Supplementary Information for

The *Mycobacterium marinum* ESX-1 system mediates phagosomal permeabilization and type I interferon production via separable mechanisms

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SI Materials and Methods

Macrophages. Bone marrow-derived macrophages from wild type C57BL/6 (B6) or *ifnar1*$^{-/-}$ (IFNAR-KO) mice were prepared as previously described (1). In brief, extracted bone marrow cells were cultured for 7 days in a macrophage medium consisting of RPMI 10% (v/v) heat-inactivated fetal calf serum (Sigma-Aldrich), 1% (v/v) glutamine (ThermoFisher) and 10% (v/v) m-CSF containing supernatant from 3T3-CSF cells.

Bacterial strains. The wild type *M. marinum* M-strain and an isogenic deletion mutant (ΔRD1) lacking the RD1 locus (2), as well as the insertional transposon mutants used have been previously described (3-5) (see Table S1). *M. marinum* was grown at 30°C in Middlebrook 7H9 broth (BD Biosciences) or on 7H10 agar plates (BD Biosciences), respectively. Both media contained 0.5% (v/v) glycerol. The 7H9 broth was supplemented with 0.05% Tween 80 (Sigma-Aldrich) and 10% ADC enrichment (BD Biosciences), and the 7H10 agar with 10% OADC enrichment (Conda Lab). Cultures were supplemented with antibiotics as described in original references (see Table S1). *M. marinum* strains carrying the pTEC15 plasmid (Addgene) encoding Wasabi were grown with 50 mg/ml Hygromycin B. For fractionation of *M. marinum* cultures they were grown in Sauton's defined medium (Teknova).

Bacterial cell fractions for Western Blot analysis. Strains were grown to $OD_{600nm} \approx$ 0.8 in 25 ml of 7H9 broth. The bacteria from 15 ml of the 7H9 culture were collected by centrifugation and washed extensively in Sauton's medium, and inoculated into 20 ml (final volume) of this medium. Two days after inoculation cultures were collected

for fractionation into the secreted fraction (culture fraction, CF), envelope fraction (ENV) and cytosolic fraction (CYT), as previously described in detail (5). For Western blot analysis of fractions, loading was normalized to the weights of the original bacterial pellets.

Macrophage infection and analysis of intracellular growth. Macrophage infections were performed as described (1). Briefly, macrophages were infected with the appropriate number of bacteria suspended in macrophage medium to obtain the desired multiplicity of infection (MOI). Two hours post infection the cells were washed with macrophage medium to remove extracellular bacteria, and remaining extracellular bacteria were killed off by incubating the cells with macrophage medium supplemented with 200 µg/ml amikacin (Sigma-Aldrich) for 2 h. Cells were then washed twice with fresh medium and incubated at 32°C with 5% CO_2 until further analysis.

For analysis of intracellular growth, infected macrophages were lysed with 0.1% (final concentration) Triton X-100 (Sigma-Aldrich) for 10 min at room temperature (RT) at the indicated time points post infection. Ten-fold serial dilutions of the lysates were plated on 7H10 agar plates for colony forming unit (CFU) analysis.

Analysis of actin tail formation. Macrophages were seeded at a density of 0.15 x 10^6 cells/ml on sterile 15 mm diameter glass coverslips pre-coated with 10 µg/ml fibronectin (Sigma-Aldrich) 24 h prior to infection. Macrophages were infected with bacteria expressing green fluorescence, either *gfp* on the chromosome for WT, ΔRD1, EspE::tn and EccCb1::tn or Wasabi fluorescence expressed from the plasmid pTEC15

(Addgene) for EspG1::tn, EspH::tn, EccA1::tn, EspI::tn, EspJ::tn, EspK::tn and EspB::tn. At appropriate time points, cells were washed gently with pre-warmed PBS and fixed with 4% paraformaldehyde (PFA) at RT for 20 min. Fixed cells were then washed with PBS and permeabilized with 0.1% Triton X-100 for 4 min at RT. Cells were blocked with PBS supplemented with 1% BSA for 1 h, and F-actin was stained using Alexa Fluor 594-conjugated Phalloidin (Invitrogen). After washes with PBS and distilled H_2O, coverslips were mounted in mowiol (Sigma-Aldrich) and analyzed with a Zeiss Axiovert 200M microscope, using a 63X objective. Images were captured with the Axiovision software and processed using the software ImageJ.

