SUPPORTING INFORMATION

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

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Table S1. Strains and plasmids used in this study

Name	Genotype	Reference
***M. marinum* strains**		
M	Wild-type *M. marinum*, parent strain	ATCC BAA-535
Δ*esxBA*	Deletion of *esxB* and *esxA*	Gift of Eric Brown (1)
ΔRD1	Δ$eccCb_1$-Δ*espK*	(2)
Δ$eccCb_1$	Deletion of $eccCb_1$	(3)
$eccCb_1$1*(Oc)*	M with $eccCb_1$1*(Oc)* allele integrated at *esx-1* locus	(3)
$eccCb_1$1*(Oc)*/$p_{MOPS}$$eccCb_1$	$eccCb_1$1*(Oc)*with $P_{MOP}$$eccCb_1$ integrated at *attB*	(3)
$eccCb_1$1*(Oc)*/p_{MOPS} $eccCb_1$1*(Oc)*	$eccCb_1$1*(Oc)* with p_{MOPS} $eccCb_1$1*(Oc)* integrated at *attB*	(3)
$eccCb_1$1*(Oc)*/p_{MOPS}*whiB6*	$eccCb_1$1*(Oc)*with p_{MOPs}*whiB6* integrated at *attB*	This study
$eccCb_1$1*(Oc)*/p*whiB6*	$eccCb_1$1*(Oc)* with p*whiB6* integrated at *attB*	This study
Δ*whiB6*	Deletion of *whiB6*	This study
Δ*whiB6*/p_{MOPS}*whiB6*	Δ*whiB6* with p_{MOPS}*whiB6* integrated at *attB*	This study
Δ*whiB6*/p*whiB6*	Δ*whiB6* with p*whiB6* integrated at *attB*	This study
Δ$eccCa_1$	Deletion of $eccCa_1$	This study
Δ$eccE_1$	Deletion of $eccE_1$	This study
whiB6-3xFL	M with *whiB6* allele tagged with 3X-FLAG at C-terminus	This study
whiB6-3xFL Δ$eccB_1$	*whiB6-3xFL* with deletion of $eccB_1$	This study
whiB6-3xFL Δ$eccCb_1$	*whiB6-3xFL* with deletion of $eccCb_1$	This study
whiB6-3xFL Δ$eccD_1$	*whiB6-3xFL* with deletion of $eccD_1$	This study

whiB6-3xFL Δ$eccE_1$	*whiB6-3xFL* with deletion of $eccE_1$	This study
whiB6-3X*FL* Δ$eccCb_1$/ p$_{MOPS}$$eccCb_1$	*whiB6-3xFL* with deletion of $eccCb_1$ and p$_{MOPS}$$eccCb_1$ integrated at *attB*	This study
whiB6-3X*FL* Δ$eccCb_1$/ p$_{MOPS}$$eccCb_1K90A$	*whiB6-3xFL* with deletion of $eccCb_1$ and p$_{MOPS}$$eccCb_1K90A$ integrated at *attB*	This study
Plasmids		
pMV306H	*int, hygR, oriE* empty vector	(4)
p*whiB6*	*MMAR_5437* with ~1kb upstream and 1kb downstream (6576114-6578335, *'5438-5436'*), *int, hygR*	This study
p2NIL	*kanR, ampR, oriE* empty vector	(5)
pGOAL19	*ampR, hygR, lacZ, sacB, oriE*	(5)
p2NIL-Δ*whiB6* GOAL	*MMAR_5437* flanking regions, *kanR, hygR, sacB, lacZ*	This study
p2NIL-*whiB6 3xFLAG* GOAL	*MMAR_5437* allele with 3' end 3xFLAG tag and flanking regions, *kanR, hygR, sacB, lacZ*	This study
p2NIL-Δ*esxBA* GOAL	*esxB* and *esxA* flanking regions, *kanR, hygR, sacB, lacZ*	This study
p2NIL-Δ$eccB_1$ GOAL	$eccB_1$ flanking regions, *kanR, hygR, sacB, lacZ*	This study
p2NIL-Δ$eccCa_1$ GOAL	$eccCa_1$ flanking regions, *kanR, hygR, sacB, lac*	This study
p2NIL-Δ$eccCb_1$ GOAL	$eccCb_1$ flanking regions, *kanR, hygR, sacB, lacZ*	(3)
p2NIL-Δ$eccD_1$ GOAL	$eccD_1$ flanking regions, *kanR, hygR, sacB, lacZ*	This study
p2NIL-Δ$eccE_1$ GOAL	$eccE_1$ flanking regions,	This study

	kanR, hygR, sacB, lacZ	
p2NIL-Δ*esxBA* GOAL	flanking regions of *esxB* and *esxA*, *kanR, hygR, sacB, lacZ*	This study
p_{MOPS}*MMAR_0039*	Parental plasmid used for PCR amplification of the p_{MOP} vector for use in plasmid construction	(6)
$p_{MOPS}eccCb_1$	$eccCb_1$ from *M. marinum* M behind the MOPS promoter	(3)
p_{MOPS}*whiB6*	*whiB6* from *M. marinum* M behind the MOPS promoter	This study
$p_{MOPS}eccCb_1K90A$	$eccCb_1$ from *M. marinum* M behind the MOPS promoter with K90A mutation in ATPase domain 2.	This study
p_{MOPS}*esxBA*	*esxB* and *esxA* from *M. marinum* M behind the MOPS promoter	This study
p_{MOPS}*esxBM98AesxA*	*esxB* with M98A mutation and wild type *esxA* from *M. marinum* M behind the MOPS promoter	This study

Table S2. Oligonucleotide primers

Primer	Sequence (5’→3’)	Application
ors219	TGGTGTCACGCTCGTGCGCGGACGATCAATTCTTC	Amplification of regions flanking the *whiB6* open reading frame (ORF), excluding the *whiB6* gene. Used for construction of the p2NIL-Δ*whiB6* GOAL suicide plasmid.
ors220	GCAGACCCCTCGCAGCAGCGGCGGACGCGGCCGGGCATCACATATCTAAGCGTTCCTCCATAAAG	
ors221	TGATGCCCGGCCGCGTCC	
ors222	GCAGTCAGGCACCGTACGCGAGTCGGTCTCATCAG	
orb99	ATCGTGGTCCTTGTAGTCGCCTGCCGATTGGGCGGTGATCC	Amplification of regions flanking the *whiB6* ORF and the *whiB6* ORF with a 3’ 3xFLAG tag. Used along with oligonucleotide primers ors219 and ors222 for construction of the p2NIL-*whiB6 3xFLAG* GOAL suicide plasmid
orb100	GGCGACTACAAGGACCACGATGGCGACTACAAGGACCACGACATCGACTACAAAGACGATGACGACAAGTGATGCCCGGCCGCGTCC	
orb14	TCAATAGCCTCGGCGGCTTC	Amplification of the *whiB6* region. Used for verification of *whiB6* merodiploid, deletion, and knock-in strains
orb15	TCGCGCTGTTTGCCTACGTG	
ors225	AGATTCCGCTGGGCGTTTGC	Amplification of a 217bp fragment of the *whiB6* gene transcript
ors226	TCTGCCAGCGACCGAAGTTG	
orb124	TTGGACGAGGCCGTCAAAG	Amplification of a 151bp fragment of the *eccCb*$_1$ gene transcript
orb125	CAGCGCCGACAATCATGTG	
otn33	ATTCAGGAGTCCAGCATGACTGCAACTGCTCTGTACG	Amplification of the *whiB6* ORF. Used for construction of the p$_{MOP}$-*whiB6* plasmid
otn34	GCCTGAGCGGTCCCGTCATGCCGATTGGGCGGTG	
OMF049	GTTGGACTCAAGACGATAGTTACCGGATAAG	Plasmid amplification from pBR322 origin.
OMF050	CTTATCCGGTAACTATCGTCTTGAGTCCAAC	
OMF075	GGATCCAGCTGCAGAATTCAGG	Sequencing confirmation of *esxB* M98A mutation in p$_{MOPS}$*esxBA* construct.
OMF096	ACGAGCGTGACACCACGATGCC	Amplification of the p2NIL vector
OMF097	ACGGTGCCTGACTGCGTTAGCAATTTAACTG	
OMF166	CGTGGTGTCACGCTCGTGCGACAACCAAATGAGGATTTGTCC	Amplification of regions flanking the *esxA* and *esxB* ORFs, excluding the *esxB* and *esxA* genes. Used for construction of
OMF167	CCCGTGACCTTAAGGGTCTTCATCTCTGCCATGCTGG	

