ -helical region near the N-terminus and a basic  $\alpha$ -helical region at its C-terminus with a central  $\beta$ -sheet region (43).

Most Wbl proteins (~90%) contain a "WhiB" domain (including all Wbl homologues in phages, and *Mtb* WhiB1, WhiB2, WhiB4, WhiB5, and WhiB6), whereas ~7% possess an additional AT-hook like domain at their C-terminus (e.g., *Mtb* WhiB3 and WhiB7). The AT-hook like domain is a DNA-binding motif with a preference for A/T-rich regions (4) (Fig. 3B). Approximately 2% of Wbl members contain an additional RNA polymerase (RNAP) sigma70r4 binding region and a clearly distinguished DNA-binding 'helix-turn-helix' (HTH) motif that binds to the -35 promoter region. A small percentage of Wbl members contain a RNAP sigma70r4t2 signature (Fig. 3B).



**FIG. 2. Phylogenetic analysis of *Mtb* (A) WhiB3, (B) WhiB6, and (C) WhiB5.** BLAST analyses were performed against the NCBI 'Nr' database. All the hits (except candidates from the same species) with  $e \geq 1 e^{-10}$  were considered significant and further used in Clustal W alignment. A maximum likelihood method was used for tree construction, and boot strap values are indicated along the tree nodes. This analysis demonstrates that *Mtb* WhiB3 constitute a separate node (arrow) (A) from *Streptomyces* spp. and is closer related to *Nocardia* and *Clostridium* spp. A similar distribution pattern and tree architecture also hold true for *Mtb* WhiB1, WhiB2, WhiB4, and WhiB7 (not shown), but not for WhiB6 (B) and WhiB5 (C), which are restricted to mycobacteria. (To see this illustration in color the reader is referred to the web version of this article at [www.liebertonline.com/ars](http://www.liebertonline.com/ars)).

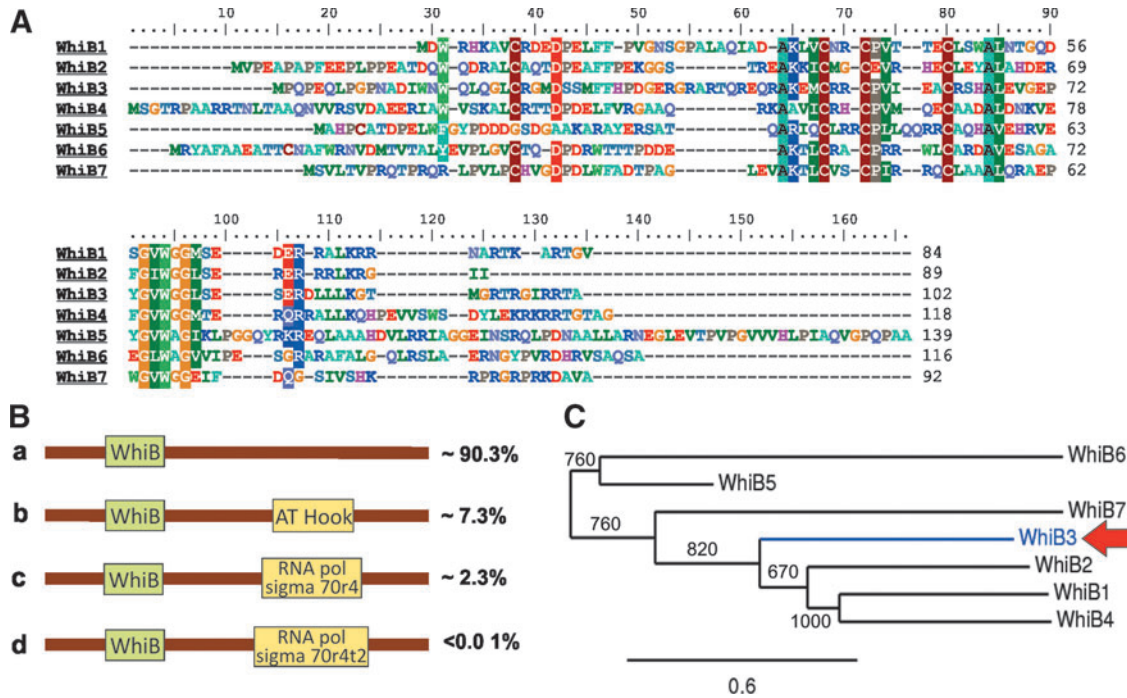
Although a systematic functional analysis of Wbl protein domains is lacking, the diversity in protein architecture may be responsible for the varied functions including redox homeostasis(40), cAMP regulation (1), oxidative stress (28), cell division (23), sporulation (16), quorum sensing (5), drug resistance (33), and virulence (44).

