ARTICLES

Secreted transcription factor controls *Mycobacterium tuberculosis* virulence

Sridharan Raghavan[1], Paolo Manzanillo[1]*, Kaman Chan[1]*, Cole Dovey[1] & Jeffery S. Cox[1]

Bacterial pathogens trigger specialized virulence factor secretion systems on encountering host cells. The ESX-1 protein secretion system of *Mycobacterium tuberculosis*—the causative agent of the human disease tuberculosis—delivers bacterial proteins into host cells during infection and is critical for virulence, but how it is regulated is unknown. Here we show that EspR (also known as Rv3849) is a key regulator of ESX-1 that is required for secretion and virulence in mice. EspR activates transcription of an operon that includes three ESX-1 components, *Rv3616c–Rv3614c,* whose expression in turn promotes secretion of ESX-1 substrates. EspR directly binds to and activates the *Rv3616c–Rv3614c* promoter and, unexpectedly, is itself secreted from the bacterial cell by the ESX-1 system that it regulates. Efflux of the DNA-binding regulator results in reduced *Rv3616c–Rv3614c* transcription, and thus reduced ESX-1 secretion. Our results reveal a direct negative feedback loop that regulates the activity of a secretion system essential for virulence. As the virulence factors secreted by the ESX-1 system are highly antigenic, fine control of secretion may be critical to successful infection.

Pathogenic microbes sense their environment and change their physiology to interact with the host. *Mycobacterium tuberculosis*, a pathogen of global importance, utilizes the ESX-1 protein secretion system to export virulence factors that disarm host macrophages[1–3]. ESX-1, also termed type VII secretion, is critical for virulence, and transports proteins from inside the bacterium across the cell envelope[1,2,4,5]. The entire ESX-1 system has been implicated in innate immune modulation, especially early after infection in macrophages[1,6–8]. Consistent with this view, ESX-1 mutants are specifically defective in the early stages of growth in mice[1,2,5]. However, the role of the major secreted substrates, ESAT-6 and CFP-10, in virulence is a matter of current debate[2,3,9,10]. Furthermore, these proteins are also potent antigens that elicit protection against tuberculosis in animal models, and are important components of vaccines currently in clinical trials[11–15].

The core machinery of the ESX-1 pathway, as well as ESAT-6 and CFP-10, are encoded at the genomic locus known as region of difference 1 (RD1), which is absent in the BCG vaccine strain[16]. Many other proteins encoded at the RD1 locus are also required for ESX-1 function, suggesting that this secretion apparatus is complex[17–19].

In addition to RD1-encoded genes, a separate locus required for ESX-1 system function has also been identified[20,21]. This locus contains the genes *Rv3616c–Rv3614c* that are required for ESX-1 system activity; the Rv3616c protein, termed EspA, is a secreted substrate of the pathway[20,21]. The discovery of this additional substrate also revealed the surprising phenomenon of mutually dependent export, in which the secretion of each substrate relies upon the secretion of the other substrates[20]. Although the mechanism underlying this co-dependence is not understood, it raises the possibility that substrates exert control over the secretion activity of the entire ESX-1 pathway. The phenomenon of mutually dependent secretion has complicated the genetic dissection of effector functions of single ESX-1 substrates, and the specific functions of the known ESX-1 substrates remain unclear.

In this work, we have identified a novel secreted substrate of the ESX-1 pathway, EspR. Surprisingly, this protein is a DNA-binding transcription factor that regulates *M. tuberculosis* genes that are required for ESX-1. The finding of a secreted DNA-binding protein reveals a novel homeostatic feedback mechanism by which the activity of a secretion system is monitored and regulated through coupling between secretion and transcription.

EspR (Rv3849) is secreted by ESX-1

In a genetic screen, we identified an *M. tuberculosis* transposon mutant that induced high levels of IL-12 from macrophages, a common phenotype of ESX-1 secretion mutants[1], and determined that the transposable element inserted 13 nucleotides upstream of the gene *Rv3849*. Immunoblotting proteins from bacterial pellets and culture supernatants demonstrated that Rv3849 was secreted from wild-type cells, and although protein levels were severely reduced in the *Rv3849*$^{-}$ mutant, a low level of Rv3849 was still present in cell lysates (Fig. 1a, lanes 1–4). Notably, ESAT-6 secretion was severely diminished in the *Rv3849*$^{-}$ mutant, with the block nearly equivalent to that of a mutant lacking the putative pore protein Rv3877 (Fig. 1a, lanes 1–4, 7 and 8). This defect was corrected upon integration of a single copy of the *Rv3849* gene into mutant bacteria (Fig. 1a, lanes 5 and 6). Importantly, Rv3849 secretion is blocked in bacteria lacking either Rv3877 (lanes 7 and 8) or ESAT-6 (encoded by *esxA*, lanes 9 and 10). Like other ESX-1 secretion mutants[1], *Rv3849*$^{-}$ mutant cells exhibited a characteristic growth defect during infection of mice (Fig. 1b). Mutant bacteria grew poorly in the lungs during the first five days post-infection but this was followed by a period in which the viable mutants recovered normal growth kinetics. Although the mutant cells established a stable infection by three weeks, the bacterial burden was approximately an order of magnitude less than the wild type (Fig. 1b). Therefore, Rv3849 is a new ESX-1 substrate that, like ESAT-6, CFP-10 and EspA, is required for the function of the entire ESX-1 system[20].

