Cell Reports

Supplemental Information

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

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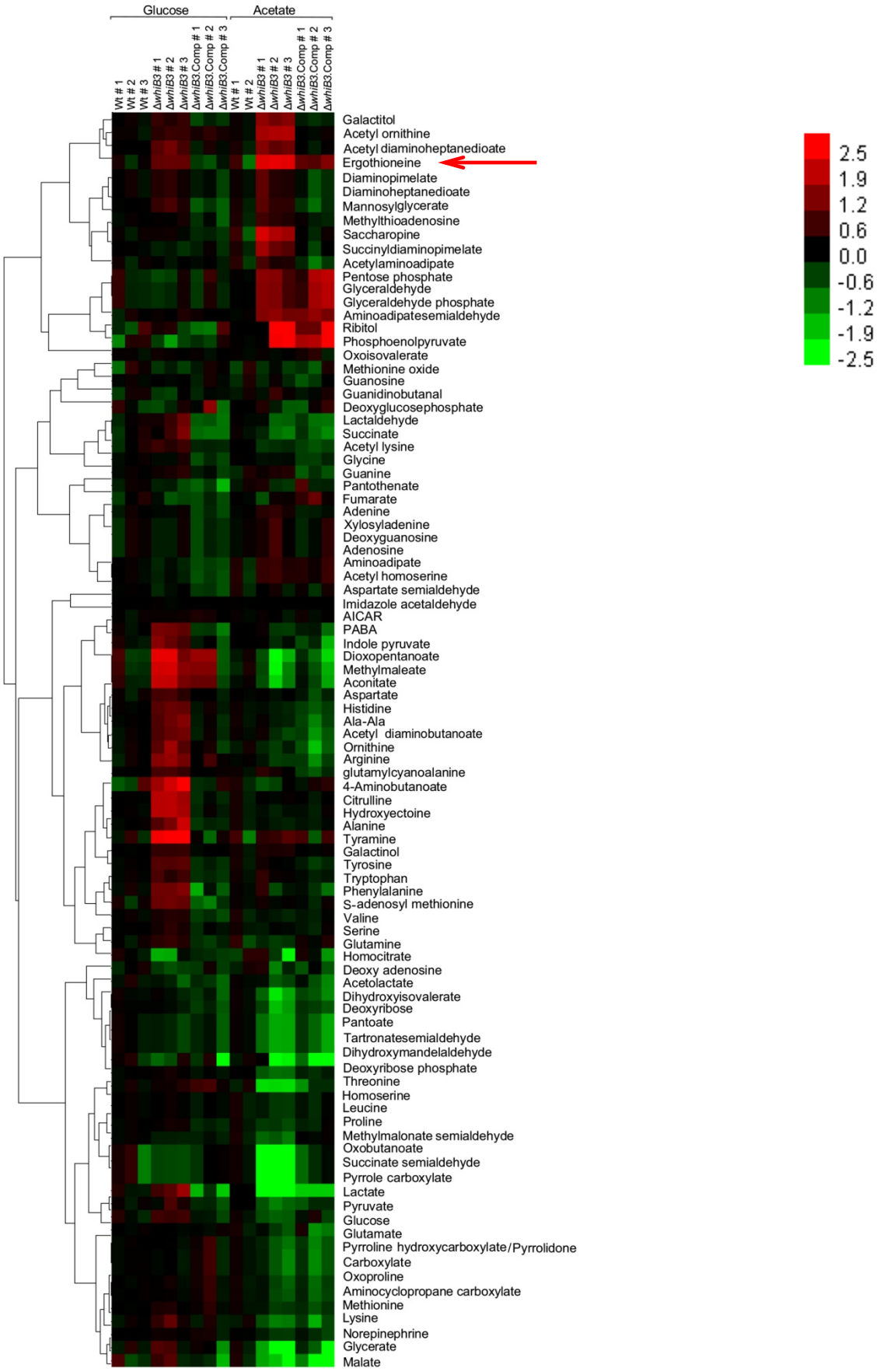

Figure S1. Liquid chromatography/mass spectrometry analysis of putative metabolite assignments. Related to Figure 1. Cell extracts were prepared from *Mtb* H37Rv (Wt), *Mtb*Δ*whiB3* and *Mtb*Δ*whiB3* complemented (Δ*whiB3*:Comp) strains cultured in the presence of glucose or acetate. Red arrow indicates EGT.

