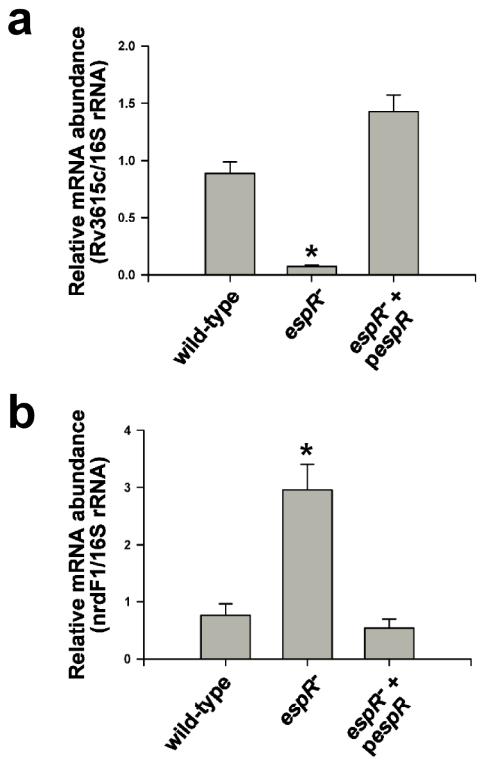
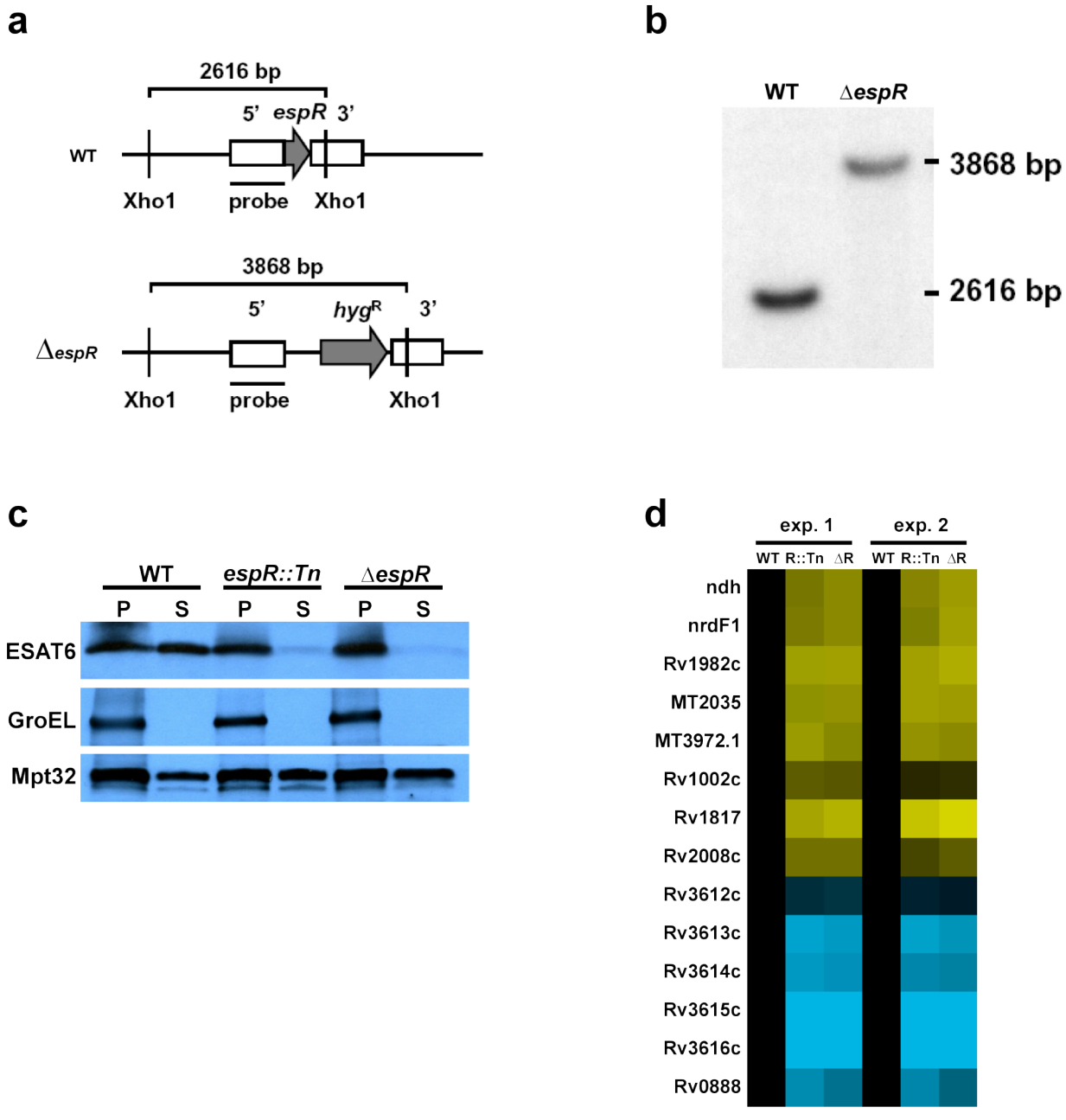
Supplementary Figures and Legends