EspR is required for ESX-1 gene expression

Although standard homology and motif searches failed to identify any putative functional domains in Rv3849, a secondary structure-based

[1]Department of Microbiology and Immunology, Program in Microbial Pathogenesis and Host Defense, University of California, San Francisco, 600 16th Street, Campus Box 2200, San Francisco, California 94143-2200, USA.
*These authors contributed equally to this work.

homology search[22] identified a significant match to the *Bacillus subtilis* transcription factor SinR, a helix–turn–helix (HTH) DNA-binding protein[23]. Modelling the three-dimensional structure of Rv3849 using the crystal structure of SinR as a template revealed considerable similarity between the two proteins, especially at the amino-terminal HTH domain (Fig. 1c). Thus, although Rv3849 is an ESX-1 secreted protein, it bears striking similarities to a DNA-binding protein.

To determine if Rv3849 is a transcriptional regulator, we used *M. tuberculosis* microarrays to compare the global transcriptional profile between wild-type and *Rv3849*$^{-}$ bacteria grown in culture. Of the 4,505 genes represented on the array, expression of only five operons, representing 14 genes, was altered in *Rv3849*$^{-}$ mutant cells (Fig. 1d, Supplementary Table 1). Three operons were activated in the *Rv3849*$^{-}$ mutant, including a putative operon containing the genes *MT2035*, *Rv1982c* and *nrdF1* (Fig. 1d). Notably, the *Rv3616c–Rv3612c* operon was one of two operons that were significantly downregulated in the *Rv3849*$^{-}$ mutant (Fig. 1d). Quantitative RT–PCR (qPCR) to measure *nrdF1* and *Rv3615c* messenger RNA levels provided additional support for the microarray data (Supplementary Fig. 1). Because our *Rv3849* mutant contains a transposon insertion in the promoter but retains an intact open reading frame, we made a deletion mutant in which the entire *Rv3849* gene was replaced by allelic exchange. Importantly, the secretion and transcriptional phenotypes of the transposon mutant are indistinguishable from those of the deletion mutant (Supplementary Fig. 2). Because the genes *Rv3616c, Rv3615c* and *Rv3614c* are required for ESX-1, this result provided a direct link between Rv3849 and the secretion system. Overall, depletion of Rv3849 led to both positive and negative effects on the expression of a small number of *M. tuberculosis* genes, including one locus critical for ESX-1 function. Given the regulatory function of Rv3849 in ESX-1 secretion, we renamed this protein EspR (ESX-1 secreted protein regulator).

As ESX-1 functions early during infection[1,8], we reasoned that EspR may activate ESX-1 secretion upon phagocytosis by macrophages. To assay EspR activity, we measured transcription of the *Rv3616c–Rv3612c* operon during infection by qPCR. *Rv3616c–Rv3612c* expression was significantly induced by two hours post-infection in wild-type bacteria (Fig. 1e), consistent with the recent finding that this operon is among a set of genes activated upon phagosomal acidification[24]. In *espR*$^{-}$ bacteria, however, *Rv3616c–Rv3612c* mRNA levels were near background two hours after infection, and although some induction occurred by four hours, the levels were much reduced compared to wild-type *M. tuberculosis* (Fig. 1e). These data suggest that EspR is induced upon phagocytosis, activating expression of downstream ESX-1 components.

EspR binds DNA and activates transcription

To determine if EspR directly regulates *Rv3616c–Rv3612c*, we measured the activity of an *Rv3616c* promoter-*lacZ* transcriptional reporter in *Mycobacterium smegmatis*, a non-pathogenic relative of *M. tuberculosis* that lacks the *Rv3616c–Rv3612c* operon but encodes a homologue of EspR. The 680-base-pair (680-bp) promoter induced slight *lacZ* expression compared to the promoter-less construct, but was strongly activated by expression of *M. tuberculosis* EspR (Fig. 2a). Deletions from the 5′ end of the promoter indicated that sequences between 427 and 520 bp upstream of *Rv3616c* were required for EspR activity (Fig. 2a). In electrophoretic mobility shift assays (EMSA), purified EspR, expressed as an N-terminal fusion with maltose binding protein (MBP–EspR), bound directly to probes that included at least 520 bp of the *Rv3616c* promoter (Fig. 2b). The 427-bp probe was weakly bound by the fusion protein (Fig. 2b), and no binding was detectable with a 320-bp probe (data not shown). Thus, EspR binds directly to the *Rv3616c* promoter and activates transcription.

