Ergothioneine Maintains Redox and Bioenergetic Homeostasis Essential for Drug Susceptibility and Virulence of *Mycobacterium tuberculosis*

Graphical Abstract

**Highlights**

- WhiB3 regulates EGT production and maintains bioenergetic homeostasis in *Mtb*
- EGT modulates drug sensitivity and protects *Mtb* from diverse oxidative stressors
- EGT is essential for *Mtb* survival in macrophages and in mice

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**In Brief**

Saini et al. report that *Mtb* WhiB3, a 4Fe-4S redox sensor protein, regulates ergothioneine (EGT) production in a carbon-source-dependent manner and that EGT is required for *Mtb* survival in the host. The authors establish that EGT maintains redox and bioenergetic homeostasis in *Mtb*, which influences drug susceptibility and pathogenicity.

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Ergothioneine Maintains Redox and Bioenergetic Homeostasis Essential for Drug Susceptibility and Virulence of Mycobacterium tuberculosis

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SUMMARY
The mechanisms by which Mycobacterium tuberculosis (Mtb) maintains metabolic equilibrium to survive during infection and upon exposure to antimycobacterial drugs are poorly characterized. Ergothioneine (EGT) and mycothiol (MSH) are the major redox buffers present in Mtb, but the contribution of EGT to Mtb redox homeostasis and virulence remains unknown. We report that Mtb WhiB3, a 4Fe-4S redox sensor protein, regulates EGT production and maintains bioenergetic homeostasis. We show that central carbon metabolism and lipid precursors regulate EGT production and that EGT modulates drug sensitivity. Notably, EGT and MSH are both essential for redox and bioenergetic homeostasis. Transcriptomic analyses of EGT and MSH mutants indicate overlapping but distinct functions of EGT and MSH. Last, we show that EGT is critical for Mtb survival in both macrophages and mice. This study has uncovered a dynamic balance between Mtb redox and bioenergetic homeostasis, which critically influences Mtb drug susceptibility and pathogenicity.

INTRODUCTION
Tuberculosis (TB) is the second most common cause of death from an infectious agent after HIV. This is largely due to the ability of Mycobacterium tuberculosis (Mtb) to remain in a dormant, drug-tolerant state for decades in humans before emerging to cause active disease in ~10% of those infected. Mtb is exposed to environments with a wide range of available carbon sources, reactive oxygen intermediates (ROIs), and reactive nitrogen intermediates (RNIs) inside the host that may cause cell death. Therefore, it is strongly anticipated that the ability of Mtb to maintain redox balance and metabolic homeostasis is critical to its pathogenicity and virulence (Kumar et al., 2011). In addition, some front-line TB drugs such as isoniazid are prodrugs that require bioreduction by Mtb for anti-mycobacterial activity (Lei et al., 2000). Thus, a fundamental challenge to global TB control is to understand the mechanisms by which Mtb adapts to diverse carbon sources and redox environments encountered in the host.

Mtb produces mycothiol (MSH; Figure 1A), which acts as a major redox couple to protect against various redox stressors and anti-TB drugs (Buchmeier et al., 2003; Rawat et al., 2007). Mtb also produces a second thiol couple, ergothioneine (EGT; Figure 1B), a sulfur-containing histidine derivative with potent antioxidant properties (Genghof, 1970; Hand and Honek, 2005). However, despite considerable effort, roles for EGT in Mtb and its potential involvement in redox homeostasis and pathogenesis remain unknown. Recently, we have shown that EGT levels in Mtb are modulated by protein phosphorylation during transition into late states of growth (Richard-Greenblatt et al., 2015), yet it is still unclear why mycobacteria produce both EGT and MSH to maintain redox homeostasis.

Redox balance is essential for energy metabolism, including glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (OXPHOS). Despite this strong interdependence between redox homeostasis and energy metabolism, very few tools are available to investigate mycobacterial bioenergetics in real time and in a noninvasive manner. Since cellular respiration involves a complex interplay of biological factors, including the availability, nature, and concentration of oxidizable substrates as well as energy demand, methods for detecting such bioenergetic perturbations in Mtb will be of great value.

We previously demonstrated that WhiB3, an Mtb 4Fe-4S cluster redox sensor and virulence protein, maintains intracellular redox homeostasis of the mycobacterial cell to provide metabolic and cellular integrity (Singh et al., 2007, 2009; Steyn et al., 2002). In this study, we examined how WhiB3 controls...
Figure 1. Metabolomic Analysis of ΔwhiB3 Demonstrates Increased Levels of EGT

(A and B) Chemical structures of (A) MSH and (B) EGT.

(C) Principal-component analysis shows that WT Mtb, ΔwhiB3, and ΔwhiB3:comp strains can be distinguished based on their overall metabolomic profiles. For metabolomic analysis, each strain was independently examined at least twice in triplicate.

(D) Volcano plot of metabolites in WT Mtb and ΔwhiB3 plotted as fold change versus significance. The green lines demarcate metabolites with a greater than 2-fold change on the x axis and a corrected p value < 0.05 (289/3,327 molecules). Metabolites with less than a 2-fold change and/or p > 0.05 are shown in gray. See also Table 1.

