



WhiB6 regulation of ESX-1 gene expression is controlled by a negative feedback loop in *Mycobacterium marinum*

Rachel E. Bosserman^{a,1,2}, Tiffany T. Nguyen^{a,1,3}, Kevin G. Sanchez^a, Alexandra E. Chirakos^a, Micah J. Ferrell^a, Cristal R. Thompson^a, Matthew M. Champion^b, Robert B. Abramovitch^c, and Patricia A. Champion^{a,4}

^aDepartment of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556; ^bDepartment of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556; and ^cDepartment of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824

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ESX (ESAT-6 system) export systems play diverse roles across mycobacterial species. Interestingly, genetic disruption of ESX systems in different species does not result in an accumulation of protein substrates in the mycobacterial cell. However, the mechanisms underlying this observation are elusive. We hypothesized that the levels of ESX substrates were regulated by a feedback-control mechanism, linking the levels of substrates to the secretory status of ESX systems. To test this hypothesis, we used a combination of genetic, transcriptomic, and proteomic approaches to define export-dependent mechanisms regulating the levels of ESX-1 substrates in *Mycobacterium marinum*. WhiB6 is a transcription factor that regulates expression of genes encoding ESX-1 substrates. We found that, in the absence of the genes encoding conserved membrane components of the ESX-1 system, the expression of the *whiB6* gene and genes encoding ESX-1 substrates were reduced. Accordingly, the levels of ESX-1 substrates were decreased, and WhiB6 was not detected in *M. marinum* strains lacking genes encoding ESX-1 components. We demonstrated that, in the absence of EccCb₁, a conserved ESX-1 component, substrate gene expression was restored by constitutive, but not native, expression of the *whiB6* gene. Finally, we found that the loss of WhiB6 resulted in a virulent *M. marinum* strain with reduced ESX-1 secretion. Together, our findings demonstrate that the levels of ESX-1 substrates in *M. marinum* are fine-tuned by negative feedback control, linking the expression of the *whiB6* gene to the presence, not the functionality, of the ESX-1 membrane complex.

regulation | protein secretion | ESX-1 | ESAT-6 | *Mycobacterium*

Bacteria use secretion systems to transport protein substrates across membranes. Gram-negative (or diderm-LPS) bacteria secrete protein across the inner membrane and outer membrane (OM) using type I–IX secretion systems (1). Protein secretion in Gram-negative bacteria is a tightly regulated process. In particular, type 3 secretion systems (T3SSs) are regulated by environmental signals and by feedback-control mechanisms (2–7). Feedback control means that secretory activity or assembly is directly linked to effector gene expression, maintaining the appropriate levels of effector gene expression (7, 8). Injectisome T3SSs are used by several pathogenic bacteria (e.g., *Shigella*, *Pseudomonas*, and *Yersinia*) to translocate protein effectors into the host cell and modulate virulence (2). In these systems, feedback control links the transcription of effector genes to secretory function (4, 5, 9). In bacteria with a single polar flagella (e.g., *Campylobacter*), feedback control links substrate gene expression to flagellar assembly (6, 7).

Mycobacteria are classified as diderm-mycolate bacteria (10–12). In addition to the cytoplasmic membrane (CM), mycobacteria have a mycolate-OM (MOM) with a lipid content that is distinct from other OMs (1, 10–12). The ESX/WSS secretion systems are a unique family of protein transporters that are thus far restricted to Gram-positive bacteria and mycobacteria (13–17). Although the ESX/WSS systems transport proteins across the CM, it is unclear

how mycobacterial ESX proteins are translocated across the MOM (18).

ESX/WSS systems are functionally diverse with roles ranging from bacterial development to conjugation, metal homeostasis, and pathogenesis in different species (19–26). In mycobacteria, as many as five related ESX systems (ESX 1–5) can be encoded within the genome, with an additional system encoded on a plasmid for some species (27–29). Generally, ESX systems include several ESX conserved components (Eccs), small secreted Esx proteins, and several less conserved ESX-associated proteins (Esp) (13, 28, 30). Many of the Ecc proteins are localized to the CM, where they interact to form the ESX membrane complex (31–33).

The ESX-1 system is required for the virulence of mycobacterial pathogens, including *Mycobacterium tuberculosis* (*M. tb*), the causative agent of human tuberculosis, and the nontubercular species *Mycobacterium marinum* (26, 34–37). Expression of ESX-1 genes from *M. tb* in ESX-1-deficient *M. marinum* strains restores function and virulence, demonstrating that the two systems are functionally

Significance

Mycobacteria use ESX systems to transport protein substrates across the cytoplasmic membrane. The ESX-1 system is required for mycobacterial pathogenesis in *Mycobacterium tuberculosis* (*M. tb*), the cause of tuberculosis (TB). Differences in the expression of genes encoding ESX substrates directly impacts *M. tb* transmission and virulence. Deletion of genes encoding ESX exporters results in reduced levels of ESX substrates in mycobacteria. Here, we define a fundamental mechanism of regulation of ESX-1 substrates in *M. marinum*, a pathogenic mycobacterial species and a model for *M. tb*. We demonstrate that the transcriptional regulation of genes encoding ESX-1 substrates is linked to the presence or absence of the ESX-1 exporter. These findings provide insight into how substrate levels are intricately controlled in mycobacteria.

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¹R.E.B. and T.T.N. contributed equally to this work.

²Present address: Department of Microbiology and Molecular Genetics, The University of Texas Health Science Center at Houston, Houston, TX 77030-1503.

³Present address: Eli Lilly and Company, Indianapolis, IN 46285.

⁴To whom correspondence should be addressed. Email: pchampion@nd.edu.

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equivalent (37–39). In the host, pathogenic mycobacteria are taken up by professional phagocytes, including macrophages (40, 41). The ESX-1 system damages the phagosomal membrane, allowing bacterial interaction with the cytosol and survival. Mycobacteria lacking functional ESX-1 systems are attenuated and are retained in the phagosome (42–47). In the laboratory, the ESX-1 system promotes the secretion of several protein substrates into the culture media during growth (11, 35, 36, 39, 48–54).

Genes encoding ESX-1 substrates are directly regulated by several transcription factors in *M. tb*, including PhoP, EspR, and WhiB6 (53, 55–59). In *M. marinum* and in *M. tb*, WhiB6 is positively autoregulated and directly regulates the transcription of genes encoding several ESX-1 substrates and components (56, 58, 60). Recently, a model was proposed for *M. marinum* in which WhiB6 senses changes in the redox status of the environment and regulates the expression of ESX-1 genes. Thus, WhiB6 integrates a potential environmental signal and modulates ESX-1 secretion in *M. marinum* (60).

It is not known if ESX systems are regulated by feedback-control mechanisms akin to those understood for the T3SS in Gram-negative bacteria. We and others have observed that, when ESX systems in divergent mycobacterial species are inactivated by genetic disruption, substrate levels are reduced in the mycobacterial cell (35, 37, 52, 54, 61–67). We therefore hypothesized that ESX systems regulate the levels of ESX substrates within the cell by using a feedback-control mechanism. To test our hypothesis, we used transcriptional profiling, genetics, and quantitative proteomics in *M. marinum* to generate mechanistic evidence that the ESX-1 exporter is regulated by negative feedback control.

Results

ESX-1 Substrate Levels Are Regulated by EccCb₁ in *M. marinum*. To define the mechanism of feedback control of the ESX-1 system in *M. marinum*, we recapitulated the observation that substrate levels are reduced in the absence of a functional ESX-1 system (35, 37, 52, 54, 61–67). We measured secretion of two major substrates, EsxA (ESAT-6) and EsxB (CFP-10), into the culture media during *in vitro* growth (Fig. 1). EsxA and EsxB were produced and secreted from the WT strain (Fig. 1, lanes 1 and 2). EccCb₁ (Ecc Cb locus 1) is a component of the ESX-1-system (35, 36). *M. tb* and *M. marinum* strains lacking EccCb₁ do not secrete ESX-1 substrates *in vitro* (35–37). The EsxA and EsxB proteins were produced in strains bearing a deletion in (Fig. 1, lane 3) or an ochre allele of *eccCb₁* [*eccCb₁(Oc)*] (68); Fig. 1, lane 5]. The *eccCb₁(Oc)* allele encodes a premature stop codon following amino acid 48 of EccCb₁, and does not produce EccCb₁ protein (68). As expected, the EsxA and EsxB proteins were not secreted from the *eccCb₁* mutant strains (Fig. 1, lanes 4 and 6). Constitutive expression of *eccCb₁* from the synthetic mycobacterial optimal promoter (Mops) on an integrating plasmid (*p_{Mops}eccCb₁*) restored the secretion of EsxA and EsxB from the *eccCb₁(Oc)* strain (Fig. 1, lane 8). Importantly, the total amount of EsxA and EsxB protein produced and secreted from the WT strain (Fig. 1, lanes 1 and 2) appeared greater than the amount of EsxA and EsxB produced in the ESX-1-deficient strains (Fig. 1, lanes 3 and 4 or 5 and 6). From these data, we conclude that the total amount of the ESX-1 substrates, EsxA and EsxB, seems reduced in the absence of a functional *eccCb₁* gene.