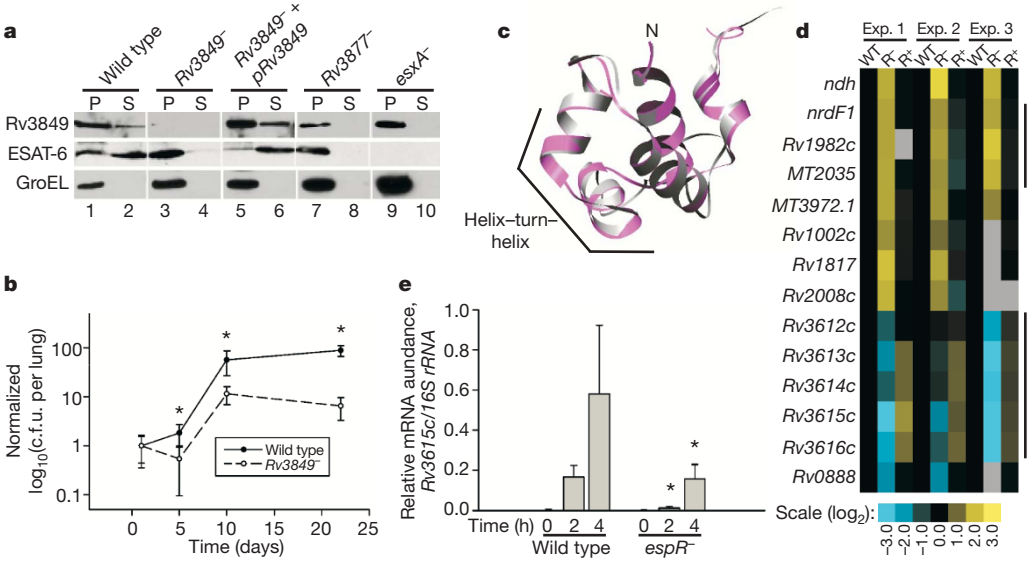

Figure 1 | Rv3849 is a secreted regulator of the ESX-1 system. a, Cell pellets (P) and culture supernatants (S) from wild-type, *Rv3849*$^{-}$, *Rv3849*$^{-}$ complemented, *Rv3877*$^{-}$, and *esxA*$^{-}$ bacteria were probed for Rv3849, ESAT-6 and GroEL by western blot. GroEL, an intracellular protein used as a lysis control, was detected in cell pellets of all strains and was absent from supernatants. **b**, C57BL/6 mice were infected with 1×10^6 colony-forming units (c.f.u.) of wild-type and *Rv3849*$^{-}$ mutant *M. tuberculosis* through the intravenous route. Bacterial growth was monitored by counting c.f.u. from lung homogenates, and values at each time point were normalized to the initial dose at 24 h post-infection. For each bacterial strain, mean ± s.d. is shown from five mice at each time point. *$P < 0.05$ between *Rv3849*$^{-}$ and wild-type. **c**, The crystal structure of SinR (violet) was used as a template to generate a structural model of Rv3849 (silver) using the program Modeller. The overlay of the N-terminal HTH domains of both proteins is shown. **d**, Genome-wide transcriptional profiles were examined using *M. tuberculosis*-specific oligonucleotide microarrays. Gene expression values in *Rv3849*$^{-}$ (R^{-}) and complemented *Rv3849*$^{-}$ (R^{+}) bacteria were divided by expression values in wild-type (WT) bacteria. Results from three independent experiments are shown. Genes with statistically significant changes in expression are shown (see Supplementary Methods for details of statistical analysis). Black, no change; yellow, increased expression; blue, decreased expression relative to wild-type; grey, missing data. Lines to the right of the cluster diagram represent probable operons. **e**, Expression of *Rv3615c* in wild-type and *espR*$^{-}$ bacteria in liquid culture (0 h) and 2 h and 4 h following infection of bone-marrow-derived macrophages was measured by quantitative PCR from amplified total *M. tuberculosis* RNA. *Rv3615c* expression was normalized to expression of *16S rRNA*. Shown are mean ± s.d. of triplicate measurements from one of 2 experiments. *$P < 0.05$ comparing wild type and *espR*$^{-}$ at 2 h and at 4 h.

