Supporting Information

Modification of a PE/PPE substrate pair reroutes an Esx substrate pair from the mycobacterial ESX-1 type VII secretion system to the ESX-5 system

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Running title: PE/PPE determine system-specificity of Esx substrates

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Table S1. Strains used in this study.

Strain	References
WT *M. marinum* M	(29)
eccCb$_1$ mutant (ESX-1 mutant)	(32)
eccC$_5$ mutant (ESX-5 mutant)	This study, complete deletion of *eccC*$_5$ in the genome of *M. marinum* M background strain

Table S2. Primers used in this study.

Primers used in this study	Sequence	Purpose of use
pe35-NheI-Fw	CCCGCTAGCATGCGATCCATGTCTTTTGA	cloning *pe35/ppe68_1mmar* into pSMT3
ppe68_Flag Rv	TCACTTGTCGTCATCGTCTTTGTAGTCCCAGT CGTCGTCGTCATC	to clone the cluster *pe35/ppe68_1/esxB_1/EsxA_1* into pSMT3
Flag_esxB Fw	GACTACAAAGACGATGACGACAAGTGAGAGT CGTTGCTAAAAAGGACTTTC	to clone the cluster *pe35/ppe68_1/esxB_1/EsxA_1* into pSMT3
esxA HA tag BamHI Rv	CCCCCGGATCCTTAAGCTAAGCATAATCAGG A ACATCATACGGATAGCCGAACATCCCCG	to clone the cluster *pe35/ppe68_1/esxB_1/EsxA_1* into pSMT3
ppe68_1 Flag_HindIII RV	CCCCCAAGCTTTCACTTGTCGTCATCGTCTTT GT AGTCCCAGTCGTCGTCGTCATC	to clone *pe35/ppe68_1* with FLAG tag into pMV
pe35 EcoRI FW	CCCCCGAATTCATGCGATCCATGTCTTTTGAC C CCG	to clone *pe35/ppe68_1* with FLAG tag into pMV
pe35_1 SWAP pe31 ss Rv	CGTTGGCGGTTTCGGTGCTCAGGTACGAGGC C GCGATCTGCCGC	to clone the secretion signal of *pe31* into *pe35_1*
pe31 ss flank ppe68_1 FW	CACCGAAACCGCCAACGCCGTGGCATCTCAA T AGTCGGCCTGCCAAC	to clone the secretion signal of *pe31* into *pe35_1*
esxB Fw esxN	GTCCTCGCAAATGGGCTTCTGACGCGCAAAG C CAC	exchange 15 aa of secretion signal of *esxB* into *esxM*
esxB Rv esxM	CTAGCCGCGGAGGACCTGCTGGGAAGCCTGC T CTTGCTGCTCGTAGTTGTTACCGGCCTGACGG	exchange 15 aa of secretion signal of *esxM* into *esxB*
ppe68_1 Flag_HindIII RV	CCCCCAAGCTTTCACTTGTCGTCATCGTCTTT GT AGTCCCAGTCGTCGTCGTCATC	to clone *pe35/ppe68_1* with FLAG tag into pMV
pe35 EcoRI FW	CCCCCGAATTCATGCGATCCATGTCTTTTGAC CC CG	to clone *pe35/ppe68_1* with FLAG tag into pMV
esxB NheI Fw	CCCCCGCTAGCATGGCAGAGATGAAGACCGA T GC	to clone *esxB_1/esxA_1* into pSMT3

Table S3. Plasmids used in this study.