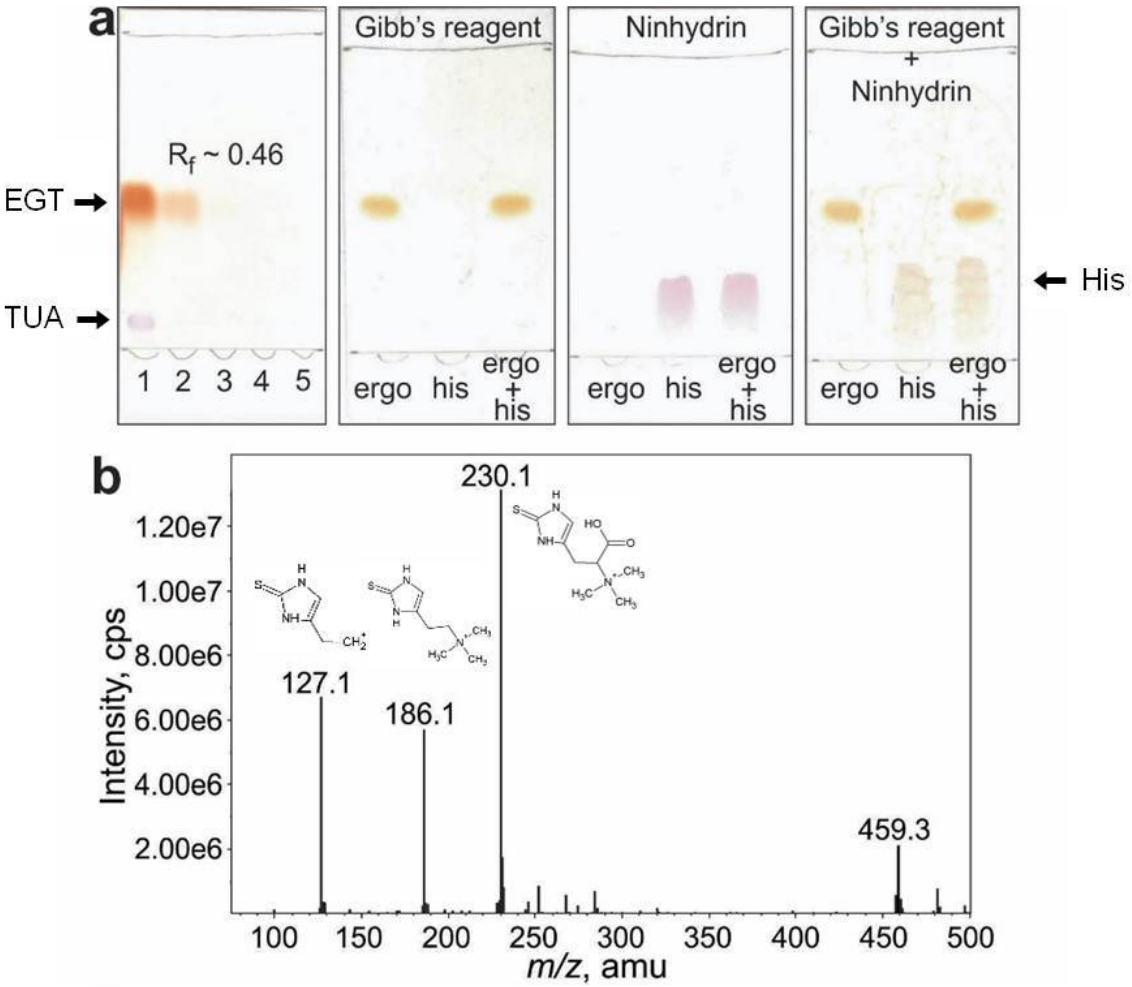

Figure S2. Detection of EGT by TLC analysis. Related to Figure 3. (a) Pure EGT and/or histidine (His) dissolved in water was spotted on a TLC plate and run in a 3:1 methanol:water solvent system followed by application of Gibb's reagent, ninhydrin or both. Left Panel: EGT (brick-red spot) runs with an R_f value of ~0.46. Thiolurocanic acid (TUA) is a breakdown product of EGT. The concentration of EGT was (lane 1) 10 mM, (lane 2) 1 mM, (lane 3) 100 µM, (lane 4) 10 µM, (lane 5) 1 µM. Center Panels: EGT and His (0.1 nmol) were visualized with Gibb's reagent and 0.25% ninhydrin in acetone, respectively. His turns violet in the presence of ninhydrin. Right Panel: Applying both compounds alters the color of His, but it is clear that the compounds have distinct Rf values. **(b)** The most abundant fragment ions of EGT were identified for use in LC-MS/MRM.

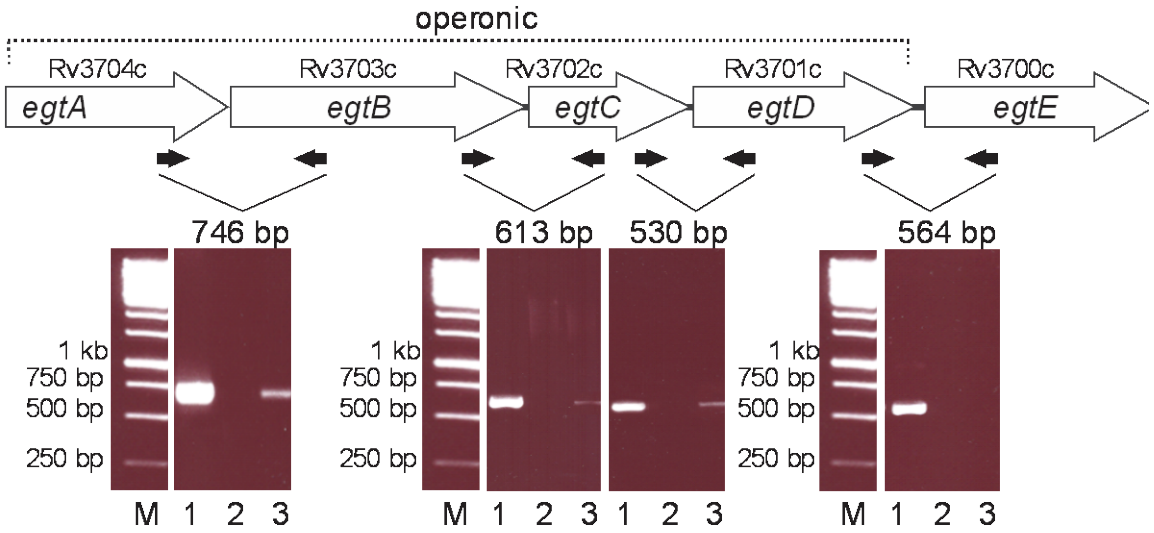

Figure S3. Organization of *egt* genes in *Mtb*. Related to Figure 4. Reverse Transcriptase-PCR analysis shows that *egtA* – *egtD* are operonic while *egtE* is not. The expected size of each amplicon is indicated above each gel image. *Mtb* CDC1551 genomic DNA (gDNA) (lane 1) was used as a positive control and mRNA that was not reverse transcribed was used as negative control (lane 2). The PCR products from cDNA (lane 3) indicate that the adjacent genes are present on the same mRNA transcript and are, thus, operonic. Primers used were: *egtA* Fwd operon, 5'-TCGGCAATGGCTGGAAATTCGCTA-3'; *egtB* Rev operon, 5'-ATCAC CATCGCGAACACGAAGCTG-3'; *egtB* Fwd operon, 5'-CTATGCCAACCTGGGCGGTCAAAC-3'; *egtC* Rev operon, 5'-TCGCGCTGACTTCGATCGGCATAC-3'; *egtC* Fwd operon, 5'-TGGACGCTCTGGG AGACACCATTG-3'; *egtD* Rev operon, 5'-TGCCGCTACCCAACTCGACCAAAG-3'; *egtD* Fwd operon, 5'-CAGCGCAGAAGAGCGGATTG-3'; *egtE* Rev operon, 5'-AGCAGATCCAACGCGTGCAG-3'. M, molecular size marker.