*Mtb* harbors seven Wbl proteins, WhiB1 (Rv3219), WhiB2 (Rv3260c), WhiB3 (Rv3416), WhiB4 (Rv3681c), WhiB5 (Rv0022c), WhiB6 (Rv3862c), and WhiB7 (Rv3197A) (Fig. 3C). Only moderate aa sequence similarity exists between members of the *Mtb* Wbl family (<70%). BLAST analysis of *Mtb* Wbl proteins against A CLAssification of Mobile genetic Elements (ACLAME), a database of mobile genetic elements such as plasmids, phages, and viruses, identified several Wbl homologues that are present on plasmids. For example, WhiB1 and WhiB4 share significant similarity ( $E\text{-value} = \leq e^{-15}$ ) with gene products from *Streptomyces* plasmids. Also, WhiB2 and WhiB3 show similarity with *Rhodococcus* plasmid-encoded gene products (Table 1). Thus, it is unlikely that *Mtb* Wbl members evolved through gene duplication events. Rather, it is tempting to hypothesize that the *Mtb* Wbl proteins have been

independently acquired from mycobacterial ancestors via lateral gene transfer from mobile elements such as plasmids or phages. The selective distribution of *Mtb* Wbl proteins in different mycobacterial species (e.g., the absence of WhiB5 in nonpathogenic species) coupled with considerable sequence heterogeneity, suggest a discrete functional role for each *Mtb* Wbl protein.

#### Genetic Regulation of *Mtb* wbl Genes

To better understand the genetic regulation of *Mtb* wbl genes, Geiman *et al.* (22) performed an elegant real-time PCR expression study that examined the differential expression of all seven *Mtb* wbl genes following exposure to a variety of environmental stresses, including detergent exposure, oxidative stress, acid exposure, nutrient starvation, ethanol, antibiotic, heat, and low iron stress. WhiB3 expression was highly increased upon acid stress, but weakly upon exposure to oxidants. WhiB7 was highly upregulated under low iron conditions, whereas whiB7 and whiB2 expression was shown to be significantly increased upon exposure to antibiotics (22).



**FIG. 3. Phylogenetic relationship among *Mtb* Wbl proteins and domain organization across species.** (A) *Mtb* Wbl protein sequences were extracted from NCBI and aligned using Clustal W. In addition to four invariant Cys residues that are a characteristic signature of the Wbl family, the small G-X-W motif, typical of fatty acid binding proteins, is also present. Note that WhiB5 lacks a Cys residue. (B) Domain architecture of the Wbl member indicates that the “WhiB” domain is the most widely represented since it is present in ~90% of Wbl members. All *Streptomyces*, mycobacterial phages, and mycobacterial WhiB1, WhiB2, WhiB4, WhiB5, and WhiB6 homologues share this architecture. (C) Phylogenetic tree was constructed using the maximum likelihood method and revealed that *Mtb* Wbl’s are clustered in two nodes comprising of WhiB5 and WhiB6 in one node, and WhiB1, WhiB2, WhiB3 (red arrow), WhiB4, and WhiB7 in another. This indicates that WhiB5 and WhiB6 are functionally distinct from the other *Mtb* Wbl’s. The numbers along the nodes denote bootstrap values, and the phylogenetic scale represents the number of differences between different sequences.

The *whiB7* finding is consistent with a previous study implicating members of the WhiB family in antimicrobial stress (33). Overall, *whiB6* appeared to be responsive to the widest variety of stress conditions (22).

Exploiting an *Mtb* transposon library screen, *whiB1* and *whiB6* were both implicated in the switchover from a slow to fast growth rate (8). In another genome-wide expression study, *whiB6* expression was consistently upregulated under prolonged hypoxia, a condition associated with dormant in-

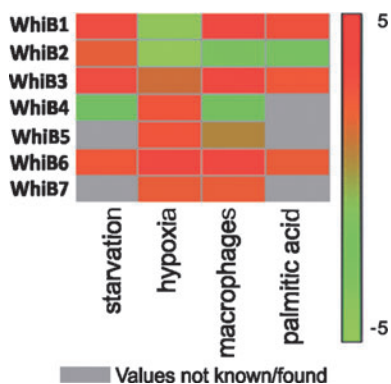
fection, whereas *whiB4* and *whiB2* were moderately upregulated (35, 36). Last, *whiB2* expression was shown to be significantly increased upon re-aeration following hypoxia, suggesting a possible role for *whiB2* in the activation of *Mtb* from the latent state (39).