	Plasmids	Characteristics	Gene origin	Reference
I	pMV::*pe35/ppe68_1WT-FLAG*	hsp60 promoter, KanR, integrative	*M. marinum* M	This study
II	pSMT3::*esxB_1/EsxA_1WT-HA*	hsp60 promoter, hygroR	*M. marinum* M	This study
III	pSMT3::*pe35/ppe68_1-FLAG /esxB_1/esxA_1-HA WT* ALL WT	hsp60 promoter, hygroR, all four genes are WT	*M. marinum* M	This study
IV	pSMT3::*pe35/ppe68_1-FLAG /esxB_1/esxA_1-HA WT* dSS PE35	hsp60 promoter, hygroR, deletion of the C-terminal 15 amino acids of PE35	*M. marinum* M	This study
V	pSMT3::*pe35/ppe68_1-FLAG /esxB_1/esxA_1-HA WT* dSS EsxB_1	hsp60 promoter, hygroR, deletion of the C-terminal 21 amino acids of EsxB_1	*M. marinum* M	This study
VI	pMV::*pe35/ppe68_1WT-FLAG* PPE68_1 SWAP	hsp60 promoter, KanR, integrative, *ppe68_1* carried $espG_5$ chaperone binding domain of *ppe18*	*M. marinum* M	This study
VII	pSMT3::*pe35/ppe68_1-FLAG /esxB_1/esxA_1-HA* SINGLE SWAP	hsp60 promoter, hygroR, *ppe68_1* carried $espG_5$ chaperone binding domain of *ppe18*	*M. marinum* M	This study
VIII	pSMT3::*pe35/ppe68_1-FLAG /esxB_1/esxA_1-HA* DOUBLE SWAP	similar to number VII, plus *esxB_1* carries secretion signal of *esxM*	*M. marinum* M	This study
IX	pSMT3::*pe35/ppe68_1-FLAG /esxB_1/esxA_1-HA* TRIPLE SWAP	similar to number VIII, plus *pe35* carries secretion signal of *pe31*	*M. marinum* M	This study
X	pSMT3::*pe35/ppe68_1-FLAG /esxB_1/esxA_1-HA* EsxB_1 SWAP	hsp60 promoter, hygroR, *esxB_1* carries secretion signal of *esxM*	*M. marinum* M	This study
XI	pSMT3::*pe35/ppe68_1-FLAG /esxB_1/esxA_1-HA* EsxB_1 PE35 SWAP	similar to number X, plus *pe35* carries secretion signal of *pe31*	*M. marinum* M	This study