EccCb₁ Regulates ESX-1 Associated Gene Expression in *M. marinum*.

Feedback control directly links substrate gene expression to the secretory status or assembly of the protein transporter (5). We reasoned that the reduced levels of ESX-1 substrates observed in the absence of a functional *eccCb₁* gene could be the result of changes in substrate gene expression. To this end, transcriptional profiling analysis of the WT, *eccCb₁(Oc)*, and *eccCb₁(Oc)* strain expressing a WT copy of the *eccCb₁* gene [*eccCb₁(Oc)/p_{Mops}eccCb₁*] was carried out to identify genes whose expression was dependent on

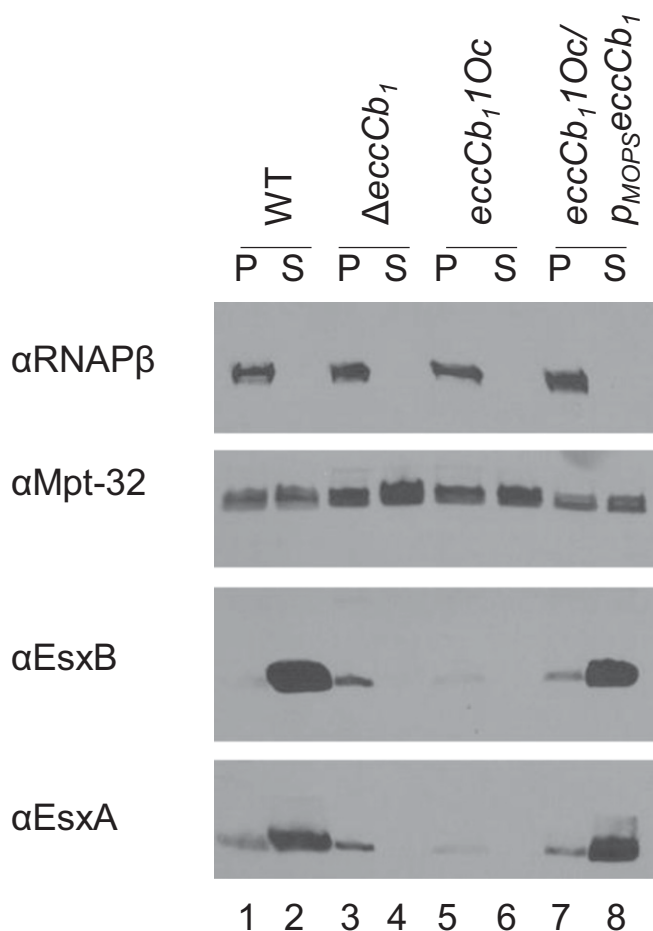


Fig. 1. ESX-1 substrates are regulated by feedback control. Western blot analysis of ESX-1 substrate production (“P,” pellet) and secretion (“S,” supernatant) during *in vitro* growth. RNAP β is a control for mycobacterial lysis. MPT-32 is a Sec-secreted loading control. The experiment is representative of three independent biological replicates.

the *eccCb₁* gene (Datasets S1 and S2). In the *eccCb₁(Oc)* strain compared with the WT strain, 74 genes were down-regulated (more than twofold; $q < 0.05$, Fig. 2A and B and Dataset S1B). Of these genes, 60 genes were not significantly down-regulated in the *eccCb₁(Oc)/p_{Mops}eccCb₁* complemented strain relative to the WT strain, indicating that expression was restored by *eccCb₁* gene expression (Dataset S2). Therefore, these 60 genes represent genes putatively induced by *eccCb₁* gene expression (Dataset S3A). These genes include the *MMAR_5437* gene, which encodes for the *M. marinum* ortholog of the *whiB6* gene from *M. tb* (60, 69), several genes encoding substrates of the ESX-1 system, along with a copy of *esxA_1* that is present elsewhere in the genome (selected ESX-associated genes are highlighted in Fig. 2C). Similarly, 92 genes were identified that were up-regulated (more than twofold; $q < 0.05$) in the *eccCb₁(Oc)* strain compared with the WT, but that were not induced in the *eccCb₁(Oc)/p_{Mops}eccCb₁* complemented strain (SI Appendix, Fig. S1, and Dataset S3B). These data demonstrate that gene expression is impacted, both positively and negatively, by the presence of the *eccCb₁* gene in *M. marinum*. Moreover, these findings support that EccCb₁ is regulating (directly or indirectly) the expression of ESX-1 associated genes.

The gene with the greatest transcriptional change in the *eccCb₁(Oc)* strain was the *whiB6* gene (*MMAR_5437*), which was down-regulated ~50 fold compared with the WT strain. WhiB6 positively regulates the expression of several genes