Fractionation of macrophages for analysis of cytosolic bacterial counts. Macrophages were infected in 6-well plates (10^6 cells/well) and incubated for 48 h with macrophage medium containing 10 µg/mL Gentamycin (Sigma-Aldrich). Supernatants were collected and centrifuged (300 g, 4 min) to pellet detached cells. Macrophages were dislodged with ice-cold PBS and split into 2 samples. One sample was used to determine the total amount of intracellular bacteria, essentially as described above. The other sample was used to determine the amount of cytosolic bacteria. For this purpose cells were incubated in 100 µl digitonin buffer (25 µg/mL digitonin [Sigma-Aldrich], 150 mM NaCl, 50 mM Hepes) for 10 min on ice, allowing for selective permeabilization of the plasma membrane. Macrophages were then pelleted by centrifugation (1000 g, 3 min) and the supernatant (cytosolic fractions) were collected. This centrifugation step was repeated 3 times before the enriched cytosolic fractions were serially diluted and plated on 7H10 agar plates for CFU analysis.

Analysis of cell-to-cell bacterial spread. Macrophages were infected in 12-well plates (0.5 x 10^6 cells/well) at the indicated MOI. At appropriate time points post infection, cells were dislodged, collected by centrifugation (200 g, 4 min) and fixed with 4% PFA for 20 min at RT. Quantification of infected cells (GFP^+ cells) was performed by flow cytometry. Data was acquired on a LSRII flow cytometer (BD Biosciences) and analyzed using the FlowJo software version 9.

Analyses of cytokine secretion and STAT activation. At appropriated time points post infection, supernatants from infected macrophages were collected and analyzed by ELISA for IFNβ (Biolegend) and IL-1β (RnD Systems) secretion.

For analysis of STAT-signaling, macrophages (0.5 x 10^6) were infected at MOI 5 for 2, 4 or 6 h. Cells were dislodged on ice and lysed in 150 µl lysis buffer (1% NP-40 [Sigma-Aldrich], 150mM NaCl [Sigma], 50mM Tris HCl [Sigma], pH8, and 1x Complete EDTA-free protease inhibitor cocktail [Roche] and 1x PhosphoSTOP Easy [Roche]). Cells were lysed for 30 min at 4°C with agitation and centrifuged for 20 min (13000 g, 4°C) to pellet cellular debris. Supernatants were analyzed by Western blot using antibodies against STAT1 and STAT2, as indicated (see Table S1).

Reverse-transcription quantitative PCR (RTqPCR) analysis of *ifnβ* gene expression. Macrophages were infected in 24-well plates (0.3 x 10^6 cells/well) and RNA was extracted 4 h post infection using the SV total RNA isolation kit (Promega), including an incubation with DNAse I to remove DNA. cDNA synthesis was performed using the GoScript reverse transcription systems (Promega). Gene expression was determined by real-time quantitative PCR (qPCR) using SSoFast

EvaGreen qPCR supermix (Biorad) on the iQ5 Real-Time PCR Detection System (BioRad). Expression of the house keeping gene *reep5* was analyzed to enable normalization of data. Specific primers used for *ifnβ* and *reep5*, respectively, are indicated in Table S1.

Analysis of Cytochrome C release. Macrophages were infected in 12-well plates (0.5×10^6 cells/well) at MOI 15 for 2, 3, 4, 5 or 6 h. As a positive control for Cytochrome C release, cells were treated with 100 ng/ml TNFα (MACS Miltenyi Biotec) and 1 µg/ml cycloheximide (Sigma-Aldrich) for 6 h. For harvest, detached cells from culture supernatants were collected by centrifugation (100 g, 2 min) and the remaining cells in the wells were detached using 250 µl ice-cold PBS. Cells were transferred in a V-bottom 96-well plate and centrifuged for 4 min (500 g, 4°C). Cell pellets were resuspended in permeabilization buffer (50 µg/ml digitonin, 100 mM KCl) for 5 min on ice. Immediately after the incubation, 100 µl of 4% PFA was added to stop the permeabilization and cells were collected by centrifugation. Macrophages were fixed for 20 min with 4% PFA at RT and then washed twice with PBS. Cells were resuspended in blocking buffer (400 µg/ml donkey gamma globulin [Jackson ImmunoResearch], 0.05% saponin [Sigma-Aldrich]) for 15 min at RT. Cytochrome C was stained overnight using a specific mouse antibody (BD Pharmingen) or a mouse isotype IgG (Biolegend) as a control, diluted in blocking buffer. After one wash with PBS supplemented with 0.05% saponin and a subsequent wash with PBS alone, cells were incubated with an anti-mouse Phycoerythrin-conjugated antibody (Biolegend) in blocking buffer for 1 h at RT, washed twice with blocking buffer and once with PBS. Analysis of Cytochrome C release was performed by flow cytometry. Data was

acquired on a LSRII flow cytometer (BD Biosciences) and analyzed using the FlowJo software version 9.

