The EspN transcription factor is an infection-dependent regulator

of the ESX-1 system in *M. marinum*

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Abstract

Bacterial pathogens use protein secretion systems to translocate virulence factors into the host and to control bacterial gene expression. The ESX-1 (ESAT-6 system 1) secretion system facilitates disruption of the macrophage phagosome during infection, enabling access to the cytoplasm, and regulates widespread gene expression in the mycobacterial cell. The transcription factors contributing to the ESX-1 transcriptional network during mycobacterial infection are not known. We showed that the EspM and WhiB6 transcription factors regulate the ESX-1 transcriptional network *in vitro* but are dispensable for macrophage infection by *Mycobacterium marinum*. In this study, we used our understanding of the ESX-1 system to identify EspN, a critical transcription factor that controls expression of the ESX-1 genes during infection, but whose effect is not detectable under standard laboratory growth conditions. Under laboratory conditions, EspN activity is masked by the EspM repressor. In the absence of EspM, we found that EspN is required for ESX-1 function because it activates expression of the *whiB6* transcription factor gene, and specific ESX-1 substrate and secretory component genes. Unlike the other transcription factors that regulate ESX-1, EspN is required for *M. marinum* growth within and cytolysis of macrophages, and for disease burden in a zebrafish larval model of infection. These findings demonstrate that EspN is an infection-dependent regulator of the ESX-1 transcriptional network, which is essential for mycobacterial pathogenesis. Moreover, our findings suggest

that ESX-1 expression is controlled by a genetic switch that responds to host

specific signals.

Importance

Pathogenic mycobacteria cause acute and long-term diseases, including human

tuberculosis. The ESX-1 system transports proteins that control the host response

to infection and promotes bacterial survival. Although ESX-1 transports proteins,

it also controls gene expression in the bacteria. In this study, we identify an

undescribed transcription factor that controls the expression of ESX-1 genes, and

is required for both macrophage and animal infection. However, this transcription

factor is not the primary regulator of ESX-1 genes under standard laboratory

conditions. These findings identify a critical transcription factor that controls

expression of a major virulence pathway during infection, but whose effect is not

detectable with standard laboratory strains and growth conditions.

Main Text

Introduction

In diverse pathogens, secretion systems transport virulence factor substrates that

promote bacterial survival in different niches within the host (1, 2). Pathogenic

mycobacteria use conserved ESAT-6 secretion (ESX) systems to facilitate

disruption of the macrophage phagosome during infection, enabling access to the

cytoplasm and toxification of the macrophage (3-5). *Mycobacterium tuberculosis*,

the causative agent of human tuberculosis, and *Mycobacterium marinum*, a

poikilothermic fish pathogen, share a conserved ESX-1 secretion system that is

required for pathogenesis (6-8). The ESX-1 system is composed of ESX core

components (Ecc's) that reside within the mycobacterial cell membrane (CM) (7,

9, 10). During macrophage infection, the ESX-1 system transports protein

substrates that are secreted components of the ESX-1 machinery (11) and

effectors that promote phagosomal damage (4, 5, 12, 13). Phagosomal damage

enables the essential interaction between pathogenic mycobacteria and the

macrophage cytoplasm (5, 14-17).

Protein secretion systems in several pathogens also regulate bacterial gene

expression (2, 18, 19). In addition to transporting proteins, we and others have

shown that ESX-1 controls gene expression globally in *M. marinum* and *M.*

tuberculosis (20-22). Loss of the ESX-1 membrane complex leads to widespread

changes in gene expression (20, 21). The gene most responsive to loss of the

ESX-1 membrane complex, *whiB6*, encodes a transcription factor that activates

ESX-1 substrate gene expression (20, 23, 24). We showed that EspM is a direct

repressor of *whiB6* gene transcription in the absence of the ESX-1 membrane

complex (20, 22). Reduced WhiB6 leads to lower levels of ESX-1 substrate gene

transcription, preventing accumulation of ESX-1 substrates in the absence of the

transport machinery (20). Genetic deletion of the *espM* gene, which is divergently

encoded from the *whiB6* gene, leads to derepression of *whiB6* expression, and

increased levels of ESX-1 substrates in *M. marinum* during *in vitro* growth (22).

Despite regulating ESX-1 substrate gene expression under laboratory

conditions, neither WhiB6 nor EspM is essential for virulence in macrophage

models of infection (20, 22). Therefore, ESX-1 may be differentially regulated in

the laboratory and at different points or niches during infection. We previously used

lacZ+ transcriptional fusions to measure the transcriptional activity of the *M.*

marinum whiB6 promoter in the presence and absence of the EspM and WhiB6

transcription factors. In the absence of both EspM and WhiB6, there was a

significant increase in *β*-galactosidase activity, ~2-fold higher than in the absence

of EspM alone (22). These data suggested that in the absence of both WhiB6 and

EspM, *whiB6* gene expression was activated. We exploited our knowledge of a

negative regulator of ESX-1 expression, EspM, as a tool to dissect the genetic

circuitry that regulates the ESX-1 regulatory network (Fig. 1A).

Results

EspN is a putative transcription factor that binds the *whiB6* promoter.

To identify regulators responsible for activation of the *whiB6* promoter in the

absence of EspM we used the *whiB6-espM* intergenic region to enrich proteins

from lysate generated from the *ΔespM M. marinum* strain. Mass spectrometry

identified MMAR_1626 as the only protein enriched for binding the *whiB6-espM*

intergenic region in the absence of *espM* (Fig. 1B) (22).

MMAR_1626 is an unstudied putative transcriptional regulator with a

predicted N-terminal winged helix-turn-helix domain, and a C-terminal sterol carrier

protein 2 (SCP2) domain (Fig. 1C) (25, 26). *MMAR_1626* gene orthologs are found

across *Mycobacterium* and other high G+C Gram-positive bacteria, including

members of the TB complex, *M. tuberculosis* (*Rv1725c*) and *M. bovis* (*Mb1754c*),

and non-pathogens, including *M. smegmatis* (27, 28)*.* Based on the following data,

we propose naming this protein EspN, according to accepted nomenclature (29).

EspN is an activator of *whiB6* gene expression in the absence of

EspM. Our data led us to hypothesize that EspN is an activator of *whiB6* gene

expression (Fig. 1A). To test this hypothesis, we generated an unmarked *espN*

deletion strain using allelic exchange (Fig. S1A and S1B) (30). The parental *M.*

marinum M strain (wild type, WT) has a *whiB6* allele with a C-terminal 3xFLAG

epitope integrated at the *whiB6* locus (20). We generated the corresponding

complementation strain by integrating a copy of the *espN* gene behind the

constitutive mycobacterial optimal promoter at the *attL* site. We tested *espN*

expression in these strains using qRT-PCR (Fig. S2A). We detected the *espN*

transcript in the WT strain, but not in the Δ*espN* strain (*P*=.0002, compared to the

WT strain, inset). *espN* was significantly overexpressed ($\geq$ 40-fold) in the

Δ*espN/pespN* strain as compared to the WT strain (*P*<.0114, Fig S2A).