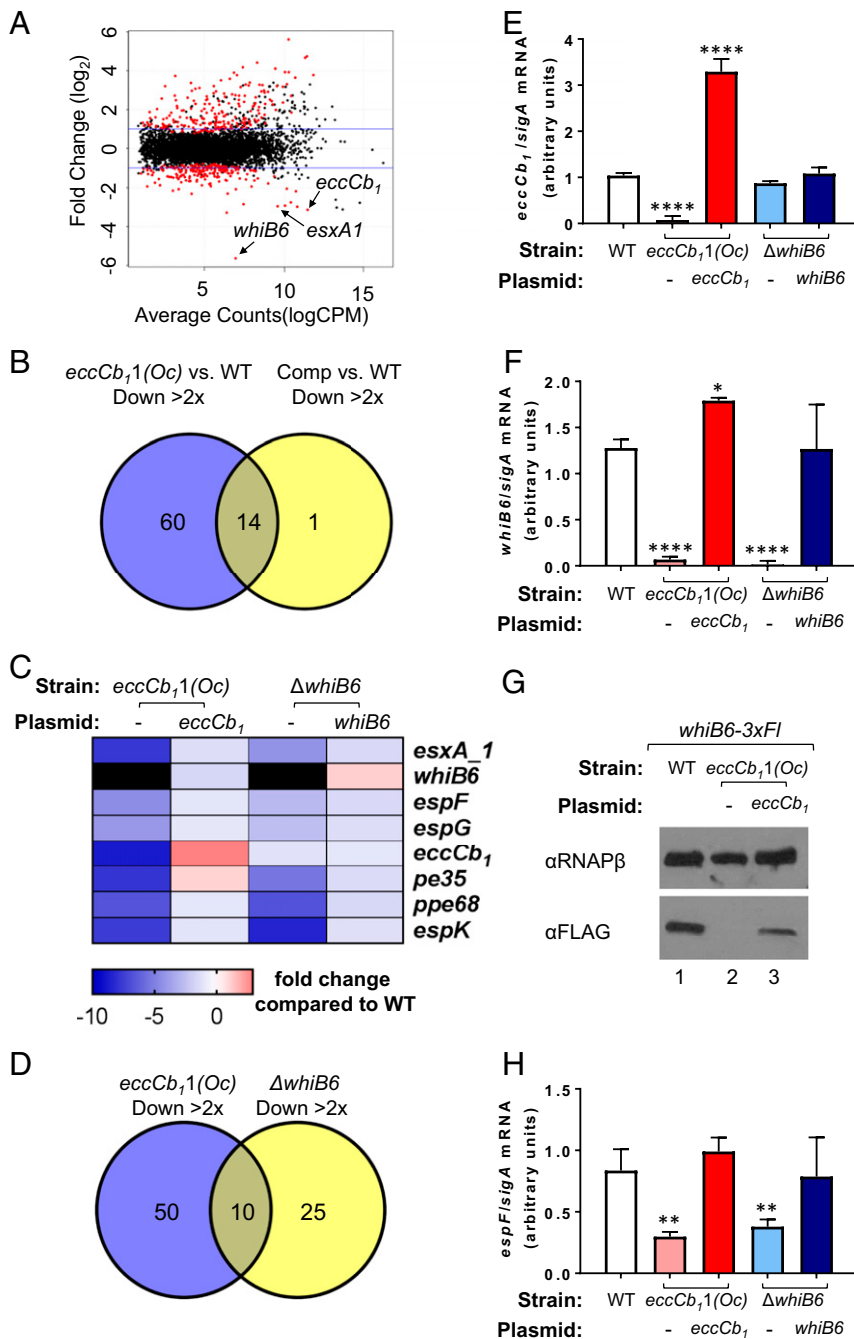


Fig. 2. EccCb₁ regulates *whiB6* and ESX-1 substrate gene expression in *M. marinum*. (A) RNA-seq magnitude–amplitude plot of WT *M. marinum* vs. the *eccCb1*(Oc) strain. Selected genes that are down-regulated in the *eccCb1*(Oc) strain (*whiB6*, *eccCb1*, and *esxA1*) are highlighted. Black dots indicate a lack of statistical significance, and red dots indicate statistical significance ($q < 0.05$). Complete data are presented in Dataset S1. (B) Venn diagram of genes down-regulated (more than twofold; $q < 0.05$) in the *eccCb1*(Oc) strain (Dataset S1C) or the complemented strain (Dataset S2C) relative to the WT strain. The putative EccCb₁ regulated genes are shown in Dataset S3A. (C) Heat map of selected ESX-associated genes that are significantly down-regulated in the *eccCb1*(Oc) strain, several of which are also differentially regulated in Δ *whiB6* strain. Genes down-regulated greater than 10 fold are indicated in black. All plasmids in Fig. 2 include the p_{Mops} promoter. (D) Venn diagram of genes down-regulated (more than twofold; $q < 0.05$) in the *eccCb1*(Oc) strain but not the complemented strain (Dataset S3A), or in the *whiB6* mutant strain but not the complemented strain (Dataset S3C), relative to the WT strain. The overlapping genes are enriched for ESX-associated genes (Dataset S3E). (E) Relative *eccCb1* gene expression by qRT-PCR. The error bars represent propagated SD. The significance was defined by using an ordinary one-way ANOVA ($P < 0.0001$). *eccCb1* levels were compared with those in the WT strain by using a Dunnett’s multiple comparison test ($****P \leq 0.0001$). (F) *whiB6* gene expression by qRT-PCR. Replicates and error bars are as in E. Significance was defined by using an ordinary one-way ANOVA ($P < 0.0001$). *whiB6* levels were compared with the WT strain by using a Dunnett’s multiple comparison test ($*P \leq 0.05$). (G) Western blot analysis of WhiB6-3xFL levels in the presence and absence of the *eccCb1* gene from whole-cell lysates. RNAP β was used as a loading control. The image is representative of three biological replicates. (H) *espF* gene transcription by qRT-PCR. Error bars represent SD. Significance was defined by using an ordinary one-way ANOVA ($P = 0.0001$). *espF* levels were compared with those in the WT strain by using a Dunnett’s multiple comparison test ($**P \leq 0.01$). For E–G, all transcripts were normalized to the levels of *sigA*. Nonsignificant differences ($P > 0.05$) are not indicated. The qRT-PCR data represent the average of two biological replicates, each with two technical replicates.

encoding ESX-1 substrates in *M. marinum* and *M. tb* (56, 59, 60). We generated a clean deletion of the *whiB6* gene in *M. marinum*. We complemented the Δ *whiB6* strain by constitutively expressing the *whiB6* gene from an integrating plasmid with the synthetic Mops promoter (Δ *whiB6*/ p_{Mops} *whiB6*). Transcriptional profiling of the WT, Δ *whiB6*, and Δ *whiB6*/ p_{Mops} *whiB6* complemented *M. marinum* strains was undertaken to identify genes with expression that is dependent on *whiB6* (Datasets S4 and S5). In the Δ *whiB6* strain compared with the WT, 51 genes were down-regulated (more than twofold; $q < 0.05$; SI Appendix, Fig. S2 A and B, and Dataset S4C). Of these genes, 35 were not significantly down-regulated in the Δ *whiB6*/ p_{Mops} *whiB6* complemented strain relative to the WT strain, indicating that expression was restored by *whiB6* gene expression (Dataset S3C). These genes represent putative WhiB6-activated genes. As

expected, several genes whose expression was activated by WhiB6 encoded ESX-1 substrates (SI Appendix, Fig. S2C), which overlapped with those identified previously (60). These data support that WhiB6 induces the expression of ESX-1 associated genes. Several genes that were down-regulated in the Δ *whiB6* mutant strain appear to be involved in redox homeostasis and metal-ion physiology. A total of 34 genes were identified that were up-regulated (more than twofold; $q < 0.05$) in the Δ *whiB6* strain compared with the WT strain, but that were not induced in the complemented strain (SI Appendix, Fig. S2, and Dataset S3D), although these genes did not appear to be enriched for specific function or well-characterized pathways.

There was a significant overlap between the genes that were down-regulated in the *eccCb1*(Oc) and Δ *whiB6* strains, but not their respective complemented strains (Fig. 2D). These 10 genes

include *whiB6*, *exxA_1*, and several genes encoding ESX-1 substrates or components (*espF*, *espG*, *pe35*, *ppe68*, and *espK*; a complete list of the overlapping genes is provided in [Dataset S3E](#)). Notably, *eccCb1* gene expression was not down-regulated in the $\Delta whiB6$ strain. However, *whiB6* gene expression was down-regulated in the *eccCb1(Oc)* strain. These data suggest that *EccCb1*, or a gene it regulates, is controlling *whiB6* gene expression or activity and that *WhiB6* is controlling expression of other coregulated genes including those encoding ESX-1 substrates.

We confirmed each of these findings by using quantitative RT-PCR (qRT-PCR). To this end, we isolated total RNA from the WT, *eccCb1(Oc)*, *eccCb1(Oc)/p_{Mops}eccCb1*, $\Delta whiB6$, and $\Delta whiB6/p_{Mops}whiB6$ strains. First, we confirmed that *eccCb1* transcript levels were significantly reduced in the *eccCb1(Oc)* strain ($P \leq 0.0001$; Fig. 2E). Constitutive expression of the *eccCb1* gene in the *eccCb1(Oc)* strain restored *eccCb1* transcript to levels significantly greater than those in the WT strain ($P \leq 0.0001$). Expression of the *eccCb1* gene was not significantly affected in the $\Delta whiB6$ or $\Delta whiB6/p_{Mops}whiB6$ strains. From these data, we conclude that the *eccCb1* gene is not regulated by *WhiB6* in *M. marinum*.

We next tested if *whiB6* gene expression was down-regulated in the *eccCb1(Oc)* strain. As shown in Fig. 2F, *whiB6* transcript levels were significantly reduced in the *eccCb1(Oc)* strain [19-fold, comparable to the RNA sequencing (RNA-seq) data; $P \leq 0.0001$] compared with the levels measured from the WT strain. Constitutive expression of the *eccCb1* gene in the *eccCb1(Oc)* strain restored *whiB6* transcript levels significantly greater than those detected in the WT strain ($P \leq 0.05$). As a control for specificity, we measured *whiB6* transcript levels in the $\Delta whiB6$ and $\Delta whiB6/p_{Mops}whiB6$ strains. The levels of *whiB6* transcript were significantly reduced in the $\Delta whiB6$ strain ($P \leq 0.0001$) and restored to WT levels in the $\Delta whiB6/p_{Mops}whiB6$ strain.

We sought to confirm that *WhiB6* protein levels reflected the observed changes in *whiB6* gene expression. Because we did not have an antibody for *WhiB6*, we integrated a *whiB6* allele encoding for a *WhiB6* protein with a C-terminal 3 \times FLAG epitope tag (*whiB6-3xFl*) into the $\Delta whiB6$ strain. The resulting strain encoded the *whiB6-3xFl* allele at the *whiB6* locus ([SI Appendix, Fig. S3](#)). The *WhiB6-3xFl* protein binds DNA and activates transcription (59). We deleted the *eccCb1* gene in the *whiB6-3xFl* strain and generated a complemented strain by introducing a plasmid constitutively expressing the *eccCb1* gene. As shown in Fig. 2G, we detected *WhiB6-3xFl* in whole-cell lysates generated from the WT strain (Fig. 2G, lane 1). Consistent with the reduced levels of *whiB6* transcription in strains lacking *EccCb1*, the *WhiB6-3xFl* protein was not detected in lysates from the $\Delta eccCb1$ strain (Fig. 2G, lane 2). Expression of the *eccCb1* gene from a plasmid restored the levels of *WhiB6-3xFl* protein (Fig. 2G, lane 3). These data indicate that *WhiB6* protein levels are also reduced in the absence of *EccCb1*.