The failure of *espR*$^-$ mutants to express *Rv3616c–Rv3612c* suggested that the obligatory role of EspR in ESX-1 secretion was to activate transcription of this operon. To test this, we attempted to generate EspR mutants that specifically blocked either its transcriptional activity or its secretion. To this end we made point mutations in the N-terminal DNA-binding region of EspR in residues conserved among EspR homologues in related actinomycetes (Fig. 2c, top). Each of these mutated proteins failed to bind the *Rv3616c–Rv3612c* promoter (Supplementary Fig. 3) and was unable to restore ESAT-6 secretion in *espR*$^-$ mutant *M. tuberculosis* (Fig. 2d, top). Likewise, since the ESX-1 secretion signal in CFP-10 resides in the carboxy terminus[25], we attempted to interrupt EspR secretion by creating a series of C-terminal truncation mutants (Fig. 2c, bottom). Each of these mutants retained full DNA-binding activity, though with variable migration of the bound probe, suggesting alterations in the physical geometry of the protein–DNA complex (Supplementary Fig. 3). Deletion of 10 or more amino acids from the C terminus of EspR led to complete inactivation of ESX-1, while deletion of just the last five amino acids did not prevent ESAT-6 secretion (Fig. 2d, bottom). Each of the variants was expressed at levels equivalent to that of the wild-type protein (data not shown). Gene expression analysis of two N-terminal and two C-terminal mutant-expressing strains showed that the ability of each mutant to restore ESX-1 secretion correlated with transcriptional control of the EspR regulon (Fig. 2e). Point mutants that failed to bind DNA did not restore wild-type expression of EspR transcriptional targets to *espR*$^-$ bacteria (Fig. 2e, lanes 4 and 5). Likewise, EspR lacking five C-terminal amino acids retained partial transcriptional activity, whereas deletion of ten amino acids completely abrogated EspR activity (Fig. 2e, lanes 6 and 7). These data demonstrate that both the DNA-binding and C-terminal domains of EspR are required for transcriptional activity and for ESX-1 function.

Ectopic expression of *Rv3616c–Rv3614c* bypasses EspR

To determine if EspR plays an obligate role in ESX-1 function in addition to its transcriptional activity, we sought to bypass EspR by ectopic expression of the *Rv3616c–Rv3612c* operon in the *espR*$^-$ mutant. If the sole function of EspR in ESX-1 secretion is to activate this single operon, then ESX-1 secretion should be active under these conditions despite the lack of EspR. Because we had shown previously that Rv3613c and Rv3612c were not required for ESX-1 (ref. 21), we expressed just Rv3616c–Rv3614c under the control of the constitutive GroEL promoter in *espR*$^-$ bacteria. In this strain, ESX-1 secretion was restored to levels similar to that of wild-type (Fig. 3a, lanes 7 and 8), demonstrating that the sole requisite function of EspR in ESX-1 secretion is the transcriptional activation of *Rv3616c–Rv3614c*. This clearly excludes a role for EspR in subsequent secretory activities, distinguishing it from other co-dependent ESX-1 substrates.

Blocking EspR secretion potentiates ESX-1 gene transcription

As the secretion of EspR is not required for ESX-1 system function, the simplest model is that EspR secretion reduces its activity by