Analysis of cellular ATP content. Macrophages infection was performed in 96-well plates (5 x 10^4 cells/well) at MOI 15. At 24 h post infection, ATP was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) following the manufacturer's protocol.

Cell fractionation for detection of cytosolic DNA. Preparation of whole cell extracts (WCE) and purified cytosolic fractions for the analysis of cytosolic DNA content was performed as previously described in detail (6). At the indicated time point post infection cells (10^6) were washed with warm PBS, dislodged with ice-cold PBS and split into 2 samples in order to prepare whole cell extract (WCE) and the cytosolic fraction, respectively. For WCE, cells were resuspended in 50 µM NaOH and boiled for 30 min to solubilize the DNA, and neutralization was achieved by adding 1 M Tris HCl (pH8) corresponding to $1/10^{th}$ of the volume. For cytosolic samples, cells were resuspended in digitonin buffer (25 µg/ml digitonin, 150 mM NaCl and 50 mM Hepes) for 10 min on ice and immediately centrifuged for 3 min (1000 g, 4°C) to pellet non-permeabilized cells. This step was repeated 3 times and the final supernatant was centrifuged for 10 min (17000 g, 4°C) to remove remaining cellular debris. DNA was extracted using the GeneJET PCR Purification kit (Thermofisher).

DNA content in the WCE and purified cytosolic fraction was analyzed by quantitative PCR. Mitochondrial (*Dloop1*), nuclear (*Tert*) and bacterial (*FurA*) DNA was analyzed using specific primers (Table 1). The cycle threshold (C_t) for the cytosolic sample was normalized to the C_t value for the corresponding WCE sample.

For host DNA (*Dloop1* and *Tert*) the ratios obtained for uninfected cell samples were used as reference to calculate the relative fold change of cytosolic DNA content in infected samples (the DDC_t method). For *M. marinum* DNA (*FurA*) the ratio obtained was normalized to that of DRD1 infected cells at 2 hours post infection.

Proteins in the WCE and purified cytosolic fraction were precipitated using trichloroacetic acid, and analyzed by Western blot using antibodies against Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the Pyruvate dehydrogenase E1 alpha subunit (PDE1α) as a mitochondrial marker (see Table S1).

Analysis of bacterial GFP expression. Bacteria were grown 4 days in 7H9 medium, collected by centrifugation (2500 g, 10 min), washed twice in PBS and fixed with 4% PFA for 20 min at RT. Bacteria were passed through a 26G3/8 needle 3 times to disrupt bacterial aggregates. GFP expression was measurement by flow cytometry. Data was acquired on a LSRII flow cytometer (BD Biosciences) and analyzed using the FlowJo software version 9. Wild type *M. marinum* not expressing GFP was used as a negative control.

Cell death assay by flow cytometry

At the indicated time points post infection, macrophages ($0.5x10^6$) were dislodged using ice cold PBS, centrifuged for 4 min (200 g, 4°C) and stained with Zombie aqua fixable viability kit (Biolegend) diluted 1/1000 in PBS during 30 min on ice. Cells were then washed with PBS 0.5% BSA and then fixed 20 min with 4% PFA at room temperature. Cells were washed with PBS 0.5% BSA and data was acquired on a LSRII flow cytometer (BD Biosciences) and analyzed using the FlowJo software version 9.

Cell death assay by measurement of Lactate dehydrogenase (LDH) release

Cell culture supernatants were collected after 24 and 48 h post infection and analyzed for LDH release using the colorimetric CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) following the manufacturer's protocol.