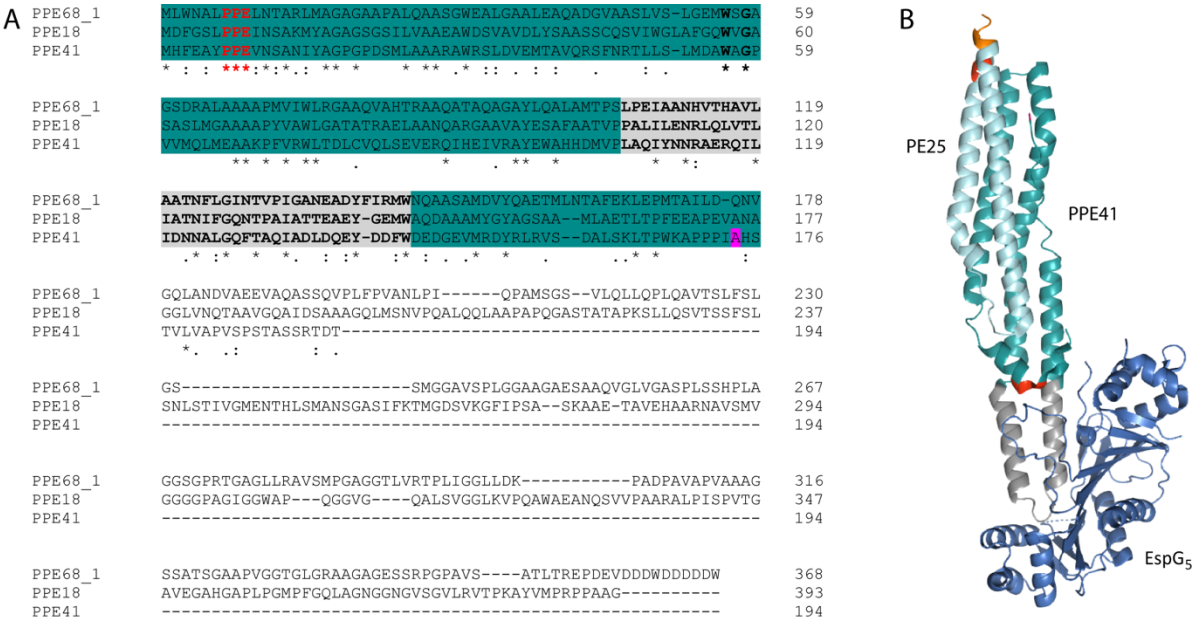

Figure S1. (A) Sequence alignment of PPE proteins used in this study. The alignment also contains PPE41 from *M. tuberculosis*, which structure is shown in (B). The conserved N-terminal domain of PPE proteins, with an approximate length of 180 amino acids, are indicated in green. The EspG binding domain that was exchanged in the study is indicated in grey. The conserved PPE motifs are depicted in red; the WxG motifs are depicted in bold and the pink amino acid indicates the most C-terminal amino acid visible in the crystal structure of the PPE41, shown in (B). (B) Solved structure of PE25-PPE41-$EspG_5$ from *M. tuberculosis* (22). PE25 protein is indicated in light blue, PPE41 in green and the $EspG_5$ chaperone in dark blue. The PE and PPE motifs are indicated in red and the general secretion motif (YxxxD/E) in the PE protein that is only partly solved, is indicated in orange. The most C-terminal amino acid of the PPE41 visible in the crystal structure is indicated in pink, similar as under (A).