OMF168	GAGATGAAGACCCTTAAGGTCACGGGGATGTTTGCTTAATCC	the p2NIL- Δ*esxBA* GOAL suicide plasmid.
OMF169	CGCAGTCAGGCACCGTGTTTCGCCTCATCGGCTCG	
OMF170	TCGTCAACACGAACAGACTTCCC	Amplification of *esxA, esxB* and near flanking sequence. Used for verification of *esxBA* deletion
OMF171	GTCATCTGGAGGTCCGGAACC	
OMF186	CGTGGTGTCACGCTCGTGAGTCAAGGTCCATGGACCTATTCACCG	Amplification of regions flanking the $eccB_1$ ORF, excluding the $eccB_1$ gene. Used for construction of the p2NIL-Δ$eccB_1$ GOAL suicide plasmid.
OMF187	CTCCGGGCTTAAGCAGGCGAAGCCCCATGTTTCAC	
OMF188	CTTCGCCTGCTTAAGCCCGGAGCACCTCAATGACG	
OMF189	ACGCAGTCAGGCACCGTGGCTTCCTCGGCCATGTTGG	
OMF190	ACTCCTGCAGGCAGCCAAAACG	Amplification of $eccB_1$ and near flanking sequence. Used for verification of the $eccB_1$ deletion
OMF191	GGCATCATCAGCATGTATGGCG	
OMF192	CGTGGTGTCACGCTCGTATTTGCCACCCGTATCGAATTCG	Amplification of regions flanking the $eccCa_1$ ORF, excluding the $eccCa_1$ gene. Used for construction of the p2NIL-Δ$eccCa_1$ GOAL suicide plasmid.
OMF193	GTCAGCGCTTCCTTAAGGGTGAATTTCTTTGTCGTCATTGAGG	
OMF194	GAAATTCACCCTTAAGGAAGCGCTGACACCATGATCC	
OMF195	ACGCAGTCAGGCACCGTAGGTCACAATGATGTGCAAGCC	
OMF196	GGAAATTTGTTCAGTTGCAGTCACC	Amplification of $eccCa_1$ and near flanking sequence. Used for verification of the $eccCa_1$ deletion and $eccCb_1K90A$ mutagenesis.
OMF197	AGGTCGATGCAATAGAACTGGACC	
OMF198	CGTGGTGTCACGCTCGTAGCGGCACCACCGAAATCACC	Amplification of regions flanking the $eccD_1$ ORF, excluding the $eccD_1$ gene. Used for construction of the p2NIL-Δ$eccD_1$ GOAL suicide plasmid.
OMF199	GGATGTTTCGAACCTTAAGGGGCAGCACTAGATCTGTCATCCGTC	
OMF200	GCTGCCCCTTAAGGTTCGAAACATCCGATTCTGAGTCACC	
OMF201	ACGCAGTCAGGCACCGTTGTCATGTGGGGATGGCTTGG	
OMF202	GTGTCGGAACAGCAGTTGGTCC	Amplification of $eccD_1$ and near flanking sequence. Used for verification of the $eccD_1$ deletion
OMF203	CTCCAGTTGCTGCTATCGGCG	

OMF204	CGTGGTGTCACGCTCGTCGTCGAGGATCTTCAGATGATCGCCG	Amplification of regions flanking the *eccE*$_1$ ORF, excluding the *eccE*$_1$ gene. Used for construction of the p2NIL-Δ*eccE*$_1$ GOAL suicide plasmid.
OMF205	GCCATCTGCTTAAGACCGGTGCTGACCCGGAACCG	
OMF206	GCACCGGTCTTAAGCAGATGGCCCTGCCCAAGTAGG	
OMF207	ACGCAGTCAGGCACCGTGTATTCATTTGGTTCAGCGTTCCGTCC	
OMF208	GGTGGTCAATGCTGTCGCGG	Amplification of *eccE*$_1$ and near flanking sequence. Used for verification of the *eccE*$_1$ deletion
OMF209	CTGTTCAAGATTTCCTGCTGATCCACC	
OMF530	CAGACCGGCGCCTCGACACTGCTGCAGACGTTGGTCATG	K90A mutagenesis of *eccCb*$_1$ in p$_{MOPS}$*eccCb*$_1$
OMF531	CAGTGTCGAGGCGCCGGTCTGGGGGGCACC	
OMF534	GACGGCCTCGTCCAACGTCG	Sequencing verification of *eccCb*$_1$*K90A* mutation.
M98AF_MM	GCTGTCCTCGCAAGCGGGCTTCTGATT	M98A mutagenesis of *esxB* in p$_{MOPS}$*esxBA*
M98AR_MM	AATCAGAAGCCCGCTTGCGAGGACAGC	
ors100	CGGTATCAGCTCACTCAAAGGCG	Amplification of the pMV306H vector
ors101	GATCCAGCTGCAGAATTCGAAG	
otn31	GAGTGAGCTGATACCGCCCTTCTCGAACGCGATCAG	Amplification of the *whiB6* ORF along with upstream and downstream regions. Used for construction of the pMV306- *whiB6* plasmid
otn32	ATTCTGCAGCTGGATCATTCGAAGCGGCCATTCGAG	
olc25	CAACGAGATAGTCGGCGAAC	Amplification of a 219bp fragment of the *espF* gene transcript
olc26	TCGTCGGAGTTCAGATAGGC	
IFNβ -F	CTGGAGCAGCTGAATGGAAAG	Amplification of IFN-β transcript. From (14).
IFNβ –R	CTTGAAGTCCGCCCTGTAGGT	
β -actin-F	AGGTGTGATGGTGGGAATGG	Amplification of actin transcript. From (14).
β -actin-R	GCCTCGTCACCCACATAGGA	

Table S3. Supplemental Information qRT-PCR analyses

qRT-PCR of *eccCb$_1$* and *whiB6* deletion strains (Figure 2E, 2F, 2H)	
Biological Replicate #1, Plate #1	
Amount of cDNA/ well	1µL (258-276ng)
Volume of Well	10µL
whiB6 NTC- Ct value	Undetermined
sigA NTC- Ct value	Undetermined
Standard Curve R2 *whiB6*	0.992143
Standard Curve R2 *sigA*	0.991773
Range of Standard Curve	500ng- 0.5ng
Biological Replicate #1, Plate #2	
Amount of cDNA/ well	1µL (258-276ng)
Volume of Well	10µL
eccCb$_1$ NTC- Ct value	Undetermined, Undetermined
sigA NTC- Ct value	Undetermined, Undetermined
Standard Curve R2 *eccCb$_1$*	0.993879
Standard Curve R2 *sigA*	0.992978
Range of Standard Curve	500ng-0.5ng
Biological Replicate #2, Plate #3	
Amount of cDNA/ well	1µL (261-271ng)
Volume of Well	10µL
eccCb$_1$ NTC- Ct value	Undetermined, Undetermined
whiB6 NTC- Ct value	Undetermined, Undetermined
sigA NTC- Ct value	Undetermined, Undetermined
Standard Curve R2 *eccCb$_1$*	0.997672
Standard Curve R2 *whiB6*	0.987426
Standard Curve R2 *sigA*	0.974346
Range of Standard Curve	500ng- 0.5ng
Biological Replicate #1 and #3, Plate #4	
Amount of cDNA/well	1µL (258-283ng)
Volume of Well	10µL
espF NTC- Ct value	Undetermined
sigA NTC- Ct value	33.7839
Standard Curve R2 *espF*	0.993876
Standard Curve R2 *sigA*	0.992978
Range of Standard Curve	500ng- 0.5ng
qRT-PCR of *ΔwhiB6* and complementation strains (Figure 3B)	
Biological Replicate #1, Plate #1	
Amount of cDNA/ well	1µL (240-260 ng)
Volume of Well	10µL
whiB6 NTC- Ct value	Undetermined
sigA NTC- Ct value	34.49105
Standard Curve R2 *whiB6*	0.9965
Standard Curve R2 *sigA*	0.9948