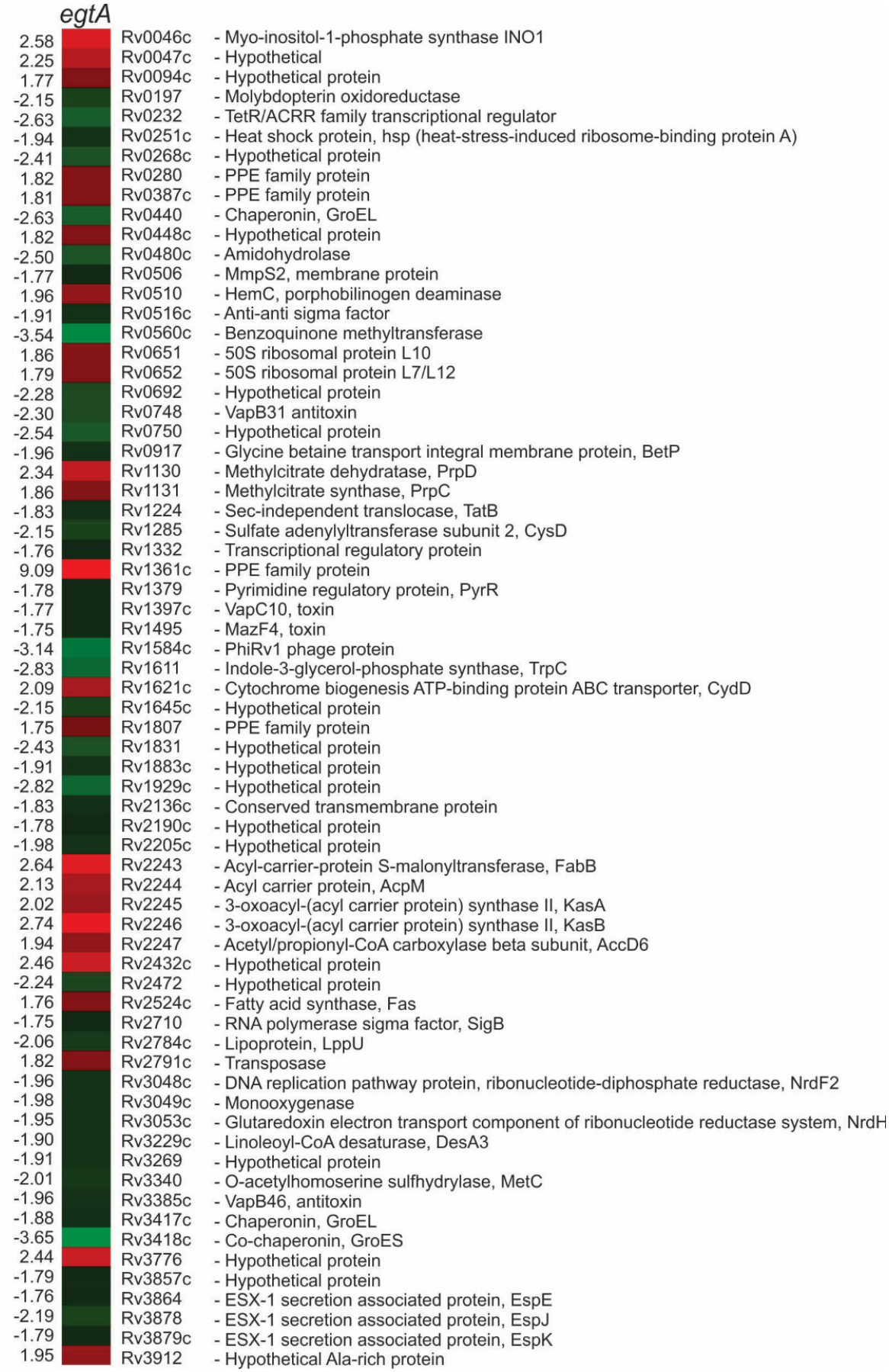

Figure S4. Genome-wide transcription profile of *egtA*:Tn under normal growth conditions. Related to Figure 6.