To evaluate the role of EspN on *whiB6* expression, we measured *whiB6*

transcript levels in the Δ*espN* and Δ*espN/pespN* strains relative to the WT strain

and Δ*eccCb$_1$* strains using qRT-PCR (Fig. 2A). The Δ*eccCb$_1$* strain does not

produce the EccCb1 protein, a cytoplasmic component of the ESX-1 system that

directly interacts with the membrane components (7). The loss of EccCb1 results

in a loss of the entire ESX-1 membrane complex (10, 20, 31), and results in

repression of *whiB6* gene expression (20, 22). *whiB6* expression in the Δ*eccCb$_1$*

strain was significantly reduced compared to the WT strain (*P*<.0001*)* due to a loss

of the ESX-1 membrane complex (20). In contrast, *whiB6* expression in the Δ*espN*

and Δ*espN*/*pespN* strains was stochastic and not significantly different from the

WT strain. These findings were confirmed using western blot analysis on *M.*

marinum cell-associated proteins (Fig. 2B). We detected WhiB6-Fl protein in the

WT strain (Fig. 2B, lane 1). Consistent with our previous data, deletion of the

eccCb₁ gene resulted in a loss of detectable WhiB6-Fl protein (Fig. 2B, lane 2).

The levels of WhiB6-Fl protein in the Δ*espN* strain or the Δ*espN*/p*espN*

complementation strains were similar to the WT strain (Fig. 2B, lanes 3 and 4).

From these data, we conclude that there was no EspN-dependent change in *whiB6*

expression in *M. marinum* under the conditions tested.

We identified both EspM, the repressor of *whiB6* expression, and EspN

using DNA affinity chromatography with the same *whiB6* promoter DNA (22). While

we identified EspM specifically bound to the *whiB6* promoter following incubation

with WT *M. marinum* lysate, the EspN protein was only specifically bound to the

whiB6 promoter following incubation with Δ*espM* lysate (22). We hypothesized that

EspN may regulate *whiB6* expression in the absence of and in opposition to the

EspM repressor. To test this hypothesis, we deleted the *espN* gene in a Δ*espM M.*

marinum strain. We also introduced an integrating plasmid constitutively

expressing the *espN* gene into the Δ*espM* strain. We confirmed the loss and

restoration of *espN* expression using qRT-PCR (Fig. S2B). The *espN* transcript

was absent from the Δ*espM*Δ*espN* strain, and significantly increased in the

Δ*espM/pespN* strain (~60-80-fold, *P*<.0001). We performed qRT-PCR to quantify

changes in *whiB6* transcript levels in the *espN* deletion and overexpression strains

compared to the Δ*espM* strain (Fig. 2C). *whiB6* was expressed in the WT strain

and was significantly reduced in the Δ*eccCb₁* strain relative to the WT strain

(*P*<.0001) (20, 22). *whiB6* expression was significantly increased in the Δ*espM*

strain (*P*<.0001), consistent with the loss of the EspM repressor (22). Both genetic

deletion and overexpression of the *espN* gene in the Δ*espM* strain resulted in a

significant reduction in *whiB6* transcript levels compared to Δ*espM* strain (*P*=.0002

and *P*=.0009*)*. The levels of *whiB6* in both strains were also significantly reduced

compared to the WT strain (*P*<.0001). We interpret these data to mean that

overexpression of *espN* in the Δ*espM* strain results in a loss of EspN function,

similar to the deletion strain (32). Moreover, we conclude that EspN is required for

the elevated levels of WhiB6 in the Δ*espM* strain.

To confirm these findings, we performed a western blot analysis on cell-

associated fractions of *M. marinum* mutants to detect changes in WhiB6 protein

(Fig. 2D). Similar to Figure 2B and our previous work, the WhiB6-Fl protein was

produced to detectable levels in the WT strain and reduced in the Δ*eccCb₁* strain

(lanes 1 and 2, Fig. 2D). WhiB6-Fl protein levels were increased in the Δ*espM*

strain (lane 3), consistent with the role of EspM as a *whiB6* repressor. Deletion or

overexpression of *espN* in the absence of EspM resulted in a loss of WhiB6-Fl

protein (lanes 4 and 5, Fig. 2D), consistent with the loss of *whiB6* expression in

Fig. 2C. Together, these data suggest that EspN activates the expression of *whiB6*

in the absence of EspM (Fig. 2E), working in opposition to EspM at the *whiB6*

promoter. Moreover, overexpression of the *espN* gene results in a loss of function

of EspN in the absence of EspM.

EspN is required for ESX-1 dependent lytic activity. *M. marinum* exhibit

contact-dependent hemolytic activity that requires the ESX-1 system. To test if

EspN was required for ESX-1 function in the absence of EspM, we measured the

membranolytic activity of the relevant *M. marinum* strains by measuring sheep red

blood cell (sRBC) lysis (Fig. 3A). Water and PBS were "no bacteria" controls. They

resulted in total sRBC lysis and baseline sRBC lysis as measured by OD_{405},

respectively (Fig. 3A). *M. marinum* lysed sRBCs in an ESX-1-dependent manner

($\Delta eccCb_1$ vs WT, $P<.0001$). Deletion or overexpression of the *espN* gene did not

significantly impact the hemolytic activity of *M. marinum* relative to the WT strain.

From these data we conclude that EspN is dispensable for ESX-1-mediated

hemolysis in the presence of EspM. The $\Delta espM$ strain lysed sRBCs to a slightly

lower level than the WT strain ($P=.0122$). The deletion or overexpression of *espN*

in the $\Delta espM$ strain abrogated hemolysis ($P<.0001$), similar to the $\Delta eccCb_1$ strain.

From these data, we conclude that EspN is essential for the lytic activity of ESX-1

in the absence of EspM.

EspN is a transcriptional activator of genes encoding for ESX-1

components and substrates. We sought to determine why EspN was necessary

for ESX-1 hemolytic activity. WhiB6 regulates the expression of ESX-1 substrates

(20, 23, 24). If EspN was essential for lytic activity solely because EspN activated

whiB6 expression, then we would expect that the hemolytic activity of the

$\Delta espM\Delta espN$ strain would phenocopy the $\Delta whiB6$ strain. Consistent with our

previous findings, the $\Delta whiB6$ strain retained hemolytic activity (20). These data

indicate that the loss of WhiB6 alone in the $\Delta espM\Delta espN$ strain is insufficient to

explain the loss of hemolytic activity.

We reasoned that the loss of hemolytic activity could be due to loss of ESX-

1 secretion (8). To test this idea, we performed western blot analysis to measure

the production and secretion of two ESX-1 substrates, EsxB and EspE. EsxB is

secreted with EsxA as an early substrate of the ESX-1 system, and is likely a

secreted component of the ESX-1 system in the mycolate outer membrane (7, 11).

The loss of EsxB results in a loss of hemolytic activity because it abrogates

secretion of the ESX-1 substrates (8, 33). EspE is a late substrate of the ESX-1

system (11). The loss of EspE results in a loss of hemolytic activity, but does not

impact the secretion of other ESX-1 substrates, except EspF (11, 33). As shown

in Figure 3B, EsxB and EspE were produced in the WT strain (lane 1, αEsxB,

αEspE) and secreted into the culture supernatant during *in vitro* growth (lane 9).

EsxB and EspE proteins were reduced in the Δ*eccCb₁* strain (lane 2), due to

repression of *whiB6* expression by EspM (22). Neither protein was secreted into

the culture supernatant (lane 10, Fig. 3B) due to a loss of the ESX-1 membrane

complex. Both EspE and EsxB were produced (lane 3, Fig. 3B) and secreted from

the Δ*espM* strain (lane 11, Fig. 3B), consistent with our prior findings (22). Deletion

or overexpression of the *espN* gene in the Δ*espM* strain resulted in a loss of EspE

production (lanes 4 and 5, Fig. 3B). Although EsxB was produced in both strains,

the secretion of EsxB was reduced compared to the WT and Δ*espM* strains (lanes

12 and 13, Fig. 3B).