Range of Standard Curve	500ng- 0.5ng
Biological Replicate #2, Plate #2	
Amount of cDNA/ well	1µL (240-260ng)
Volume of Well	10µl
whiB6 NTC- Ct value	36.623
sigA NTC- Ct value	32.6121
Standard Curve R2 *whiB6*	0.9965
Standard Curve R2 *sigA*	0.9948
Range of Standard Curve	500ng- 0.5ng
Biological Replicate #3, Plate #3	
Amount of cDNA/ well	1µL (240-260 ng)
Volume of Well	10µL
whiB6 NTC- Ct value	Undetermined
sigA NTC- Ct value	37.2156
Standard Curve R2 *whiB6*	0.9965
Standard Curve R2 *sigA*	0.9948
Range of Standard Curve	500ng- 0.5ng
qRT-PCR of *eccCb*$_1$1*(Oc)* strains (Figure 3C)	
Biological Replicate #1, Plate #1	
Amount of cDNA/ well	1µL (240-260 ng)
Volume of Well	10µL
whiB6 NTC- Ct value	Undetermined
sigA NTC- Ct value	34.49105
Standard Curve R2 *whiB6*	0.9965
Standard Curve R2 *sigA*	0.9948
Range of Standard Curve	500ng- 0.5ng
Biological Replicate #2, Plate #2	
Amount of cDNA/ well	1µL (240-260ng)
Volume of Well	10µl
whiB6 NTC- Ct value	36.623
sigA NTC- Ct value	32.6121
Standard Curve R2 *whiB6*	0.9965
Standard Curve R2 *sigA*	0.9948
Range of Standard Curve	500ng- 0.5ng
Biological Replicate #3, Plate #3	
Amount of cDNA/ well	1µL (240-260 ng)
Volume of Well	10µL
whiB6 NTC- Ct value	Undetermined
sigA NTC- Ct value	37.2156
Standard Curve R2 *whiB6*	0.9965
Standard Curve R2 *sigA*	0.9948
Range of Standard Curve	500ng- 0.5ng
qRT-PCR of *eccCb*$_1$1*(Oc)* strains (Figure 3D)	
Biological Replicate #1 and #2, Plate #1	
Amount of cDNA/ well	2µL (476-516ng)

Volume of Well	20μL
esxA NTC- Ct value	Undetermined, Undetermined
sigA NTC- Ct value	33.4768, 32.806
Standard Curve R2 *esxA*	0.931961
Standard Curve R2 *sigA*	0.941728
Range of Standard Curve	1000ng- 1ng
Biological Replicate #3 and #4, Plate #2	
Amount of cDNA/ well	2μL (482- 520ng)
Volume of Well	20μL
esxA NTC- Ct value	39.1811, Undetermined
sigA NTC- Ct value	33.1723, 33.124
Standard Curve R2 *esxA*	0.99553
Standard Curve R2 *sigA*	0.999086
Range of Standard Curve	1000ng- 1ng
qRT-PCR of ESX-1 deletion strains (Figure 4B)	
Biological Replicate #1, Plate #1	
Amount of cDNA/ well	1μL (264-283ng)
Volume of Well	10μL
whiB6 NTC- Ct value	Undetermined, Undetermined
sigA NTC- Ct value	Undetermined, Undetermined
Standard Curve R2 *whiB6*	0.995919
Standard Curve R2 *sigA*	0.991811
Range of Standard Curve	500ng- 0.5ng
Biological Replicate #2, Plate #2	
Amount of cDNA/ well	1μL (266-277ng)
Volume of Well	10μl
whiB6 NTC- Ct value	Undetermined
sigA NTC- Ct value	Undetermined
Standard Curve R2 *whiB6*	0.990176
Standard Curve R2 *sigA*	0.993175
Range of Standard Curve	500ng- 0.5ng
Biological Replicate #3, Plate #3	
Amount of cDNA/ well	1μL (255-278ng)
Volume of Well	10μL
whiB6 NTC- Ct value	NA[a]
sigA NTC- Ct value	Undetermined
Standard Curve R2 *whiB6*	0.949229
Standard Curve R2 *sigA*	0.990418
Range of Standard Curve	500ng- 0.5ng
qRT-PCR of IFN-β and Actin from *M. marinum* infected RAW cells (Fig 5C)	
Biological Replicate #1 and #2, Plate #1	
Amount of RNA/ well	2μL (50ng)
Volume of Well	10μL

ifnβ NTC- Ct value	Undetermined, Undetermined, Undetermined, Undetermined
actin NTC- Ct value	35.2191, Undetermined, 37.4071, 35.3412
Standard Curve R2 *ifnβ*	0.992737
Standard Curve R2 *actin*	0.973715
Range of Standard Curve	100ng- 0.1ng
Biological Replicate #3 and #4, Plate #2	
Amount of RNA/ well	2µL (50ng)
Volume of Well	10µL
ifnβ NTC- Ct value	Undetermined, Undetermined, Undetermined, Undetermined
actin NTC- Ct value	Undetermined, Undetermined, Undetermined, Undetermined
Standard Curve R2 *ifnβ*	0.98824
Standard Curve R2 *actin*	0.95823
Range of Standard Curve	100ng- 0.1ng
Biological Replicate #5 and #6, Plate #3	
Amount of RNA/ well	2µL (50ng)
Volume of Well	10µL
ifnβ NTC- Ct value	Undetermined, Undetermined, Undetermined, Undetermined
actin NTC- Ct value	Undetermined, Undetermined Undetermined, Undetermined
Standard Curve R2 *ifnβ*	0.97006
Standard Curve R2 *actin*	0.93929
Range of Standard Curve	100ng- 0.1ng

a. NA: NTC was not analyzed for this primer pair.

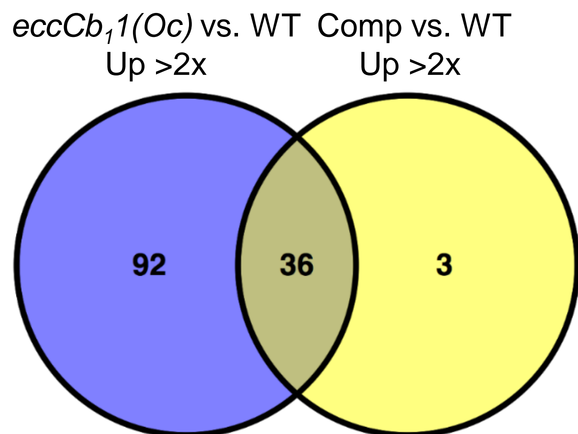


Figure S1: Comparison of genes upregulated in the *eccCb*$_1$1*(Oc)* strain compared to the wild-type and complemented strains.