Supplementary Figure 1



Supplementary Figure 1. *Rv3615c* and *nrdF1* are regulated by EspR. (A) *Rv3615c* expression was measured in total RNA harvested from mid-log phase *M. tuberculosis* by quantitative real-time PCR in each strain and normalized to *16S rRNA* expression. Shown are averages and standard deviations of triplicate measurements from two experiments. *P<0.05 between *espR*$^-$ and wild-type. **(B)** *nrdF1* expression was measured as in (A). Shown are averages and standard deviations of triplicate measurements from two experiments. *P<0.05 between *espR*$^-$ and wild-type. These data support the array results shown in Fig. 1D.

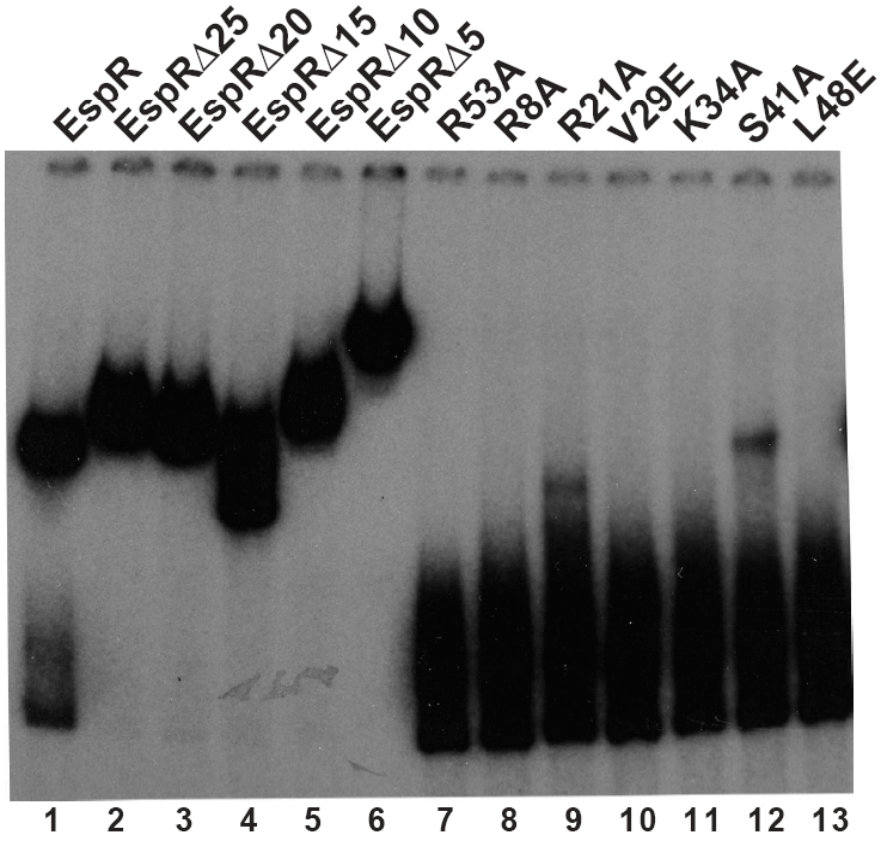
Supplementary Figure 2



Supplementary Figure 2. *EspR* deletion mutant phenocopies *EspR* transposon mutant. (A) Map of the *espR* region in wild-type and the Δ*espR* mutant showing the restriction sites and probe location for southern blot. **(B)** Genomic DNA from wild-type and Δ*espR* strains was digested with XhoI, and the blot was probed with a 5' flank sequence, revealing a 2616 bp fragment for wild-type and a 3868 bp fragment for the