Reduced EsxB secretion could be due to reduced levels of the ESX-1

membrane complex. EccCb₁ is a cytoplasmic component of the ESX-1 system that

directly interacts with the membrane components (7). The loss of EccCb₁ results

in a loss of the entire ESX-1 membrane complex (20). We measured EccCb₁ levels

by western blot analysis (Fig. 3B). EccCb₁ protein was detected in the cell-

associated proteins from the WT strain and absent from the Δ$eccCb_1$ strain (lanes

1 and 2, lower band, Fig. 3B). The Δ$espM$ strain produced EccCb$_1$ protein (lane 3,

Fig. 3B). In the absence of EspM and EspN, EccCb$_1$ protein levels were reduced

to levels lower than the WT strain (lanes 4 and 5, Fig. 3B).

Based on the findings in Figure 3B, we tested if EspN regulated the

expression of *espE* or the membrane complex in the absence of EspM. To

determine if loss of EspE protein was due to decreased *espE* expression, we

performed qRT-PCR (Fig. 3C). Deletion of the *eccCb₁* gene led to a significant

reduction in *espE* expression relative to the WT strain (P<.0001), consistent with

Fig. 4A and our previous findings (22, 34). Deletion of *espM* significantly increased

espE gene expression relative to the WT strain (P=.0010)(22). These data confirm

that EspM represses the expression of the *espE* gene in *M. marinum,* consistent

with our prior RNA sequencing results (22)*.* Deletion or overexpression of *espN* in

the Δ*espM* strain resulted a complete loss of *espE* gene expression, significantly

lower than both the Δ*espM* (P<.0001) and the WT strains (P<.0001). From these

data we conclude that EspN is required for expression of *espE* in the Δ*espM* strain

(Fig. 3E).

WhiB6 positively regulates *espE* gene expression. Importantly, deletion of

the *whiB6* gene resulted in a significant reduction (P=.0003), but not a loss of the

espE transcript. Therefore, the loss of WhiB6 from the Δ*espM*Δ*espN* strain was

insufficient to cause the measured loss of *espE* expression.

The *eccCb₁* gene is downstream of genes that encode for three other ESX-

1 components (Fig. 3D, *eccA, eccB, eccCa₁).* It is not known if the *ecc* genes in

the ESX-1 locus are operonic. To test if the observed loss of $EccCb_1$ protein was

due to reduced expression of the *ecc* genes, we measured *eccA* expression using

qRT-PCR (Fig. 3D). We measured a significant reduction in the levels of *eccA* in

the $\Delta eccCb_1$ strain as compared to the WT strain ($P<.0001$). Deletion of *espM* led

to a significant increase in *eccA* levels compared to the WT strain ($P=.0012$).

Together, the reduction of *eccA* levels in the $\Delta eccCb_1$ and the increased levels of

eccA in the $\Delta espM$ strain suggest that EspM represses expression of the *eccA*

gene (Fig. 3E). The deletion or overexpression of the *espN* gene in the $\Delta espM$

strain significantly reduced *eccA* expression as compared to the $\Delta espM$ ($P<.0001$)

and the WT strains ($P<.0001$). Interestingly, the levels of *eccA* transcript in the

$\Delta whiB6$ strain, although significantly reduced from the $\Delta espM$ strain ($P=.0026$),

were not significantly different from the WT strain. From these data, we conclude

that EspN activates *eccA* gene expression and EspM represses *eccA* gene

expression. Moreover, the regulation of *eccA* by EspN is independent of WhiB6

(Fig. 3E).

The EspM N-terminus post-transcriptionally regulates EspN. Our findings

suggest that EspN directly activates *whiB6* expression, and is either a direct or

indirect activator of *espE* and *eccA* expression. Importantly, our data show that

EspN activates ESX-1-gene expression only in the absence of EspM (Fig. 3E).

Therefore, we sought to understand how EspN was connected to our established

ESX-1 transcriptional network (Fig. 1A). We first tested if EspM and EspN regulate

each other at the transcriptional level. We measured the expression of the *espN*

gene in the $\Delta espM$ strain, and the *espM* gene in the $\Delta espN$ strain (Fig. S4A and

B). We did not observe any significant change in *espN* expression in the Δ*espM*

strain, or in *espM* expression in the Δ*espN* strain. From these data, we conclude

that EspM and EspN do not regulate each other transcriptionally under the

conditions tested.

The EspM protein consists of 4 putative domains, including an N-terminal

fork-head associated domain (FHA) and two helix-turn-helix (HTH) domains

separated by a helical bundle domain (Fig. 4A) (35). The FHA domain alone

(EspM$_{NT}$ in Fig. 4A) does not bind DNA *in vitro* (22). The two HTH domains with

the helical bundle (EspM$_{CT}$ in Fig. 4A) are sufficient to bind the *whiB6* promoter *in*

vitro and to repress *whiB6* expression in *M. marinum* (22, 35)*.* To further

understand the functions of the EspM domains, we expressed the *espM, espM$_{NT}$*

and *espM$_{CT}$* genes including a C-terminal V5 epitope tag in the Δ*espM* strain. All

EspM-V5 proteins were expressed in *M. marinum* (lanes 4-6, Fig. 4A)*.* Both the

EspM full-length and EspM$_{CT}$ proteins resulted in multiple bands as detected by

the αV5 antibody, consistent with N-terminal processing events. The EspM$_{NT}$

protein resulted in a single size appropriate band (lane 5, Fig. 4A). Consistent with

our prior findings, expression of the EspM-V5 protein reduced the WhiB6-Fl protein

and significantly reduced the *whiB6* transcript relative to the Δ*espM* strain (lane 4

vs 5, Fig. 4B, and Fig. S5A) (35). Expression of the EspM-V5 protein also resulted

in reduced EccCb$_1$, EspE, and EsxB protein levels (lane 4) compared to the Δ*espM*

strain. We measured significant reductions in the expression of the *eccA* and *espE*

genes (Fig. 4B and Fig. S5B and C). Expression of the *espM$_{CT}$* gene resulted in a

loss of detectable WhiB6-Fl protein and further reductions of the EccCb$_1$, EspE

and EsxB proteins compared to the ΔespM and ΔespM/pespMV5 strains (lanes 6

vs 3 and 4, Fig. 4A). Likewise, we measured significant reductions in the levels of

whiB6, eccA and *espE* gene expression relative to the Δ*espM* strain. Interestingly,

expression of the *espM$_{NT}$* gene in the Δ*espM* strain resulted in a loss of EspE

protein and a reduction of the WhiB6-Fl and EccCb$_1$ protein as compared to both

the Δ*espM* and the WT strains. Although the EspM$_{NT}$ does not bind the *whiB6*

promoter *in vitro* (22), we measured significant reductions in the expression of the

whiB6, espE, and *eccA* genes compared to the Δ*espM* and complemented strains

(Fig. 4B and Fig. S5). None of the strains tested exhibited significant reductions in

espN expression.

These data suggested that the Δ*espM/pespM$_{NT}$*-V5 strain effectively

phenocopied the Δ*espM*Δ*espN* strain (Fig. 3B). To test if the Δ*espM/espM$_{NT}$-V5*

strain further phenocopied the Δ*espM*Δ*espN* strain, we performed sRBC lysis

assays on the strains in Figures 4A and 4B. We reasoned that if the

Δ*espM/pespM$_{NT}$*-V5 phenocopied the Δ*espM*Δ*espN* strain, it would be non-

hemolytic (Fig. 3A). Expression of the *espM-V5* or *espM$_{CT}$-V5* genes in the Δ*espM*

strain did not result in a significant change in hemolytic activity (Fig. 4B). However,

expression of the *espM$_{NT}$-V5* abrogated hemolytic activity of the Δ*espM* strain

(*P<.0001*). From these data, we conclude that the expression of the EspM$_{NT}$

protein phenocopies the loss of the EspN protein in the Δ*espM* strain. Together

our data support a model by which the EspM FHA domain negatively regulates

EspN post-transcriptionally.