When comparing the microarray expression profiles of several clinical *Mtb* strains within macrophages (27), it became clear that the *wbl* expression profiles are highly variable. Figure 4 depicts a summary of the relative *wbl* expression

TABLE 1. SIMILARITY OF *Mtb* WBL PROTEINS WITH MOBILE GENETIC ELEMENTS

Protein name	Query length	Hit length	Plasmids	Host organism	E value*	Identity (%)
WhiB1	84	105	SAP1	<i>Streptomyces avermitilis</i> MA-4680	4.00E-21	63
WhiB2	89	89	pREL1	<i>Rhodococcus erythropolis</i> PR4	9.00E-20	66
WhiB3	102	119	pRHL1	<i>Rhodococcus</i> sp. RHA1	8.00E-19	50
WhiB4	118	268	SCP1	<i>Streptomyces coelicolor</i>	5.00E-15	50
WhiB6	116	117	pMKMS01	<i>Mycobacterium</i> sp. KMS	3.00E-19	53
			Phages			
WhiB1	84	86	gp94	<i>Mycobacterium smegmatis</i>	3.00E-12	61
WhiB2	89	215	gp65	<i>Mycobacterium smegmatis</i>	5.00E-20	44
WhiB4	118	215	gp68	<i>Mycobacterium smegmatis</i>	5.00E-10	41

\**Mtb* Wbl proteins were used in a BLAST analysis against the ACLAME database that contains mobile genetic elements such as plasmids and phages. Only hits with  $e^{-10}$  were considered as significant. The presence of Wbl proteins on plasmids and phages suggest that *Mtb wbl* genes were laterally acquired. WhiB5 and WhiB7 did not yield any significant hits.



**FIG. 4. Relative transcription profiles of *Mtb wbl* genes.** Graphic representation of the independent *Mtb wbl* transcription profiles extracted from published studies (9, 35, 45). *WhiB3* and especially *whiB6* appear to be induced under a wide range of conditions including starvation, hypoxia, and within macrophages. Expression of *whiB4* was repressed under nutrient-starved conditions and upon infection of macrophages, whereas *whiB4* expression was slightly induced during hypoxia. Notably, *whiB1* and *whiB2* appears to be strongly repressed under hypoxic conditions.

profiles obtained from the literature. Noticeably, *whiB6* appears to be prominently upregulated under several conditions, consistent with the report by Geiman *et al.*, (22). *WhiB4* expression was upregulated under hypoxia, but downregulated during starvation and within macrophages. Surprisingly, starvation in the presence of oxygen leads to increased *whiB4* expression (9), suggesting a possible role in *Mtb* reactivation. *Mtb whiB3* appeared to be upregulated under a range of conditions.

### Metabolic Regulation of *Mtb WhiB3*

#### Metabolism

*Mtb WhiB3* senses fluctuations in the intracellular redox environment associated with  $O_2$  depletion, including the metabolic switchover to the favored *in vivo* carbon source, fatty acids (40). This has important consequences for understanding how *Mtb* persists within the host, as it is widely believed that fatty acids serve as a main source of carbon and energy during long-term infection.

Several lines of evidence implicate *WhiB3* as a metabolic regulator. First, *WhiB3* is essential for maintaining bacterial cell shape and size (40, 41). Second, *WhiB3* controls the anabolism of complex virulence lipids such as polyacyltrehaloses (PAT), diacyltrehaloses (DAT), sulfolipids (SL-1), and phthiocerol dimycocerosates (PDIM), which requires large quantities of reducing equivalents (NAD[P]H). Third, *WhiB3* was shown to maintain redox homeostasis during infection of macrophages (40). Fourth, the *Mtb ΔwhiB3* mutant showed substantially decreased production of the methyl-branched polar lipids PAT, DAT, and SL-1, but increased production of PDIM, and to a lesser extent triacylglycerol (TAG) (40). *Mtb ΔwhiB3* grows on much higher concentrations of propionate compared to wild-type (wt) *Mtb*, and accumulates PDIM and TAG during intramacrophage growth, suggesting that the increased resistance to propionate toxicity is because this intermediate is channeled into PDIM via the methyl-malonyl CoA (MMCoA) pathway

and into TAG (40). These metabolic analyses implicate *WhiB3* in core intermediary metabolism and are reminiscent of studies executed on FNR and ArcA, which regulate key enzymes in the tricarboxylic acid cycle (TCA) cycle under carbon source starvation conditions (31, 38).

#### Protein networks and *Wbl* function

Originally, *Mtb WhiB3* was discovered through a yeast two-hybrid screen using SigA as bait (44). Recently, an *E. coli* two-hybrid system was exploited and showed that 113 proteins interacted with *WhiB3* and 52 with *WhiB7* (average number of interacting neighbors  $\sim 6$ ). As is typically the case with microbial protein linkage maps, many interactions are difficult to explain in the context of what is biologically known about *WhiB3* (or *WhiB7*) function, and await future verification. Also, the frequency of false positives and negatives, often due to “sticky” interacting proteins (a common observation in yeast two-hybrid screens) is unknown. Nonetheless, the potentially large number of proteins interacting with *WhiB3* and *WhiB7* is difficult to ignore and may point to biological properties that are distinct among *Mtb Wbl* proteins. Lastly, it is uncertain to what extent post-translational modification (e.g., assembly of the *WhiB3* and *WhiB7* Fe-S clusters) of *Mtb* proteins expressed in *E. coli* influences protein-protein interaction. Thus, although an admirable resource, these data await further verification and should be interpreted with caution in order to generate accurate knowledge on the biological function of *Mtb Wbl* proteins.