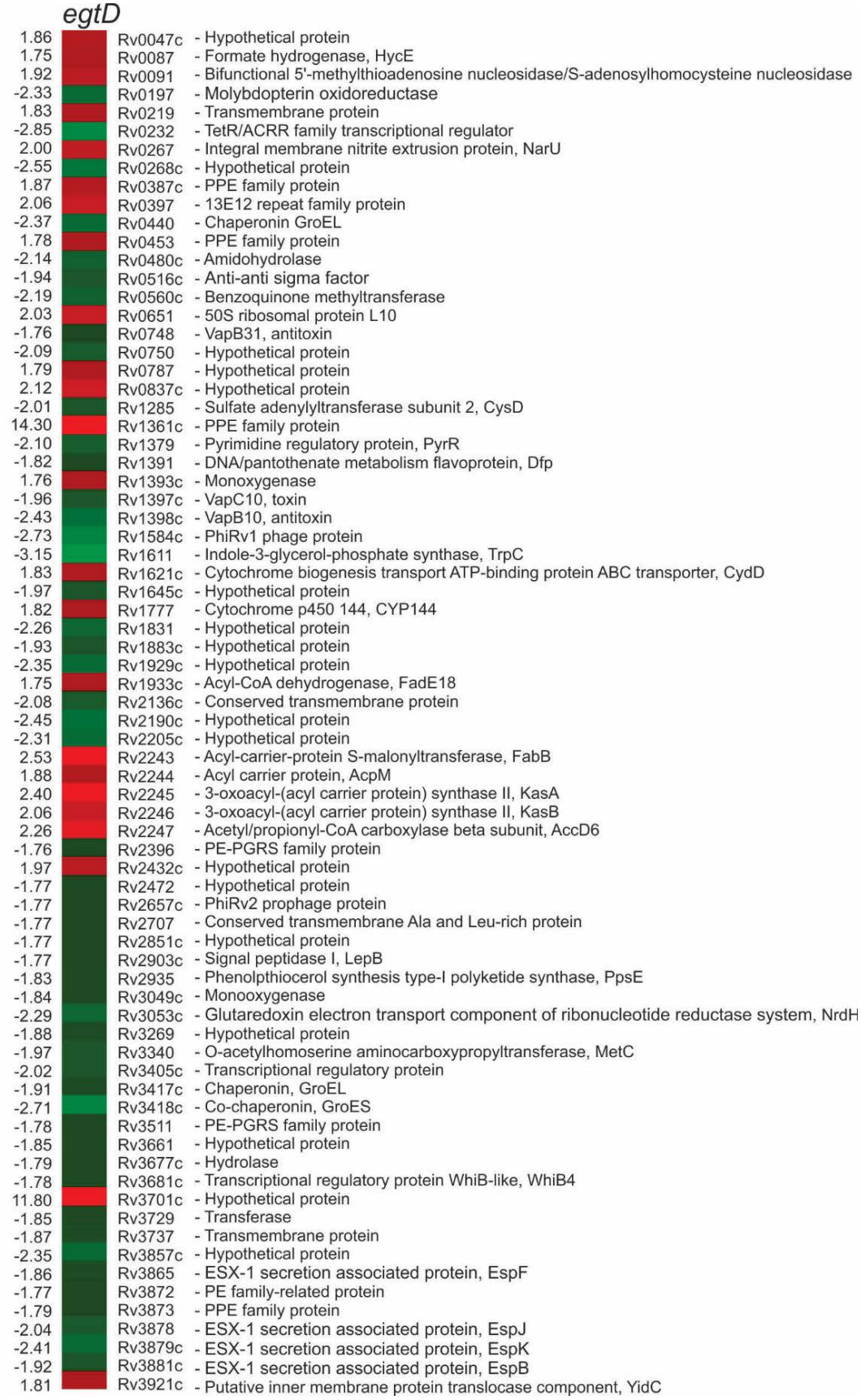

Figure S5. Genome-wide transcriptional profile of *egtD*:Tn under normal growth conditions. Related to Figure 6.

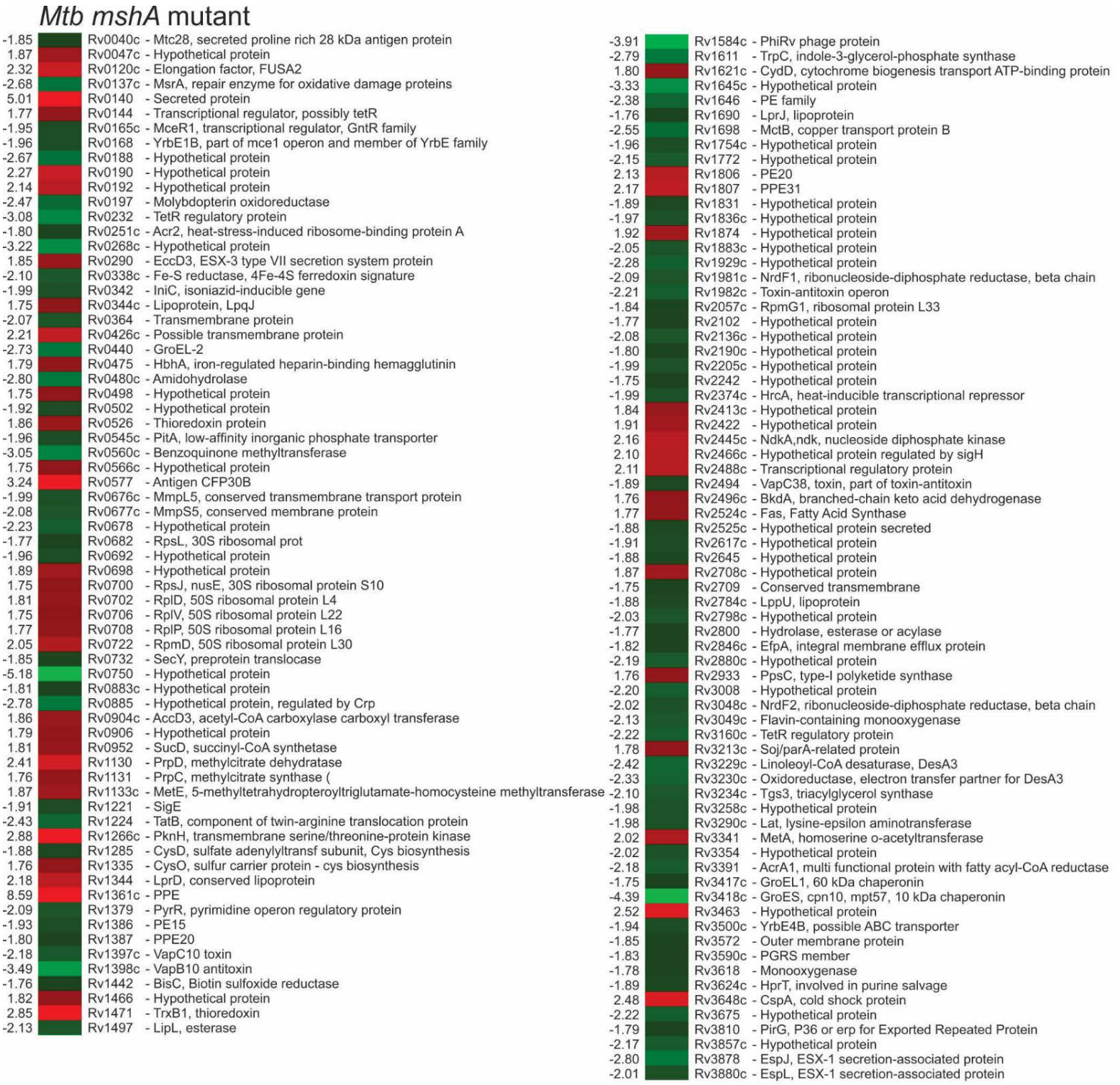

Figure S6. Genome-wide transcriptional profile of Δ*mshA* under normal growth conditions. Related to Figure 6.

Table S1. Total metabolite profiling of Δ*whiB3* assessed by pathway enrichment analysis using MetPA. Related to Figure 1 and Table 1.