EspN is required during *M. marinum* infection of macrophages and

zebrafish. Our data support a model in which EspM and EspN function in

opposition to regulate WhiB6 and the ESX-1 system (Fig. 3E). EspM represses

whiB6 expression in the absence of the ESX-1 system, and the genes encoding

ESX-1 components (22). In the absence of EspM, our data support that EspN

activates *whiB6* and ESX-1 gene expression. However, under *in vitro* growth

conditions, the presence of EspM obscures the regulatory role of EspN (Fig. 2).

We previously found that EspM was dispensable for *M. marinum* virulence in a

macrophage model (22). Therefore, we hypothesized that there may be signals

governing the switch between EspM and EspN in the host that may be absent

during *in vitro* growth.

M. marinum cause macrophage cytolysis in an ESX-1-dependent manner.

Strains lacking the ESX-1 system are retained in the phagosome and are non-

cytolytic (4). We infected with *M. marinum* (MOI of 4) and imaged 24 hours post-

infection (hpi) to measure macrophage cytotoxicity using ethidium homodimer

(EthD-1) staining (Fig. 5A). EthD-1 is a membrane-impermeable fluorescent

molecule that specifically stains DNA in cells with permeabilized cell membranes,

reflecting the cytolytic activity of *M. marinum* (8, 36) . As shown in Figure 5A,

infection with WT *M. marinum* resulted in macrophage cytotoxicity. Uninfected

macrophages and macrophages infected with the Δ*eccCb$_1$* strain exhibited

significantly less cytotoxicity than infection with the WT strain (*P*<.00001). The

Δ*espN* strain showed a statistically significant decrease in macrophage cytotoxicity

compared to the WT strain, similar to the Δ*eccCb$_1$* ESX-1-compromised mutant

strain ($P<.0001$). Cytotoxicity was partially complemented by overexpressing *espN*

in the Δ*espN* strain ($P<.0001$). The Δ*espM*Δ*espN* and Δ*espM*/p*espN* strains were

non-cytolytic, similar to the Δ*eccCb₁* strain (Fig. S6).

To test if *espN* was required for mycobacterial growth in macrophages, we

performed CFU analysis (Fig. 5B). As published previously, the WT *M. marinum*

strain grew within macrophages during the course of the infection. In contrast, the

Δ*eccCb₁* strain was attenuated for growth in macrophages, and resulted in CFUs

that were significantly different from the WT strain at 72 and 96 hpi. Consistent

with the cytotoxicity data, the Δ*espN* strain was attenuated for growth in the

macrophage as compared to the WT strain, similar to the Δ*eccCb₁* strain.

Strikingly, expression of the *espN* gene restored growth of *M. marinum* similar to

the WT strain. From these data we conclude that the ESX-1 transcriptional network

and EspN are required for *M. marinum* growth and cytotoxicity during macrophage

infection.

We next tested if EspN was important for animal infection. To examine the

in vivo consequences of EspN deletion, we made constitutively fluorescent

versions of the WT, Δ*espN,* and complemented *M. marinum* strains. Using the

zebrafish larval model of mycobacterial infection, we monitored infection burden

over five days of infection as measured by a validated Fluorescence Pixel Count

(FPC) assay (Fig. 5C and 5D) (37, 38). Bacterial burden was substantially reduced

at five days post-infection. We found that *in vivo*, Δ*espN* had 3.4-fold reduced

burden compared to WT at five days post-infection ($P=.00012$) and was

complemented by constitutive expression of the *espN* gene. (Fig. 5C and 5D).

From these data, we conclude that EspN is required during zebrafish infection by

M. marinum.

Discussion

Our prior studies hinted at the existence of an additional activator of the ESX-1

system (22). We used our knowledge of a negative regulator of ESX-1 expression,

EspM, as a tool to dissect the genetic circuitry that regulates the ESX-1 regulatory

network *in vivo*, arriving at a novel ESX-1 regulator that specifically functions in the

host. We discovered and characterized EspN, an infection-dependent

transcriptional regulator of the ESX-1 system. Our data supports that EspN and

EspM function as a switch that regulates expression of both the ESX-1 secretion

machinery and specific substrates. In the absence of the ESX-1 system, EspM

represses the expression of the *whiB6* gene as well as genes encoding the ESX-

1 components (EccA and others) and substrates. The result is reduced substrate

and component gene expression in the absence of the secretory machinery,

preventing substrate accumulation (20, 22). EspM also directly or indirectly

regulates additional genes in *M. marinum* (22). In the presence of the ESX-1

system, WhiB6 activates the expression of the ESX-1 substrate genes and other

genes, allowing production of substrates during active secretion (20, 23, 24).

The presence of EspM masked a role for EspN-dependent regulation of

ESX-1 genes *in vitro*. In the absence of EspM, EspN is essential for ESX-1 activity.

We showed that EspN is required for expression of the *espE* gene and contributes

to the expression of the *eccA* gene, allowing optimal production of the ESX-1

membrane complex and secretion of the ESX-1 substrates. Unlike deletion of

espM or *whiB6* (20, 22), deletion of *espN* attenuated the cytolytic activity and

growth of *M. marinum* during macrophage infection, suggesting that, during

infection, the transcriptional balance regulating the ESX-1 network is altered in the

absence of EspN. Moreover, deletion of both *espM* and *espN* from *M. marinum*

resulted in a loss of cytolytic activity during macrophage infection. EspN was

likewise necessary for robust infection in a zebrafish *in vivo* model. Together, these

data demonstrate that the ESX-1 transcriptional network is required during

infection and identify EspN as a novel and critical regulator of this network.

Our data demonstrate that the ESX-1 system is differentially regulated

during *in vitro* growth and in the host. We propose that under *in vitro* growth

conditions, EspM represses expression of the ESX-1 system. Therefore, the

molecular mechanisms governing protein secretion by ESX-1 have been defined

primarily under conditions in which ESX-1 gene expression is repressed. Further

understanding of EspN could allow us to study ESX-1 under induced conditions in

the laboratory, potentially allowing the identification of new secretory components

or substrates.

Our data support that EspN and EspM comprise a genetic switch that is

functionally operant early in infection, including *in vivo* during zebrafish infection,

where only innate immunity is present. While we do not yet understand the

molecular nature of the switch, EspM and EspN likely cannot occupy the *whiB6,*

eccA or *espE* promoters at the same time. Moreover, it is likely that a host-specific

signal may regulate the switch including oxidative stress, pH and other

phagosomal cues. Interestingly, *espN* is divergently transcribed from a putative

methyltransferase gene (*MMAR_1627*) (27). We do not yet know if the genes

surrounding *espN* in *M. marinum* are important for regulation of ESX-1.

Interestingly, methylation has been shown to control genetic switches in bacteria

(39, 40).

Expression of the ESX-1 system under *in vitro* conditions varies in diverse

strains (24). Consistent with our model for a host-induced activation of ESX-1

genes by EspN, ESX-1 gene expression is upregulated in the host (41). The

differential regulation of virulence in the host was suggested using transcriptomic

studies in *M. marinum* in a variety of environments (42). The differential regulation

of protein secretion systems under laboratory conditions and in the host is a

common theme in protein secretion in bacterial pathogens. For example, some

clinical *Vibrio cholerae* strains have active Type VI systems under laboratory

conditions, while the pandemic strains only activate their Type VI systems in the

host (43). The specialized Type III secretion systems in *Vibrio* species are

regulated by bile salts (44), and by Ca^{2+} and host cell contact in *Pseudomonas*

(45-47).