(E) Independent validation of EGT production in WT Mtb, ΔwhiB3, and ΔwhiB3:comp based on LC-MS analysis after growth on medium containing 5 mM acetate. Error bars denote SEM.

(F) Untargeted metabolite profiling assessed by MetPa enrichment analysis, based on changes in the relative abundance of metabolites (see also Figure S1 and Table S1), indicate that WhiB3 has a profound effect on Mtb metabolism, especially carbon catabolism, bioenergetic metabolism, amino acid metabolism, the pentose phosphate pathway (PPP), and glycolysis.

(legend continued on next page)
redox and bioenergetic homeostasis in *Mtb* to moderate virulence. We used a combination of metabolomic, bioenergetic, and transcriptomic approaches and established links between WhiB3 and bioenergetic homeostasis and EGT, a major redox buffer. We characterized the genetic architecture of the EGT biosynthesis operon in *Mtb* and assessed the contribution of EGT in protection against oxidative stress, in antimycobacterial drug susceptibility, and in bioenergetic homeostasis. Further, we examined a link between *Mtb* central carbon catabolism and EGT production and the relationship between EGT and MSH biosynthesis. Using genome-wide expression analysis of genetically defined mutants of MSH and EGT biosynthesis, we identified differentially regulated genes common to all *Mtb* mutants. Finally, using macrophages and a mouse model of infection, we establish that maintaining redox balance and bioenergetic homeostasis is essential for *Mtb* virulence.

**RESULTS**

**WhiB3 Regulates EGT Production in *Mtb***

Since *Mtb* WhiB3 is an intracellular redox sensor (Singh et al., 2009), we sought to identify redox-responsive metabolites regulated by WhiB3. We analyzed the metabolomes of *Mtb* (H37Rv), ΔwhiB3 and the corresponding *whiB3*-complemented strain (ΔwhiB3:comp), under rigorously controlled conditions (de Carvalho et al., 2010). Metabolites were separated by high-performance liquid chromatography (HPLC) and detected by electrospray ionization coupled to mass time-of-flight mass spectrometry [liquid chromatography-tandem mass spectrometry (LC-MS/MS)] as previously described (Rhee et al., 2011). Among the statistically significant differences detected (Figures 1C, 1D, and S1) was the increased production of EGT in ΔwhiB3. Independent validation showed a 7.3-fold increase in EGT levels in ΔwhiB3 (Figure 1E), and complementation of *whiB3* restored the EGT content to near wild-type levels (Figure 1E).

Next, we performed Metabolic Pathway Analysis (MetPa), which combines pathway enrichment analysis with pathway topology, to detect metabolic differences between *Mtb* and ΔwhiB3 (Everts et al., 2014; Nandakumar et al., 2014). This analysis highlighted changes in the abundance of metabolites of biochemical pathways in ΔwhiB3, including glycolysis, the pentose phosphate pathway, the TCA cycle, and several amino acid biosynthesis pathways (Figure 1F; Tables 1 and S1). The pyruvate node is of particular significance as it is a key intermediate of many amino acid metabolism pathways, glycolysis, and the TCA cycle (Figure 1G; Table S1).

Overall, these metabolomic data demonstrate that WhiB3 modulates the production of EGT. Further analyses revealed that WhiB3 also modulates the production of several amino acids, which act as anaplerotic and cataplerotic substrates and converge on glycolysis and the TCA cycle to affect energy metabolism (Figure 1G).

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Metabolic Pathway Analysis (MetPa) combines pathway enrichment analysis with pathway topology analysis to identify the most relevant pathways under the specified conditions. MetPa uses the KEGG metabolic pathways as the backend knowledge base and integrates univariate analysis, over-representation analysis, Global Test, GlobalAncova, and network topology analysis into pathway analysis. The total/maximum importance of each pathway = 1; pathway impact value = cumulative % from the matched metabolic nodes. Pathways with the most significant change (p ≤ 0.05) are listed. KEGG, Kyoto Encyclopedia of Genes and Genomes; TCA, tricarboxylic acid. See also Table S1.

WhiB3 Maintains Bioenergetic Homeostasis in *Mtb*

Next, we optimized extracellular flux analysis previously applied only to eukaryotic cells, for the study of *Mtb*. In this method, fluorescence sensors measure dissolved extracellular oxygen and proton concentration in real time in a microtransient chamber formed when the probes are in the measurement position. Inhibitors, modulators, or substrates can be added during the run through delivery ports in the cartridge. This technology allowed us to accurately measure the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of *Mtb* cells in real time in a noninvasive manner (Figure 2A). The OCR and ECAR are direct and highly sensitive measures of cellular metabolic activity (Ferrick et al., 2008). ECAR gives a measure of H+ extrusion associated with carbon catabolism via glycolysis and the TCA cycle, whereas OCR is indicative of cellular respiration due to OXPHOS. The cell energy phenotype can be deduced from a phenogram (plotting of OCR versus ECAR) that illustrates metabolic shifts between substrate oxidation and glycolysis. We used pyruvate, identified as a key glycolytic intermediate (Figure 1G; Table S1), as the sole carbon source in extracellular flux analyzer (XF) bioenergetic assays. We used carbonyl cyanide m-chlorophenylhydrazone (CCCP), a membrane uncoupler, to examine the capacity of WhiB3 to maintain bioenergetic homeostasis.