Enriched pathways and metabolites	KEGG Compound number
Arginine and Proline metabolism	
L-glutamine	C00064
Citrulline	C00327
L-Aspartic acid	C00049
L-Arginine	C00062
S-Adenosyl methionine	C00019
Fumaric acid	C00122
γ-aminobutyric acid	C00334
Ornithine	C00077
N-acetylornithine	C00437
L-Proline	C00148
Pyrroline hydroxycarboxylic acid	C04281
Pyruvic acid	C00022
Pantothenate and CoA biosynthesis	
2-acetolactate	C00900
2, 3 dihydroxy methyl butanoate	C04039
L-Valine	C00183
L-Aspartic acid	C00049
Pantoate	C00522
2-ketoisovaleric acid	C00141
Pantothenic acid	C00864
Pyruvic acid	C00022
Butanoate metabolism	
γ-aminobutyric acid	C00334
Succinic acid semialdehyde	C00232
Succinic acid	C00042
Fumaric acid	C00122
2 acetolactate	C00900
R-malate	C00497
Pyruvic acid	C00022
Glycine, serine and threonine metabolism	
L-Aspartic acid	C00049
L-homoserine	C00263
L-Aspartate 4-semialdehyde	C00441
L-threonine	C00188
2-ketobutyric acid	C00109
Glycine	C00037
Glyceric acid	C00258
L-Serine	C00065
L-Tryptophan	C00078
Pyruvic acid	C00022
Cysteine and methionine metabolism	
L-Serine	C00065
L-Aspartic acid	C00049
L-homoserine	C00263
L-Methionine	C00073
S-Adenosyl methionine	C00019
L-Aspartate 4-semialdehyde	C00441
2-ketobutyric acid	C00109
5-Methylthioadenosine	C00170
Pyruvic acid	C00022

Alanine, aspartate and glutamine metabolism	
L-Aspartic acid	C00049
Fumaric acid	C00122
Glutamine	C00064
4-aminobutanoate	C00334
Succinate semialdehyde	C00232
Succinate	C00042
TCA cycle	
Succinic acid	C00042
Fumaric acid	C00122
cis-Aconitate	C00417
Phosphoenolpyruvate	C00074
Pyruvic acid	C00022
Lysine biosynthesis	
Aspartate	C00049
L-Homoserine	C00263
L-Aspartate 4-semialdehyde	C00441
N-Succinyl-LL-2,6-diaminoheptanedioate	C04421
LL-2,6-Diaminopimelate	C00666
L-lysine	C00047

Table S2. Determination of drug susceptibility of Wt, *egt* and *mshA* mutant mycobacterial strains against anti-TB drugs.* Related to Figure 4.

***Mtb* Strain**	**Rifampicin**	**Isoniazid**	**Bedaquiline**	**Clofazimine**
Wt	0.03	0.03	0.25	1.0
egtA:Tn	0.015	0.006	0.125	0.65
egtD:Tn	0.015	0.01	0.125	0.50
Δ*mshA*	0.015	0.006	0.008	0.0625

* The values indicated in the table represent MIC (µg/ml) obtained by CFU assays.

Table S3. Quantitative RT-PCR analysis of Wt and complemented strains under normal growth conditions. Related to Figure 6C.

Gene ID	Wt	*egtA*:TnC	*egtD*:TnC	Δ*mshA*:comp
Rv0232	1.42	1.26	1.05	1.23
Rv0440	1.05	0.92	1.23	1.07
Rv0560c	0.77	0.70	0.68	0.96
Rv0750	1.40	1.59	1.19	1.39
Rv1285	0.79	0.50	0.72	0.47
Rv1361c	0.57	0.49	0.46	0.43
Rv1621c	0.03	0.04	0.03	0.03
Rv1645c	0.61	0.44	0.50	0.38
Rv3418c	1.55	1.05	1.4	1.48
Rv3878	1.85	1.168	1.81	1.16

Gene expression values are normalized against *sigA* expression as the reference internal control. Each value is the average of three independent measurements performed in duplicate.

Table S4. Primer sequences used for quantitative RT-PCR analysis. Related to Figure 6.

Primer ID	Primer Sequence (5'- 3') F, forward; R, reverse
sigA F	GTGATTTCGTCTGGGATGAAGA
sigA R	TACCTTGCCGATCTGTTTGAG
Rv0232 F	CGGCAGCTTGAACGTCATA
Rv0232 R	TGGCGATGTCGGCAATAAA
Rv0440 F	ACAATTGCGTACGACGAAGA
Rv0440 R	GGGCCCAATGTCACCTTTA
Rv0560c F	CAGTTCGTTTACCGGCTATGA
Rv0560c R	CGAATACCAACACGAAGTAGGA
Rv0750 F	GACTGCGTCATCCACATCAT
Rv0750 R	GCCGATATCCAATGCCAGAT
Rv1285 F	CGACGAAGTTATCGCTACCC
Rv1285 R	GTCACGGTCTGTATCGGATTT
Rv1361c F	GTGAACTCTGGAAAGCCATCT
Rv1361c R	TCGACACGTGGTTGTTGAG
Rv1621c F	CCCTACTTCACCGGCTATTT
Rv1621c R	AGCACCATGAAGATCGGTATC
Rv1645c F	GTCATCAAGGTCGCCGAATA
Rv1645c R	CGCATGCTCATCCCATACA
Rv3418c F	TGGCGAAGGTGAACATCAA
Rv3418c R	TTGGCGGTGTCAGGAATG
Rv3878 F	CGAACAGCATCCAACATGAAC
Rv3878 R	CGAATGCATACTGGCTCAAAC
sigB F	ACTGTTACACACCGACATCC
sigB R	CCCGAATAGTTTGCCGATTTG
sigH F	GAAGGCCTATGCGGGATTT
sigH R	GCGATAGCTGTTGATGTAGGT

Supplemental Experimental Procedures

Materials

Middlebrook 7H9, 7H10 and 7H11 media was purchased from Difco, USA. The XF96 microtiter plates and cartridges for extracellular flux analysis were obtained from Seahorse Bioscience, USA. All drugs and chemicals were purchased from Sigma, USA unless otherwise specified.

***Mtb* strains and culturing conditions**

Mtb strains were grown at 37°C in 7H9 (broth) or 7H10/7H11 (agar) media with 1x ADST (albumin saline enrichment (albumin-NaCl) with 0.05% tyloxapol). Where indicated, cultures were supplemented with glucose, sodium acetate, sodium propionate, sodium pyruvate, glucose, sodium acetate, sodium propionate, cholesterol, sodium stearate, sodium myristate, sodium palmitate or behenic acid to a final concentration of 50 µM. Glycerol was excluded from growth media containing acetate, succinate or sodium propionate. The CDC1551 *egtA*:Tn and *egtD*:Tn strains were grown in the presence of 25 µg/ml kanamycin (Kan), while H37Rv Δ*whiB3* and CDC1551 Δ*mshA* were grown in the presence of Hygromycin B (Hyg; 50 µg/ml). All complemented strains were grown in the presence of Hyg (50 µg/ml) and Kan (25 µg/ml).