One interpretation of our findings is that EspN activity or *espN* expression

is responsive to host-specific signals. Notably, there is evidence of infection-

dependent regulation of *M. tuberculosis espN* (Rv1725c). Transcriptional profiling

of bacteria from infected macrophages isolated from mouse infections revealed

significant upregulation of *espN* transcript compared to broth-grown culture (48).

At an early timepoint one week post infection using a mouse intravenous infection

model, a TnSeq screen identified a ~2- fold decrease in representation of

transposon insertions in *M. tuberculosis Rv1725c* [*P*=.0039, (49)].   However, at

later timepoints (four weeks and eight weeks) this effect was no longer present

(49, 50). These results and our work with *M. marinum* suggest complex regulatory

circuits and genetic requirements operate at discrete stages of infection. It is also

possible that EspN may be differentially localized in the mycobacterial cell in

response to the host environment. SCP2 domains mediate interaction with

membranes rich in acidic phospholipids (51, 52), and may serve to mediate EspN

interaction with the mycobacterial cell membrane under specific conditions. SCP2

domains also mediate lipid transfer of sterols such as cholesterol and fatty acids

(53, 54), which are an energy source for mycobacterial pathogens during infection

(55, 56). Experiments focused on the C-terminal SCP-2 domain may reveal how

EspN senses and responds to the host environment.

Alternatively, host-specific cues may remove the EspM repressor from the

whiB6 promoter. Our data suggests that EspM regulates EspN post-

transcriptionally through its N-terminus, which contains an FHA domain. Proteins

with FHA domains regulate other Gram-negative secretion systems post-

transcriptionally (57-59). Our data also support that EspM may be processed *in*

vitro. EspM cleavage might remove it from the promoter, similar to the cI repressor

from λ phage (60). Alternatively, cleavage may liberate the N-terminus to regulate

EspN activity, allowing the EspM C-terminus to bind DNA and repress gene

expression.

Interestingly, EspN is required during infection while EspM is dispensable.

EspN-dependent regulation of ESX-1 may be essential for virulence. Alternatively,

EspN could regulate additional genes important for mycobacterial pathogenesis.

Defining the regulatory targets of EspN, and how the EspM and EspN regulons

compare, will likely require measuring global gene expression during specific

stages of infection.

We were surprised that the overexpression or deletion of *espN* in the

absence of *espM* resulted in the same phenotypes in *M. marinum.* It is well-

established that genetic overexpression can result in loss of function phenotypes

through a variety of molecular mechanisms (32). For example, *espN*

overexpression could result in EspN aggregation or mislocalization. Transcription

factor multimerization is well described as a regulatory mechanism in bacterial

gene expression (60, 61). However, in higher organisms, transcription factors can

form aggregate-like bodies that serve as functional regulatory mechanisms which

mimic loss of function (62). If EspN forms multimeric aggregates when the *espN*

gene is overexpressed, aggregation may prevent the interaction with DNA that

mediates transcriptional activation. Alternatively, EspN may be mislocalized under

overexpression conditions. Regardless of this mechanism, the same *M. marinum*

strain (Δ*espN*/p*espN*) exhibited a loss of *espN* function under laboratory conditions

and complementation of *espN* function in macrophages and animals. These data

further support that EspN activity responds to host specific signals.

Other transcription factors regulate the ESX-1 system; however, to our

knowledge, EspN is the first ESX-1 regulator that specifically functions in the host.

The known transcription factors regulate ESX-1 substrates, not the ESX-1

components. Several transcription factors regulate *whiB6* and the *espACD* operon

which encodes secreted substrates essential for ESX-1 secretion in *M.*

tuberculosis (63-66). PhoPR and MprAB regulate *whiB6* expression directly and

through the EspR regulator (67-71), in response to pH or stress, both of which are

important for survival in the acidified phagosome (72-74). However, the *espACD*

operon is dispensable for ESX-1 secretion and pathogenesis in *M. marinum,* and

regulation of *whiB6* by PhoPR has not been reported (11, 75). Although this may

suggest divergence in ESX-1 regulation between *M. marinum* and in *M.*

tuberculosis, the ESX-1 transcriptional network is conserved (20, 21, 76).

Widespread ESX-1-dependent gene expression has been reported in both

mycobacterial species (20, 21, 76). Moreover, we showed that EspM and the

regulatory substrates, EspE and EspF, are functionally conserved in *M.*

tuberculosis (22, 33). Future research will be aimed at defining the ESX-1

transcriptional network in *M. tuberculosis.*

Overall, this study further defined the regulatory network underlying control

of the ESX-1 secretion system and demonstrated its importance during infection.

We have, for the first time, identified an infection-dependent transcriptional

activator responsible for regulating both the ESX-1 components and substrates.

Our study will serve as a foundation for understanding the molecular complexities

of ESX-1 regulation in the host. We are now poised to define the molecular

mechanisms underlying how the ESX-1 system senses and responds to a

changing host environment

Materials and Methods

Bacterial strains were derived from the *M. marinum* M parental strain (ATCC BAA-

535), and were maintained as previously described (20, 22, 33, 77). Nomenclature

follows the conventions proposed by Bitter et al (29). Genetic deletions were

performed using allelic exchange as previously described (20, 22, 33, 77, 78).

Hemolytic activity was measured against sRBCs as described previously (20, 22,

33, 77). Cell-associated and secreted mycobacterial proteins were isolated and

analyzed as described in (77, 79). Protein levels were measured using western

blot analysis. RNA extraction was performed from *M. marinum* using the Qiagen

RNeasy kit, followed by qRT-PCR relative to the levels of *sigA* as described

previously (22, 35). Macrophage (RAW 264.7) cytotoxicity was measured using

Ethidium homodimer uptake following infection by *M. marinum* as described in (34,

77). Mycobacterial CFUs were performed similarly to (79). Protein modeling was

performed using Robetta and Pfam as indicated. Zebrafish larvae infections with

M. marinum were performed and bacterial burden was measured using fluorescent

pixel counts as (37). Statistical analysis was performed using GraphPad Prism v.

9 or R within the latest version of R Studio IDE. Detailed methods are in the

Supplementary Material.