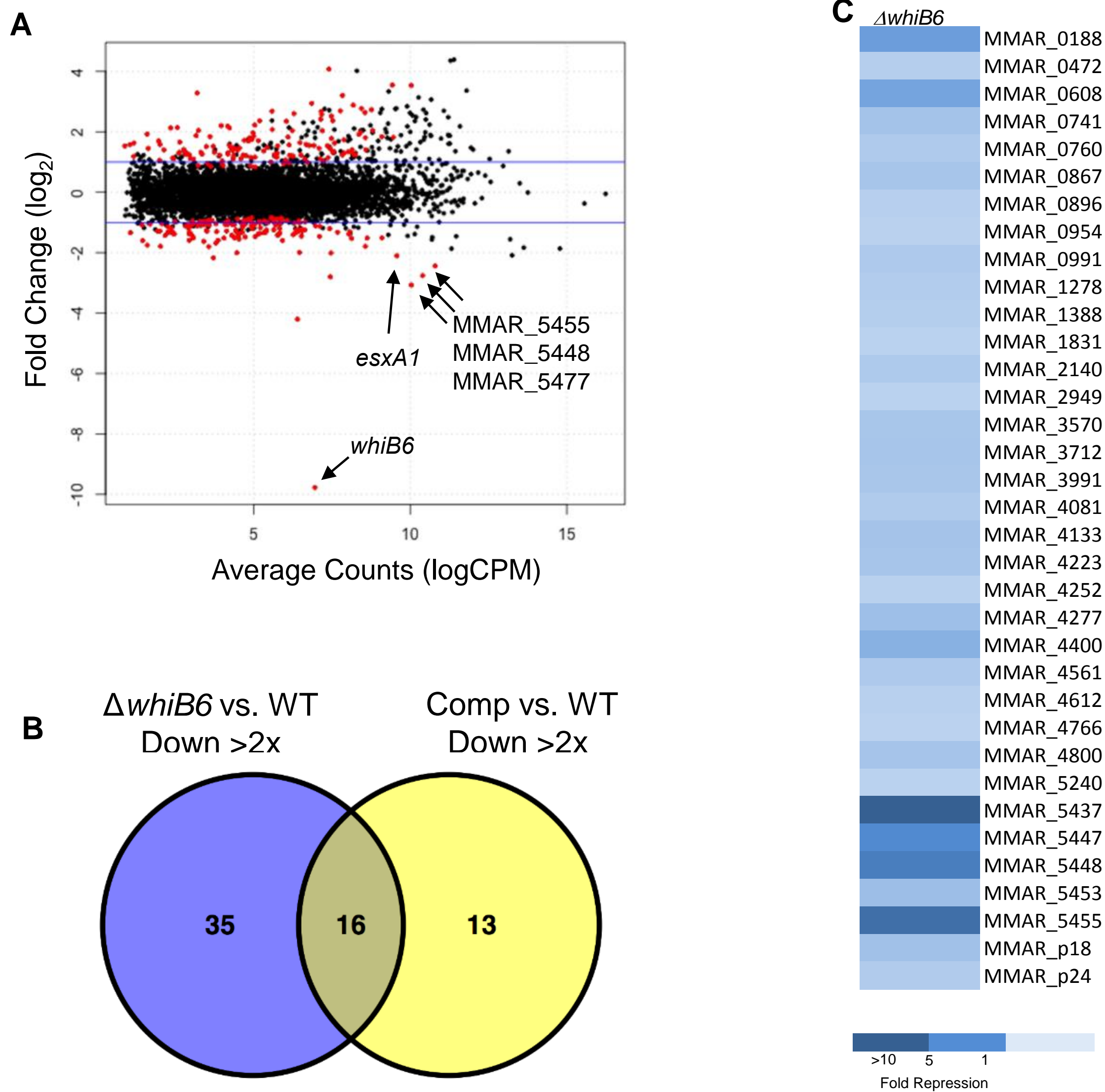


Figure S2. Transcriptome analysis of WT, Δ*whiB6* and complemented *M. marinum* strains

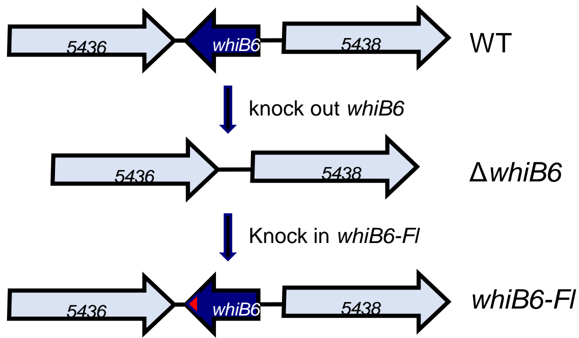


Figure S3: Schematic of the *whiB6* knock-out and knock-in strains.

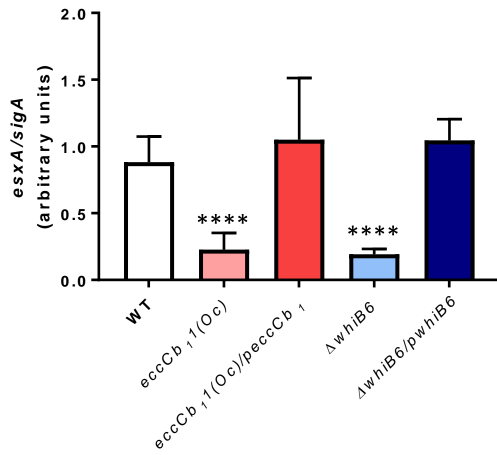
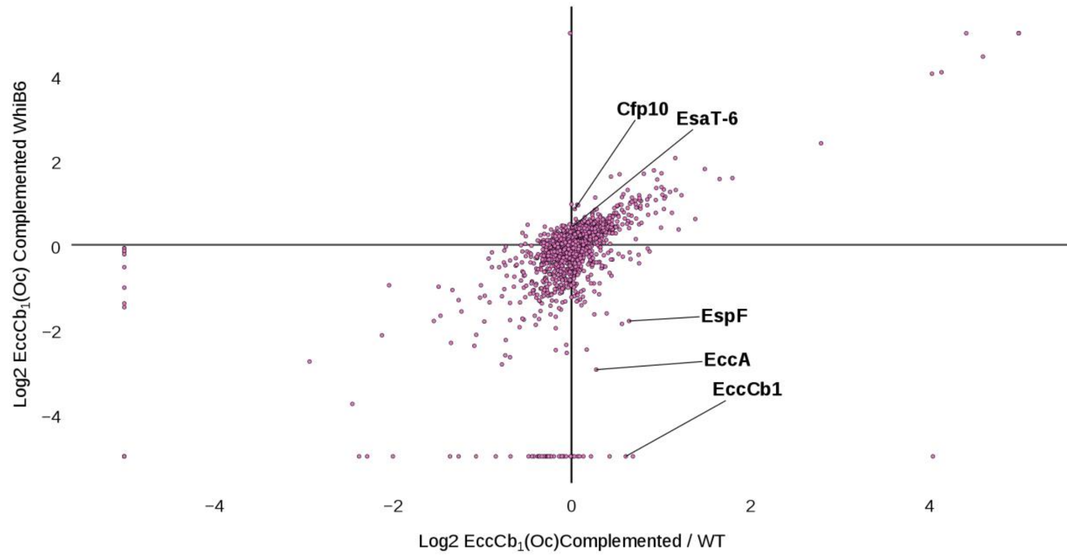


Figure S4: Effect of the *eccCb*$_1$ and *whiB6* genes on the expression levels of the *esxA* gene

A



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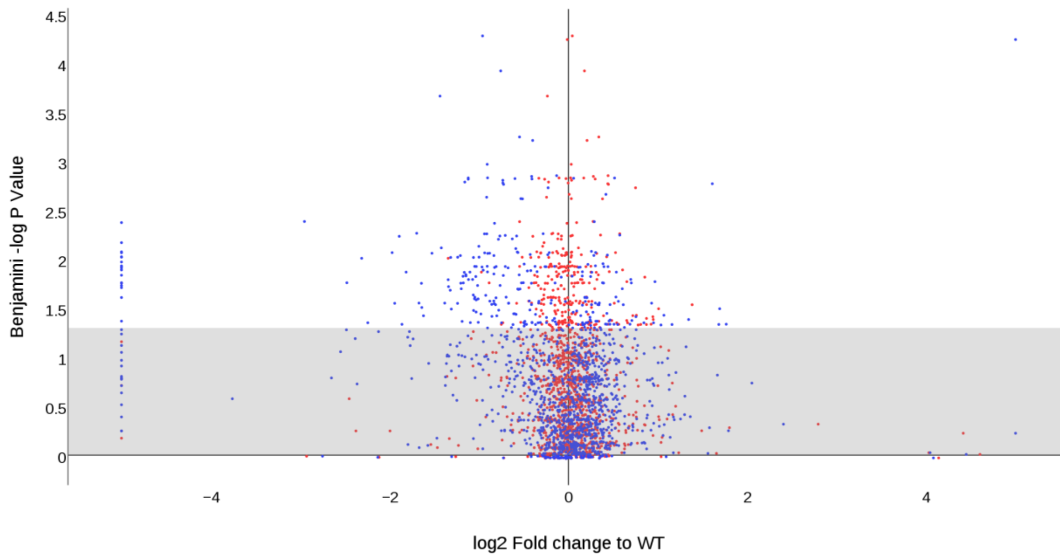

Figure S5: Proteins restored by expression of *whiB6* or *eccCb$_1$* in the *eccCb$_1$1(Oc)* strain.