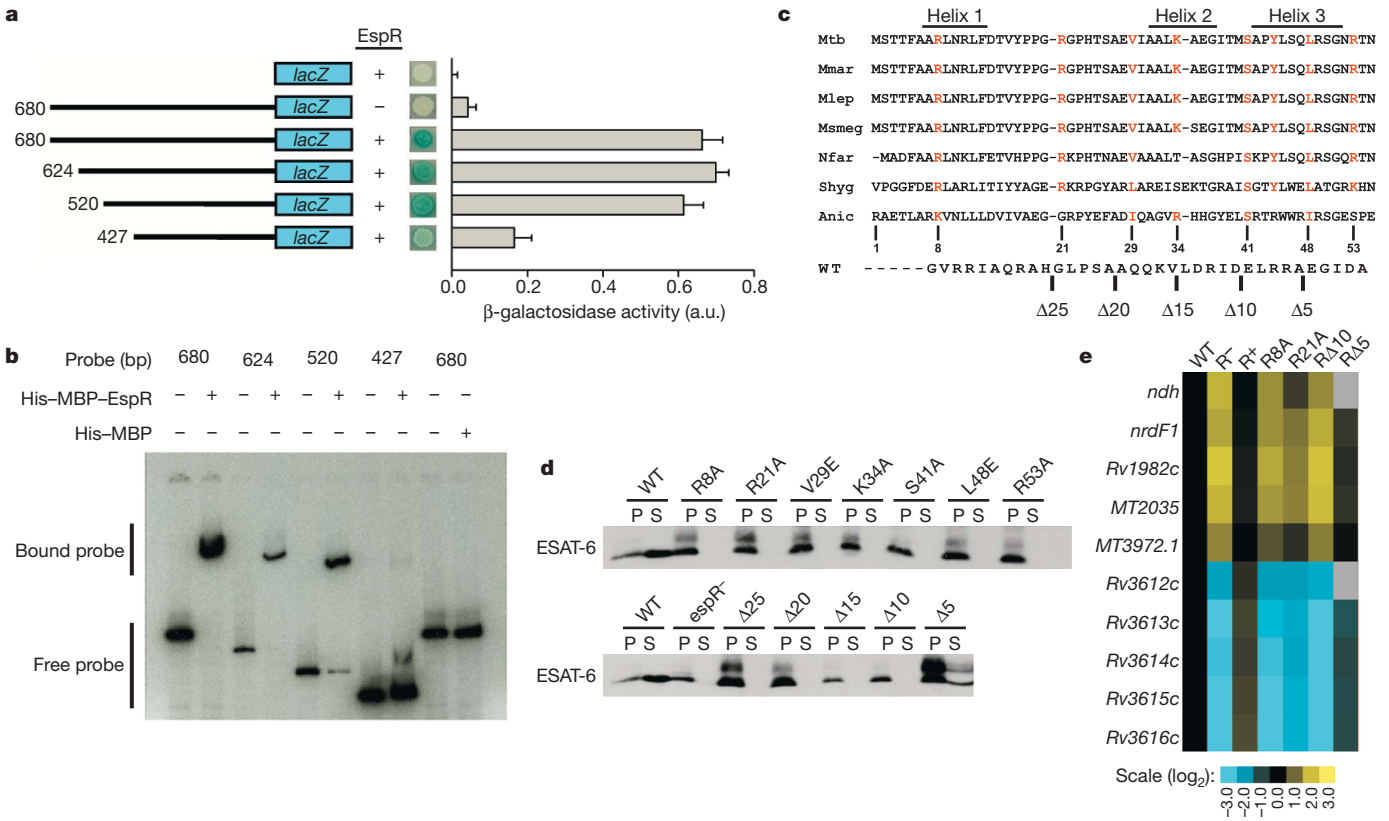

Figure 2 | EspR binds and activates the *Rv3616c–Rv3612c* promoter. a, *M. tuberculosis* EspR was expressed in *M. smegmatis* strains carrying *lacZ* reporter constructs with different length *Rv3616c* promoter fragments. Activity was monitored by plating strains on media containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and by quantitative β-galactosidase assay. Shown are mean + s.d. of triplicate measurements from one of 3 experiments. **b**, Binding of EspR to the *Rv3616c* promoter was assayed by EMSA using purified MBP–EspR fusion protein, or MBP alone, and radiolabelled DNA fragments. **c**, N-terminal sequences of EspR homologues of other actinomycetes were aligned and the highly conserved residues targeted for mutagenesis are highlighted in red (top). A C-terminal deletion series of EspR was also generated (bottom). **d**, Secretion of ESAT-6 from *espR*$^-$ bacteria expressing mutant forms of EspR was assayed by western blot analysis of cell pellets (P) and culture supernatants (S). **e**, Expression of the EspR regulon in the *espR*$^-$ mutant (R^-) expressing either wild-type *espR* (R^+) or the indicated mutants was monitored by microarray. Gene expression in each strain was divided by expression level in wild-type (WT) bacteria. Black, no change; yellow, increased expression; blue, decreased expression relative to wild type; grey, missing data.

decreasing the intracellular concentration of the activator. This model predicts that impairment of EspR secretion should result in increased intracellular EspR and resultant increases and decreases in the expression of its positive and negative transcriptional targets, respectively. Although we were unable to isolate a mutant EspR that was specifically impaired for secretion, we serendipitously found that addition of an N-terminal 3×Flag epitope tag to EspR blocked its secretion (Fig. 3b). Using antibodies that recognize EspR, Flag–EspR was detected exclusively in cell pellets, whereas the residual EspR expressed from the mutated chromosomal gene was effectively secreted (Fig. 3b, lanes 3 and 4). Notably, the Flag epitope had little effect on DNA binding to the *Rv3616c–Rv3612c* promoter (Supplementary Fig. 4). Importantly, blocking EspR secretion increased EspR activity: genes activated by EspR were more highly expressed and negatively regulated genes were even further repressed (Fig. 3c). Likewise, titration of intracellular EspR levels using a conditional promoter system led to corresponding increases in *Rv3615c* expression, showing that EspR activity can be modulated by its intracellular concentration (Fig. 3d, e and Supplementary Fig. 5). This result is consistent with the model that secretion of EspR functions to limit its intracellular activity, functionally coupling transcription and secretion in the ESX-1 system.