To confirm that ESX-1 substrate gene expression was reduced in the *eccCb1(Oc)* strain, we measured expression of the *espF* (*MMAR_5440*) and *exxA* substrate genes (11, 37, 51). As shown in Fig. 2H and [SI Appendix, Fig. S4](#), we observed a significant decrease in *espF* (3.66-fold) and *exxA* (3.87-fold) transcript levels in the *eccCb1(Oc)* strain compared with the WT strain ($P \leq 0.01$ for *espF* and $P \leq 0.0001$ for *exxA*). Constitutive expression of the *eccCb1* gene in the *eccCb1(Oc)* strain restored *espF* and *exxA* transcript levels to WT levels. The expression of the *espF* and *exxA* genes is positively regulated by *WhiB6* in *M. marinum* (60). We measured a significant decrease in *espF* (2.99-fold) and *exxA* (4.57-fold) transcript levels in the $\Delta whiB6$ strain ($P \leq 0.01$ for *espF* and $P \leq 0.0001$ for *exxA*). Constitutive expression of the *whiB6* gene in the $\Delta whiB6$ strain restored *espF* and *exxA* gene expression levels to WT levels. From these data, we conclude that, in the absence of *EccCb1*, *espF* and *exxA* gene expression is

reduced, consistent with the RNA-seq data presented in Fig. 2 and the Western blot analysis data presented in Fig. 1. Together, these data demonstrate that *EccCb1* regulates *whiB6* and ESX-1 substrate gene expression in *M. marinum*.

Constitutive *whiB6* Gene Expression Is Sufficient to Bypass *EccCb1* Regulation of ESX-1 Genes. We sought to define how *EccCb1* regulates *whiB6* and ESX-1 gene expression. We hypothesized that *EccCb1* regulated *whiB6* gene expression at the level of transcription (Fig. 2), and that, in the absence of *eccCb1*, ESX-1 substrate gene expression was down-regulated by the loss of *WhiB6*. We reasoned that we could test this hypothesis by expressing *whiB6* from a constitutive promoter (*p_{Mops}whiB6*) or its native promoter (*pwhiB6*; Fig. 3A) in the absence of *eccCb1*. We hypothesized that, if the *whiB6* promoter was the target of feedback control, we could bypass regulation by *EccCb1* and restore ESX-1 substrates by expressing *whiB6* from the constitutive promoter, but not the native promoter.

The native expression plasmid was generated by introducing the *whiB6* ORF and ~1,000 bp upstream and downstream of the gene into an integrating plasmid (Fig. 3A). As shown in Fig. 3B, expression of *whiB6* from the constitutive or native promoters restored the levels of *whiB6* transcript to WT levels in the $\Delta whiB6$ strain. Similar to Fig. 2, *whiB6* transcript levels were significantly reduced in the strain expressing the *eccCb1(Oc)* allele ($P < 0.0001$; Fig. 3C) compared with the levels measured from the strain expressing *eccCb1*. Constitutive, but not native, expression of the *whiB6* gene restored *whiB6* transcript levels in the *eccCb1(Oc)* strain. The levels of *whiB6* expression in the strains with constitutive expression of the *eccCb1(Oc)* allele and native *whiB6* gene expression were not significantly different. Together, these data demonstrate that the *whiB6* promoter is likely the target of regulation by feedback control.

We compared the levels of *EsxA* protein in the *eccCb1(Oc)* strain expressing the *eccCb1(Oc)* allele [*p_{Mops}eccCb1(Oc)*, vector control], the *eccCb1* gene (*p_{Mops}eccCb1*, complemented), or the *whiB6* gene from a constitutive promoter (*p_{Mops}whiB6*) or its native promoter (*pwhiB6*). As shown in Fig. 3D, relative to the levels of *EsxA* protein in the *eccCb1(Oc)* strain (Fig. 3D, lane 1), expression of the *eccCb1* gene increased the level of *EsxA* (Fig. 3D, lane 2). Constitutive expression of the *whiB6* gene (Fig. 3D, lane 3) in the *eccCb1(Oc)* strain restored *EsxA* levels. Native expression of the *whiB6* gene did not restore *EsxA* levels in the *eccCb1(Oc)* strain (Fig. 3D, lane 4). From these results, we conclude that constitutive expression of the *whiB6* gene was sufficient to restore *EsxA* levels in *M. marinum* in the absence of *EccCb1*.

We further characterized the impact of constitutive expression of *whiB6* in the *eccCb1(Oc)* strain. We hypothesized that restoration of *whiB6* expression was increasing expression from the *exxA* gene. Consistent with this hypothesis, *exxA* gene expression was significantly reduced in the *eccCb1(Oc)* strain expressing the *eccCb1(Oc)* allele ($P \leq 0.0001$) compared with the WT strain (Fig. 3E). Constitutive expression of the *eccCb1* gene or the *whiB6* gene increased *exxA* expression levels, even though both were still significantly different from the WT strain ($P = 0.0432$ and $P = 0.0077$, respectively). From these data, we conclude that expression of *whiB6* from a constitutive promoter was sufficient to restore *exxA* gene expression in the absence of *EccCb1*.

WhiB6 regulates the expression of several genes encoding ESX-1 substrates in *M. marinum* (60) (Fig. 2). We sought to determine if constitutive expression of the *whiB6* gene was sufficient to restore levels of known ESX-1 substrates in the *eccCb1(Oc)* strain. We performed global proteomics on whole-cell lysates of *M. marinum* WT, *eccCb1(Oc)*, *eccCb1(Oc)/p_{Mops}eccCb1*, and *eccCb1(Oc)/p_{Mops}whiB6* ([SI Appendix, Fig. S5](#), and [Dataset S6](#)). We identified 2,586 proteins at a 1% false discovery rate. Protein quantification was performed by using