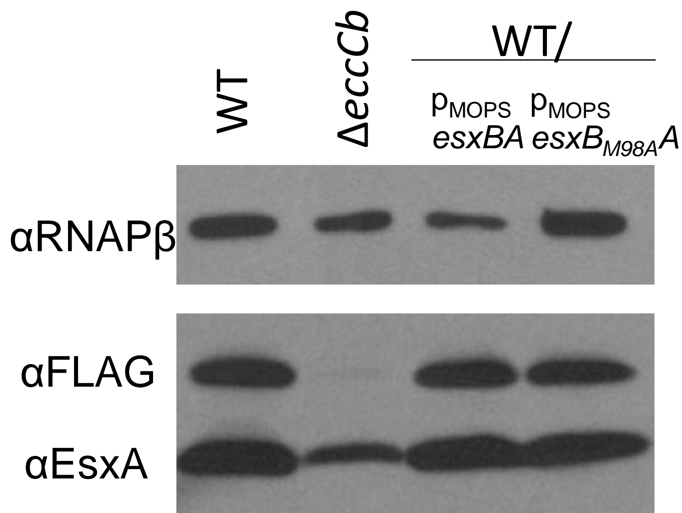

Figure S6: Accumulation of EsxBA is not sufficient to control the levels of WhiB6.

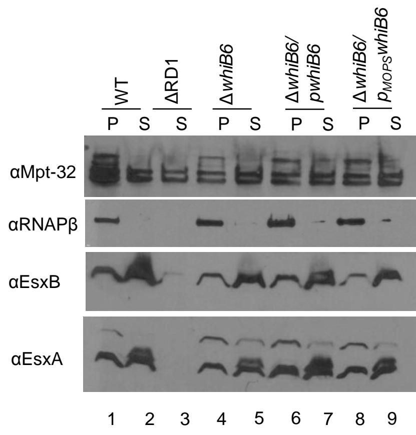

Figure S7: ESX-1 secretion is not abrogated in the Δ*whiB6* strain

Supplementary Figure Legends

Figure S1. Comparison of genes upregulated in the *eccCb₁1(Oc)* strain compared to the wild-type and complemented strains. Venn diagram of genes upregulated (>2-fold, q<0.05) in the *eccCb*$_1$1*(Oc)* strain (Dataset 1,B) or the complemented strain (Table Dataset 2,B), relative to the WT strain. The 92 genes specifically upregulated in the ochre mutant are putative ochre regulated genes (Dataset 3, B).

Figure S2. Transcriptome analysis of WT, Δ*whiB6* and complemented *M. marinum* strains. A) RNA-seq magnitude-amplitude plot of WT *M. marinum* vs. the *Δwhib6* mutant strain. Highlighted genes show selected genes that are downregulated in the *Δwhib6* mutant strain, including the *whiB6* gene, which was undetectable in the mutant. Black dots are not statistically significant and red dots are statistically significant (q<0.05). Complete gene expression data are presented Dataset 4. Venn diagram of genes downregulated or upregulated (>2-fold, q<0.05) in the *Δwhib6* mutant strain (Dataset 4, B and C) or the *Δwhib6* complemented strain (Dataset 5B and C), relative to the WT strain. The 35 genes specifically downregulated and 34 genes specifically upregulated in the *Δwhib6* mutant are putative *whiB6* regulated genes (Dataset 3, C and D). **C.** Heatmap showing the 35 genes that are downregulated specifically in the *Δwhib6* mutant.

Figure S3: Schematic of the *whiB6* knock-out and knock-in strains. Schematic of the *whiB6* (*MMAR_5437*) locus in the *M. marinum* genome. Details of the construction for the Δ*whiB6* and *whiB6-Fl* strains are found in the Supplementary Methods.

Figure S4: Effect of the *eccCb*$_1$ and *whiB6* genes on the expression levels of the *esxA* gene *esxA* gene transcription normalized to the levels of *sigA* as measured by qRT-PCR. Data represents the average of three biological replicates, with a total of 8 technical replicates. Error bars represent standard deviation. Significance was defined using an ordinary one way ANOVA (P=0.0001). *esxA* levels were compared to those in the WT strain using a Dunnett's multiple comparison test, where **** means P≤ 0.0001. Nonsignificant differences (P>0.05) are not indicated.

Figure S5: Proteins restored by expression of *whiB6* or *eccCb*$_1$ in the *eccCb*$_1$*1(Oc)* **A**. Plot of fold change in Complemented *eccCb*$_1$1*(Oc)*/p$_{MOPS}$*eccCb*$_1$/ WT *vs* fold change in *eccCb*$_1$1*(Oc)*/p$_{MOPS}$*whiB6*/ WT. Data were acquired by LC/MS/MS proteomics and quantified using label-free quantification (MaxQuant) Highlighted are ESX-1 gene products which are restored to WT levels by overexpression of *whiB6* in an *eccCb*$_1$1*(Oc)* background [Cfp10 (EsxB), Esat6 (EsxA)] and genes that are not (EspF, $EccA_1$,). The $EccCb_1$ does not complement with expression of *whiB6*, which is why it is (-2^5) on that axis. Points along axis (+/- 2^5) fold change were identified in only one of the respective strains significantly and change in expression was capped at 32-fold for clarity. Dataset 6 contains all raw and integrated peak-areas. **B**. Volcano plot of individual fold/WT changes for significance from WT. Blue = *eccCb*$_1$1*(Oc)*/p$_{MOPS}$*whiB6* / WT Red = *eccCb*$_1$1*(Oc)*/p$_{MOPS}$*eccCb*$_1$/ WT. Benjamini-hochberg corrected α=0.05 is shown. Dataset 6 lists all proteins with Rank-P value significance (P benjamini >= P).

Figure S6: Accumulation of EsxBA is not sufficient to control the levels of WhiB6. Constitutive expression of *esxBA* or *esxBM98AEsxA* in the WT strain does not lead to reduced levels of WhiB6-Fl protein. The data is representative of three biological replicates.

Figure S7: **ESX-1 secretion is not abrogated in the Δ*whiB6* strain.** Western blot analysis of ESX-1 substrate production (p, pellet) and secretion (s, supernatant) during *in vitro* growth. RNAPβ serves as a control for mycobacterial lysis. MPT-32 is secreted independently of ESX-1, and serves as a loading control. The experiment shown is representative of three experiments on independent biological replicates.

Figure S8: The Δ*whiB6* strain is cytolytic in a macrophage model. Cytolysis of RAW 264.7 cells 24 hours post infection with *M. marinum,* MOI=5. Calcein-AM labels live cells, EthD-1 labels permeabilized cells. Scale bar, 50 μm. The images shown are representative of three biological replicates, each with three technical replicates.

Supplementary Dataset Descriptions

Dataset 1. Genes regulated in the *eccCb*$_1$1*(Oc)* (ochre) strain compared to the WT strain.

A. All genes *eccCb*$_1$1*(Oc)*/WT fold change

B. Genes up >2 fold, q<0.05 in *eccCb*$_1$1*(Oc)*/WT

C. Genes down >2 fold, q<0.05 in *eccCb*$_1$1*(Oc)*/WT

Dataset 2. Genes regulated in the *eccCb*$_1$1*(Oc)* complemented (comp) strain compared to the WT strain.