(G) EGT is linked to the metabolism of histidine (His) as it is derived from hercynine (Her), and MSH biosynthesis is regulated by NADPH produced by the PPP. Glycolysis and the TCA cycle both feed into the electron transport chain to regulate the electrical (Δψ) and concentration (ΔpH) gradients across the membrane, which maintain bioenergetic homeostasis. Amino acids acting as anaplerotic and cataplerotic substrates are boxed.
homeostasis upon uncoupling of OXPHOS from ATP production. In the presence of pyruvate, \( \text{D whiB3} \) had a significantly lower OCR than WT and \( \text{D whiB3:comp} \) (Figure 2B). After addition of CCCP (8 \( \mu \text{M} \); M7), both the WT and \( \text{D whiB3:comp} \) strains responded to the uncoupling stress by increasing OCR (Figure 2B). The OCR and ECAR data from these experiments were plotted to generate a phenogram, which provides a visualization of the overall metabolic profiles of the different strains (Figure 2C).

Our data show that \( \text{D whiB3} \), in contrast to WT \( \text{Mtb} \), did not respond with an increase in OCR or ECAR to re-establish membrane potential (M7, Figure 2C), indicating that \( \text{D whiB3} \) is bioenergetically deficient.

In sum, we optimized extracellular flux analysis for the study of \( \text{Mtb} \) bioenergetics. Adapting this technology toward \( \text{Mtb} \) has broad implications for studying bioenergetics of microbial pathogens in general. The results demonstrate that in the absence of WhiB3, \( \text{Mtb} \) cannot respire efficiently when the glycolytic intermediate pyruvate is the sole carbon source. This suggests that the absence of WhiB3 leads to dysfunctional respiration and confirms its involvement in maintaining bioenergetic homeostasis.

EGT Detection Using TLC

The study of EGT biosynthesis in bacteria has been hampered by the fact that EGT quenches the fluorescence of bimane derivatives, thereby making routine detection of EGT by HPLC analysis difficult. We therefore developed a rapid thin-layer chromatography (TLC)-based radioactive labeling assay and showed that pure EGT has an Rf value of 0.46 (Figure S2A). TLC analysis of concentrated \( \text{M. smegmatis} \) (Msm) and \( \text{Mtb} \) extracts revealed bands with Rf values consistent with that of pure EGT (Figures 3A and S2A). LC-MS/MS analysis of these bands and an EGT standard revealed virtually identical retention times and confirmed them to be EGT (Figures 3B, 3C, and S2B). An earlier study in \( \text{Claviceps purpurea} \) showed that [2-\( ^{14}\text{C} \)]-acetate could be used to label EGT (Heath and Wildy, 1956). However, we could not detect labeled EGT after TLC analysis of extracts from \( \text{Msm} \) cultured in the presence of [2-\( ^{14}\text{C} \)]-acetate even though UV exposure revealed a band at the same position as pure EGT, indicating that EGT was present (Figure 3D). However, by using [\( ^{35}\text{S} \)]-Cys as a precursor, we observed a labeled spot after TLC analysis with the same Rf value as the EGT identified by LC-MS/MS analysis (Figure 3E).
Altogether, we have developed a simple radioactive TLC-based assay that facilitates the rapid, routine examination of EGT in mycobacteria and potentially other EGT-producing bacteria. Further, this method revealed differences in the biosynthetic pathways of either EGT or EGT precursors between fungi and mycobacteria.

**WhiB3-Mediated Regulation of EGT Production Is Dependent on Central Carbon Metabolism**

Since WhiB3 initiates the metabolic switchover to the preferred in vivo carbon source (fatty acids) and integrates redox signals with core intermediary metabolism (Singh et al., 2007, 2009), we sought to establish whether the type of carbon source influences WhiB3-mediated regulation of EGT production. LC-MS/MS was used to quantify intracellular EGT in *Mtb, ΔwhiB3,* and *ΔwhiB3:comp* grown in physiologically relevant concentrations (50 μM [Lee et al., 2013]) of carbon sources, including glucose, lipids, and lipid precursors. As shown in Figure 3F, *ΔwhiB3* had significantly increased levels of EGT compared to WT or *ΔwhiB3:comp* when grown in glucose, acetate, cholesterol, behenate, stearate, myristate, or palmitate and no difference when grown in propionate. EGT levels in WT *Mtb* ranged from 40 to over 300 ng/10^8 cells with higher (>200 ng/10^8 cells) levels occurring in cells grown in myristate (C-14), palmitate (C-16), and stearate (C-18). *ΔwhiB3* grown in the same C-14, C-16, and C-18 fatty acids produced even higher levels of EGT (>400 ng/10^8 cells). We observed EGT levels < 120 ng/10^8 cells in WT *Mtb* grown on glucose, propionate (C-3), acetate (C-2), behenate (C-22), and cholesterol (C-27). *ΔwhiB3* grown in these substrates had similarly lower EGT levels albeit significantly higher than the WT, with the exception of glucose, where levels were > 250 ng EGT/10^8 cells. In sum, these data demonstrate that WhiB3 regulates EGT production in response to catabolism of a diverse set of fatty acid precursors and cholesterol.