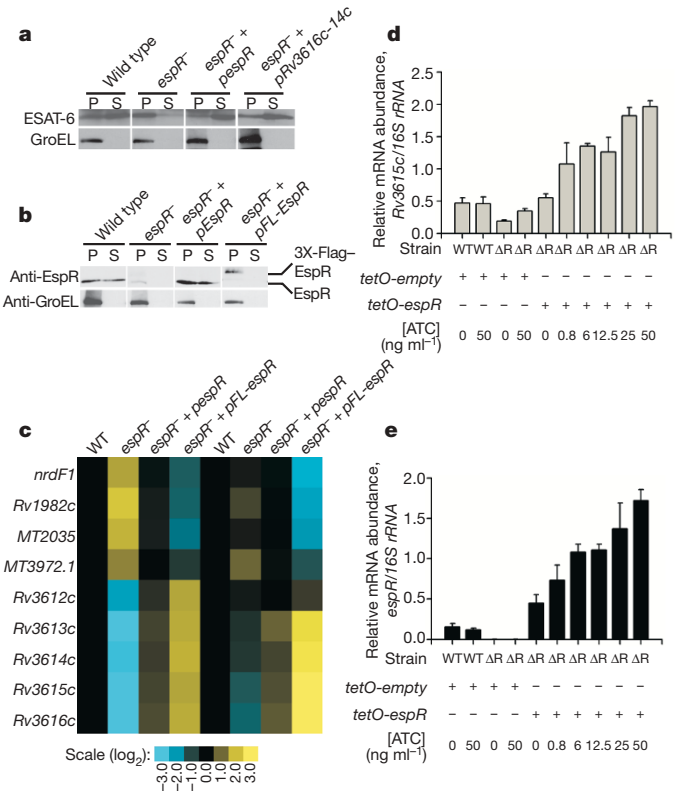

Figure 3 | Examining the role of EspR secretion in ESX-1 function. **a**, *Rv3616c–Rv3614c* were expressed in *espR*$^-$ bacteria under the control of the constitutive *GroEL* promoter. ESAT-6 and GroEL were analysed by western blot in cell pellets (P) and culture supernatants (S) of wild-type bacteria, *espR*$^-$ bacteria, complemented *espR*$^-$ bacteria, and *espR*$^-$ bacteria constitutively expressing *Rv3616c–Rv3614c*. **b**, Cell pellets (P) and culture supernatants (S) of wild-type bacteria and *espR*$^-$ bacteria expressing N-terminally Flag-tagged EspR were probed by western blot for EspR and GroEL. Arrows indicate endogenous EspR and 3×-Flag–EspR. **c**, Microarray analysis comparing expression of EspR regulon genes in the strains used in **b**. Black, no change; yellow, increased expression; blue, decreased expression relative to wild-type; grey, missing data. Two replicate experiments are shown. **d**, **e**, EspR was placed under the transcriptional control of the Tet-inducible promoter and introduced on an integrating plasmid into wild-type (WT) and Δ*espR* (ΔR) bacteria. Total RNA was harvested from mid-log phase *M. tuberculosis* grown in the presence of the indicated concentration of anhydrous tetracycline (ATC). *Rv3615c* (**d**) and *espR* (**e**) expression were measured by quantitative real-time PCR and normalized to *16S rRNA* expression. Shown are mean + s.d. of triplicate measurements from one representative experiment of three.

Discussion

We have discovered a simple transcriptional feedback mechanism by which secretion of the DNA-binding transcription factor EspR via the ESX-1 pathway negatively regulates this virulence factor secretion system. Secretion of transcriptional regulators is extremely rare in biology, with only three other examples in prokaryotes[26–29], none of which bind directly to DNA. In eukaryotes, HIV Tat[30] and members of the homeobox family[31] may be secreted, although the functional importance of their export is unclear. Our data are consistent with the model depicted in Fig. 4, in which the flux of protein secretion through ESX-1 cycles between low- and high-activity states driven by the EspR-regulated activation of *Rv3616c–Rv3612c* and inhibited by secretion of the regulator.

In *M. tuberculosis*, tight control of the ESX-1 system during infection may be achieved through integration of multiple regulators, including both EspR and the two-component regulator PhoP (refs 32, 33). Appropriate activity and timing of ESX-1 secretion is probably critical for survival, as this system is both an essential virulence determinant as well as a major source of potent immunostimulatory antigens. Given the early virulence role of ESX-1 secretion and the antigenicity of ESX-1 substrates to the adaptive immune response, it