A provocative finding by one group was that several *Wbl* proteins in their apo-forms demonstrated nonspecific protein disulfide reductase activity (2, 3, 20, 21). The authors proposed that the Fe-S cluster blocks the four active Cys residues until endogenous oxidants destroys the Fe-S cluster, which then leads to the formation of an intramolecular disulfide bond. Subsequent reduction of the disulfide bond (through a yet unknown mechanism) stimulates disulfide reductase activity. This debate was fueled further when independent groups were unable to detect such activity with *WhiB1* or the *S. coelicolor* orthologue of *WhiB3* (13, 42), and also when it was reported that *Mtb WhiB1* interacts with GlgB (an  $\alpha$ -1,4-glucan branching enzyme) to reduce the intramolecular GlgB disulfide bond (20). Although these enzymatic studies are in contrast with increasing evidence suggesting that the *Wbl* proteins are regulatory DNA-binding proteins (37, 40, 42), or transcription factors as was recently demonstrated for *Mtb WhiB1* (42), it cannot be discounted that *WhiB1* may function as a highly selective reductase.

In sum, in the past few years novel genome-wide technologies (46) and comprehensive biochemical analyses (3) have been employed to characterize the *Mtb Wbl* proteins. However, the methodology and exact experimental conditions should be critically evaluated in order to yield accurate information on the biological functions of *Wbl* proteins.

#### The *WhiB3* Fe-S Cluster

To date, biochemical evidence suggests that all *Wbl* proteins contain Fe-S clusters, and it is implicitly assumed that this prosthetic group plays a crucial role in *Wbl* protein function. However, evidence suggests that the Fe-S clusters from different *Mtb Wbl* proteins vary in their behavior towards  $O_2$  exposure (3) (Fig. 5). Furthermore, the fact that Fe-S

cluster proteins are stable under anaerobic conditions and unstable under aerobic conditions directly implicates the Fe-S clusters in Wbl function. Host factors may also react with Fe-S clusters. For example, Fe-S cluster enzymes in the TCA cycle are known to be inhibited by  $O_2^{\bullet-}$  and by high  $pO_2$  (26). In addition, NO can react with the Fe-S clusters of TCA cycle enzymes, leading to the formation of a protein-bound dinitrosyl-iron complex (DNIC), which can alter enzymatic activity (17). Also, in elegant biochemical studies on *Streptomyces* WhiD (the homologue of *Mtb* WhiB3) and *Mtb* WhiB1, protein stability in the presence of  $H_2O_2$  and  $O_2^{\bullet-}$  (13) and NO (14) was examined. The reaction of *S. coelicolor* WhiD and *Mtb* WhiB1 with NO was particularly interesting as each reacted  $10^4$ -fold faster with NO than  $O_2$  in a multiphasic reaction containing eight NO molecules per [4Fe-4S] cluster (14). Using electron paramagnetic resonance spectroscopy, Singh *et al.* (41) demonstrated that  $O_2$  exposure can cause cluster degradation, and that *Mtb* WhiB3 can function as a sensor of the two dormancy gases  $O_2$  and NO.

What protects the WhiB3 Fe-S cluster against oxidation and eventually complete loss of the cluster during aerobic growth? One possibility is that a mycobacterial redox buffer in the cytoplasm protects cellular Fe-S clusters. Using methyl-mycythiol, it was shown that the extent of cluster loss was substantially less compared to untreated *Streptomyces* WhiD samples (13). Methyl-mycythiol also reduced the sensitivity of native WhiD against low pH. Lastly, Cys and thioredoxin were also shown to be protective against cluster loss due to  $O_2$  exposure (13). This provides theoretical evidence for how mycobacteria may maintain cytoplasmic Fe-S cluster integrity during aerobic growth.

An important physicochemical factor that may influence Fe-S cluster assembly (and thus WhiB3 function) is the acid dissociation constant ( $pK_a$ ) of residues adjacent to the conserved Cys residues and factors that may modify their properties. For example, WhiB3 Cys residues are flanked by multiple arginine residues (Cys<sub>23</sub>-Arg<sub>24</sub>-Cys<sub>53</sub>-Arg<sub>54</sub>-Arg<sub>55</sub>-Cys<sub>56</sub>-Cys<sub>62</sub>-Arg<sub>63</sub>). At neutral pH, Arg residues located close to Cys residues can significantly lower the  $pK_a$  of Cys, resulting in Cys thiolates, which are highly susceptible to oxidation. Not surprisingly, *Mtb whiB3* was highly upregulated when exposed to acidic stress (22). In sum, the inherent biochemical properties of the 4Fe-4S cluster allow WhiB3 to function as a sensitive redox switch that responds to a range of environmental signals implicated in virulence.