**The Mtb EGT Operon**

In vitro reconstitution studies using protein preparations of *Eriinia tasmaniensis* and *Msm* (Seebeck, 2010), as well as a recent genetic study in *Msm* (Sao Emani et al., 2013), have provided biochemical and genetic evidence for EGT production in *Msm*. Analysis of the *Mtb* genome led us to predict that locus Rv3704c–Rv3700c constitutes the EGT biosynthetic operon. Figure 4C shows the genetic organization and biochemical pathway involved in EGT production and the link between EGT and MSH biosynthesis in *Mtb*. Examination of EGT production in *Mtb* CDC1551 and corresponding transposon mutants that disrupt *egtA* or *egtD* (hereafter referred to as *egtA:Tn* and *egtD:Tn*) revealed that *Mtb* *egtA:Tn* and *egtD:Tn* do not produce...
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EGT (Figure 4A). Production of EGT in egtA:Tn was successfully complemented with the addition of the complete egt gene cluster (Rv3704c–Rv3700c, egtA:TnC) and partially complemented in egtD:Tn with the addition of egtD (egtD:TnC; Figure 4B). The arrangement of the egt genes, with egtA/egtB and egtC/egtD having overlapping stop and start codons (5'-GTGA-3' and 5'-ATGA-3', respectively), suggested that they may be operonic. RT-PCR analysis established that egtA through egtD are indeed operonic, while egtE is not (Figure S3). In sum, we have confirmed the genetic components and operonic organization (Rv3704c–Rv3700c) responsible for EGT biosynthesis in Mtb.

**EGT and Mycobacterial Redox Balance**

Next, to examine the biological significance of EGT, and whether EGT and MSH are linked metabolically, we measured EGT levels in Mtb defective in MSH production. We noticed substantially increased EGT levels in ΔmshA compared to WT (Figure 4D). HPLC analyses of reduced MSH (2MSH) and oxidized (disulfide) MSH (MSSM) levels in Mtb CDC1551, egtA:Tn, and egtD:Tn indicated that the MSH/MSSM ratio does not differ in the Mtb EGT mutants (Figure 4E).

We then examined the endogenous reactive oxygen species (ROS) levels in Mtb strains defective in EGT or MSH production. Flow cytometry analysis of WT, egtA:Tn, and ΔmshA following exposure to the ROS-responsive dye CellROX Green revealed that egtA and mshA mutants have a significantly increased percentage of cells that produce ROS under normal growth conditions (Figure 4F). Compared to WT, egtA:Tn had 25-fold and ΔmshA had 8-fold more ROS-producing cells. Further, the egtA:Tn strain had 3-fold more ROS-producing cells compared to ΔmshA. The increased EGT levels observed in ΔmshA (Figure 4D) likely mitigate the endogenous ROS in this mutant. We conclude that while a lack of MSH substantially increases levels of EGT, loss of EGT does not lead to a corresponding increase in MSH/MSSM, which may be the cause of increased endogenous ROS levels observed in the egtA mutant. These data establish a physiological link between MSH and EGT production in Mtb and confirm their role in controlling endogenous ROS.

**Role of EGT in Protection against Oxidative Stress**

The role of MSH in protecting mycobacteria from oxidative stress is well documented (Buchmeier et al., 2003; Miller et al., 2007). However, we know little about the role of EGT in combating oxidative stress in Mtb. We investigated the ability of EGT to protect Mtb under various conditions of oxidative stress. We observed that both egtA:Tn and egtD:Tn were significantly more sensitive than WT upon exposure to hydrogen peroxide (H$_2$O$_2$), paraquat (PQT), menadione (MND), and cumene hydroperoxide (CHP) (Figure 4G). Survival of egtA:Tn and egtD:Tn was reduced most significantly by CHP and MND. Compared to egtA:Tn, viability of egtD:Tn was significantly decreased following exposure to H$_2$O$_2$ or PQT (Figure 4G). We noted that egtA:Tn is susceptible only at the higher concentration of PQT. Complementation of the mutants restored protection against the oxidants, and viability was similar to that of WT Mtb. Altogether, these findings provide evidence that EGT protects Mtb from a diverse set of oxidative stressors.