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References

1. E. R. Green, J. Mecsas, Bacterial Secretion Systems: An Overview. *Microbiol Spectr* **4**
(2016).
2. K. R. Nicholson, P. A. Champion, Bacterial secretion systems: Networks of pathogenic
regulation and adaptation in mycobacteria and beyond. *PLoS Pathog* **18**, e1010610
(2022).
3. P. S. Manzanillo, M. U. Shiloh, D. A. Portnoy, J. S. Cox, Mycobacterium Tuberculosis
Activates the DNA-Dependent Cytosolic Surveillance Pathway within Macrophages. *Cell*
Host Microbe **11**, 469-480 (2012).
4. R. Simeone *et al.*, Phagosomal rupture by Mycobacterium tuberculosis results in toxicity
and host cell death. *PLoS Pathog* **8**, e1002507 (2012).
5. D. Houben *et al.*, ESX-1-mediated translocation to the cytosol controls virulence of
mycobacteria. *Cell Microbiol* **14**, 1287-1298 (2012).
6. D. M. Tobin, L. Ramakrishnan, Comparative pathogenesis of Mycobacterium marinum
and Mycobacterium tuberculosis. *Cell Microbiol* **10**, 1027-1039 (2008).
7. S. A. Stanley, S. Raghavan, W. W. Hwang, J. S. Cox, Acute infection and macrophage
subversion by Mycobacterium tuberculosis require a specialized secretion system. *Proc*
Natl Acad Sci U S A **100**, 13001-13006 (2003).
8. L. Y. Gao *et al.*, A mycobacterial virulence gene cluster extending RD1 is required for
cytolysis, bacterial spreading and ESAT-6 secretion. *Mol Microbiol* **53**, 1677-1693 (2004).
9. K. M. Guinn *et al.*, Individual RD1-region genes are required for export of ESAT-6/CFP-
10 and for virulence of Mycobacterium tuberculosis. *Mol Microbiol* **51**, 359-370 (2004).
10. E. N. Houben *et al.*, Composition of the type VII secretion system membrane complex.
Mol Microbiol **86**, 472-484 (2012).
11. R. M. Cronin, M. J. Ferrell, C. W. Cahir, M. M. Champion, P. A. Champion, Proteo-
genetic analysis reveals clear hierarchy of ESX-1 secretion in Mycobacterium marinum.
Proc Natl Acad Sci U S A **119**, e2123100119 (2022).
12. W. H. Conrad *et al.*, Mycobacterial ESX-1 secretion system mediates host cell lysis
through bacterium contact-dependent gross membrane disruptions. *Proc Natl Acad Sci U*
S A **114**, 1371-1376 (2017).
13. F. Sayes *et al.*, Multiplexed Quantitation of Intraphagocyte Mycobacterium tuberculosis
Secreted Protein Effectors. *Cell Rep* **23**, 1072-1084 (2018).
14. R. Wassermann *et al.*, Mycobacterium tuberculosis Differentially Activates cGAS- and
Inflammasome-Dependent Intracellular Immune Responses through ESX-1. *Cell Host*
Microbe **17**, 799-810 (2015).
15. R. O. Watson, P. S. Manzanillo, J. S. Cox, Extracellular M. tuberculosis DNA Targets
Bacteria for Autophagy by Activating the Host DNA-Sensing Pathway. *Cell* **150**, 803-815
(2012).
16. J. Smith *et al.*, Evidence for pore formation in host cell membranes by ESX-1-secreted
ESAT-6 and its role in Mycobacterium marinum escape from the vacuole. *Infect Immun*
76, 5478-5487 (2008).
17. N. van der Wel *et al.*, M. tuberculosis and M. leprae translocate from the phagolysosome
to the cytosol in myeloid cells. *Cell* **129**, 1287-1298 (2007).
18. E. D. Brutinel, T. L. Yahr, Control of gene expression by type III secretory activity. *Curr*
Opin Microbiol **11**, 128-133 (2008).

19. S. N. Joslin, D. R. Hendrixson, Activation of the Campylobacter jejuni FlgSR two-
component system is linked to the flagellar export apparatus. *J Bacteriol* **191**, 2656-2667
(2009).
20. R. E. Bosserman *et al.*, WhiB6 regulation of ESX-1 gene expression is controlled by a
negative feedback loop in Mycobacterium marinum. *Proc Natl Acad Sci U S A*
10.1073/pnas.1710167114 (2017).
21. A. M. Abdallah *et al.*, Integrated transcriptomic and proteomic analysis of pathogenic
mycobacteria and their esx-1 mutants reveal secretion-dependent regulation of ESX-1
substrates and WhiB6 as a transcriptional regulator. *PLoS One* **14**, e0211003 (2019).
22. K. G. Sanchez *et al.*, EspM Is a Conserved Transcription Factor That Regulates Gene
Expression in Response to the ESX-1 System. *mBio* **11** (2020).
23. Z. Chen *et al.*, Mycobacterial WhiB6 Differentially Regulates ESX-1 and the Dos Regulon
to Modulate Granuloma Formation and Virulence in Zebrafish. *Cell Rep* **16**, 2512-2524
(2016).
24. L. Solans *et al.*, A specific polymorphism in Mycobacterium tuberculosis H37Rv causes
differential ESAT-6 expression and identifies WhiB6 as a novel ESX-1 component. *Infect*
Immun **82**, 3446-3456 (2014).
25. R. D. Finn *et al.*, Pfam: the protein families database. *Nucleic Acids Res* **42**, D222-230
(2014).
26. C. UniProt, UniProt: the universal protein knowledgebase in 2021. *Nucleic Acids Res* **49**,
D480-D489 (2021).
27. A. Kapopoulou, J. M. Lew, S. T. Cole, The MycoBrowser portal: a comprehensive and
manually annotated resource for mycobacterial genomes. *Tuberculosis (Edinb)* **91**, 8-13
(2011).
28. S. A. Shiryev, J. S. Papadopoulos, A. A. Schaffer, R. Agarwala, Improved BLAST
searches using longer words for protein seeding. *Bioinformatics* **23**, 2949-2951 (2007).
29. W. Bitter *et al.*, Systematic genetic nomenclature for type VII secretion systems. *PLoS*
Pathog **5**, e1000507 (2009).
30. T. Parish, N. G. Stoker, Use of a flexible cassette method to generate a double unmarked
Mycobacterium tuberculosis tlyA plcABC mutant by gene replacement. *Microbiology* **146**
(Pt 8), 1969-1975 (2000).
31. V. J. van Winden *et al.*, Mycosins Are Required for the Stabilization of the ESX-1 and
ESX-5 Type VII Secretion Membrane Complexes. *MBio* **7** (2016).
32. G. Prelich, Gene overexpression: uses, mechanisms, and interpretation. *Genetics* **190**,
841-854 (2012).
33. A. E. Chirakos, K. R. Nicholson, A. Huffman, P. A. Champion, Conserved ESX-1
substrates EspE and EspF are virulence factors that regulate gene expression. *Infect*
Immun 10.1128/IAI.00289-20 (2020).
34. E. A. Williams *et al.*, A Nonsense Mutation in Mycobacterium marinum That Is
Suppressible by a Novel Mechanism. *Infect Immun* **85** (2017).
35. K. G. Sanchez, R. J. Prest, K. R. Nicholson, K. V. Korotkov, P. A. Champion, Functional
Analysis of EspM, an ESX-1-Associated Transcription Factor in Mycobacterium marinum.
J Bacteriol 10.1128/jb.00233-22, e0023322 (2022).
36. G. M. Kennedy, G. C. Hooley, M. M. Champion, F. M. Medie, P. A. Champion, A novel
ESX-1 locus reveals that surface associated ESX-1 substrates mediate virulence in
Mycobacterium marinum. *J Bacteriol* **196**, 1877-1888 (2014).
37. K. Takaki, J. M. Davis, K. Winglee, L. Ramakrishnan, Evaluation of the pathogenesis and
treatment of Mycobacterium marinum infection in zebrafish. *Nat Protoc* **8**, 1114-1124
(2013).
38. J. W. Saelens *et al.*, An ancestral mycobacterial effector promotes dissemination of
infection. *Cell* https://doi.org/10.1016/j.cell.2022.10.019 (2022).
39. H. N. Lim, A. van Oudenaarden, A multistep epigenetic switch enables the stable
inheritance of DNA methylation states. *Nat Genet* **39**, 269-275 (2007).
40. I. Cota *et al.*, OxyR-dependent formation of DNA methylation patterns in OpvABOFF and
OpvABON cell lineages of Salmonella enterica. *Nucleic Acids Res* **44**, 3595-3609 (2016).