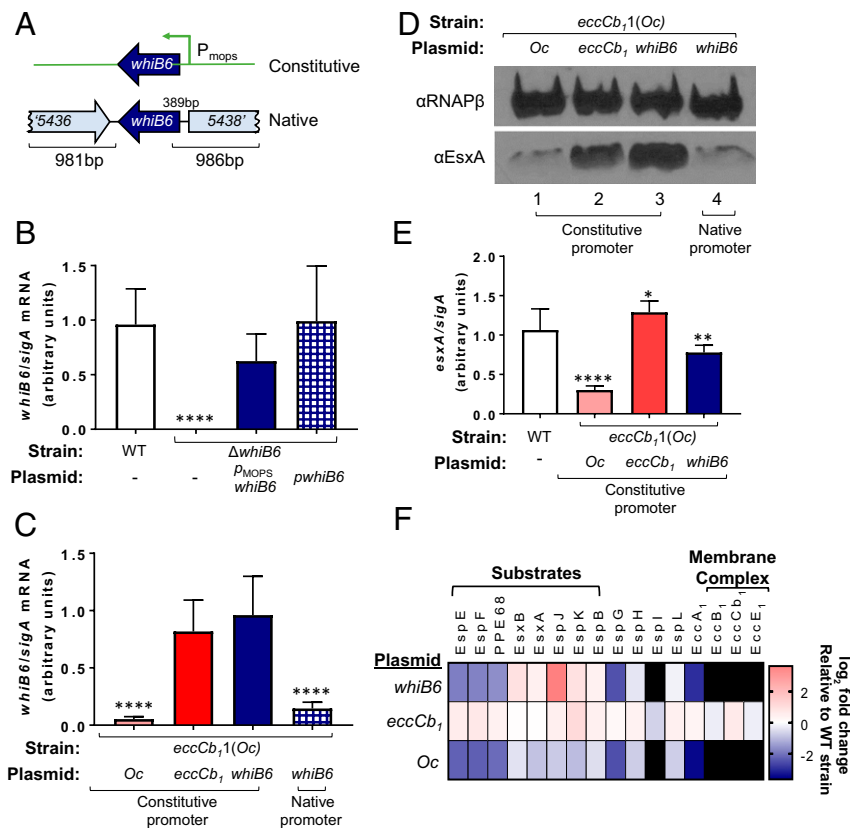


Fig. 3. Constitutive expression of *whiB6* is sufficient to bypass *EccCb1*-mediated regulation of ESX-1 genes. (A) Schematic of the constitutive (P_{Mops} *whiB6*) and native (*pwhiB6*) *whiB6* expression constructs. (B) *whiB6* gene transcription by qRT-PCR. The *whiB6* transcript was normalized to *sigA*. Data represent the average of three biological replicates, each performed in technical triplicate and normalized by using an interplate calibrator. Error bars represent the SD. Significance was defined by using an ordinary one-way ANOVA ($P < 0.0001$). *whiB6* levels were compared with those in the WT strain by using a Šidák's multiple comparison test ($****P < 0.0001$). Nonsignificant differences not indicated. "Oc" is the *eccCb1(Oc)* allele for Fig. 3. (C) *whiB6* gene transcription measured as in B. Plasmid-borne genes expressed by the constitutive P_{Mops} promoter or the native promoter as indicated. Significance was defined by using an ordinary one-way ANOVA ($P < 0.0001$). *whiB6* levels were compared across all strains by using a Tukey's multiple comparison test. Significance compared with the *eccCb1(Oc)* strain with the P_{Mops} *eccCb1* plasmid are shown. There were no significant differences between the *eccCb1(Oc)* strains bearing the P_{Mops} *eccCb1* and the P_{Mops} *whiB6* plasmids ($P = 0.5851$) or between the *eccCb1(Oc)* strains bearing the P_{Mops} *eccCb1(Oc)* and *pwhiB6* plasmids ($P = 0.7704$). (D) Western blot analysis of EsxA levels in whole-cell lysates generated from the *eccCb1(Oc)* strain. Plasmid-borne genes expressed by the constitutive P_{Mops} promoter (lanes 1–3) or the native promoter (lane 4) are indicated. RNAP β was used as a loading control. The image shown is representative of three biological replicates. (E) *esxA* gene transcription by qRT-PCR. The *esxA* transcript was normalized to *sigA*. Data represent the average of four biological replicates, each with two technical replicates. Error bars represent the propagated SD. Significance was in comparison with WT as in C ($***P \leq 0.01$, $*P \leq 0.05$, and $****P \leq 0.0001$). In this panel, all genes were expressed from the constitutive promoter. (F) Quantification of ESX-1-associated proteins by using label-free proteomics. The strains are the same as E. All protein levels are represented as the \log_2 fold change compared with those measured in the WT strain. Black indicates that the proteins were quantified in the WT strain only. The experiment was performed on biological duplicates, each with two technical replicates.

label-free-quantification (LFQ), which integrates the peak area of peptides corresponding to each protein (70).

We measured the levels of 20 ESX-1 proteins in the WT strain (Fig. 3F). Four proteins (MycP3, EccD₁, PE35, and EccCa₁) were confidently identified in only the WT strain, and the levels of these proteins were not measured in the *eccCb1(Oc)* strains. We identified 16 ESX-1 proteins (eight substrates and eight components) with reduced levels in the *eccCb1(Oc)* strain expressing the *eccCb1(Oc)* allele compared with the WT strain. We were surprised to find that, in addition to substrates encoded by *WhiB6*-regulated genes, several protein components of the ESX-1 system, whose gene expression is not regulated by *WhiB6*, were also reduced or were undetected in the *eccCb1(Oc)* strain compared with the WT strain. Interestingly, two proteins (EccB₁ and EccE₁) whose levels were reduced in the *eccCb1(Oc)* strain form the membrane complex with EccCb₁ in the CM (56). All 16 proteins were restored to at least WT levels by expression of the *eccCb1* gene in the *eccCb1(Oc)* strain. From these data, we conclude that, in the absence of EccCb₁, the levels of ESX-1

substrates and components, including the membrane complex, are reduced.

Constitutive expression of *whiB6* in the *eccCb1(Oc)* strain restored the levels of a subset of ESX-1 substrates (EsxA, EsxB, EspJ, EspK, and EspB) and components (EspH and EspL) to levels at or greater than the WT strain. From these data, we conclude that the loss of *WhiB6* gene expression, which results in a loss of *WhiB6*, causes the reduced levels of ESX-1 substrates in the *eccCb1(Oc)* strain (as observed in Fig. 1).

Interestingly, constitutive expression of *whiB6* failed to restore the levels of three ESX-1 substrates (EspE, EspF, and PPE68), despite the fact that the *espE*, *espF*, and *ppe68* gene expression is regulated by *WhiB6* (60) (Fig. 2C). These data indicate that EspE, EspF, and PPE68 are also regulated independently of *WhiB6* transcriptionally or posttranscriptionally.

As expected, constitutive expression of *whiB6* also failed to restore the levels of EccCb₁ in the *eccCb1(Oc)* strain. Interestingly, five additional components, two of which are in the ESX-1 membrane complex (EccB₁, EccE₁) were not restored by

constitutive expression of the *whiB6* gene. These data indicate that reduced levels of EspG, EspI, EccA₁, EccB₁, and EccE₁ in the *eccCb1(Oc)* strain is independent of *whiB6* gene expression.

Together, these data suggest that transcriptional regulation of *whiB6* results in a reduction of ESX-1 substrates in the absence of EccCb₁. Moreover, the loss of EccCb₁ impacts the levels of components of the ESX-1 system, including the membrane complex, through an undescribed mechanism.

The ESX-1 Membrane Complex Regulates *whiB6* Gene Expression in *M. marinum*. Based on the data in Fig. 3F, the loss of EccCb₁ in the *eccCb1(Oc)* strain caused a reduction or loss of proteins that form the ESX-1 membrane complex (Fig. 4A) and other components. We hypothesized that regulation of *whiB6* transcription was not specific to *eccCb1*. We tested if the deletion of other ESX-1 associated genes reduced *whiB6* gene expression. Deletion of the *esxB* genes, which encode for the EsxB and EsxA substrates, abrogates ESX-1 secretion (26, 35–37, 52). As shown in Fig. 4B, deletion of the *esxB* genes significantly reduced *whiB6* transcript levels compared with the WT strain ($P < 0.05$), but the levels of *whiB6* gene transcription were significantly greater in the Δ *esxB* strain compared with those measured in the Δ *eccCb1* strain. Accordingly, we detected WhiB6-3xFl protein in the cell-associated fractions generated from the Δ *esxB* strain (Fig. 4C). In contrast to the Δ *esxB* strain, deletions in the *eccCb1*, *eccCa1*, *eccE1*, *eccD1*, or *eccB1* genes (components of the membrane complex) significantly reduced *whiB6* gene expression (Fig. 4B) or WhiB6-3xFl protein levels (Fig. 4C) compared with the WT strain, and similar to the *eccCb1(Oc)* strain (Fig. 2F). From these data, we conclude that loss of genes encoding ESX-1 components, but not the EsxB substrates, promotes reduced *whiB6* gene expression.

Deletion of the *esxB* genes abrogated ESX-1 secretion but did not phenocopy the changes in *whiB6* transcription and protein levels measured in the Δ *eccCb1* strain. Therefore, secretory status of the ESX-1 system was not likely to be the signal regu-

lating *whiB6* gene expression. Because *whiB6* expression was not greatly affected in the absence of *esxB*, EsxB accumulation could be the signal for feedback regulation. To mimic EsxB accumulation, we constitutively expressed *esxB* genes encoding WT or EsxB proteins that cannot be secreted [EsxBM98A (71)] and measured WhiB6-3xFl levels. Expression of the WT or mutant EsxB proteins in the *whiB6-3xFl* strain did not appreciably reduce the levels of WhiB6-3xFl (SI Appendix, Fig. S6). Therefore, accumulation of EsxB is likely not the signal for feedback regulation.