**EGT and Antimycobacterial Drug Susceptibility**

The redox status and metabolic state of Mtb can affect its sensitivity to anti-TB drugs (Baek et al., 2011; Kumar et al., 2011; Nanadakumar et al., 2014). Here, we tested the hypothesis that lack of EGT in Mtb modulates susceptibility to front-line anti-TB drugs. To address this, we used a colony-forming unit (CFU)-based viability assay to determine drug susceptibilities. We observed that, compared to WT Mtb, the minimal inhibitory concentration (MIC) of rifampicin (RIF), isoniazid (INH), bedaquiline (BDQ), and clofazimine (CFZ) was significantly reduced for the egtA:Tn, egtD:Tn, and ΔmshA strains (Table S2). At MIC values obtained for WT Mtb, the percentage survival of egtA:Tn, egtD:Tn, and ΔmshA was < 40% and < 25% of WT Mtb in the presence of RIF and INH, respectively (Figure 4H). Similarly, against CFZ and the recently developed drug BDQ, survival of egtA:Tn and egtD:Tn strains was reduced to < 70% and < 50% of WT Mtb, respectively. Genetic complementation of the egtA/egtD and mshA mutants reduced drug susceptibility to near wild-type levels. As shown in Figure 4H, drug susceptibilities of the egt mutants against INH and RIF were comparable to that of ΔmshA (MIC = 0.015 and 0.006 μg/ml, respectively). Of note, ΔmshA was significantly more susceptible to BDQ and CFZ compared to WT Mtb and the egtA:D mutants, revealing a greater role for MSH in protecting against these drugs. These data demonstrate that the lack of EGT results in increased susceptibility to anti-TB drugs and indicate that EGT and MSH are not fully redundant and have overlapping but distinct functional roles in Mtb physiology.

Figure 4. Genetic Characterization of the EGT Biosynthetic Operon in Mtb

(A) TLC and autoradiographic analyses of $^{35}$S-cysteine-fed egtA:Tn, egtD:Tn, and the corresponding WT strain (Mtb CDC1551) indicate that disruption of egtA or egtD abrogates EGT production.

(B) Genetically complemented strains egtA:TnC and egtD:TnC produce EGT. Complementation in egtD:TnC appeared to be partial.

(C) Model depicts a putative link between the MSH and EGT pathways via Acetyl-Cys and Cys (indicated by dotted arrows).

(D) TLC and autoradiographic analyses of $^{35}$S-cysteine-fed Mtb CDC1551 and its corresponding ΔmshA strain (duplicate samples) show increased EGT production in the mutant lacking MSH.

(E) HPLC measurement of reduced (MSH) and oxidized (MSSM) mycothiol. MSH/MSSM ratio is presented as the mean ± SD of three independent cultures.

(F) Flow-cytometric measurement of endogenous ROS with CellROX Green dye in Mtb revealed that the egtA:Tn and ΔmshA mutant had significantly more ROS-producing cells relative to WT (***p < 0.001, **p < 0.05, and *p < 0.05, respectively) or corresponding complemented strains.

(G) EGT protects Mtb against oxidative stress. Following exposure to H$_2$O$_2$, PQT, MND, and CHP, cells were plated and CFUs enumerated. Percentage survival was determined relative to untreated (UT) cells for each strain. Each column represents the mean ± SD (n = 4, *p < 0.05, **p < 0.01, and ***p < 0.001) compared to the WT strain.

(H) Lack of EGT increases susceptibility of Mtb to RIF, INH, BDQ, and CFZ. Graphs show, at MIC values obtained for WT Mtb, the percentage survival (CFUs) of egtA:Tn, egtD:Tn, and ΔmshA relative to the WT strain (see also Table S2). Complementation restored drug sensitivities to near WT levels. Each column represents the mean ± SD (n = 4, *p < 0.05, **p < 0.01, and ***p < 0.001) compared to WT strain.

See also Figure S3 and Table S2.
EGT and MSH Are Critical for Bioenergetic Homeostasis in Mtb

Since reduction/oxidation reactions (redox) and energy metabolism are intricately linked, we tested the hypothesis that redox buffers MSH and EGT are essential for maintaining bioenergetic homeostasis. Extracellular flux analysis showed alterations in the bioenergetic profiles (OCR and ECAR) of mshA and egtA mutant strains when using glucose or acetate as the sole carbon source. Genetic complementation of these mutants restored the bioenergetic phenotype comparable to WT, suggesting that lack of either EGT or MSH alters respiration in Mtb (Figures 5A–5L). The OCR (M1) of egtA:Tn and ΔmshA grown in acetate was higher than that of their respective WT strains (Figures 5D and 5J). However, addition of CCCP (M7) resulted in an increase in OCR (Figures 5D and 5J).
Importantly, the substantially different bioenergetic profiles higher OCR and ECAR than the WT and complemented strains at clear shifts occur from low OCR and ECAR in the mutants at M1 to highlighted in the phenograms in Figures 5C, 5F, 5I, and 5L, where

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Figure 6. Whole-Genome Transcriptional Profiling of WT Mtb, egta:A.Tn, egtD:Tn, and ΔmshA

(A) Illustration of the overlap of the transcriptomic response of Mtb mutants defective in EGT and MSH production (Venn diagram). Also shown are selected subsets of differentially regulated genes in each strain classified according to function. Green: up-regulation, red: downregulation. See Figures S4–S6 for a detailed list of differentially regulated genes.

(B) Microarray analysis of egta:A.Tn, egtD:Tn, and ΔmshA grown under normal conditions identified a common set of 25 genes, 22 of which were downregulated. Genes displaying at least 1.75-fold variation in expression, with a q value of 1%, were considered to be differentially regulated. The q value is the equivalent of the p value after multiple-testing correction (see Supplemental Experimental Procedures).

(C) Quantitative real-time PCR analysis of a subset (10 of 25) of genes under normal growth conditions to validate microarray profiles. Expression of sigA was used as an internal control, and gene expression values were normalized to WT (Manganelli et al., 1999).