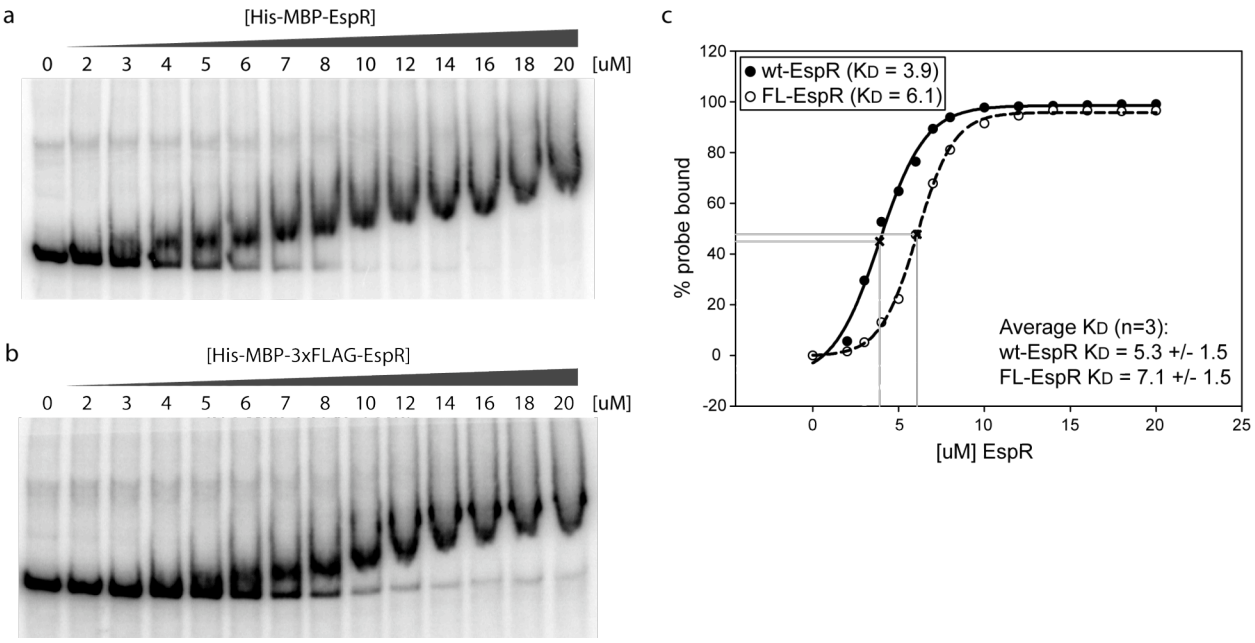
mutant. **(C)** Cell pellets (P) and culture supernatants (S) from wild-type, *espR::Tn* (R::Tn), and Δ*espR* (ΔR) bacteria were probed for ESAT-6, GroEL, and Mpt32 by western blot. **(D)** The genome-wide transcriptional profiles were examined using *M. tuberculosis*-specific oligonucleotide microarrays. Gene expression values in *espR::Tn* and Δ*espR* bacteria were divided by expression values in wild-type bacteria in three experiments. Genes with statistically significant changes in expression are shown (see Supplementary Methods for details of statistical analysis). Expression in wild-type (WT), *espR::Tn* mutant, and Δ*espR* bacteria from two independent experiments are shown. Black represents no expression change, yellow represents increased expression, and blue represents decreased expression relative to wild-type.