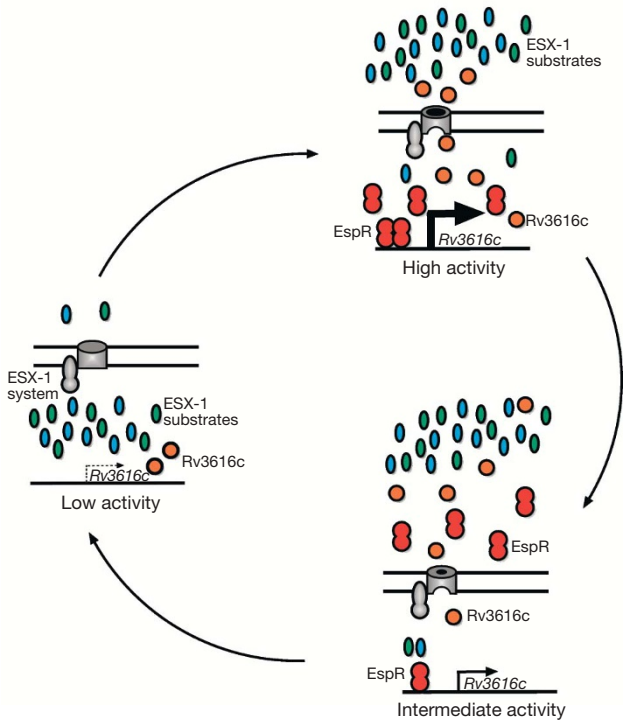

Figure 4 | Model of EspR regulated activation of ESX-1 secretion and negative feedback through export via the ESX-1 pathway. ESX-1 function cycles between low-activity and high-activity states (left and top right, respectively) and is activated by EspR transcriptional activity but limited by EspR secretion. Activation of EspR by unknown stimuli leads to increased transcription (thick black arrow) expression of Rv3616c (orange circles) and other genes in the operon (not shown). Rv3616c accumulation leads to high activity of ESX-1, perhaps via interactions with membrane-bound ESX-1 secretion components (grey), leading to secretion of substrates, including Rv3616c. Secretion of EspR decreases the intracellular concentration of the activator and subsequently lowers Rv3616c transcription, depicted here (thin arrow, bottom right) as an intermediate-activity state. Eventually, this negative feedback loop returns the ESX-1 system back to the low-activity state transcription (dashed arrow).

is intriguing to speculate that *M. tuberculosis* may balance the costs and benefits of ESX-1 activity by inducing secretion immediately after phagocytosis infection, through EspR, and then inactivating it as the infection progresses via secretion of the activator. Notably, it has been shown[34] that the number of ESAT-6 reactive T cells waned during chronic *M. tuberculosis* infection, a finding consistent with this idea. Understanding how key virulence factors and antigens are regulated during infection deepens our understanding of tuberculosis pathogenesis, and may affect the design of new vaccine strains with enhanced immunogenicity.

METHODS SUMMARY

All strains and plasmids used in this study are described in Supplementary Table 2. Mouse infections, bacterial protein preparation and analysis, RNA preparation, and microarray hybridization were performed as previously described[1,21,35,36]. EMSAs were performed by incubating purified protein with approximately 10^5 c.p.m. of radiolabelled probe in the presence of excess unlabelled non-specific DNA before running on a non-denaturing 6% polyacrylamide gel. For qPCR, *M. tuberculosis* cells were isolated from bone-marrow-derived macrophages, total bacterial RNA was isolated and amplified using a Bacterial Amplification Kit (Ambion), reverse transcribed and used as template for PCR in the presence of SYBR Green. Oligonucleotides used for gene amplification are indicated in the Supplementary Methods and expression of individual mRNAs was normalized to expression of *16S rRNA*.

Rv3849 was modelled using the program Modeller (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/).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.R. performed the secretion assays, virulence studies, protein modeling and β-galactosidase assays. S.R. and P.M. performed the microarray experiments and data analysis. EMSAs were performed by S.R. and K.C. C.D. and P.M. created the Δ*espR* mutant and performed the Tet-inducible *espR* experiment. The project was planned and the manuscript written by S.R. and J.S.C.

Author Information All microarray data are available in the Gene Expression Omnibus (GEO) database via accession number GSE11696. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to J.S.C. (Jeffery.Cox@ucsf.edu).

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* initiation codon. 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. All mice were housed and treated humanely, as described in an animal care protocol approved by the UCSF Institutional Animal Care and Use Committee. *M. tuberculosis* cultures were grown to mid-log phase, washed and resuspended in PBS-Tween. Mice were infected intravenously through the tail-vein with 1×10^6 c.f.u. Lungs, spleens and livers were collected, homogenized, and plated for bacterial colonies[37]. Statistical significance was determined by analysis of variance (ANOVA; $\alpha = 0.05$) and two-tailed *t*-test assuming unequal variance between two groups (wild-type and *Rv3849*$^{-}$) for each time point.

Macrophage infections. Macrophages were derived from bone marrow progenitors by culturing with macrophage-colony stimulating factor (M-CSF), frozen at $-80\,^{\circ}$C and plated one day before infection[38]. *M. tuberculosis* cells 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 h, macrophages were washed with PBS and incubated in fresh media at 37 $^{\circ}$C.