A. All genes comp/WT fold change

B. Genes up >2 fold, q<0.05 in comp/WT

C. Genes down >2 fold, q<0.05 in comp/WT

Dataset 3. Comparison of mutant and complemented vs WT strains (from the Venn Diagrams).

A. Genes down >2X, q<0.05 in *eccCb*$_1$1*(Oc)* /WT but not differentially regulated in *eccCb*$_1$*1(Oc)* comp/WT

B. Genes up >2 fold, q<0.05 in *eccCb*$_1$1*(Oc)* /WT, but not in *eccCb*$_1$1*(Oc)* comp/WT

C. Genes down >2 fold, q<0.05 in Δ*whib6*/WT but not down in complemented strain

D. Genes up >2 fold, q<0.05 in Δ*whib6*/WT but not up in the complemented strain.

E. Genes down in *eccCb*$_1$1*(Oc)* and Δ*whiB6,* but not the complemented strains.

Dataset 4. Genes regulated in the *ΔwhiB6* strain compared to the WT strain.

A. All genes in WT, Δ*whiB6* and complemented strains, fold change calculated for Δ*whiB6*/WT

B. Genes up >2 fold, q<0.05 in Δ*whib6*/WT

C. Genes down >2 fold, q<0.05 in Δ*whib6*/WT

Dataset 5: Genes regulated in the *ΔwhiB6* complemented (comp) strain compared to the WT strain

A. All genes in WT, Δ*whiB6* and complemented strains, fold change calculated for Comp/WT.

B. Genes up >2 fold, q<0.05 in comp/WT

C. Genes down >2 fold, q<0.05 in comp/WT

Dataset 6: Raw and processed mass spectrometry data for quantification of changes of ESX-1-associated proteins

Supplementary Methods

Bacterial strains and growth conditions

All *M. marinum* strains were maintained at 30°C in Middlebrook 7H9 defined broth (Sigma-Aldrich, St. Louis, MO) supplemented with 0.5% glycerol and 0.1% Tween-80 (Fisher Scientific, Pittsburgh, PA) or on Middlebrook 7H11 defined agar (Sigma-Aldrich) plates supplemented with 0.5% glycerol and 0.5% glucose. Agar plates and broth were supplemented with kanamycin (20 µg/ml; IBI Scientific, Peosta, IA) or hygromycin (50 µg/ml; EMD Millipore, Billerica, MA) where appropriate.

DH5α *E. coli* strains were maintained at 37ºC in Luria Bertani (LB) Broth or on LB agar (Amresco, Solon, OH) plates. Media was supplemented with kanamycin (50 µg/ml), hygromycin (200 µg/ml), and ampicillin (200 µg/ml; Thermo Fisher, Waltham, MA) where appropriate.

Mycobacterial strain construction

All plasmid preparations were performed using the AccuPrep Plasmid Miniprep DNA extraction Kit (Bioneer, Alameda, CA). All DNA constructs and *M. marinum* strains were confirmed by PCR followed by DNA sequencing analysis in the Genomics and Bioinformatics Core Facility at the University of Notre Dame. All oligonucleotide primers used to generate and confirm DNA constructs and strains were purchased from Integrated DNA Technologies (IDT, Coralville, IA)

<u>(i) Generation of the ΔwhiB6 strain</u>

The Δ*whiB6 M. marinum* strain was generated the using allelic exchange method by Parish and Stoker (5) as described previously (3). The p2NIL-Δ*whiB6* knockout plasmid was generated using the FastCloning approach (7). Approximately 1.5kb upstream of the annotated *whiB6* gene [Mycobrowser,(8)] was amplified using

oligonucleotide primers ors219 and ors220 (Table S2). Approximately 1.4 kb downstream of the *whiB6* gene was amplified using oligonucleotide primers ors221 and ors222. The primers were designed to retain only the initial start and stop codon of the *whiB6* open reading frame. The p2NIL plasmid was amplified using oligonucleotide primers ors116 and ors117, exactly as described in (3). The upstream and downstream PCR products were introduced into the p2NIL vector using FastCloning, as previously described (3). The p2NIL plasmid was a gift from Tanya Parish (Addgene plasmid # 20188) (5). The resulting plasmid with insert was confirmed by restriction digestion and DNA sequencing analysis using oligonucleotide primers p2NILPstFwd and p2NILRev (3). The pGOAL19 cassette was introduced as previously described (3). pGOAL19 was a gift from Tanya Parish (Addgene plasmid # 20190)(5). The p2NIL-Δ*whiB6* knockout construct was UV treated (100 mJ UV light cm^{-2} ,CL-1000 Ultraviolet Crosslinker, UVP, Upland, CA) and introduced into *M. marinum* M competent cells by electroporation and the resulting blue colonies were selected exactly as in (3). Integration of the plasmid into the genome (merodiploid) was confirmed by PCR amplification of the region using oligonucleotide primers orb14 and orb15. White sucrose-resistant colonies were selected and the loss of the *whiB6* open reading frame was screened by PCR and DNA sequencing analysis using oligonucleotide primers orb14 and orb15.

(ii) Generation of the *whiB6-3xFLAG* strain

The *whiB6-3xFL* knock-in construct was generated by amplifying the *whiB6* open reading frame and its upstream region using oligonucleotide primers ors219 and orb99. The downstream region was amplified using oligonucleotide primers orb100 and ors222. An in-frame 3xFLAG tag [(5’

GGCGACTACAAGGACCACGATGGCGACTACAAGGACCACGACATCGACTACAAAG ACGATGACGACAAGTGA 3') encoding a glycine linker and the tag DYKDHDG-DYKDHDI-DYKDDDDK- STOP] was added to the 3' end of the *whiB6* gene. The knock-in construct was generated using 3-part FAST cloning by mixing the up and downstream PCR products with amplified p2NIL vector, described above. The p2NIL-*whiB6-3xFLAG* knock-in plasmid was confirmed by restriction digestion and DNA sequencing analysis using oligonucleotide primers p2NILPstFwd and p2NILRev. The pGOAL19 cassette was introduced as previously described (3). The p2NIL-*whiB6-3xFLAG* knock-in construct was UV treated as above and introduced into *M. marinum* Δ*whiB6* competent cells by electroporation as above. Integration of the plasmid into the genome was confirmed using oligonucleotide primers orb14 and orb15. White sucrose-resistant colonies were screened and the introduction of the *whiB6-3xFl* allele was confirmed by PCR analysis and sequencing using oligonucleotide primers orb14 and orb15.

(iii) Generation of ESX-1-deficient strains

Knock-out constructs for generating deletions of the *esxBA,* $eccB_1$, $eccCa_1$, $eccD_1$ and $eccE_1$ genes were constructed using the p2NIL system. The p2NIL vector was linearized with ScaI (New England BioLabs [NEB], Ipswich, MA) and used as a template for amplification with Q5 polymerase (NEB) and primers OMF096 and OMF097. Amplified vector was treated with DpnI (NEB) heat-killed and used in subsequent FastCloning reactions.

Amplifications from *M. marinum* strain M genomic DNA were done using Phusion polymerase (NEB) in the supplied GC buffer supplemented with 4% DMSO. Primers OMF166 and OMF167 were used to amplify the first 6 codons of *esxB* plus 1592 upstream

bases while primers OMF168 and OMF169 were used to amplify the last 5 codons of *esxA* and 1549 downstream bases. Primers OMF186 and OMF187 were used to amplify the first 5 codons of $eccB_1$ plus 1668 upstream bases while primers OMF188 and OMF189 were used to amplify the last 6 codons of $eccB_1$ plus the 1608 downstream bases. Primer OMF192 and OMF193 were used to amplify the first 7 codons of $eccCa_1$ plus 1800 upstream bases while primers OMF194 and OMF195 were used to amplify the last 5 codons plus 1583 downstream bases. Primers OMF198 and OMF199 were used to amplify the first 7 codons of $eccD_1$ plus 1731 upstream bases while primers OMF200 and OMF201 were used to amplify the final 7 codons of $eccD_1$ plus 1603 downstream base pairs. Primers OMF204 and OMF205 were used to amplify the first 15 codons of $eccE_1$ and 1690 upstream bases while OMF207 and OMF207 were used to amplify the last 7 codons and 1597 downstream bases. Retaining additional 5' codons of $eccE_1$ was done to minimize potential effects on the overlapping 3' region of the adjacent $MycP_31$ ORF.