For metabolic labeling and extraction of EGT from mycobacteria, cells were cultured on mixed cellulose ester membranes placed on solid media for four weeks and promptly transferred from plates to -80°C (de Carvalho et al., 2010). Briefly, mycobacteria grown to early- to mid-log phase in MB7H9 were set to an OD_{600} of 0.8-1.0 and 400 µl was spread onto membranes, which were then placed onto MB7H10 plates. To determine the effect of the carbon source on EGT production, *Mtb* cells were cultured in MB7H9, 10% OADC, 0.2% glycerol and 0.05% tyloxapol containing one of the following carbon sources for up to 4 days: glucose, sodium acetate, sodium propionate, cholesterol, sodium stearate, sodium myristate, sodium palmitate or sodium behenate to a final concentration of 50 µM.

Cloning and Operonic Analysis of Genes Involved in EGT Biosynthesis in *Mtb*

To complement the *egt* transposon (Tn) mutants, *egt* genes were PCR amplified from *Mtb* DNA and cloned into the *Nco*I/*Hin*d III sites of pMV762 as described in Supplementary Methods. The operonic organization of genes was investigated by isolating total RNA from a log-phase culture of Wt *Mtb* CDC1551. RNA was reverse transcribed and the resulting cDNA was used as a PCR template with primer pairs designed to amplify appropriate regions of the *egt* genes (Figure S3).

Liquid chromatography/mass spectrometry (LC/MS) for metabolome analysis

Metabolites were separated on a Cogent Diamond Hydride Type C column as detailed previously (de Carvalho et al., 2011). An Agilent Accurate Mass 6220 TOF spectrometer coupled to an Agilent 1200 LC system was used. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution using an isocratic pump with a 100:1 splitter. This configuration achieved mass errors of approximately 5 parts-per-million (ppm), mass resolution ranging from 10,000-25,000 (over *m/z* 121-955 amu), and 5 $\log_{10}$ dynamic range. Detected ions were deemed metabolites on the basis of unique accurate mass-retention time identifiers for masses exhibiting the expected distribution of accompanying isotopomers. EGT was confirmed by demonstrating co-elution with a chemical standard.

Bioenergetic analysis of *Mtb*

An XF96 Extracellular Flux Analyzer was used for all bioenergetic assays. Assays used unbuffered 7H9 media (pH 7.35). Bacilli were grown for 24 hrs prior to the start of the experiment in7H9 medium supplemented only with 0.05% tyloxapol. Prior to addition of a carbon source, the OCR were allowed to stabilize. Three OCR and ECAR measurements were made after the sequential addition of a carbon source (50 µM) or CCCP (1 and 8 µM final concentration respectively) through the drug ports of the sensor cartridge. The OCR and ECAR data points are representative of the average OCR and ECAR during 4 min of continuous measurement in the transient micro-chamber, with the error being calculated from the OCR and ECAR measurements taken from three replicate wells by the Wave Desktop 2.2 software (Seahorse Bioscience). The transient micro-chamber is automatically re-equilibrated between measurements through the up and down mixing of the probes of the XF96 sensor cartridge in the wells of the XF cell culture microplate.

TLC analysis of EGT

Radiolabeled extracts were concentrated to dryness and resuspended in 50-100 µl acetonitrile:methanol:50 mM ammonium acetate (40:40:20) [AMAA]. At least 30,000 cpm in a maximum of 15 µl was loaded on silica gel TLC plates and developed in a 3:1 methanol:water solvent system. For each culture condition, the strains were grown for the same number of days before extraction. Radiolabeled EGT (identified by its R_f value; see below) was visualized by phosphorimaging using a Storm 820 phosphorimager (GE Healthcare). Quantitative analysis was performed using ImageQuant (GE Healthcare). The R_f value of EGT in this solvent system was determined by TLC analysis of pure EGT followed by application of 0.2% 2,6-dichloroquinonechloroimide (Gibb's reagent) in ethanol, which makes EGT appear brick-red. The R_f value of pure histidine was also determined after visualization with 0.25% ninhydrin in acetone, with which histidine appears violet.

Metabolic labeling and extraction of EGT from mycobacterial strains

For filter culturing, mycobacteria grown to early- to mid-log phase in 7H9 were set to an OD_{600} of 0.8-1.0 in 400 µl and inoculated onto mixed cellulose ester membranes, which were then placed onto 7H10 plates containing the same carbon source as the broth. For radioactive labeling, 2-3 µCi/ml of ^{35}S-cysteine (American Radiolabeled Chemicals) was spread on the plates prior

to the placement of the membranes. Alternatively, mycobacterial cells were cultured in broth in the presence of ^{35}S-Cys. The cells were grown on plates until a confluent film was achieved, and growth times varied for different carbon sources. Bacteria were metabolically quenched by immersion into AMAA pre-cooled to -40°C. For metabolome analysis, metabolites were extracted by mechanical lysis followed by clarification and filtration with a 0.22 µm filter. Extracts of radioactively labeled bacteria were prepared by heating at 90°C for 20 min prior to clarification and filtration. When necessary, bacterial biomass of individual samples was determined by measuring residual protein content. For broth culturing, mycobacteria grown to mid- to late-log phase in the presence of 2-3 µCi/ml ^{35}S-cysteine or 5-10 µCi of [2-^{14}C]-acetate were pelleted, washed twice with medium and, after the addition of AMAA or water, extracts were prepared by heating as above.

Measurement of EGT levels in *Mtb* and mouse lungs using LC-MS/MS

Actively growing *Mtb* H37Rv, H37RvΔ*whiB3* and complemented strains were washed and used to inoculate cultures in 7H9 medium with a starting OD_{600}=0.2. Each culture was grown in a single carbon source (50 µM) with tyloxapol. Following four days of growth, 2 ml of culture was used for EGT extraction and quantification using standard addition. Cells were washed twice in an equal volume of 1x PBS and were resuspended in 2 ml of 70% acetonitrile with 25 ng/ml of the internal standard 1-methyl-4-phenylpyridinium (MPP+). Cells were disrupted with a MagNAlyser (Roche) and 0.1 mm silica beads (BioSpec) at a speed of 7000 rpm for 60 s intervals followed by 2 min of rest at -20°C (repeated four times). The extract was then filter sterilized using 0.22 µm nylon polypropylene Spin-X® Centrifuge Tubes prior to further analysis by LC-MS. Intracellular EGT levels were normalized to CFU and each sample was analyzed in triplicate.