(D) Fold change in gene expression upon exposure to oxidative stress (CHP) relative to expression in unexposed cells. Expression of sigB (Rv2710) and sigH (Rv2232c) was used as a positive control to evaluate oxidative stress response. *Sole gene (Rv2136c) unaffected by any uncoupler, respiratory inhibitor, membrane potential, or ΔPh modulator (Boshoff et al., 2004). See also Figures S4–S6 and Tables S3 and S4.

highlighted in the phenograms in Figures 5C, 5F, 5I, and 5L, where clear shifts occur from low OCR and ECAR at the WT and complemented strains at M7. Importantly, the substantially different bioenergetic profiles of genes, we carried out whole-genome transcriptomic profiling and compared the gene expression profiles of Mtb strains deficient in egta (Figure S4), egtD (Figure S5), and mshA (Figure S6). Compared to WT Mtb, 139 genes are differentially expressed in upon addition of an uncoupler suggest electron transport chain dysfunction in the mutants resulting in greater OCR and ECAR in order to reestablish their membrane potentials. The unexpectedly high OCR observed in the egtaA complemented strain at the higher concentration of CCCP (M7, Figure S5) could be due to aberrant behavior of constitutive hsp60 promoter in the epimonal plasmid of the complement and has been observed previously in metabolic studies (Hartman et al., 2014). In sum, our data support the paradigm that alterations in redox status can modulate bioenergetic functions in Mtb cells.

Identification of Differentially Regulated Genes in EGT- and MSH-Deficient Mtb Genes that contribute toward maintaining endogenous redox balance in Mtb are not well defined. To identify this core subset...
ΔmshA, and 68 and 74 genes in the egtA and egtD mutant strains, respectively (Figure 6A). Notably, a common set of 25 genes was differentially regulated across all three strains (Figure 6B). The changes in gene expression observed in the microarray analysis were validated independently in egtA:Tn, egtD:Tn, and ΔmshA by quantitative real-time PCR analysis on a select subset of 10 genes (Figure 6C). Quantitative real-time PCR analysis of egtA:Tn, egtD:Tn, and ΔmshA:comp yielded gene expression values comparable to WT (Table S3).

Importantly, 24 of the 25 differentially regulated genes shared by the EGT- and MSH-deficient strains are differentially regulated by inhibitors of respiration (KCN, thioridazine, and 2,4-dinitrophenol), uncouplers (CCCP and N,N'-dicyclohexylcarbodiimide), or compounds that target an energized membrane (vanilomycin) or ΔpH (nigericin) (Boshoff et al., 2004). Further, exposure of WT and all three mutant strains to oxidative stress (CHP) resulted in substantially increased expression of the same subset of 10 genes used in the microarray validation (Figure 6D). This observation underscores the importance of this core set of genes in redox regulation in Mtb in the absence of EGT and MSH under conditions of increased oxidative stress.

These data demonstrate that a lack of EGT or MSH results in differential expression of genes involved in nucleotide and sulfur metabolism, secretion, toxin/anti-toxins, lipid metabolism, and cytochrome biogenesis, likely as a compensatory response to the loss of redox couples. The regulation of trx genes by MSH, but not by EGT, is possibly an indicator of distinct chemical and functional requirements of mycobacterial redox buffers.

**EGT Biosynthesis by Mtb Is Required for Survival in Macrophages and Mice**

We next examined the role of EGT in intracellular Mtb survival and virulence. We observed significantly reduced survival of egtA:Tn and egtD:Tn in RAW264.7 macrophages at 5 days post-infection compared to WT Mtb (Figure 7A). Complementation of the egtA/egtD mutants restored intracellular survival to near WT levels. To determine the role of EGT in Mtb pathogenicity, mice were infected with WT, egtD:Tn, egtA:Tn, or the complemented strains in two independent experiments. We observed significantly reduced bacillary burdens in the lungs of mice infected with egtD:Tn or egtA:Tn compared to mice infected with WT Mtb. Complementation of egtD:Tn and egtA:Tn successfully restored bacillary load to WT levels. The egtD:Tn showed ~4-fold decrease in lung bacillary burden, whereas the egtA:Tn showed a 4-log10 reduction (Figures 7B and 7C). Multiple lesions were observed in the lungs of mice infected with WT and egtA:TnC strains compared to lungs of mice infected with egtA:Tn (Figure 7F). Histopathological analysis of lung sections from mice infected with WT Mtb, egtA:TnC, and egtD:TnC strains revealed large areas of alveolar consolidation due to lymphocyte-rich granulomatous infiltration (Figure 7G). In addition, multinucleated giant cells were observed in and around granulomas in the lungs of these mice. In contrast,
egTA:Tn- and egTD:Tn-infected lungs showed diffuse areas of lymphocyte infiltration and exhibited visible alveolar spaces with partial lung parenchyma. Although it is known that EGT accumulates in organs that encounter oxidative stress (Cheah and Halliwell, 2012), it is not known whether infection by Mtb promotes accumulation of host-derived EGT or whether Mtb itself contributes significantly to the host EGT pool. Infection of macrophages with WT Mtb, egTD:Tn, egTA:Tn, and complemented strains (egTD:TnC and egTA:TnC) showed no significant difference in intracellular EGT concentrations (data not shown). Also, in mice infected with WT Mtb, egTD:Tn, egTA:Tn, or the complemented strains, we observed no statistically significant differences in lung EGT concentrations (Figures 7D and 7E). Altogether, our findings suggest that Mtb is not a significant contributor to host EGT during infection. In sum, independent disruption of two different genes within the EGT biosynthetic pathway results in attenuation in vivo and provides strong evidence for a role of EGT in Mtb intracellular survival and virulence.