Supplementary Figure 3



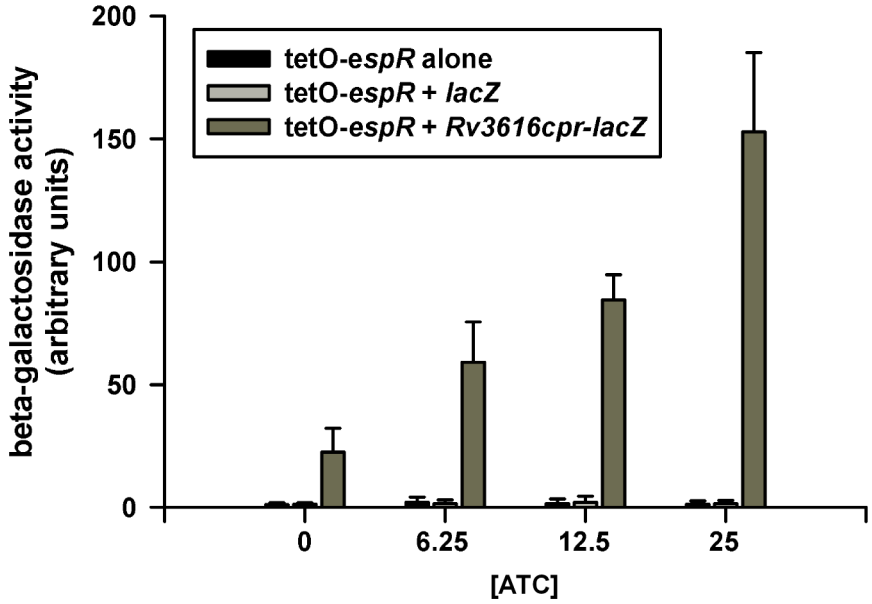
Supplementary Figure 3. N-terminal point mutants fail to bind DNA, whereas C-terminal truncation mutants retain DNA-binding activity. (A) EMSAs were performed as described in Figure 2, using C-terminal EspR truncations and N-terminal point-mutated EspR and the 680bp *Rv3616c* promoter fragment. N-terminal point mutations, but not C-terminal truncations, severely reduce DNA binding of EspR.

Supplementary Figure 4



Supplementary Figure 4. 3xFLAG-EspR binds *Rv3616c-Rv3612c* promoter DNA comparably to wild-type EspR. EMSA was performed, as in Figure 2, by incubating the 680bp *Rv3616c* radiolabeled promoter fragment with purified MBP-EspR **(A)** or MBP-3xFLAG-EspR (**B)** fusion protein for 30 minutes at room temperature, followed by separation of protein-DNA complexes on a non-denaturing 6% polyacrylamide gel and visualization of probe mobility retardation by autoradiography. **(C)** DNA-protein complexes were quantified by densitometry and plotted showing binding curves and calculated dissociation constants for MBP-EspR (solid line) and MBP-3xFLAG-EspR (dashed line).

Supplementary Figure 5



Supplementary Figure 5. Increased expression of EspR causes increased expression of an *Rv3616c promoter-lacZ* reporter gene in *M. smegmatis*. *M. smegmatis* expressing the indicated *lacZ* reporter construct and *tetO-espR* were plated on media containing X-gal and the indicated amount of anhydrous tetracycline (ATC). Quantitative liquid β-galactosidase activities in the corresponding strains are shown.