Gently homogenized mouse lung samples were treated with collagenase and DNase for 22 h followed by the addition of an equal volume of water and vortexing. Samples were centrifuged and particulates removed using a 0.22 micron filter, after which 50 µl of filtrate was treated with 150 µl of acetonitrile to precipitate proteins. Samples were centrifuged at 16 000 *x g* for 12 minutes at 20°C. The supernatant was diluted 10-fold in 80% acetonitrile and analysed by LC-MS/MS on a triple quadrupole mass spectrometer (AB SCiex QTrap 5500) using atmospheric pressure ionization with positive electrospray. The LC system consisted of an Agilent 1260 Binary LC Pump, an auto sampler, and an Agilent ZORBAX HILIC Plus silica Column (100 mm X 2.1 mm; particle size, 3.5 µm) at 30°C. The solvent for isocratic chromatography (flow rate 200 µl/min) was acetonitrile (76%) and water (24%), with 0.1 % formic acid. For quantification by multiple reaction monitoring (MRM), the optimal collision energy (CE) for nitrogen-induced fragmentation in the second quadrupole was determined for EGT and MPP^+. From the product ion spectra, the following fragments were selected for MRM (*m/z* parent, *m/z* fragment, and CE): EGT 230, 127 and 27 V; MPP^+ 171, 129 and 45 V. For each analyte, the area of the intensity versus the time peak was integrated and divided by the area of MPP^+ peak to give the EGT response ratio. Standard addition calibration curves (R^2 >0.99) were constructed for each sample and were used to determine the concentration of EGT in *Mtb* or mouse lung tissue.

Microarray analysis

Mtb RNA was obtained from exponentially growing CDC1551 (wild type), CDC1551Δ*mshA*, *egtA*:Tn and *egtD*:Tn strains cultured in 7H9 medium supplemented with ADST (Albumin Dextrose Saline Tyloxapol) using RNApro (MP Biomedicals, USA) as per manufacturer's instructions. The quality of RNA was examined by running the samples on Experion gene chips (BioRad). Expression analyses were performed by the Center for Applied Genomics (www.cag.icph.org) at the Public Health Research Institute (Newark, NJ) as described previously (Kumar et al., 2008). These microarrays consist of 4,295 70-mer oligonucleotides representing 3,924 open reading frames (ORFs) from *M. tuberculosis* strain H37Rv (http://www.sanger.ac.uk) and 371 unique ORFs from strain CDC1551 (http://www.tigr.org) that are absent in the H37Rv strain. The complete gene list and array layout can be found at www.cag.icph.org/downloads_page.htm). Total RNA (0.5 to 1 µg) from each sample was used for each microarray with three biological replicates for each strain. The detailed labeling and hybridization protocol can be obtained at www.cag.icph.org/downloads_page.htm. Total RNA was prepared in triplicate and used for cDNA synthesis, which was performed using random primers and labeled with cyanine-3 or cyanine-5 dUTP (Perkin Elmer) to generate cyanine 3 or cyanine 5 dUTP labeled probes. Hybridization was performed overnight. For each pair of samples in each experiment, dye flips were performed. After washing, the arrays were dried by centrifugation (100 x *g*, 2 min) and scanned using GenePix4000B scanner (Molecular Devices). The images were processed using GenePix 5.1 software. The ratio of the mean median intensity of Cy5 over the mean median intensity of Cy3 was determined for each spot and the fold change values were calculated. Gene differentially regulated by at least 1.75-fold, with a *q* value of 1% were considered significant. The *q* value is the equivalent of the *p* value after multiple-testing correction. Functional classification and pathway annotations were performed using TubercuList dataset (http://genolist.pasteur.fr/TubercuList) and the KEGG database.

Quantitative RT-PCR Analysis

Validation of gene expression profiles generated by microarray analysis was performed by quantitative real time PCR (qRT-PCR) using SsoAdvanced SYBR green supermix (BioRad, USA) with the BioRad CFX96 detection system according to the manufacturer's instructions. Wt *Mtb*, *egtA*:Tn, *egtD*:Tn, *ΔmshA* and the corresponding complemented strains were grown to mid-log phase and total RNA was isolated using RNApro (MP Biomedicals, USA) according to the manufacturer's instructions. Extracted RNA was further treated with DNase (Thermo Scientific, USA) to remove any traces of genomic DNA. For oxidative stress experiments, mid-log phase cultures of Wt *Mtb*, *egtA*:Tn, *egtD*:Tn and *ΔmshA* mutants were exposed to CHP (0.5 mM, Sigma, USA) for 2 hours prior to isolation of total RNA. The integrity of the RNA was determined by measuring the ratio of UV absorbance at 260 nm and 280 nm. 100 ng of total RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (BioRad, USA). qRT-PCR reactions were performed in duplicate using 3 independent biological replicates according to the manufacturer's recommendations. Relative fold changes in gene expression were determined using the $2^{\Delta Ct}$ method, (Schmittgen and Livak 2008) where Ct values were normalized against *sigA* expression as an internal reference control (Manganelli, et al.,

1999). Gene expression values in Figure 6C were normalized against Wt values. For oxidative stress experiments (Figure 6D), gene expression values for each strain were also normalized against untreated cultures subsequent to *sigA* normalization. Primers used are listed in Table S4.

Endogenous ROS determination of *Mtb* cells

Exponentially growing wild type CDC1551, Δ*mshA*, *egtA*:Tn and the complemented strains cultured in 7H9 medium supplemented with OADC were treated with CellRox Green (Life Technologies) to a final concentration of 5 µM for 30 min at 37ºC. The cells were pelleted and the supernatant was discarded. The cells were washed with OADC-7H9 medium to remove any extracellular CellRox Green. The cells were resuspended in media and analyzed on an Guava easyCyte 8HT flow cytometer using the ExpressPlus Module, collecting 5000 events at a flow rate of 0.59 µl/s at an excitation/emission wavelengths of 485/565 nm.