The p2NIL-Δ$eccB_1$, p2NIL-Δ$eccCa_1$, p2NIL-Δ$eccD_1$, p2NIL-Δ$eccE_1$, p2NIL-Δ*esxBA* plasmids were generated using the FastCloning method (7). Briefly, 2 µl each of upstream and downstream amplification products were mixed with 1 µl of amplified p2NIL vector and introduced into chemically competent Top10 *E. coli* and plated on LB agar with 50 µg/mL kanamycin (IBI). Plasmids were evaluated by restriction digest with BamHI (NEB) and XhoI (NEB). The pGOAL cassette was introduced as previously described (3).

(iv) Generation of ESX-1-deficient strains in the *whiB6-3XFLAG* strain

The *whiB6-3xFLAG* Δ*eccCb*$_1$ strain was created using the p2NIL-Δ*eccCb*$_1$ knockout plasmid (3). The p2NIL-Δ*eccCb*$_1$ knockout plasmid was UV treated and introduced into *whiB6-3xFLAG* competent cells by electroporation as described previously. The deletion strain was generated as exactly as previously described for the Δ*eccCb*$_1$ *M. marinum* strain (3). Confirmation of the deletion of the *eccCb*$_1$ gene was achieved by PCR followed by DNA sequencing analysis using oligonucleotide primers orb72 and orb73. The *whiB6-3xFLAG* Δ*eccB*$_1$, *whiB6-3xFLAG ΔeccD*$_1$, and *whiB6-3xFLAG* Δ*eccE*$_1$ strains were created using the p2NIL-Δ*eccB1,* p2NIL-Δ*eccD*$_1$, and p2NIL-Δ*eccE*$_1$, knock-out plasmids described above. The resulting strains were confirmed by PCR analysis and DNA sequencing analysis using oligonucleotide primers OMF90 and OMF91, OMF202 and OMF203, and OMF208 and OMF209, respectively (see Table S2).

Generation of whole cell lysates and secreted protein fractions

ESX-1 secretion assays were performed exactly as described previously (9). Briefly, *M. marinum* strains were grown in Sauton's defined liquid media for 48 hours. To generate whole cell lysates, cells were collected by centrifugation, resuspended in phosphate buffered saline (PBS) and mechanically lysed. Secreted protein fractions (culture filtrates) were generated by filtering culture supernatants through a 0.22μm filter followed by concentration using a 3kDa Amicon filter (EMD Millipore, Billerica, MA). All protein concentrations were determined using the Micro-BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL), according to the manufacturer's instructions as described previously (10). Protein fractions were analyzed by immunoblot analysis.

Generation of *esxBA* and *eccCb*$_1$ mutant expression plasmids.

Mutagenesis of the p_{MOPS}*eccCb₁* construct for the $EccCb_1K90A$ mutation was carried out through two separate PCR amplifications of the wild-type expression plasmid with primers OMF049, OMF0531 and OMF050, OMF0530 and Phusion (NEB) polymerase in GC buffer and 4% DMSO. PCR products were evaluated by gel electrophoresis and then incubated with DpnI (NEB). Equivalent volumes (0.5 µl each) of digested products were mixed and transformed into chemically competent DH5α *E. coli*. Transformations were plated on LB agar with 200 µg/ml hygromycin (Calbiochem). Three isolated colonies were picked from the plate and grown overnight in 5 ml shaking cultures of LB broth with 200 µg/ml hygromycin. Plasmids were purified using miniprep spin columns (Bioneer) and sequenced using primer OMF197 at the Notre Dame Sequencing Core. Plasmid preps of resultant primers were checked by sequencing with the primer OMF534. Mutagenesis of the p_{MOPS}*esxBA* construct was similarly carried out by PCR amplification using the combinations OMF049, esxBM98AR_MM and OMF050, esxBM98AF_MM. Resultant plasmids were purified and sequenced using the primer OMF075.

Confirmed plasmids were introduced into the WT and Δ$eccCb_1$ *whiB6-3xFL* strains of *M. marinum* by electroporation followed by plating on 7H11 (Hardy Diagnostics) with 50 µg/ml hygromycin. After two weeks outgrowth colonies were picked and streak purified on 7H11 plates. Presence of plasmid was checked by PCR with MOPS-Fwd and MOPS-RevHyg primers.

Immunoblot analysis

Unless otherwise specified, 20µg of cell lysate and secreted proteins were separated on 4-20% Criterion or Mini-Protean TGX Tris-HCl precast polyacrylamide Gels

(Bio-Rad). The proteins were transferred to a nitrocellulose membrane at 100V for 1 hour. Nitrocellulose membranes were incubated with primary antibodies against: RNA polymerase subunit β (RpoB; 1:10,000; ab12087; Abcam, Cambridge, UK), ESAT-6 (EsxA; 1:5,000; HYB 076-08-02; Thermo Fisher, Waltham, MA), and Monoclonal Anti-FLAG M2 (1:5000; Sigma). The following reagents were obtained through BEI Resources, NIAID, NIH: Polyclonal Anti-*Mycobacterium tuberculosis* CFP10 (Gene Rv3874) (antiserum, Rabbit), NR-13801and Polyclonal Anti-*Mycobacterium tuberculosis* Mpt32 (Gene Rv1860) (antiserum, Rabbit), NR-13807 as previously described. The nitrocellulose membrane was then washed three times with PBS-T and incubated with secondary antibodies. Secondary antibodies were resuspended in 5% milk in 1X PBS-T and used at a concentration of 1:5,000. Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin secondary antibody (Bio-Rad Laboratories) was utilized for detection of RpoB, EsxA, and FLAG. HRP-conjugated goat anti-rabbit IgG secondary antibody (BioRad Laboratories) was utilized for detection of EsxB and Mpt32. All proteins were detected using the LumiGLO Chemiluminescent Substrate Kit (KPL, Gaithersburg, MD) and developed on film as previously indicated (3). The number of replicates for each assay are presented in the legend for each figure.

Quantitative proteomics

Mycobacterial strains were grown in Sauton's medium. Whole cell lysates were digested with trypsin (Promega, Madison, WI). Whole cell lysates of cytosolic proteins were generated for proteomics as described above. 50μg of soluble protein from two biological replicates was extracted and precipitated with MeOH-$CHCl_3$. Dried protein pellets were denatured and re-suspended in 50% 2,2,2 TFE 50mM ammonium

bicarbonate (Sigma) reduced with DTT and alkylated with iodoacetamide. After dilution of the TFE with ammonium bicarbonate 1ug of sequencing grade trypsin (Promega) was added to each sample and digestion was allowed to proceed overnight at 37C. Following digestion the peptide mixtures were acidified and desalted using C18 spin columns (Protea, Morgantown, WV) according to manufacturer's instructions and dried in a vacuum concentrator. 1µg of each digest was analyzed in biological and technical duplicate by LC/MS/MS quantitative proteomics. 1µg was injected onto a C18BEH 100mm x 100µm column (Waters, Billerica, MA) running at 800nl/min. a 100min gradient from 5-32% H_2O-ACN (0.1% Formic acid) was used. MS/MS was performed on an orbitrap QExactive HF (Thermo San Jose, CA) instrument running a TOP20 Data-dependent acquisition (11).