Supplementary Methods

Bacterial strains and plasmids

All *M. tuberculosis* strains used in this study are derived from the wild-type Erdman strain. The *Rv3849*⁻ mutant carries a Tn*5370* transposon insertion 13 nucleotides upstream of the Rv3849 intiation codon. The full coding sequence of *Rv3849* plus 200 nucleotides upstream were cloned into the integrating plasmid pMV306-Kan to generate the p*Rv3849* (p*EspR*, pSR211) complementation construct. N-, and C- terminal Rv3849 mutants were generated by site-directed mutagenesis of pSR211 and transformed into *M. tuberculosis*. 3X-Flag-EspR was generated by fusion PCR insertion of a 3X-Flag epitope tag upstream of EspR in pSR211. GroEL promoter-driven *Rv3616c-Rv3614c* (pSR403) was generated by fusion PCR insertion of GroEL promoter upstream of *Rv3616c* in pJAM69. See Supplementary Table 2 for list and descriptions of strains and plasmids used in this study.

Mouse infection

C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All mice were housed and treated humanely as described in an animal care protocol approved by the UCSF Institutional Animal Care and Use Committee. For infections, *M. tuberculosis* cultures were grown to mid-log phase, washed and resuspended in PBS-Tween. Mice were infected intravenously through the tail-vein with 1e6 colony-forming-units. Lungs, spleens, and livers were harvested, homogenized, and plated for bacterial colonies at specified time points as previously described[1]. Statistical significance was determined by ANOVA ($\alpha = 0.05$) and two-tailed t-test assuming unequal variance between two groups (wild-type and *Rv3849*⁻) for each timepoint.

Macrophage infections

Macrophages were derived from bone marrow progenitors by culturing with macrophage-colony stimulating factor (M-CSF), as previously described[2], and frozen at -80 ° C. Frozen cells were plated one day prior to infection. *M. tuberculosis* cultures were grown in Middlebrook 7H9 supplemented with oleic acid–albumin–dextrose-catalase (OADC), glycerol, and 0.05% Tween-80 to mid-log phase. Inocula were washed in PBS, diluted into DMEM supplemented with 10% Horse Serum, and added to macrophages at a multiplicity of infection of 10. Following phagocytosis for 2 hours, macrophages were washed with PBS and incubated in fresh media at 37 ° C.

Protein Preparation and Analysis

Concentrated culture supernatants were collected as previously described[1,2]. *M. tuberculosis* strains were grown to mid-log phase in Middlebrook 7H9 media supplemented with 0.05% Tween-80, diluted to back to OD600=0.05 in Sauton's media supplemented with 0.05% Tween-80, and grown to mid-log phase. Cells were washed, diluted to OD600=0.05 in Sauton's media supplemented with 0.005% Tween-80 and grown for 5 days in roller bottles. Culture supernatants were harvested by centrifugation, filter sterilized, and concentrated. Fifteen micrograms of cell lysates and culture supernatants were separated by SDS-PAGE, and specific proteins were visualized by immunoblotting with antibodies against Rv3849 (mouse polyclonal, ab43676, Abcam inc.), Mpt32 (generated at University of Texas, Southwestern Medical Center, Center for Proteomics Research, Antibody Production Core), ESAT-6 (Hyb 76-8), or GroEL (HAT5) (kind gifts of P. Andersen, Statens Serum Institute, Copenhagen Denmark).

Protein purification

Coding sequence of wild-type and mutated Rv3849 was cloned by ligation-independent cloning into pLIC-HMK (a gift of Dr. James Berger, University of California, Berkeley). Plasmids were transformed into *E. coli* BL21/DE3/pLysS, and protein expression was induced for 4 hours with 1mM IPTG. His-tagged MBP-EspR fusion protein was purified on a Ni-NTA agarose column (Qiagen Inc.).