### ***Mtb* WhiB3 DNA-Binding and Transcriptional Regulation**

Since the identification of a Wbl protein in *Streptomyces* spp. in 1992, it has been hypothesized that Wbl proteins bind DNA due to the presence of putative DNA-binding motifs (16). Initial evidence became stronger when it was demonstrated that *Mtb* WhiB3 physically interacts with the principle sigma factor, SigA (RpoV) (44). However, it took nearly two decades to provide experimental proof that a Wbl protein, WhiB3, binds DNA (40). The evidence came from characterizing the DNA-binding properties of apo- and holo- forms of WhiB3 to the upstream sequence of lipid biosynthesis genes *pks2* and *pks3* (40). Important findings were: (i) the redox state of reconstituted holo-WhiB3 did

not affect DNA binding, (ii) oxidized apo-WhiB3 bound DNA much stronger than either holo-WhiB3 or reduced apo-WhiB3, and (iii) the oxidized apo-WhiB3 form demonstrated the strongest DNA binding. It was further demonstrated that WhiB3 regulates *Mtb pks2* and *pks3* genes in a redox-dependent manner, which provides further proof of a role for WhiB3 in the redox-dependent regulation of virulence factors (40). In another study, an *E. coli* bacterial one-hybrid system was used to screen for *Mtb* regulatory proteins that bind to promoter DNA (25). *Mtb* WhiB3 was shown to bind to the promoter regions of several *in vivo* induced genes. Although an important resource, the use of *E. coli* as surrogate host to examine *Mtb* DNA-binding proteins has the same drawbacks as the *E. coli* protein-protein interaction technology (see Protein Networks and Wbl Function) and awaits independent verification.

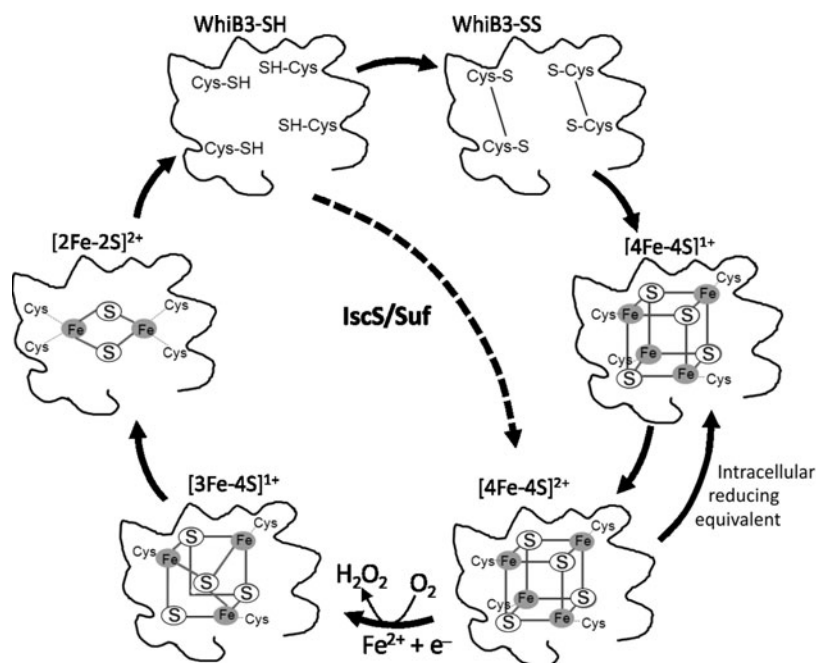
Since the WhiB3 4Fe-4S cluster reacts with NO to form a DNIC complex, any structural or redox-mediated changes associated with complex formation could potentially affect DNA binding and/or transcriptional regulation of WhiB3 target genes (Fig. 6). Notably, while the redox sensing capacity of WhiB3 is based on the presence of 4Fe-4S cluster and Cys residues, there is no clearly defined DNA-binding domain. Nonetheless, the C-terminus of WhiB3 contains a weak AT-hook motif TMGRTRGIRRTA, which is prevalent in eukaryotic nuclear proteins that nonspecifically interact with DNA (4). AT-hook motifs are known to interact with the minor groove of AT-rich DNA sequences, which in *Mtb* are typically restricted to the promoter regions. Similar to other AT-hook-containing proteins (4), the AT-hook like domain may enable WhiB3 to nonspecifically bind, or "coat" DNA as a protection against free radical-mediated damage.

Collectively, these WhiB3 studies suggested that the Wbl proteins interact strongly with DNA in their oxidized apo-forms and weakly in the holo-forms. It was subsequently shown that *Mtb* apo-WhiB1 (42), and *Mtb* apo-WhiB2 and its mycobacteriophage homologue apo-WhiBTM4 bind DNA strongly, as opposed to the holo-form of these proteins that bind DNA weakly or not at all (37). Interestingly, *Mtb* WhiB2 and WhiBTM4 bind to a conserved promoter sequence upstream of *whiB2* to regulate its transcription (37).

Recent studies have shown that oxidized *Mtb* apo-WhiB1 as well as nitrosylated WhiB1 bind DNA, and that DNase I footprinting of apo-WhiB1 bound to *whiB1* promoter DNA revealed a 37-bp protected region (42). Importantly, transcriptional analyses demonstrated that apo-WhiB1 repressed *whiB1* transcription *in vitro*, and provided evidence for the first time that a Wbl member functions as a transcription factor (42).