Another possibility consistent with our data was that the ESX-1 membrane complex regulates *whiB6* gene expression. We sought a mutation in an ESX-1 component that abolished secretory activity, but did not affect the levels of the ESX-1 membrane proteins. The EccCb₁ protein has two ATPase domains, referred to as ATPase domain 2 and 3 (72, 73). The K90 residue is required for the ATP binding by ATPase domain 2. Mutation of EccCb₁K90 to A or T resulted in a stable but nonfunctional protein in *M. tb* (72, 73). We generated the *M. marinum* *eccCb1K90A* allele and constitutively expressed it in the *whiB6-3xFl* Δ *eccCb1* strain. As expected, the WT strain expressed the WhiB6-3xFl protein and secreted the EsxA substrate (Fig. 4D, lane 1). Deletion of *eccCb1* resulted in the loss of WhiB6-3xFl protein and EsxA secretion (Fig. 4D, lane 2). WhiB6-3xFl levels and EsxA secretion were restored by expression of *eccCb1* in the Δ *eccCb1* strain (Fig. 4D, lane 3). Even though EsxA was not secreted from the Δ *eccCb1* strain expressing EccCb₁K90A allele, WhiB6-3xFl was detected (Fig. 4D, lane 4).

We next determined how the EccCb₁K90A protein affected the levels of ESX-1 membrane proteins by using LFQ proteomics. As shown in Fig. 4E, we were unable to detect the EccCb₁ protein in the Δ *eccCb1* strain. The EccCb₁ protein was identified at similar levels in the Δ *eccCb1*/p_{Mops}*eccCb1K90A* and the complemented strains (log₂ fold change = 0.227). The levels of the ESX-1 conserved component proteins were reduced in the Δ *eccCb1* strain compared with the complemented strain. EccA₁ was reduced (log₂

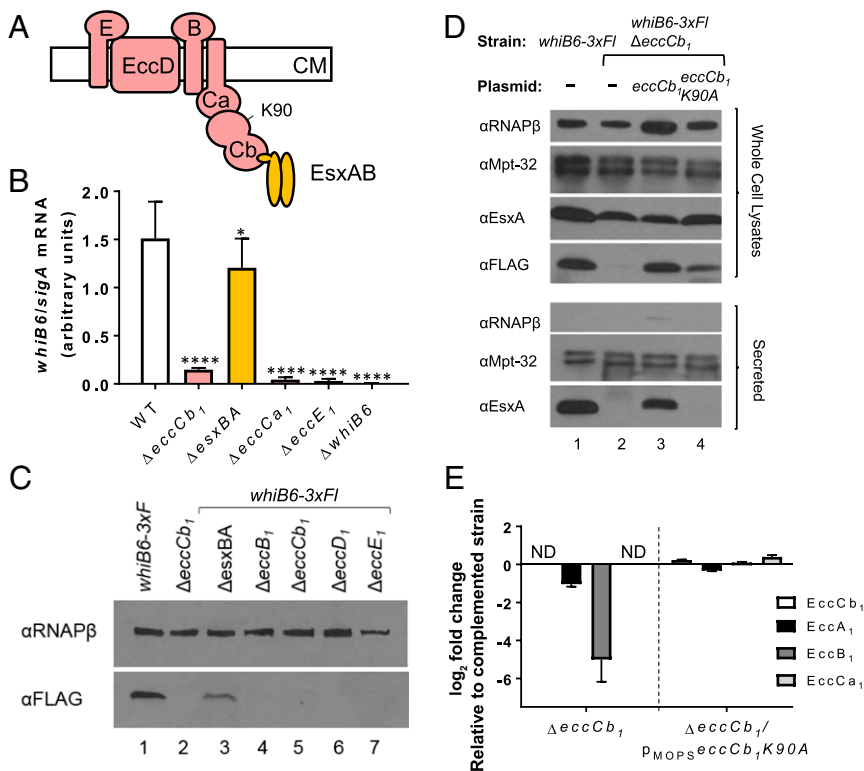


Fig. 4. The membrane components regulate *whiB6* gene expression in *M. marinum*. (A) Schematic representation of the ESX-1 membrane complex (pink) (31–33). The EsxA and EsxB substrates are shown in gold. The K90 residue is shown. (B) Relative *whiB6* gene expression by qRT-PCR, normalized to *sigA* expression. Data shown represents three biological replicates, including seven technical replicates. Error bars represent propagated SD. Significance was defined by using an ordinary one-way ANOVA ($P < 0.0001$). *whiB6* levels were compared with those in the WT strain by using a Dunnett's multiple comparison test ($*P \leq 0.05$ and $****P \leq 0.0001$). (C) Western blot analysis of WhiB6-3xFl levels in whole-cell lysates generated from strains lacking specific ESX-1-associated genes. Lanes 1 and 3–7 are strains with the *whiB6-3xFl* allele; lane 2 is WT for *whiB6*. RNAP β is used as a loading control. *eccCb1* genes are behind p_{Mops}. (D) Western blot analysis of whole-cell lysates (Upper) and secreted fractions (Lower) generated from the *whiB6-3xFl* Δ *eccCb1* strains expressing the WT *eccCb1* or *eccCb1K90A* alleles. RNAP β serves as a lysis control. MPT-32 serves as a loading control. (E) Fold change in the levels of ESX-1 membrane proteins from LFQ proteomics. Three technical replicates and two biological duplicates were integrated and averaged. SE (percent coefficient of variation) was calculated for each technical triplicate. Propagation of error was performed to determine error for fold change. ND, not detected in the Δ *eccCb1* strain. Protein ratios are reported as the log₂ fold change compared with the complemented strain. Significance was determined as in Fig. 3.

fold change = -1.037) in the $\Delta eccCb_1$ strain and slightly less so (\log_2 fold change = -0.321) in the $\Delta eccCb_1/p_{Mops}eccCb_1K90A$ strain compared with the complemented strain. The EccCa₁ protein, which interacts directly with EccCb₁ (35), was not detected in the $\Delta eccCb_1$ strain. The EccB₁ protein was identified but at too low a level to quantify in the $\Delta eccCb_1$ strain. In contrast, both proteins were present in the $\Delta eccCb_1/p_{Mops}eccCb_1K90A$ strain and the complemented strain at similar levels (\log_2 fold changes = 0.393 and 0.099, respectively). We were unable to detect EccD₁, and EccE₁ was not reliably quantified in this analysis. These data indicate that expression of EccCb₁K90A did not result in a significant reduction in the levels of the ESX-1 conserved components. Taken together, these data indicate that the presence or assembly of the ESX-1 membrane complex, not secretory activity, is the signal that regulates *whiB6* gene expression.

Feedback Control Functions to Fine-Tune ESX-1 Gene Expression. Genetic deletion does not reflect a physiological trigger for ESX-1 feedback control. Deletion of individual ESX-1 genes resulted in loss of *whiB6* gene expression (Fig. 4 B and C). However, in strains bearing mutation or deletion of *eccCb_1* or deletion of *whiB6*, substrate gene expression was reduced, not abrogated. We therefore reasoned that characterizing ESX-1 secretion levels from the $\Delta whiB6$ strain could inform our understanding of feedback control. It was previously reported by Chen et al. (60) that replacement of the *whiB6* gene with the hygromycin resistance gene (*whiB6::hyg*) in *M. marinum* resulted in a loss of ESX-1 secretion.

However, Chen et al. observed that *whiB6::hyg M. marinum* retained hemolytic activity and virulence, both of which require a functional ESX-1 system. We hypothesized that feedback control functions to fine-tune the levels of ESX-1 secretion, rather than shutting the system off.

Consistent with the report by Chen et al. (60), the hemolytic activity of the $\Delta whiB6$ strain was slightly, but significantly ($P \leq 0.0001$), reduced compared with the WT strain (Fig. 5A). Expression of the *whiB6* gene from the constitutive or native promoters restored hemolytic activity to WT levels. In contrast, the $\Delta RD1$ strain, which bears a deletion of several ESX-1 genes and is ESX-1-deficient, had hemolytic activity similar to the PBS (i.e., no bacteria) control. These data indicate that the ESX-1 system in the $\Delta whiB6$ strain is functional.