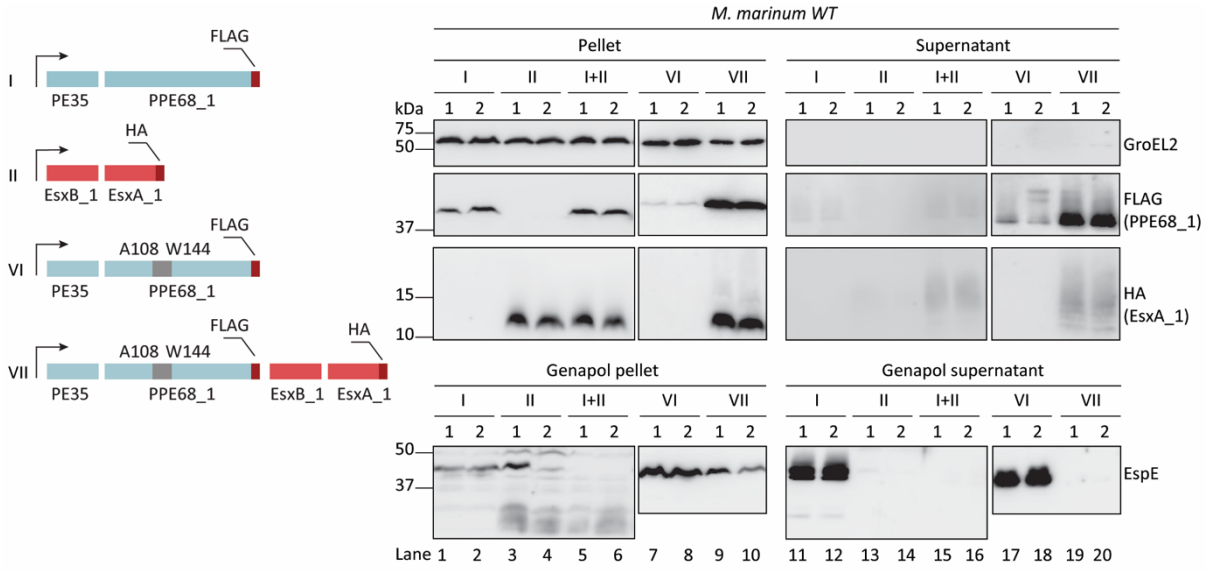

Figure S2. Overexpression of EsxA_1 interferes with EspE secretion and this secretion is not restored by co-overexpressing or rerouting of PE35/PPE68_1 via ESX-5. A schematic representation of the different constructs used are shown on the left. The genes encoding the *M. marinum* ESX-1 substrates PE35/PPE68_1 (in blue), PE35/PPE68_1 containing an $EspG_5$ chaperone binding domain (in blue and grey) and EsxB_1/EsxA_1 (in pink) are either expressed from separate *hsp60* promoters (I, II, VI) or co-expressed under the same *hsp60* promoter (VII). Constructs I and VI are expressed from integrative pMV plasmids, while constructs II and VII are expressed from multicopy pSMT3 vectors. Immunoblot analysis of the cell pellet and culture supernatant fractions of WT *M. marinum* using an HA antibody to detect EsxA_1-HA, a FLAG-antibody to detect PPE68_1-FLAG, an EsxA antibody, detecting both endogenous and exogenous EsxA paralogues, and a GroEL2 antibody to detect the intracellular control protein GroEL2. Surface-localized EspE was extracted from bacterial cell pellets using Genapol X-080, resulting in Genapol pellet and supernatant fractions. These fractions were immune-stained with EspE antibodies. Equivalent OD units of cell pellets or Genapol pellet (0.2 OD unit) and culture supernatants or Genapol supernatants (0.5 OD unit) are shown. Numbers indicate two independent *M. marinum* colonies carrying the same construct.

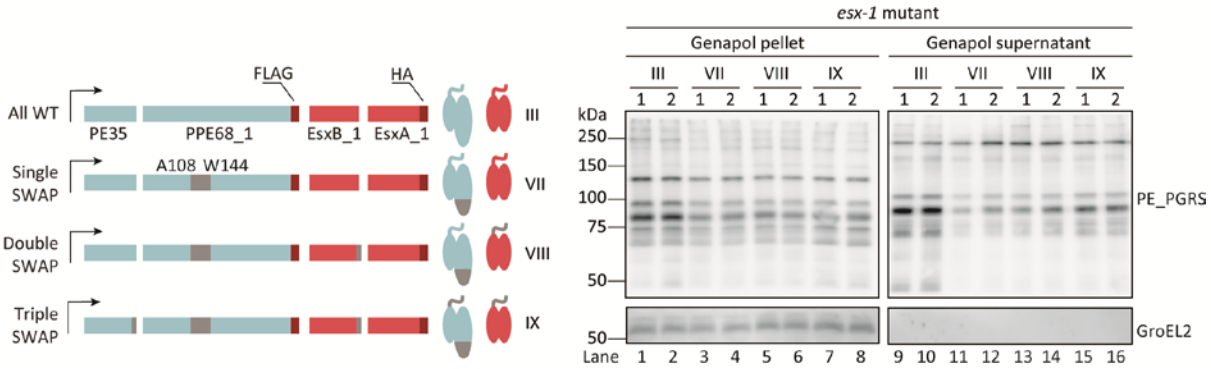

Figure S3. Rerouted PPE68_1 and EsxA_1 compete with endogenous ESX-5 substrates for secretion. Immunoblot analysis of the surface-localized ESX-5 dependent PE_PGRS substrates in the *esx-1* mutant strain overexpressing PE35/PPE68_1/EsxB_1/EsxA_1 variants. The fractions of enriched cell surface proteins (Genapol supernatant) were collected from the bacterial cell pellets (Genapol pellet) using Genapol X-080. Equivalent OD units were loaded; 0.2 OD for pellet and 0.5 OD for Genapol supernatants. Numbers indicate two independent *M. marinum* colonies carrying the same construct.