Electrophoretic mobility shift assay

Radio-labeled probe was generated by treating PCR amplified *Rv3616c* promoter sequences with T4 polynucleotide kinase in the presence of ^{32}P-g-ATP, followed by purification over a Sephadex G-50 spin column. EMSA was performed by incubating purified protein (20uM) with approximately 10^5 cpm of probe (approximately 50nM) for 30 minutes at room temperature in reaction buffer containing 10mM Tris pH8.0, 50mM NaCl, 1mM EDTA, 5% glycerol, 1mM DTT, 0.1 mg/ml salmon sperm DNA (Invitrogen Inc.), 25 µg/ml polydI:dC (Sigma Inc.) prior to running on a non-denaturing 6% polyacrylamide gel. The 680 base pair *Rv3616c* promoter fragment begins at nucleotide position 4057055 in the H37Rv genome sequence (http://genolist.pasteur.fr/TubercuList/).

Microarrays

M. tuberculosis RNA preparation and microarray hybridization was performed as previously described[3]. Briefly, total *M. tuberculosis* RNA was purified by bead-beating bacterial pellet in Trizol reagent (Invitrogen), followed by chloroform extraction, isopropanol precipitation, DNAse treatment, and cleanup over an RNEasy mini column (Qiagen Inc.). 3-5 µg of total RNA

was reverse transcribed in the presence of amino-allyl UTP and Cy3- or Cy5-labeled. Competitive hybridizations between Cy5-labeled experimental cDNA and Cy3-labeled reference cDNA were performed for 24 hours at 63° C. Seventy-mer oligonucleotides representing all predicted open reading frames in *M. tuberculosis* were purchased from Qiagen Inc. and spotted onto poly-lysine-coated glass sides. Hybridization data was deemed of high quality if it met numerous quality control criteria, including but not limited to minimum spot intensity in each channel, minimum foreground-background differential in each channel, linear hybridization across a spot in each channel. The Rv3849 regulon was defined as genes that exhibited at least 2-fold dependence on Rv3849 in at least two out of three experiments. Array results from three independent experiments were analyzed using the SAM (Significance Analysis of Microarrays) statistical package[4] to determine significantly induced or repressed genes. The analysis was performed with a false discovery rate of 0.01. All microarray data is available in the Gene Expression Omnibus (GEO) database via accession number GSE11696.

Quantitative PCR

1 to 3 µg of total *M. tuberculosis* mRNA was reverse transcribed and used as template for PCR in the presence of SYBR Green on an Opticon Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). Oligonucleotides for amplification of *Rv3615c* were: oSR603 5'gagcgtctcggtgtactg3' and oSR604 5'cgtgtcgttgaactgtgagc3'. Oligonucleotides for amplification of *nrdF1* were: oSR601 5'caacctgggataccagcctg3' and oSR 602 5'cattacgtatgagcttcc3'. Oligonucleotides for amplification of *16S rRNA* were: oAL63 5'atgctacaatcgccggtaca3' and oAL64 5'gcgttgctgatctgcgatta3'. Oligonucleotides for amplification of *espR* were: oPM501 5'aaccgcctgttcgacacggtttat3' and oPM502 5'ttcctgagcgtagctgtgataggt3'. For each sample, expression of *Rv3615c*, *nrdF1*, or *espR* was normalized to expression of *16S rRNA*.

For qPCR using RNA isolated from *M. tuberculosis* inside macrophages, bacterial RNA was isolated by first lysing macrophages in guanidine isothiocyanate buffer[5]. Intact bacteria were then pelleted and washed in PBS + 0.05% Tween-80, followed by bead-beating in Trizol, chloroform extraction, and cleanup over an RNeasy mini column. Total bacterial RNA was then amplified using a Bacterial Amplification Kit (Ambion Inc.), and *Rv3615c* expression was measured in amplified RNA as above. Statistical significance was determined by ANOVA (α = 0.05) and two-tailed t-test assuming unequal variance between two groups (wild-type and *Rv3849*$^-$) bacteria.