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

Oxidative stress exposure: Wild type CDC1551, *egtD*:Tn, *egtA*:Tn and their respective complemented strains were exposed to various oxidative stress agents. Briefly, all the mycobacterial strains were grown to 0.6-0.8 OD_{600} in 7H9 medium supplemented with 1X ADST. For each strain, 2 x 10^6 cells were transferred into each well of a 96-well plate. Oxidative stress agents hydrogen peroxide (1 mm and 5 mM), paraquat (1 mM and 5 mM), menadione (0.5 mM and 1 mM) and cumene hydrogen peroxide (0.5 mM and 1 mM) were added to respective wells and incubated at 37^{o}C for 6 hrs. Control cells did not receive any stress agent. The cells were subsequently plated on 7H11 agar plates supplemented with albumin dextrose sodium chloride (ADS). Colonies were enumerated after 4 weeks of incubation at 37^{o}C. The survival of mycobacterial strains was expressed as percentage survival in comparison to control untreated cells. Experiment was performed in duplicate.

Susceptibility of mycobacterial strains to anti-TB drugs: Wild type CDC1551, *egt*D:Tn, *egt*A:Tn, Δ*msh*A and their respective complemented strains were exposed to various anti-TB drugs. Briefly, actively growing (0.6-0.8 OD_{600}) mycobacterial strains were transferred into 96 well plates (1 x 10^5 cells per well) containing varying concentrations of different anti-TB drugs. Control cells did not receive any drug. All the cultures were incubated at 37^{o}C standing for 7 days. Bacterial cultures were serially diluted and plated on 7H11 agar plates. Colonies were counted after 4 weeks of incubation at 37^{o}C. The survival of mycobacterial strains was expressed as percentage survival in comparison to treated controls.

Cloning and complementation

To complement the *Mtb* CDC1551 *egt* transposon (Tn) mutants, *egt* genes were PCR amplified from *Mtb* and cloned into the *Nco*I/*Hin*d III sites of pMV762 (Steyn et al., 2003). The *egtA* mutant was complemented with the entire *egt* gene cluster (F primer 5'-TGACTCATGACGCTTGCCGCCATGACC-3', R primer 5'-TATGAAGCTTAAG GCCGGCTGAGGCTAAC-3') and the *egtD* mutant with *egtD* (F primer 5'-CAGTCATGAGAGTGTCGGTTGCCAACCATC-3', R primer 5'-TATGAAGCTTGCCGGCGCGGCTCACTT G-3'). The PCR products were digested with *Bsp*HI and *Hin*d III, ligated into pMV762 and transformed into DH10B *E. coli* cells. Individual clones were sequenced and plasmid DNA from correct clones was transformed into the *Mtb egt* mutants. Restoration of EGT-production was confirmed via radioactive TLC assay. For animal experiments, plasmids capable of stable integration were generated. The *egtD* gene (966 bp) and *hsp60* promoter (376 bp) were excised as a single fragment (1341 bp) from pMV762.*egtD* using *Hind* III and *Xba* I and ligated with *Hind* III and *Xba* I-digested pML1342 (Huff et al., 2010). The ligation mixture was transformed into DH5α competent cells and transformants were selected on LB hygromycin agar plates. Clones were confirmed by DNA sequencing. The pML1342.hsp60*egtD* construct was electroporated into *Mtb egtD*:Tn and transformants selected on 7H11 kanamycin and hygromycin plates. Transformants were confirmed by PCR as well as reverse transcriptase PCR.

Macrophage infections

Mtb cells were cultured to mid-log phase (OD_{600} ~ 0.8) in 7H9 medium, centrifuged and washed once in PBS Tween (PBST). Cells were subsequently centrifuged at low speed (150 *g*, 5 min) to remove cell aggregates, and then lightly sonicated. *Mtb* cells were further diluted in PBST before macrophage infection. RAW264.7 cells were infected at a multiplicity of infection (MOI) of 1:10 (macrophages:*Mtb*). Infected cells were washed 3 times after 4 h to remove extracellular *Mtb* cells and incubation was continued (37°C, 5 % CO_2) with medium changes every 36 h. Macrophages were lysed with 0.5 % Triton X-100 and intracellular *Mtb* cells were enumerated by plating diluted cell lysates on 7H10 agar and counting colonies after 4 weeks at 37°C (Crystal, 1991).

Animal infection studies and quantitation of EGT

Four- to 8-week-old BALB/c mice were housed within a BSL-3 vivarium with food and water *ad libitum*. All manipulations were approved by the UAB and UKZN Institutional Animal Care and Use Committees. To examine EGT levels during *Mtb* infection, and to examine the contribution of *Mtb* to total EGT levels in the infected mouse lung, BALB/c mice were infected with *Mtb* CDC1551, *egtA*:Tn, *egtD*:Tn or the respective complemented strains. At five weeks post infection, the number of viable bacteria present in the lungs was assessed by plating serial dilutions of whole-organ homogenates on 7H11-OADC agar plates. EGT levels in the lung were determined by LC/MS as described above.

Supplemental References

Crystal, R.G. (1991). Oxidants and respiratory tract epithelial injury: pathogenesis and strategies for therapeutic intervention. The American Journal of Medicine *91*, 39S-44S.

de Carvalho, L.P., Darby, C.M., Rhee, K.Y., and Nathan, C. (2011). Nitazoxanide Disrupts Membrane Potential and Intrabacterial pH Homeostasis of *Mycobacterium tuberculosis*. ACS Medicinal Chemistry Letters *2*, 849-854.

Huff, J., Czyz, A., Landick, R., and Niederweis, M. (2010). Taking phage integration to the next level as a genetic tool for mycobacteria. Gene *468*, 8-19.

Kumar, A., Deshane, J.S., Crossman, D.K., Bolisetty, S., Yan, B.S., Kramnik, I., Agarwal, A., and Steyn, A.J. (2008). Heme oxygenase-1-derived carbon monoxide induces the *Mycobacterium tuberculosis* dormancy regulon. J Biol Chem *283*, 18032-18039.

Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F.R., and Smith, I. (1999). Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. Molecular Microbiology *31*, 715-724.

Schmittgen, T.D. and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative CT method. Nature Protocols *3*, 1101-1108.

Steyn, A., Joseph, J., and Bloom, B. (2003). Interaction of the sensor module of *Mycobacterium tuberculosis* H37Rv KdpD with members of the Lpr family. Molecular Microbiology *47*, 1075.