Protein preparation and analysis. *M. tuberculosis* strains were grown to mid-log phase in Middlebrook 7H9 media supplemented with 0.05% Tween-80, diluted to $A_{600} = 0.05$ in Sauton's media supplemented with 0.05% Tween-80, and grown to mid-log phase[37,38]. Cells were washed, diluted to $A_{600} = 0.05$ in Sauton's media supplemented with 0.005% Tween-80 and grown for 5 days in roller bottles. Culture supernatants were collected 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), Mpt32 (generated at University of Texas, Southwestern Medical Center, Center for Proteomics Research, Antibody Production Core), ESAT-6 (Hyb 76-8), or GroEL (HAT5) (gifts from P. Andersen).

Protein purification. Coding sequence of wild-type and mutated *Rv3849* was cloned into pLIC-HMK (gift from J. Berger). Plasmids were transformed into *E. coli* BL21/DE3/pLysS, and expression was induced for 4 h with 1 mM IPTG. His-tagged MBP–EspR protein was purified using Ni-NTA agarose (Qiagen).

EMSA. *Rv3616c* promoter sequences were radiolabelled with T4 polynucleotide kinase in the presence of ^{32}P-gamma-ATP, and purified using Sephadex G-50 spin columns. Approximately 10^5 c.p.m. of probe (approximately 50 nM) was incubated with purified protein (20 μM) for 30 min at room temperature in reaction buffer containing 10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, 0.1 mg ml^{-1} salmon sperm DNA (Invitrogen), 25 μg ml^{-1} polydI:dC (Sigma) before running on a non-denaturing 6% polyacrylamide gel. The 680-BP *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[39]. 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). 3–5 μg of total RNA was reverse transcribed in the presence of amino-allyl UTP and Cy3- or Cy5-labelled. Competitive hybridizations between Cy5-labelled experimental cDNA and Cy3-labelled reference cDNA were performed for 24 h at 63 $^{\circ}$C using whole-genome oligonucleotide arrays (Qiagen). Hybridization data were deemed of high quality if they met numerous quality control criteria, including but not limited to minimum spot intensity in each channel, minimum foreground-background differential in each channel, and linear hybridization across a spot in each channel. The Rv3849 regulon was defined as genes that exhibited at least twofold dependence on Rv3849 in at least two out of three experiments. Array results from three independent experiments were analysed using the SAM (Significance Analysis of Microarrays) statistical package[40] to determine significantly induced or repressed genes. The analysis was performed with a false discovery rate of 0.01.

Quantitative PCR. 1–3 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). Oligonucleotides for amplification of *Rv3615c* were: oSR603 5′-GAGCGTCTCGGTGTACTG-3′ and oSR604 5′-CGTGTCGTTGAACTGTGAGC-3′, *nrdF1*: oSR601 5′-CAACCT GGGATACCAGCCTG-3′ and oSR 602 5′-CATTACGTATGAGCTTCC-3′, *16S rRNA*: oAL63 5′-ATGCTACAATCGCCGGTACA-3′ and oAL64 5′-GCGTTGCTGATCTGCGATTA-3′, *espR*: oPM501 5′-AACCGCCTGTTCG ACACGGTTTAT-3′ and oPM502 5′-TTCCTGAGCGTAGCTGTGATAGGT-3′. 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[41]. Intact bacteria were pelleted and washed in PBS-Tween, followed by bead-beating in Trizol, chloroform extraction, and cleanup over an RNeasy mini column. Total bacterial RNA was amplified using a Bacterial Amplification Kit (Ambion), and *Rv3615c* expression was measured in amplified RNA as above. Statistical significance was determined by ANOVA ($\alpha = 0.05$) and two-tailed *t*-test assuming unequal variance between two groups (wild-type and *Rv3849*$^{-}$) bacteria.

***lacZ* reporter assays.** *Rv3616c* promoter fragments were inserted upstream of *lacZ* in the plasmid pYUB76, the promoter–*lacZ* fusions were moved into the integrating plasmid pMV306-Kan and transformed into *M. smegmatis*. *M. tuberculosis espR* was cloned downstream of the inducible acetamidase promoter[42]. Owing to sufficient leakiness of the acetamidase promoter, inducer was not used to overexpress *espR*. Doubly-transformed *M. smegmatis* clones were spotted onto plates containing X-gal to monitor *lacZ* expression. For liquid β-galactosidase assays, *M. smegmatis* cultures were grown to $A_{600} = 0.6$ and enzyme activity was measured by the method of ref. 43. 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 gift from S. Ehrt[44]. In *M. tuberculosis*, Δ*espR* bacteria carrying either an empty *tetO* vector or an integrated plasmid with *tetO* driving Δ*espR* expression were grown in liquid culture to $A_{600} = 0.4$ then induced with 0.8–50 ng ml^{-1} of anhydrous tetracycline (ATC, Sigma). At 24 h post-induction, total bacterial RNA was harvested as described for microarrays and analysed 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 8 h with 0.8–25 ng ml^{-1} of ATC, and β-galactosidase activity was measured by the method of ref. 43.

Structural modelling. Rv3849 was modelled using the program Modeller (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/).

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