lacZ reporter assays

Rv3616c promoter fragments were PCR cloned upstream of *lacZ* in the plasmid pYUB76, then promoter-*lacZ* fusions were shuttled into the integrating plasmid pMV306-Kan, and reporter constructs were transformed into *M. smegmatis*. *M. tuberculosis espR* was cloned downstream of the inducible acetamidase promoter[6], and reporter strains were transformed with this expression construct. Due to sufficient leakiness of the acetamidase promoter, inducer was not used to overexpress *espR.* Doubly-transformed *M. smegmatis* clones were grown in liquid culture and then spotted onto plates containing X-gal to measure *lacZ* expression. For liquid beta-galactosidase assays, *M. smegmatis* cultures were grown to OD600=0.6 and beta-galactosidase activity was measured by the method of Timm et al[7]. β-galactosidase activity in each strain was normalized by subtraction of background activity measured in the strain carrying a plasmid with *lacZ* without an upstream promoter.

Tetracycline inducible expression of EspR

Tetracycline inducible vectors optimized for use in mycobacteria were a generous gift of Sabine Ehrt[8]. In *M. tuberculosis*, *espR*Δ bacteria carrying either an empty *tetO* vector or an integrated plasmid with *tetO* driving *espR*Δ expression were grown in liquid culture to OD600=0.4 then induced with 0.8-50 ng/ml of anhydrous tetracycline (ATC, Sigma Inc.). At 24 hours post-induction, total bacterial RNA was harvested as described for microarrays and analyzed by qPCR for *espR* and *Rv3615c* expression. For *M. smegmatis* experiments, wild-type bacteria containing an integrated copy of the 710 bp *Rv3616c*promoter-*lacZ* reporter were transformed with an episomal *tetO-espR* plasmid. Doubly transformed bacteria were induced for 8hrs with 0.8-25ng/ml of ATC, and beta-galactosidase activity was measured by the method of Timm et al[7].

Structural modeling

Rv3849 was modeled using the program Modeler (http://salilab.org/modeller/) with the crystal structure of SinR as a template (PDB: 1B0N). The Rv3849 model was viewed and manipulated using the program Chimera (http://www.cgl.ucsf.edu/chimera/).

Supplementary Tables

Supplementary Table 1. Numeric array data corresponding to Figure 1c.

	exp. 1			exp. 2			exp. 3		
Gene Name	WT	*espR*⁻	compl.	WT	*espR*⁻	compl.	WT	*espR*⁻	compl.
ndh	0	1.771	-0.141	0	2.403	-0.218	0	1.856	-0.108
nrdF	0	1.661	-0.141	0	1.473	-0.618	0	1.666	-0.278
Rv1982c	0	1.661	ND	0	1.593	-0.808	0	2.146	-0.448
Rv3612c	0	-1.269	-0.041	0	-0.287	0.312	0	-2.194	0.472
Rv3613c	0	-1.799	0.969	0	-0.837	0.932	0	-3.274	0.672
Rv3614c	0	-1.269	0.969	0	-0.667	0.942	0	-2.894	0.602
Rv3615c	0	-3.039	1.419	0	-1.717	0.842	0	-4.094	0.682
Rv3616c	0	-2.289	1.079	0	-1.197	0.962	0	-3.814	0.762
MT2035	0	1.691	-0.221	0	1.443	-0.958	0	1.866	-0.508
MT3972.1	0	1.701	0.269	0	1.493	-0.178	0	1.296	-0.038
Rv0888	0	-1.859	-0.211	0	-1.777	-0.168	0	ND	-0.218
Rv1002c	0	1.431	0.219	0	1.043	-0.648	0	ND	0.142
Rv1817	0	2.031	0.189	0	1.693	0.272	0	ND	0.032
Rv2008c	0	1.861	-0.061	0	1.353	-1.108	0	ND	ND

Data are $\log_2$ values normalized to wild-type. ND (no data) indicates high quality data was not acquired for the gene in that experiment.

Supplemental Table 2. Strains and Plasmids used in this study.