41. P. Aiewsakun *et al.*, Transcriptional response to the host cell environment of a multidrug-
resistant Mycobacterium tuberculosis clonal outbreak Beijing strain reveals its pathogenic
features. *Sci Rep* **11**, 3199 (2021).
42. E. M. Weerdenburg *et al.*, Genome-wide transposon mutagenesis indicates that
Mycobacterium marinum customizes its virulence mechanisms for survival and replication
in different hosts. *Infect Immun* **83**, 1778-1788 (2015).
43. S. T. Miyata *et al.*, The Vibrio Cholerae Type VI Secretion System: Evaluating its Role in
the Human Disease Cholera. *Front Microbiol* **1**, 117 (2010).
44. P. Li *et al.*, Bile salt receptor complex activates a pathogenic type III secretion system.
Elife **5** (2016).
45. E. A. Williams McMackin, L. Djapgne, J. M. Corley, T. L. Yahr, Fitting Pieces into the
Puzzle of Pseudomonas aeruginosa Type III Secretion System Gene Expression. *J*
Bacteriol **201** (2019).
46. D. W. Frank, The exoenzyme S regulon of Pseudomonas aeruginosa. *Mol Microbiol* **26**,
621-629 (1997).
47. J. Kim *et al.*, Factors triggering type III secretion in Pseudomonas aeruginosa.
Microbiology (Reading) **151**, 3575-3587 (2005).
48. D. Pisu, L. Huang, J. K. Grenier, D. G. Russell, Dual RNA-Seq of Mtb-Infected
Macrophages In Vivo Reveals Ontologically Distinct Host-Pathogen Interactions. *Cell*
Rep **30**, 335-350 e334 (2020).
49. C. M. Sassetti, E. J. Rubin, Genetic requirements for mycobacterial survival during
infection. *Proc Natl Acad Sci U S A* **100**, 12989-12994 (2003).
50. C. M. Smith *et al.*, Host-pathogen genetic interactions underlie tuberculosis susceptibility
in genetically diverse mice. *Elife* **11** (2022).
51. H. Huang, J. M. Ball, J. T. Billheimer, F. Schroeder, The sterol carrier protein-2 amino
terminus: a membrane interaction domain. *Biochemistry* **38**, 13231-13243 (1999).
52. E. S. Liedhegner, C. D. Vogt, D. S. Sem, C. W. Cunningham, C. J. Hillard, Sterol carrier
protein-2: binding protein for endocannabinoids. *Mol Neurobiol* **50**, 149-158 (2014).
53. H. Y. Lin, M. Y. Pang, M. G. Feng, S. H. Ying, A peroxisomal sterol carrier protein 2
(Scp2) contributes to lipid trafficking in differentiation and virulence of the insect
pathogenic fungus Beauveria bassiana. *Fungal Genet Biol* **158**, 103651 (2022).
54. M. Galano, S. Venugopal, V. Papadopoulos, Role of STAR and SCP2/SCPx in the
Transport of Cholesterol and Other Lipids. *Int J Mol Sci* **23** (2022).
55. A. K. Pandey, C. M. Sassetti, Mycobacterial persistence requires the utilization of host
cholesterol. *Proc Natl Acad Sci U S A* **105**, 4376-4380 (2008).
56. W. Lee, B. C. VanderVen, R. J. Fahey, D. G. Russell, Intracellular Mycobacterium
tuberculosis exploits host-derived fatty acids to limit metabolic stress. *J Biol Chem* **288**,
6788-6800 (2013).
57. J. S. Lin *et al.*, TagF-mediated repression of bacterial type VI secretion systems involves
a direct interaction with the cytoplasmic protein Fha. *J Biol Chem*
10.1074/jbc.RA117.001618 (2018).
58. J. S. Lin *et al.*, Fha interaction with phosphothreonine of TssL activates type VI secretion
in Agrobacterium tumefaciens. *PLoS Pathog* **10**, e1003991 (2014).
59. M. Pallen, R. Chaudhuri, A. Khan, Bacterial FHA domains: neglected players in the
phospho-threonine signalling game? *Trends Microbiol* **10**, 556-563 (2002).
60. A. D. Johnson *et al.*, lambda Repressor and cro--components of an efficient molecular
switch. *Nature* **294**, 217-223 (1981).
61. A. Ramalinga, J. L. Danger, N. Makthal, M. Kumaraswami, P. Sumby, Multimerization of
the Virulence-Enhancing Group A Streptococcus Transcription Factor RivR Is Required
for Regulatory Activity. *J Bacteriol* **199** (2017).
62. Y. S. Huoh *et al.*, Dual functions of Aire CARD multimerization in the transcriptional
regulation of T cell tolerance. *Nat Commun* **11**, 1625 (2020).
63. J. M. Chen *et al.*, EspD Is Critical for the Virulence-Mediating ESX-1 Secretion System in
Mycobacterium tuberculosis. *J Bacteriol* **194**, 884-893 (2012).

64.　P. A. Champion, M. M. Champion, P. Manzanillo, J. S. Cox, ESX-1 secreted virulence
factors are recognized by multiple cytosolic AAA ATPases in pathogenic mycobacteria.
Mol Microbiol **73**, 950-962 (2009).
65.　S. M. Fortune *et al.*, Mutually dependent secretion of proteins required for mycobacterial
virulence. *Proc Natl Acad Sci U S A* **102**, 10676-10681 (2005).
66.　J. A. MacGurn, S. Raghavan, S. A. Stanley, J. S. Cox, A non-RD1 gene cluster is
required for Snm secretion in Mycobacterium tuberculosis. *Mol Microbiol* **57**, 1653-1663
(2005).
67.　G. Cao *et al.*, EspR, a regulator of the ESX-1 secretion system in Mycobacterium
tuberculosis, is directly regulated by the two-component systems MprAB and PhoPR.
Microbiology **161**, 477-489 (2015).
68.　S. B. Walters *et al.*, The Mycobacterium tuberculosis PhoPR two-component system
regulates genes essential for virulence and complex lipid biosynthesis. *Mol Microbiol* **60**,
312-330 (2006).
69.　V. Anil Kumar *et al.*, EspR-dependent ESAT-6 Protein Secretion of Mycobacterium
tuberculosis Requires the Presence of Virulence Regulator PhoP. *J Biol Chem* **291**,
19018-19030 (2016).
70.　B. Blasco *et al.*, Functional dissection of intersubunit interactions in the EspR virulence
regulator of Mycobacterium tuberculosis. *J Bacteriol* **196**, 1889-1900 (2014).
71.　S. Raghavan, P. Manzanillo, K. Chan, C. Dovey, J. S. Cox, Secreted transcription factor
controls Mycobacterium tuberculosis virulence. *Nature* **454**, 717-721 (2008).
72.　B. K. Johnson *et al.*, The Carbonic Anhydrase Inhibitor Ethoxzolamide Inhibits the
Mycobacterium tuberculosis PhoPR Regulon and Esx-1 Secretion and Attenuates
Virulence. *Antimicrob Agents Chemother* **59**, 4436-4445 (2015).
73.　J. Gonzalo-Asensio *et al.*, Evolutionary history of tuberculosis shaped by conserved
mutations in the PhoPR virulence regulator. *Proc Natl Acad Sci U S A* **111**, 11491-11496
(2014).
74.　J. J. Baker, B. K. Johnson, R. B. Abramovitch, Slow growth of Mycobacterium
tuberculosis at acidic pH is regulated by phoPR and host-associated carbon sources. *Mol*
Microbiol **94**, 56-69 (2014).
75.　A. E. Chirakos, A. Balaram, W. Conrad, P. A. Champion, Modeling Tubercular ESX-1
Secretion Using Mycobacterium marinum. *Microbiol Mol Biol Rev* **84** (2020).
76.　C. Sala *et al.*, EspL is essential for virulence and stabilizes EspE, EspF and EspH levels
in Mycobacterium tuberculosis. *PLoS Pathog* **14**, e1007491 (2018).
77.　R. E. Bosserman, K. R. Nicholson, M. M. Champion, P. A. Champion, A New ESX-1
Substrate in Mycobacterium marinum That Is Required for Hemolysis but Not Host Cell
Lysis. *J Bacteriol* **201** (2019).
78.　R. E. Bosserman, C. R. Thompson, K. R. Nicholson, P. A. Champion, Esx Paralogs Are
Functionally Equivalent to ESX-1 Proteins but Are Dispensable for Virulence in
Mycobacterium marinum. *J Bacteriol* **200**, e00726-00717 (2018).
79.　A. E. Chirakos, K. R. Nicholson, A. Huffman, P. A. Champion, Conserved ESX-1
Substrates EspE and EspF Are Virulence Factors That Regulate Gene Expression. *Infect*
Immun **88** (2020).
80.　M. Baek *et al.*, Accurate prediction of protein structures and interactions using a three-
track neural network. *Science* **373**, 871-876 (2021).
730
731