Protein Identification

RAW files were searched and quantified using MaxQuant (v1.5.7.4) A current FASTA of the *M. marinum* DB3 combined with common lab contaminants was used for spectral-mass matching (12). FDR was calculated using the target-decoy method of Elias et al. (13), A 1% peptide and protein FDR cutoff were applied. Oxidation of Met, Deamidation NQ, pyroglutamic/aspartic, and Protein acetyl-NT were considered as variable modifications. Default search parameters for Orbitrap instruments were used for remaining settings. Quantification of changes in protein abundance were measured using Label Free Quantification (LFQ) from the same data sets. Default peptide inferences were used and only deamidation (N,Q) was allowed as a variable modification for quantified peptides. At least 2 integrated peptides were required for quantification with default 3/6 nearest/average neighbors necessary. Matching of quantification between samples was

required and LFQ normalization (median response-factor) was used to correct sample differences. Complete quantification and identification results are available as supplementary data (table S9). 2,587 proteins were identified at a 1% FDR, of which 2,532 were *M. marinum* in origin.

Quantitative data reduction was performed by taking the LFQ integrated intensities and calculating the ratio of each protein in a mutant background to the protein abundance determined from WT cells. All four technical and biological replicates were integrated and averaged when available. 55% of the identified proteins had quantitative measurements in all 8 replicates, 31% had data in 4 (typically identified in WT only). In cases where proteins were quantified in less than 8 samples the remaining set of biological and technical replicates were averaged together to determine the mean fold-change. In less than 1% of the cases at least two quantitative events were measured, but in separate technical replicates. In these cases the measurement of replicate 1 was used to ratio replicate 2. These were only passed if the technical replicate-ratio had the same variance as proteins which were quantified across all eight samples. In cases where a protein was reliably quantified in only a single sample type (WT, but not $EccCb_1$) these samples were given ratios of 32-fold or 1/32 fold depending on the detected sample. This is approximates the dynamic range of an LFQ experiment and prevents a large distribution of artificial large (nearly infinite) ratios to be calculated. The statistical significance of fold-change was calculated using a Welch's modified test for significance. 2-tailed comparisons of inequal variance. A Benjamini-hochberg corrected P value was calculated for each quantified value to correct for significance in multiple-comparisons testing (alpha

0.1). Fold change and -logP values were graphed using Plotly (Plotly Technologies Incorporated).

For Figure 4E, fold-change in the levels of ESX-1 membrane proteins from quantitative label-free proteomics. Fold-change was determined as the ratio of the normalized peak area for multiple peptides assigned to each protein. Three technical replicates and two biological duplicates were integrated and averaged. Standard error (% coefficient of variation) was calculated for each technical triplicate. Propagation of error was performed to determine error for fold-change. Protein ratios are reported as the log2 fold change as compared to levels measured in the complemented strain. Significance was determined as above.

RNA extraction and preparation

M. marinum cultures were grown and lysates were generated as described for ESX-1 secretion assays and bacterial lysates were generated in Qiagen RLT buffer (Qiagen, Hilden, Germany) supplemented with 1% β-mercaptoethanol. Total RNA from bacterial lysates was isolated using the RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. RNA from two biological replicates of each sample were prepared for sequencing.

qRT-PCR analysis

1μg of RNA was treated with RNase-free DNase I (Novagen, San Diego, CA). cDNA was synthesized using random hexamers (IDT) and the Superscript II (Invitrogen), ProtoScript II (NEB) or iScript (Bio-Rad) reverse transcriptase enzyme according to the manufacturer's instructions. cDNA was quantified using a Nanodrop 2000 (Thermo Scientific, Waltham, MA) and diluted to 250-280ng/μL. The standard curve was generated

using cDNA from the wild-type strain. Reactions were prepared using the indicated amount of cDNA (see Table S3), 250nM of each oligonucleotide primer, and the SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA). *whiB6* gene expression was analyzed using oligonucleotide primers ors225 and ors226. *eccCb*$_1$ gene expression was analyzed using oligonucleotide primers ors124 and ors125. *esxA* gene expression was analyzed using oligonucleotide primers esxA-3' and esxA-5'. *espF* gene expression was analyzed using oligonucleotide primers olc25 and olc26. *sigA* was used as the reference gene and was amplified using oligonucleotide primers sigA-F and sigA-R (3). All samples were run on an Applied Biosystems 7500 FAST Real-Time PCR System using the following cycle conditions: 50°C for 2min., 95°C for 10min., followed by 40 cycles at 95°C for 15sec. and 60°C for 1min. After the 40 cycles, a dissociation step was also added consisting of 95°C for 15sec., 60°C for 1min., 95°C for 15sec., and 60°C for 15sec. Supplemental information regarding the qRT-analysis (e.g. amount of cDNA, NTCs, R^2 value of standard curve, and reaction volume) is reported in Table S3.

When applicable (Figure 3B, 3C and S4), measurements were normalized using the TATAA Interplate Calibrator (IPC) SYBR (tataabiocenter). The Cq values for each read were normalized as indicated in the user manual. A standard curve was created with concentrations of 500, 50, 5, and 0.5 ng/µL of cDNA, with R^2 with no more variance than 0.90-1.1. Using this standard curve, we related the IPC Cq values to levels of DNA using the following equation:(1.2) $\frac{\text{Cq}-y\text{ intercept}}{\text{Slope}} = \text{Log Quantity of DNA}$. Finally, the amount of DNA and its relative abundance was determined as (1.3) $10^{\text{Log Quantity of DNA}} = \text{Amount of DNA}$, $\frac{\text{Amount of gene DNA}}{\text{Amount of SigA DNA}} = \text{Relative abundance}$.

Cytotoxicity assays

RAW 264.7 murine macrophages (ATCC TIB-71) were maintained as previously described (3, 9). Cells were plated in 24-well plates (Grenier Bio-one, Monroe, MC) at a cell density of 5 x 10^5 cells per well 24 hours prior to infection. Cells were infected with 2.5 x 10^6 cells of *M. marinum*. Uptake of the bacteria was allowed to proceed for 2 hours at 37°C with 5% CO_2. The cells were then treated with 100 ug/mL gentamycin (RPI, Mount Prospect, IL) for an additional 2 hours. The cells were then washed three times with PBS and fresh media was added and the cells were incubated for 24 hours at 37°C with 5% CO_2. Staining and imaging of macrophages was performed exactly as previously described (3, 9). Three independent biological replicates (three wells per strain) of the macrophage infections were imaged. Ten fields were imaged per well and ImageJ (3) was used to count the number of permeabilized (EthD-1 stained) cells for each field by adjusting the color threshold to the default parameters and then analyzing the number of particles in each field. The average and standard deviation are reported.

Cytokine assays

Quantitative real time PCR (qRT-PCR) was performed as in (9). Briefly, macrophages were plated at a cell density of 5 x 10^5 cells per well in a 24 well plate 24 hours prior to infection. Cells were then infected with 2.5 x 10^6 *M. marinum* cells and incubated for 2 hours at 37°C with 5% CO_2. Cells were then washed three times with PBS and fresh media was added. RNA was extracted 4 hours post infection using TRIzol Reagent (Life Technologies, Carlsbad, CA). The Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA) was used according to manufacturer’s instructions. Quantitative real time PCR was performed as previously described (3, 9) with a 10 µl reaction volume, 100 nM primers,

and 50 ng of DNA as template. The level of beta interferon mRNA was normalized to that of the level of actin mRNA using the following primers: IFNβ -F (5′-CTGGAGCAGCTGAATGGAAAG-3′), IFNβ -R (5′-CTTGAAGTCCGCCCTGTAGGT-3′), β -actin-F (5′-AGGTGTGATGGTGGGAATGG-3′) and β -actin-R (5′-GCCTCGTCACCCACATAGGA-3′) from (14). Three independent experiments containing two biological replicates were performed in technical duplicate.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 7 for the qRT-PCR and hemolysis assays. For qRT-PCR and hemolysis assays, significance was determined using a one-way ordinary ANOVA followed by a Dunnett's, Sidak's or Tukey's multiple comparison test as indicated in each figure legend. P values are listed in the text and in the Figure legends.

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