Strain/Plasmid	Genotype/description	Source/ref.
M. tuberculosis		
Erdman	Wild-type	W.R. Jacobs, Jr.
espR::Tn (Rv3849::Tn)	Erdman *espR::*Tn*5370,* Hyg^R	This study
*espR*Δ	Erdman *espR*Δ, Hyg^R	This study
Rv3877::Tn	Erdman *Rv3877::*Tn*5370,* Hyg^R	1
SSM6 (*esxA*$^-$)	Erdman Δ*esxA*, Hyg^R	1
SRM211	*espR::Tn* + pSR211, Kan^R	This study
SRM413	*espR::Tn* + pSR403, Kan^R	This study
SRM211Flag	*espR::Tn* + pSR211Flag, Kan^R	This study
CDM501	*espR*Δ + pCD501, Kan^R, Hyg^R	This study
CDM502	*espR*Δ + pCD502, Kan^R, Hyg^R	This study
M. smegmatis		
mc^{2}155	Wild-type	9
SRMS1	*mc^{2}155* + pSR221, Kan^R	This study
SRMS2	*mc^{2}155* + pSR223, Hyg^R	This study
SRMS3	SRMS2 + pSR221, Kan^R, Hyg^R	This study
SRMS4	SRMS2 + pSR241, Kan^R, Hyg^R	This study
SRMS5	SRMS2 + pSR242, Kan^R, Hyg^R	This study
SRMS6	SRMS2 + pSR243, Kan^R, Hyg^R	This study
SRMS7	SRMS2 + pSR244, Kan^R, Hyg^R	This study
PMMS501	*mc^{2}155* + pSR241 + pPM501, Kan^R, Hyg^R	This study
PMMS502	*mc^{2}155* + pSR221 + pPM501, Kan^R, Hyg^R	This study
Plasmids		
pMV306.Kan	*int, oriE*, Kan^R	W.R. Jacobs, Jr.
pMV261.Hyg	*oriE, oriM,* Hyg^R	W.R. Jacobs, Jr.
pSR211	pMV306.Kan + 200bp-promoter-*Rv3849*	This study
pSR211-N1	pSR211, R8A substitution in EspR	This study
pSR211-N2	pSR211, R21A substitution in EspR	This study
pSR211-N3	pSR211, V29E substitution in EspR	This study
pSR211-N4	pSR211, K34A substitution in EspR	This study
pSR211-N5	pSR211, S41A substitution in EspR	This study
pSR211-N7	pSR211, L48E substitution in EspR	This study
pSR211-N8	pSR211, R53A substitution in EspR	This study
pSR211-C1	pSR211, C-terminal D25 in EspR	This study
pSR211-C2	pSR211, C-terminal D20 in EspR	This study
pSR211-C3	pSR211, C-terminal D15 in EspR	This study
pSR211-C4	pSR211, C-terminal D10 in EspR	This study
pSR211-C5	pSR211, C-terminal D5 in EspR	This study
pSR211-Flag	pSR211, N-terminal 3X-Flag in EspR	This study
pSR403	pMV306.Kan + GroELpromoter-*Rv3616c-Rv3614c*	This study
pSR221	pMV306.Kan + no promoter-*lacZ*, Kan^R	This study
pSR241	pMV306.Kan + 680bp-*Rv3616c*promoter-*lacZ*, Kan^R	This study
pSR242	pMV306.Kan + 624bp-*Rv3616c*promoter-*lacZ*, Kan^R	This study
pSR243	pMV306.Kan + 520bp-*Rv3616c*promoter-*lacZ*, Kan^R	This study

pSR244	pMV306.Kan + 427bp-*Rv3616c*promoter-*lacZ*, Kan^R	This study
pSR223	pMV261.Hyg + Ace-promoter-*Rv3849*, Hyg^R	This study
pCD501	pMV306.Kan + *tetO* empty vector, Kan^R	This study
pCD502	pMV306.Kan + *tetO-espR*, Kan^R	This study
pPM501	pMV261.Hyg + *tetO-espR*, Hyg^R	This study

Supplementary Notes

References for Supplementary Information

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