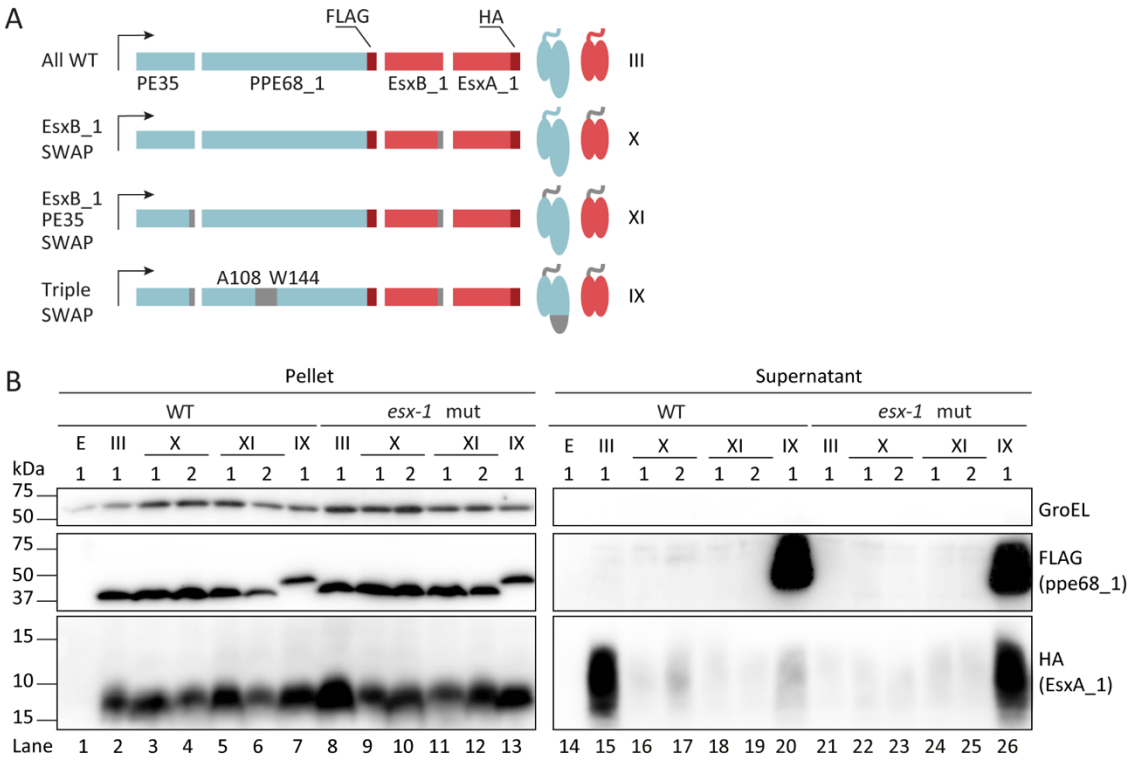

Figure S4. EsxA_1 is not rerouted to ESX-5 when the C-terminal domains of EsxB_1 and PE35 are replaced with equivalent domains of EsxM and PE31, respectively. (A) Schematic representation of the different constructs used in the secretion analysis. The introduced sequences of the ESX-5 substrates EsxM, PE31 and PPE18 are indicated in grey. The FLAG-tag on PPE68_1 and HA-tag on EsxA_1 are indicated in red. (B) Immunoblot analysis of EsxA_1 as detected with an HA antibody, PPE68_1 as probed with a FLAG antibody, and intracellular GroEL2 by a GroEL2 antibody, in pellet and supernatant fractions. Different derivatives of PE35/PPE68_1/EsxB_1/EsxA_1 were tested in WT *M. marinum* and an *eccCb*$_1$ mutant (*esx-1* mutant). Equivalent OD units of cell pellets (0.2 OD unit) and culture supernatants (0.5 OD unit) are shown. Numbers indicate two independent *M. marinum* colonies carrying the same construct. The minor HA signals that could be observed in the supernatants of the WT strain carrying EsxB_1 SWAP (construct X) and PE35 EsxB_1 SWAP (construct XI) were comparable to the signals in the supernatant fractions of the *esx-1* mutant carrying the ALL WT construct (construct III). These were therefore considered background signals, probably the result of minor cell lysis.