DISCUSSION

The aim of this study was to examine how Mtb WhiB3 maintains intracellular redox homeostasis and bioenergetics to regulate virulence. Here, we discovered that the virulence factor WhiB3 is a regulator of bioenergetic homeostasis and controls the production of a major redox buffer EGT in a carbon-source-dependent manner to maintain redox balance. Furthermore, by successfully adapting extracellular flux analysis technology, we have shown that a lack of EGT or MSH significantly alters Mtb respiratory activity, which establishes a direct link between redox homeostasis and bioenergetic metabolism. Importantly, we provide evidence that redox and bioenergetic homeostasis contribute to Mtb survival in macrophages and in mice and are critical components in drug susceptibility and sensitivity to oxidative stress. The identification of bioenergetic deficiencies in Mtb mutants that display no apparent phenotypic defects when grown in vitro, but that demonstrate attenuation upon mouse infection, points to bioenergetic homeostasis as a largely equal, as evidenced by differences in Mtb survival in the

The ability of EGT to protect against oxidative stress caused by CHP, PQT, H2O2, and MND confirms that Mtb produces EGT to maintain redox balance. Not all oxidative stresses are equal, as evidenced by differences in Mtb survival in the

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presence of various oxidants and the fact that a broad spectrum of free radical reactions exists, ranging from very reducing (e.g., $E^o = -1.800$ mV for CO$_2$/CO$_2^*$) to very oxidizing (e.g., $E^o = 2.310$ mV for HO*/H$_2$O) (Suettnner, 1993). The production of both EGT and MSH to counter free radicals may partially explain the remarkable success of 

Mtb as an intracellular pathogen that persists for decades in the oxidative environment of the lung. This work also establishes a relationship between EGT and MSH production. Notably, the increased EGT levels and reduced endogenous ROS (Figure 4F) in smshA reveal the presence of a compensatory mechanism wherein EGT production is increased to mitigate endogenous oxidative stress in the absence of MSH. Collectively, the data support that the level of EGT in the cell, which is modulated by WhiB3 and the availability of carbon sources, serves as a buffer against redox stress such as O$_2^*$ associated with cellular metabolic activity. The implication of this finding is significant as carbon sources such as fatty acids or oxidation products can modulate redox balance and thus drug susceptibility during infection by varying EGT levels.

To understand the effects of EGT or MSH deficiency on global gene expression in 

Mtb, we performed transcriptomic analysis of egta, egtd, and msha mutants and identified 25 differentially regulated genes common to all three mutants. Our quantitative real-time PCR validation underscored the accuracy of our microarray-based gene expression analysis. Of these 25 genes, 24 are also modulated by respiration inhibitors, uncouplers, or compounds that target an energized membrane (Bosshoff et al., 2004), implicating EGT and MSH in maintenance of bioenergetic homeostasis. Our observations elucidate a bioenergetics-centered model of WhiB3-mediated redox balance through EGT and represent a paradigm for redox homeostasis in prokaryotes.

Upregulation of an operon involved in mycolic acid biosynthesis (Rv2243-Rv2247) in the egta/egtd mutants and of the Rv1130-Rv1131 operon (encoding PrpD and PrpC in the methylocitrate cycle) in the msha and egta mutants suggests that perturbations in redox balance due to lack of EGT or MSH affect biosynthesis of different classes of lipids, which may act as sinks for reducing equivalents (Singh et al., 2009). An important dissimilarity between the expression profiles of egta/egtd and msha mutants is that thioredoxin B (trxB1, Rv1471) and a putative thioredoxin-like gene (trx, Rv0526) are upregulated in the msha mutant, but not in the egta/egtd mutants. This observation is consistent with the requirement of MSH for thioredoxins as reducing partners (Attarian et al., 2009). The majority of the differentially regulated genes (22 of 25) in the egta/egtd and msha mutants are downregulated. Interestingly, 17 of the 22 downregulated genes are upregulated (2- to 5-fold) during 

Mtb infection of macrophages or under nutrient deprivation conditions in vitro (Schnappinger et al., 2003; Betts et al., 2002). It is therefore reasonable to posit that redox buffers EGT or MSH may also modulate the survival of 

Mtb inside macrophages and thus influence the outcome of infection.

Our in vitro and in vivo infection studies clearly show that EGT-deficient 

Mtb strains are attenuated and establish that EGT is critical for the intracellular survival and virulence of 

Mtb (Figures 7A–7C and 7F). These results provide important insights into the role of EGT in 

Mtb virulence in addition to its role in drug susceptibility, both of which appear to be distinct from MSH (Vilcheze et al., 2008). The observation that egta and egtd contribute to different degrees to intracellular survival is supported by the finding that different intermediates of EGT biosynthesis may perform antioxidant functions (Song et al., 2015) and feed into different pathways of central carbon metabolism. Consequently, disruption of different steps in the EGT biosynthetic pathway in the egta and egtd mutants likely explains the differences in attenuation (Figures 7A–7C, 7F, and 7G).