In sum, it is clear that the oxidation states of holo- or apo-Wbl proteins alter their DNA binding and likely also transcriptional activity. Physiological modifications to either WhiB3 Cys residues (*e.g.*, oxidized, reduced) or the Fe-S cluster (*e.g.*, the apo- or holo-states, the Fe-S cluster oxidation state, nitrosylation, etc) represent a flexible switch that allows the microbial cell to rapidly respond to a wide range of environmental conditions (Fig. 6). Since virtually all DNA-binding proteins influence transcription to some extent, this suggests that the Wbl proteins are transcription regulators with DNA binding capability.

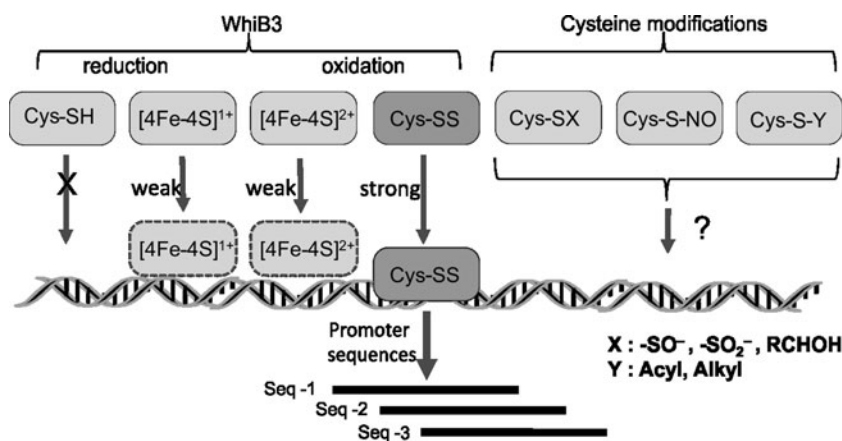
**FIG. 5. Diagrammatic representation of WhiB3 Fe-S cluster loss and assembly.** In the presence of high concentrations of oxygen, WhiB3 [4Fe-4S]<sup>1+</sup> cluster is oxidized to [4Fe-4S]<sup>2+</sup> and is subsequently converted into a [3Fe-4S]<sup>1+</sup> with the simultaneous generation of H<sub>2</sub>O<sub>2</sub>. The [3Fe-4S]<sup>1+</sup> further yields [2Fe-2S]<sup>2+</sup> intermediates, followed by a complete loss of the cluster wherein WhiB3 either exists in a reduced WhiB3-SH state, or oxidized WhiB3-SS state. *Mtb* IscS converts apo-WhiB3 to WhiB3 [4Fe-4S]<sup>2+</sup>. As suggested for *Mtb* WhiB1 (42), it is tempting to speculate that thioredoxin and mycothiol may be involved in stabilizing the WhiB3 [4Fe-4S] cluster.



### Role of WhiB3 in *Mtb* Disease

The first *in vivo* genetic complementation studies identified the principle sigma factor SigA (RpoV) as the first mycobacterial virulence factor (12). A caveat of this finding is that *sigA* is an essential gene, thereby making it exceedingly difficult to study. In this regard, WhiB3 was identified as a putative regulatory protein that interacts with SigA (44). This was determined by using a yeast two-hybrid screen

to examine the independent interactions of wt SigA or the mutated allele of SigA (Arg<sub>515</sub>-His) with WhiB3. Noticeably, the WhiB3:SigA interaction was strong, whereas the SigA(Arg<sub>515</sub>-His):WhiB3 interaction was significantly decreased. This finding suggests that the SigA:WhiB3 interaction is necessary for full virulence and that the Arg<sub>515</sub>-His mutation contributes to the attenuation observed in the avirulent *M. bovis* strain. It was hypothesized that the disruption of this interaction by the SigA Arg<sub>515</sub>-His mutation



**FIG. 6. Model depicting the mechanisms of *Mtb* WhiB3 DNA binding.** WhiB3 DNA binding is influenced by the redox state of the Fe-S cluster (in the case of holo-WhiB3), or alternatively by the oxidation states of the Cys residues (in the case of apo-WhiB3). As previously demonstrated (40), oxidized apo-WhiB3 binds DNA strongly, whereas reduced apo-WhiB3 does not bind DNA at all, and holo-WhiB3 ([4Fe-4S]<sup>1+</sup> or [4Fe-4S]<sup>2+</sup>) binds DNA weakly. It is reasonable to assume that the redox state of the mycobacterial cytoplasm would influence protonation and deprotonation of the WhiB3 Cys residues to affect DNA binding. Since *Mtb* experiences gradients of *in vivo* signals, an alteration in the concentration of effector molecules may lead to differential responses. It is therefore proposed that WhiB3 may sense and differentiate between effector molecules and their concentrations Fe-S cluster intermediates (3Fe-4S, 2Fe-2S), oxidation states of the Cys thiols, or via post-translational modifications of the Cys residues. These redox modifications likely dictate WhiB3 binding and modulation of its target genes. Since Cys residues within a protein can be modified to -SH, disulfide (-SS-), sulfenate (-SO<sup>-</sup>), sulfinate (-SO<sub>2</sub><sup>-</sup>), or s-nitrosylated states, it is likely that these modifications could also modulate DNA-binding activity.

caused alterations in expression of virulence genes under WhiB3 control (44).