Figures and Tables

Figure 1. EspN is a putative transcription factor that binds the *whiB6* promoter A. Schematic
of the ESX-1 transcriptional network. In the presence of the ESX-1 system, WhiB6 is positively
autoregulated, and positively regulates the expression of the ESX-1 substrates, and other genes.
In the absence of the ESX-1 system, EspM directly represses *whiB6* gene expression, reducing
ESX-1 substrate levels. In the absence of WhiB6 and EspM, an unknown activator upregulates
whiB6 expression (?). **B.** Mass spectrometry analysis of the DNA affinity chromatography showing
enrichment of the MMAR_1626 and HupB proteins. The HupB protein binds nonspecifically to both
DNA probes. The scale represents the log2 intensity of MS peak arears. a.u. arbitrary units. The
data was published in Sanchez et al (22). **C.** The predicted domain structure of MMAR_1626, which
we renamed EspN. Modeled using RoseTTAFold from Robetta (80). Model confidence: 0.80.
wHTH: winged helix turn helix, SCP2: sterol carrier protein 2.
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Figure 2. EspN activates *whiB6* expression in the absence of EspM. A. Relative qRT-PCR analysis of *M. marinum* strains compared to *sigA* transcript levels. Statistical analysis was performed using a one-way ANOVA (* *P*=.0359) followed by a Dunnett's multiple comparisons test relative to the WT strain. Significance between the WT and Δ*eccCb₁* strain was determined using an unpaired students t-test. **** *P*<.0001. **B.** Western blot analysis of *M. marinum* cell-associated proteins. RpoB is a loading control. All strains include a *whiB6-3xFl* allele at the *whiB6* locus (20). **C.** qRT-PCR of the *whiB6* transcript relative to *sigA*. Significance was determined using a one-way ordinary ANOVA (*P*=.0001), followed by a Tukey's multiple comparison test. Significance shown is relative to the Δ*espM* strain, with additional statistics of interest discussed in the text. **** *P*<.0001, *** *P*=.0002 for Δ*eccCb1*, ** *P*=.0010, *** *P*=.0002 for Δ*espM*/p*espN*, *** *P*=.0009 for Δ*whiB6*. **D.** Western blot analysis of 10μg of *M. marinum* whole cell lysates. RpoB serves as a loading control. All strains include a *whiB6-3xFl* allele at the *whiB6* locus (20) **E**. Schematic summarizing findings from Figure 2.

Figure 3. EspN activates expression of ESX-1 components and substrates. A. Sheep red
blood cell lysis (sRBC) measuring hemolytic activity of *M. marinum.* Statistical analysis was
performed using a one-way ordinary ANOVA followed by a Dunnett's multiple comparisons test
relative to the WT strain. **** *P*<0.0001 *,* *** *P*=.0003. **B.** Western blot of 10µg of *M. marinum* cell-
associated proteins. All strains include a *whiB6-Fl* allele. RpoB is a control for lysis. MPT-32 is a
loading control for the secreted fractions. **C.** Relative qRT analysis the *espE* transcript compared
to *sigA* transcript levels in *M. marinum*. Statistical analysis was performed using a one-way ordinary
ANOVA (*P*<.0001) followed by a Tukey's multiple comparison test. Significance is shown relative
to the Δ*espM* strain. *** *P*=0.0010 (WT)*,* **** *P*<.0001*,* *** *P*=0.0003 (Δ*whiB6*). **D.** Relative qRT
analysis of the *eccA* transcript compared to *sigA* transcript levels in *M. marinum*. Statistical analysis
was performed using a one-way ordinary ANOVA (*P*<.0001) followed by a Tukey's multiple
comparison test. Significance shown relative to the Δ*espM* strain. ** *P*=.0012 (WT), *P*=.0026
(ΔwhiB6), **** *P*<.0001*,* *** *P*=0.0003. **E.** Schematic summarizing the results of Figure 3, and the
contributions of EspN, EspM and WhiB6 to regulating ESX-1 gene expression.
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Figure 4. The EspM N-terminus negatively regulates EspN. A. Western blot of *M. marinum* whole cell lysates. All strains include a *whiB6-Fl* allele. EspM complementation strains are tagged at the C-terminus with a V5 tag. RpoB is a loading control. EspE is an ESX-1 substrate. 10µg of

protein was loaded in each lane. **B.** Relative qRT-PCR analysis of *M. marinum* strains compared
to *sigA* transcript levels. The values were normalized to those in the ΔespM strain and plotted as
the log2 fold change relative to the ΔespM strain as a heat map. The qRT-PCR data plotted in this
heat map are shown in Fig. S5A-D. **C.** Sheep red blood cell lysis (sRBC) measuring hemolytic
activity of *M. marinum.* Statistical analysis was performed using a one-way ANOVA (*P*<.0001*)*
followed by a Dunnett's multiple comparisons test (**** *P*<.0001).
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Figure 5. EspN is required for pathogenesis A. Macrophage cytolysis as measured by EthD-1 staining 24h post-infection with *M. marinum* at an MOI of 4. Statistical analysis was performed using a one-way ANOVA followed by a Dunnett's multiple comparisons test relative to the WT strain. (**** *P*<.0001) Each dot represents the number of EthD-1-stained cells in a single field. A total of 10 fields were counted using ImageJ for each well. Processing of 3 wells was performed for each biological replicate. A total of 90 fields were counted for each strain. **B.** Colony forming units (CFU) of *M. marinum* strains MOI = 0.2. The data points are the average of three independent biological replicates. Significance was determined using an ordinary 2 way ANOVA (*P*<.0001) followed by a Tukey's multiple comparison test compared to the WT strain. The significance shown is compared to the WT strain at 96-hours post infection (*P*<.0001). The Δ*eccCb₁* and Δ*espN* strains were also significantly different from the WT strain at 72 hpi (*P*=.0021 and *P*=.0024, respectively). **C.** *M. marinum* burden in zebrafish infection measured using bacterial mCerulean fluorescence. Data are comprised of two biological replicates with 20-30 independent infections per replicate. Statistical analyses were performed using one-way ANOVA followed by a Tukey multiple comparison of each group to the WT strain (*** *P*=.00012; * *P*=.021). **D.** Representative images of zebrafish infected with an initial dose of 150-200 fluorescent bacilli for (1.) WT, (2.) Δ*espN*, or (3.) Δ*espN*/p*espN* at 5 days post-infection. Scale bar is 500µm.