We next measured secretion in the absence of the *whiB6* gene. Clean deletion of the *whiB6* gene did not abrogate ESX-1 substrate secretion in vitro (*SI Appendix, Fig. S7*). However, with 20 μ g of secreted protein loaded for analysis, the levels of EsxA secretion appeared reduced in the $\Delta whiB6$ strain compared with the WT and *whiB6/p_{Mops}whiB6* complemented strains. We verified the secretion of EsxA and EsxB by loading a series of concentrations of the secreted fraction (15 μ g and 5 μ g; Fig. 5B). Deletion of the *whiB6* gene resulted in a reduction, but not a loss of EsxA and EsxB secretion (Fig. 5B, lanes 5 and 6). The levels of EsxA and EsxB in the secreted fraction were restored by *whiB6* expression from the constitutive or native promoters (Fig. 5B, lanes 7–10). We conclude that deletion of the *whiB6* gene does not abrogate ESX-1 function. Moreover, constitutive

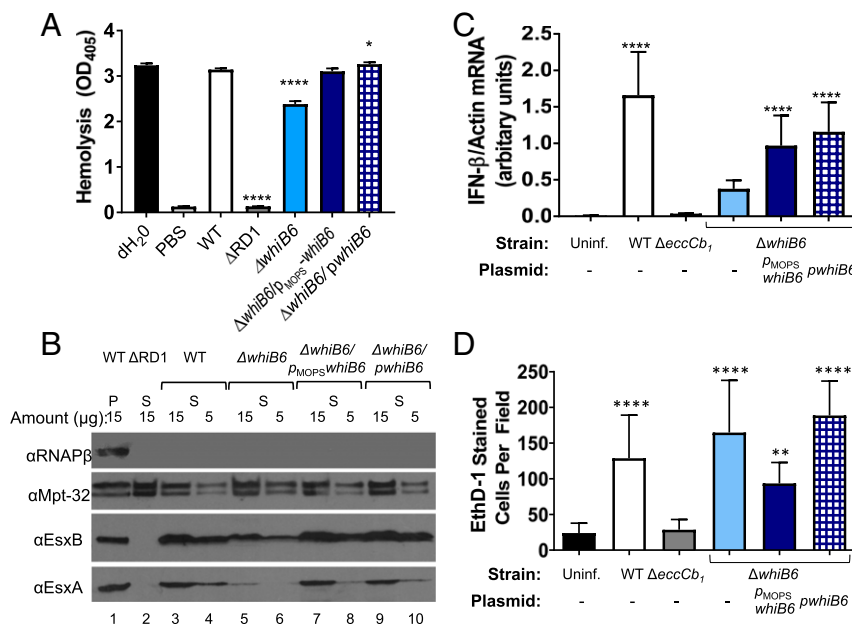


Fig. 5. Feedback control fine-tunes ESX-1 function. (A) Sheep RBC (sRBC) hemolysis assay performed on three biological replicates, each in technical triplicate. The data represent the average between the three biological replicates. Error bars represent the propagated SD. Significance was determined by using an ordinary one-way ANOVA ($P < 0.0001$). The levels of hemolysis were compared with those of the WT strain by using a Dunnett's multiple comparison test ($****P \leq 0.0001$ and $*P \leq 0.05$). Nonsignificant changes ($P > 0.05$) are not indicated. (B) Western blot analysis of EsxA and EsxB secretion. Lane 1 is whole-cell lysates from the WT strain. Lanes 2–10 are supernatant fractions. The amount loaded in each lane is indicated in micrograms. RNAPβ is a lysis control. MPT-32 serves as a loading control. (C) qRT-PCR measuring the induction of the type I IFN response by RAW 264.7 cells 4 h after infection with *M. marinum* at an MOI of 5. IFN-β expression was normalized to actin expression. Data are representative of six biological replicates, each with two technical replicates. Significance was determined by using an ordinary one-way ANOVA ($P < 0.0001$) followed by a Tukey's multiple comparison test. Significance is relative to the uninfected strain. The levels of IFN-β induction were not significantly different from the $\Delta eccCb_1$ and $\Delta whiB6$ strains ($P > 0.9999$ for $\Delta eccCb_1$ and $P = 0.1021$ for $\Delta whiB6$). (D) Quantification of cytolysis of RAW 264.7 cells. Five random fields were counted from each of three wells. The 15 resulting counts were averaged. The error bar represents SD between these counts. The experiment shown is representative of three biological replicates. Significance was determined by using an ordinary one-way ANOVA ($P < 0.0001$) followed by a Tukey's multiple comparison test. Significance shown is based on the comparison against the uninfected strain ($**P = 0.001$). Nonsignificant changes are not shown. The number of EthD-1 cells was not significantly different between the WT and the $\Delta whiB6$ strains.

or native expression of *whiB6* functionally complements the $\Delta whiB6$ strain.

To determine the physiological relevance of the feedback control, we tested how reduced ESX-1 function affected virulence of *M. marinum* in a macrophage infection model. In the macrophage, the ESX-1 system promotes cytosolic access, which leads to induction of the type I IFN, IFN- β , and lysis of the macrophage (42, 46, 47, 74). We infected RAW 264.7 cells with *M. marinum* at a multiplicity of infection (MOI) of 5 and measured induction of IFN- β 4 h after infection by using qRT-PCR. As shown in Fig. 5C, the WT *M. marinum* strain significantly induced expression of IFN- β compared with the uninfected control ($P = 0.0001$). Induction of IFN- β by the $\Delta eccCb_1$ and $\Delta whiB6$ strains was not significantly different from the uninfected control. Expression of *whiB6* from the constitutive or the native promoter in the $\Delta whiB6$ strain restored induction of IFN- β relative to the uninfected control ($P < 0.0001$). The levels of induction between the WT and the $\Delta whiB6/p_{Mops}whiB6$ strain, but not the $\Delta whiB6/p_{whiB6}$ strains, were significantly different from each other ($P = 0.0166$ and $P = 0.1425$, respectively). These data indicate that the $\Delta whiB6$ strain exhibits attenuated induction of IFN- β transcript compared with the WT strain.

We next measured macrophage cytolysis 24 h after infection (SI Appendix, Fig. S8). Ethidium homodimer (EthD-1) is a nucleic acid stain that is taken up only by cells with damaged cell membranes. In permeabilized cells, EthD-1 binds DNA and emits a red fluorescent signal. We counted the number of red cells per field to measure the cytolytic activity of each *M. marinum* strain (Fig. 5D; images in SI Appendix, Fig. S8). Infection of the RAW 264.7 cells with the WT, $\Delta whiB6$, $\Delta whiB6/p_{Mops}whiB6$, and $\Delta whiB6/p_{whiB6}$ strains caused significant increases in cytolysis (red cells, $P < 0.0001$ for WT, $\Delta whiB6$, and $\Delta whiB6/p_{whiB6}$ strains, and $P = 0.0010$ for $\Delta whiB6/p_{Mops}whiB6$) compared with the $\Delta eccCb_1$ strain and the uninfected control. The cytolytic activities of the WT and $\Delta whiB6$ strains were not significantly different from each other ($P = 0.2706$). Together, these data indicate that the $\Delta whiB6$ strain was attenuated for ESX-1 function during early stages, but not during later stages, of infection in the macrophage model. We interpret these data to mean that feedback control, which results in a loss of WhiB6, functions to fine-tune ESX-1 secretion in *M. marinum*.

Discussion

Our study demonstrates that the ESX-1 system in *M. marinum* is regulated by negative feedback control, linking the expression of the *whiB6* gene and genes encoding ESX-1 substrates to the status of the ESX-1 membrane complex. Collectively, our experimental data support a model for feedback regulation of ESX-1 in *M. marinum* that fine-tunes the levels of ESX-1 substrates. When the levels of ESX-1 components, most likely the membrane complex, are reduced, *whiB6* gene expression is down-regulated. Because expression of *whiB6* from a constitutive promoter, but not from the native promoter, restored *whiB6* expression and ESX-1 substrate levels in the absence of *eccCb_1*, the *whiB6* promoter is likely the target of feedback control. Reduced *whiB6* gene expression, which leads to a loss of WhiB6 protein, results in the down-regulation of genes encoding ESX-1 substrates and a reduction of the substrate pool within the cell.

Our model agrees with published evidence from other groups. Several groups have demonstrated that substrate levels are reduced or do not accumulate in various strains lacking a functional ESX-1 exporter in *Mycobacterium smegmatis* (61), a distantly related nonpathogenic species, or in *M. tb* (35, 62) and *M. marinum* (37, 52, 54). Conversely, substrate accumulation has also been reported in *M. marinum* (51, 52), *M. tb* (50), and *M. smegmatis* (75). In all three species, there is a *whiB6* ortholog (MSMEG_0051, Rv3862c) encoded upstream of the ESX-1

system (56). Lack of substrate accumulation has also been observed in strains deficient for ESX-3 (63) or ESX-5 export (64–66, 76). Genes encoding the ESX-5 system are also regulated by transcription factors (21, 77), which sense and respond to the environment, and may be targets of feedback control. Together, these observations suggest that ESX systems in different mycobacterial species are regulated by feedback control, although further study is required.