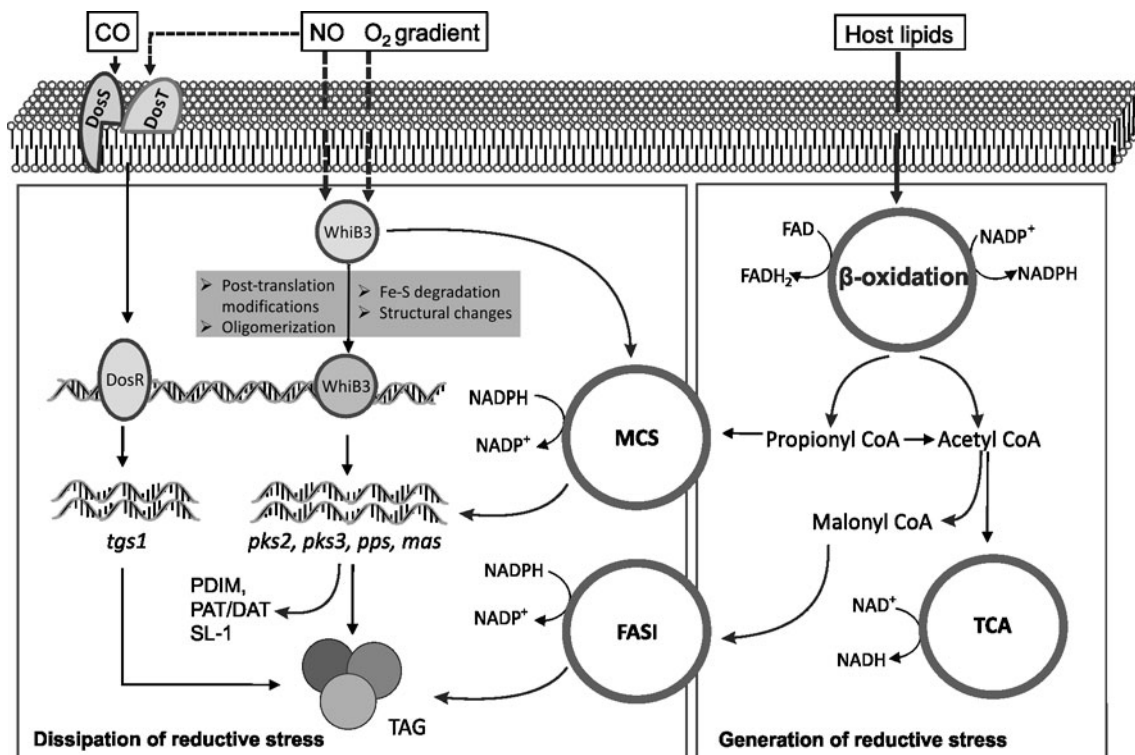
Further studies demonstrated that deletion of *whiB3* in *Mtb* and *M. bovis* caused no phenotype *in vitro*, but led to the loss of virulence in mouse and guinea pig models of TB (44). The authors proposed that virulence lipids were responsible for triggering the severe pathology. Indeed, subsequent lipid analyses by the same authors (40) have shown that the lack of WhiB3 was strongly associated with decreased production of the virulence lipids SL-1, PAT, and DAT, albeit with increased synthesis of PDIM (see section on Metabolic Regulation of *Mtb* WhiB3). The fact that the levels of some lipids are upregulated and others downregulated makes it difficult to accurately predict the exact contribution of each lipid species towards virulence. The identification of gene products under WhiB3 control that are responsible for the differences in host lung pathology is eagerly awaited.

### *Mtb* WhiB3 Modulates Oxido-Reductive Stress

*In vitro* metabolic labeling studies performed under defined thiol-specific oxidizing (diamide) or reducing (dithiothreitol) conditions demonstrated that *Mtb* WhiB3 regulates the production of the lipids PAT, PDIM, and TAG in a redox-dependent manner. Furthermore,

*Mtb* *WhiB3* is able to survive in the presence of toxic concentrations of propionate *in vitro* and within macrophages (40). A related finding was that reductive stress (dithiothreitol) exposure upregulated TAG anabolism. Earlier studies of *Mtb* showed that TAG production is highly upregulated under hypoxic conditions and exposure to NO and CO (29, 30). Oxygen, CO, and NO are modulatory ligands of the heme sensor kinases DosT and DosS, which modulate DosR, the regulator of the 47-member Dos dormancy regulon, which includes the *tgs1* gene that controls TAG biosynthesis (45). It was recently suggested that TAG is the prime carbon source utilized upon reactivation from a nonreplicating persistent state (15). Thus, these findings provide a link between the intracellular WhiB3 pathway and the two-component DosR/S/T signaling pathway.