In summary, the findings in this study reveal new mechanisms by which 

Mtb perceives and relays metabolic signals to maintain redox balance and bioenergetic homeostasis. These findings establish important roles for EGT in oxidative stress protection, drug susceptibility, and virulence in 

Mtb, which are unlike MSH. The discovery of an EGT/MSH hierarchy of redox defense mechanisms with distinct physicochemical properties to counteract a spectrum of host-generated free radicals and metabolites has significant implications for how 

Mtb maintains redox and bioenergetic homeostasis during persistence.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**

All 

Mtb strains used in this study, unless otherwise specified, were grown at 37°C in GIBCO Middlebrook (MB)7H9 (broth) or MB7H10/MB7H11 (agar) media supplemented with 0.2% glycerol, 1x ADST (albumin saline enrichment [albumin-NaCl] with 0.05% tyloxapol) as described in Supplemental Experimental Procedures.

**Liquid Chromatography-Mass Spectrometry for Metabolome Analysis**

Metabolites were separated on a Cogent Diamond Hydride Type C column and analyzed by LC-MS as described in Supplemental Experimental Procedures.

**Bioenergetic Analysis of Mtb**

OCR and ECAR of 

Mtb strains adhered to Cell-Tak-coated XF cell culture microplates (Seahorse Bioscience) at 2 x 10^6 bacilli/well were measured using an XF96 Extracellular Flux Analyzer as described in Supplemental Experimental Procedures.

**TLC and LC-MS/MS Analysis of EGT**

Cell extracts containing radiolabeled EGT were concentrated to dryness and resuspended in 50–100 µl of acetonitrile:methanol:50 mM ammonium acetate (40:40:20) (AMAA). At least 30,000 cpm in a maximum of 15 µl was loaded onto silica gel TLC plates and developed in a 3:1 methanol:water solvent system as described in Supplemental Experimental Procedures. To validate TLC analysis of EGT, concentrated, unlabeled Msm extract was loaded on a silica gel TLC plate and developed as above. The suspected EGT spot was visualized under UV light, scraped from the plate, and extracted four times with 50 µl AMAA. It was then concentrated to dryness, resuspended in 20 µl AMAA, and examined by an AB Sciex API-3200 Triple Quadrupole coupled to a Waters Acquity LC system. Data were collected in the positive electrospray mode. EGT levels in 

Mtb and mouse lungs were also determined using LC-MS/MS as described in Supplemental Experimental Procedures.

**Measurement of Endogenous ROS in Mtb**

The percentage of ROS-producing 

Mtb cells was determined using CellROX Green (Life Technologies) and flow cytometry as described in Supplemental Experimental Procedures.

**Determination of Mtb Susceptibility to Oxidants and Anti-TB Drugs**

Mtb cells were cultured as described above. Mtb strains were exposed to oxidizing agents (6 hr) or various concentrations of anti-TB drugs (7 days). Cells were plated on MB7H11 agar plates, colonies were enumerated after 4 to
5 weeks of incubation at 37°C, and percentage survival was determined. Additional details are provided in Supplemental Experimental Procedures.

Microarray Analysis

*Mtb* RNA was isolated and prepared as described in Supplemental Experimental Procedures.

Quantitative Real-Time PCR Analysis

Total RNA was isolated from mycobacterial cells and quantitative real-time PCR analysis was performed as described in Supplemental Experimental Procedures.

*Mtb* Infection Studies and Quantitation of EGT

For in vitro infection studies, RAW264.7 macrophages were infected with *Mtb* strains at a MOI of ten. Mice were used as our in vivo model of infection. Experimental details are outlined in Supplemental Experimental Procedures.

Statistics

Statistical computations were performed with Prism 6.0 software (GraphPad). Pairwise comparison was performed by Student’s t test for normally distributed data. Multiple comparisons were performed using one-way ANOVA (Tukey’s test) module of GraphPad.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is EMBL ArrayExpress: E-MTAB-4099.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.056.

AUTHOR CONTRIBUTIONS

Conceptualization, V.S., L.G., and A.J.C.S.; Investigation, mass spectrometric and metabolomic analyses (J.H.A., H.E., and K.R.); quantitation of EGT and MSH (L.G., Y.A.-G., and H.B.); XF assays (D.L.); ROS determination (B.M.C.); transcriptomic profiling and analysis (V.S.); macrophage assays (K.C.C.); animal studies at UAB (V.S., V.P.R., K.C.C., and J.N.G.) and KRITH (J.M. and V.P.R.), and histopathological analysis (V.S. and V.P.R.); Validation, V.S. and K.C.C.; Data Curation, V.S., D.L., and K.R.; Writing, V.S., B.M.C., L.G., J.N.G., and A.J.C.S.; Funding Acquisition, A.J.C.S. All authors discussed the results and commented on the manuscript.

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