Our study also indicates that feedback control is not regulated by transcription alone. Although *espF* gene expression is regulated by WhiB6, expression of *whiB6* in the absence of *eccCb_1* did not restore EspF protein (Fig. 3). Therefore, it is likely that posttranscriptional regulation is also contributing to feedback control. Indeed, the idea of posttranscriptional regulation of the levels of ESX substrates and secretion itself has been raised before. For example, the levels of the ESX-5 substrate LipY appear to be posttranscriptionally regulated (64). Moreover, the MycP1 protease regulates ESX-1 secretion posttranscriptionally. Loss of protease activity does not disrupt ESX-1 function, but results in increased levels of secreted ESX-1 substrates. Thus, the protease activity of MycP1 negatively regulates the secretion of, but not the levels of, the ESX-1 substrates within the cell (32, 78).

In the absence of the *eccCb_1* gene, we did not detect the components of the ESX-1 system that reside in the membrane (EccCa₁, EccE₁). It was previously demonstrated that five Ecc proteins (EccE, EccD, EccB, EccCa, and EccCb), from the ESX-1 and ESX-5 systems, form a complex in the CM (32, 33). The structure of the ESX-5 membrane complex from *Mycobacterium xenopi* was recently solved (31). Our data indicate that, in *M. marinum*, the loss of a single component of this complex (EccCb₁) promotes the loss or reduction of several membrane components, in line with our previous study (52). These findings agree with reports that the deletion of other Eccs (mycosins or EccE) lead to reduced levels of the other components in the CM (31–33).

Our findings are inconsistent with a subset of those reported by Chen et al. (60). In that report, the authors demonstrated that disruption of the *whiB6* gene in *M. marinum* M strain abolished the secretion of ESX-1 substrates in vitro (60). The data in Fig. 5B contradict this conclusion. We observed that clean deletion of the *whiB6* gene resulted in a reduction, but not a loss, of ESX-1 activity. Indeed, we observed secretion of EsxA and EsxB and hemolysis in the $\Delta whiB6$ strain to levels significantly higher than in ESX-1-deficient strains. It is likely that in the study of Chen et al. (60), too little protein was analyzed to detect protein secretion by Western blot analysis. Secretion at lower than the levels of detection by Western blot analysis are sufficient to promote hemolysis and virulence (63, 79–81). The H37Rv *M. tb* strain is virulent despite reduced levels of ESX-1 secretion (56). Likewise, the $\Delta whiB6$ strain retained virulence in the macrophage model of infection. The reduced levels of ESX-1 secretion in the $\Delta whiB6$ strain may have resulted in delayed ESX-1-associated virulence in the macrophage model. Chen et al. reported that the *whiB6::hyg* *M. marinum* strain retained virulence in a zebrafish model of infection (60). Our study indicates that feedback control functions as a dial to fine-tune ESX-1 protein secretion, rather than as a switch.

Our findings are reminiscent of established mechanisms of feedback regulation used in controlling T3SS in Gram-negative pathogens (reviewed in refs. 2, 4, 5). We found that, like injectisome systems, posttranscriptional mechanisms are also involved (82). Our model is also similar to feedback regulation of the flagellar T3SS system in *Campylobacter jejuni*. In *C. jejuni*, the assembly of the T3SS and the surrounding apparatus in the CM regulates substrate gene expression (6, 7, 83). Flagellar proteins localized beyond the CM are substrates of the T3SS (9). Importantly, mutations that abolish T3 secretory activity but allow the assembly of the apparatus promote substrate gene expression (7). Because the EccCb₁K90A protein abolished ESX-1 secretion, but

did not appreciably reduce the levels of WhiB6 or the other ESX-1 membrane proteins, the presence or assembly of the ESX-1 membrane complex likely regulates *whiB6* gene expression. In T3SS, feedback control fine-tunes effector and substrate pools (8). Feedback control promotes the maintenance of the effector pool, preventing depletion or accumulation of effectors within the cell that could impact bacterial survival (8). Our findings also indicate that feedback control is a widespread regulatory mechanism of bacterial protein secretion, spanning at least the evolutionarily divergent T3SS and ESX-1 systems.

More work is needed to further elucidate this signaling pathway. It is unclear what physiological conditions would lead to a reduction in ESX-1 components. The ESX-1 membrane complex could represent a regulatory checkpoint in the assembly of the ESX-1 exporter (7). When the checkpoint is successfully reached, *whiB6* and ESX-1 substrate gene expression are increased. The ESX-1 substrates may contribute to assembly of the functional ESX-1 apparatus (73). If the membrane complex forms incorrectly, the levels of the ESX-1 membrane proteins could be reduced, leading to a loss of *whiB6* gene expression and reduced substrate gene expression. Alternatively, the loss of the complex may be a response to an upstream signal. It is unknown how the loss of the membrane complex signals to reduce *whiB6* transcription. The EccCb₁ protein is not likely regulating *whiB6* gene expression directly. Because WhiB6 is positively autoregulated (60), WhiB6 may sense the loss of the ESX-1 membrane complex directly. WhiB6 is one of seven WhiB-like (Wbl) transcription factors in *Mycobacterium* (84, 85). Wbl transcription factors sense and respond to diatomic gasses and redox signals (e.g., NO and O₂) (86). WhiB6 senses changes in redox homeostasis in *M. marinum* (60). However, we do not think WhiB6 senses the ESX-1 membrane complex directly. Constitutive *whiB6* expression bypassed feedback regulation of ESX-1 substrates (Fig. 3). These data strongly suggest that the WhiB6 protein is functional in the absence of ESX-1 (60, 86). The assembly of the T3S flagellar apparatus regulates substrate gene expression through the FlgSR two-component system (7, 83, 87). There is likely a regulatory pathway that connects the ESX-1 membrane complex to *whiB6* gene expression. We envision a negative regulator that represses *whiB6* transcription or a positive regulator that no longer activates *whiB6* transcription in the absence of *eccCb₁* and the membrane complex. Indeed, *whiB6* is directly regulated at the level of transcription in *M. tb* by the PhoPR two component system, by WhiB3, and by the orphan response regulator Rv0818 (56). These regulators may be candidates for the signal transduction pathway connecting the ESX-1 membrane complex to the *whiB6* promoter in *M. marinum*.

In conclusion, we have identified a signaling pathway in *M. marinum* that regulates the levels of ESX-1 substrates. To our knowledge, this is the first report of a regulatory pathway that links the Ecc proteins to expression of ESX substrate genes at the ESX-1 locus. Our study explains, at least in part, the fundamental mechanism underlying how ESX-1 substrate pools are fine-tuned and why substrates do not accumulate in the absence of a functional ESX-1 exporter.

Materials and Methods

All *M. marinum* strains used in this study were generated from the M strain (American Type Culture Collection BAA-535) using the allelic exchange as described previously (68). All strains and plasmids are described in *SI Appendix, Table S1*, and all oligonucleotide primers are listed in *SI Appendix, Table S2*. ESX-1 secretion assays were performed as previously described (88). Mycobacterial whole-cell lysates were prepared for bottom-up proteomics and analyzed by LC/MS/MS as in previous publications (44, 45). Database identification and LFQ were performed as described previously (70). RNA was purified by using the RNeasy Mini Kit, and quality was assessed by using an Agilent Bioanalyzer 2100 and the Agilent RNA 6000 Nano Reagent kit (16S rRNA chip) at the University of Notre Dame Genomics Facility. RNA-seq was performed as described previously (89, 90), and analyzed by using the SPARTA software package (91). Genes with an average log₂ cpm < 5 were filtered from the final differential gene expression lists (*Datasets S1–S5*). The transcriptional profiling data are available at the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE99632). Details regarding cDNA synthesis and qRT-PCR analysis are reported in *SI Appendix, Tables S2 and S3*. Hemolysis assays were performed as described previously (88), except bacterial cells were incubated with sRBCs for 1.5 h at 30 °C. Readings were performed on a SpectraMax M5 plate reader (Molecular Devices) and analyzed by using SoftMax Pro-5 software (Molecular Devices). Biological and technical replicates are indicated in the figure legends. Macrophage infections and cytotoxicity assays were performed exactly as described previously (88). Counts were performed exactly as described previously (69) by using ImageJ. The cytokine assays were performed exactly as described previously (69, 88), with details reported in *SI Appendix, Tables S2 and S3*. Statistical analyses are described in the appropriate figure legends. A full explanation of study methods is provided in *SI Appendix, SI Materials and Methods*.

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