During intracellular growth, *Mtb* utilizes host fatty acids as a primary energy source *via*  $\beta$ -oxidation, wherein a large quantity of toxic propionate is generated. WhiB3 appears to play a central role in dissipating propionate toxicity and regulates the metabolic switchover between the *in vitro* carbon source (*e.g.*, glucose) and the *in vivo* carbon source (*e.g.*, acetate) (40, 41). Furthermore,  $\beta$ -oxidation is accompanied with the generation of high concentrations of reducing equivalents such as NADH and NADPH (Fig. 7).



**FIG. 7. Diagram illustrating the role of *Mtb* WhiB3 in redox regulation.** *Mtb* WhiB3 is a DNA-binding protein that senses changes in the environmental gases such as NO and gradients of O<sub>2</sub>. WhiB3 also senses or responds to fluctuating levels of reducing equivalents (NADH) that are generated by the TCA cycle (respiration), and NADPH that accumulates during ( $\beta$ -oxidation) of host fatty acids. If not properly balanced, the bacillus will experience reductive stress (18). As a result, WhiB3 channels these excess reducing equivalents into virulence lipid anabolic pathways, which requires NADPH as cofactor. Thus, virulence lipid anabolism (*e.g.*, SL-1, PDIM, PAT, DAT, and trehalose dimycolate) functions as an electron sink or reserve storage material, which is available for future use. This is also, in part, a mechanism to maintain intracellular redox balance. WhiB3 also overlaps with the DosR/S/T signaling pathway since both regulates TAG synthesis.



Extraordinarily high levels of NAD(P)H, but not NAD(P)<sup>+</sup> were identified in *Mtb* isolated from infected mouse lungs, demonstrating that *Mtb* experiences reductive stress *in vivo* (11). In macrophages, *Mtb*Δ*whiB3* was shown to experience more severe reductive stress upon infection compared to *Mtb*. These observations point to a major role for WhiB3 in dissipating excessive reductive stress (40) [for a review on reductive stress see (18)]. While the mechanism of how WhiB3 maintains redox balance is largely unknown, a likely explanation is that excess reducing equivalents are assimilated *via* lipid anabolism (Fig. 7).

### Conclusion and Future Directions

Understanding the mechanisms of how *Mtb* is able to persist in host tissue for decades without causing disease, and the development of new therapeutic strategies against latent TB depends on an advanced understanding of the role of redox homeostasis in *Mtb* pathogenesis. Novel paradigms are urgently needed, and the Wbl protein family represents an unexplored avenue of research. This poorly characterized group of Fe-S cluster proteins influences a diverse range of biological events including redox homeostasis (40), cAMP regulation (1), oxidative stress (28), cell division (23), drug resistance (33), and virulence (44), and makes them ideal candidates for further study.

Notably, the existence of more than 1600 Wbl homologues across numerous bacterial species and the fact that the ds DNA siphoviridae bacteriophage family contains Wbl homologues may shed light on the mechanism of horizontal and vertical gene transfer of *wbl* gene products. Subsequent to the original WhiB3 virulence study (44), a rapid surge in Wbl studies followed, particularly in the area of biochemistry (3, 13, 14, 41, 42), which has established a broad mechanistic framework for understanding the mode of action of Wbl members. Combining these mechanistic findings with *in vivo* *Mtb* virulence studies should lead to a better understanding of the biological function of these proteins. Demonstrating that *Mtb* WhiB3 binds DNA (40) was an important finding and has paved the way for studying how other Wbl proteins may bind DNA. Although technically challenging, obtaining the crystal structure of any Wbl protein would significantly contribute to the field. The identification of *Mtb* WhiB3 as a metabolic redox sensor (41) is particularly interesting as the concept of reductive stress emerged from these findings (18).

In sum, the Wbl family represents an important group of *Mtb* proteins of which WhiB3 represents the prevailing paradigm. Employing genome-wide technologies geared towards identifying new pathways and metabolites should lead to increased understanding of the biological function of Wbl proteins.

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### Author Disclosure Statement

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#### Abbreviations Used

Aa = amino acid  
DAT = diacyltrehalose  
DNIC = dinitrosyl-iron complex  
Fe-S = iron-sulfur  
MMCo = methyl-malonyl CoA  
*Mtb* = *Mycobacterium tuberculosis*  
PAT = polyacyltrehalose  
PDIM = phthiocerol dimycocerosates  
pO<sub>2</sub> = partial pressure of O<sub>2</sub>  
RNS = reactive nitrogen species  
ROS = reactive oxygen species  
SL = sulfolipids  
TAG = triacylglycerol  
TB = tuberculosis  
TCA = tricarboxylic acid cycle  
Wbl = WhiB-like