Statistical analysis. Statistical analyses were performed using the software GraphPad Prism version 7. A 1-way ANOVA with Dunnett's test or a 2-way ANOVA with Tukey's test were used for multiple comparisons, and an unpaired t-test for pairwise comparisons, as indicated in figure legends. A $p<0.05$ was considered significant; *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

SI Figures

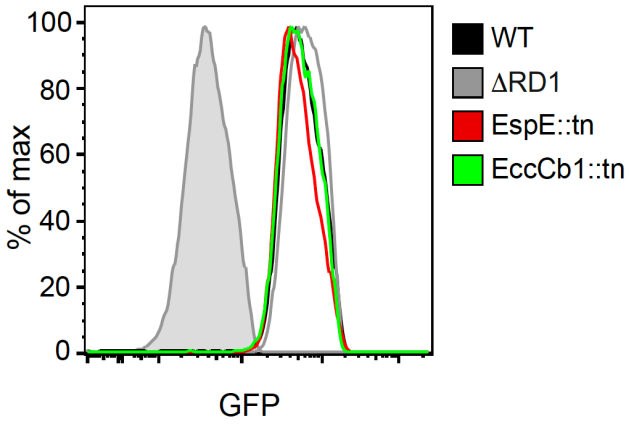

Fig. S1. WT, ΔRD1, EspE::tn and EccCb1::tn *M. marinum* strains encoding *gfp* chromosomally exhibit comparable GFP fluorescence. The histogram shows GFP fluorescence intensity (x axis) and cell counts (y axis) of the indicated *M. marinum* strains assessed by flow cytometry. WT *M. marinum* not expressing GFP was used as a negative control (grey shaded histogram).

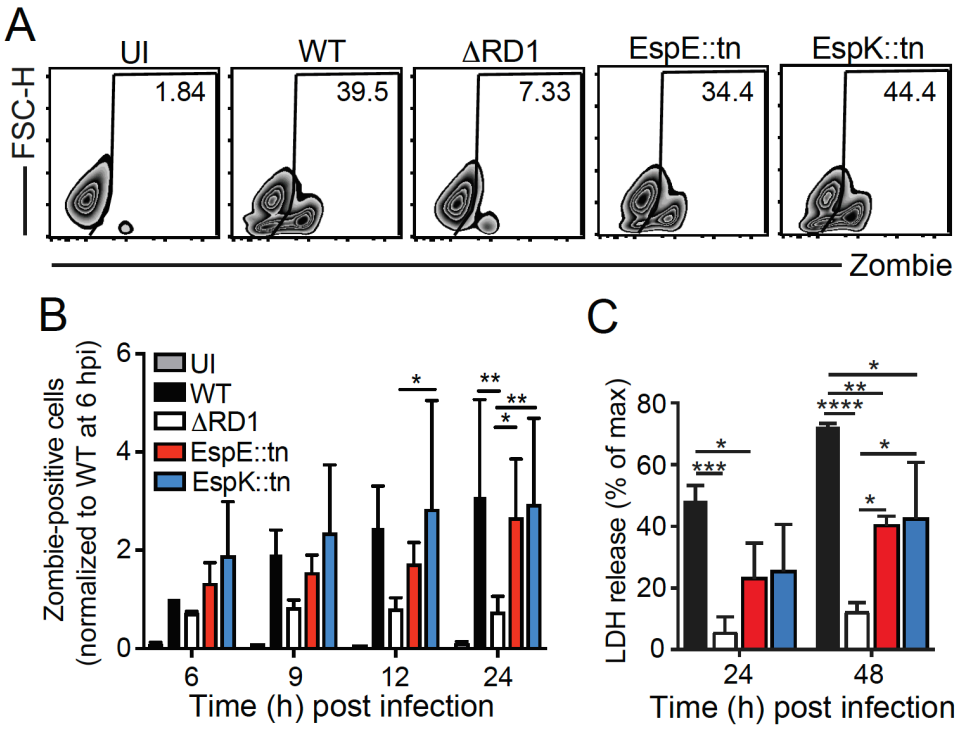

Fig. S2. Analysis of cell death during infection of macrophages. B6 macrophages were infected at MOI 15 with WT, ΔRD1, EspE::tn or EspK::tn *M. marinum*, or uninfected (UI) for control. (*A and B*) Macrophages were analyzed for cell death by flow cytometry at the indicated time points post infection using a Zombie Aqua viability kit. *(A)* Representative plots of Zombie+ macrophages at 24 hpi. (*B*) Quantification of Zombie^{+} cells normalized to WT infection at 6 hpi. Results (mean ± SD) from 4 independent experiments. 2-way ANOVA, *p<0.05, **p<0.01. (*C*) Lactate dehydrogenase (LDH) release was measured in the supernatants of macrophages as indicated. Results (mean ± SD) from 3 independent experiments. 2-way ANOVA, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

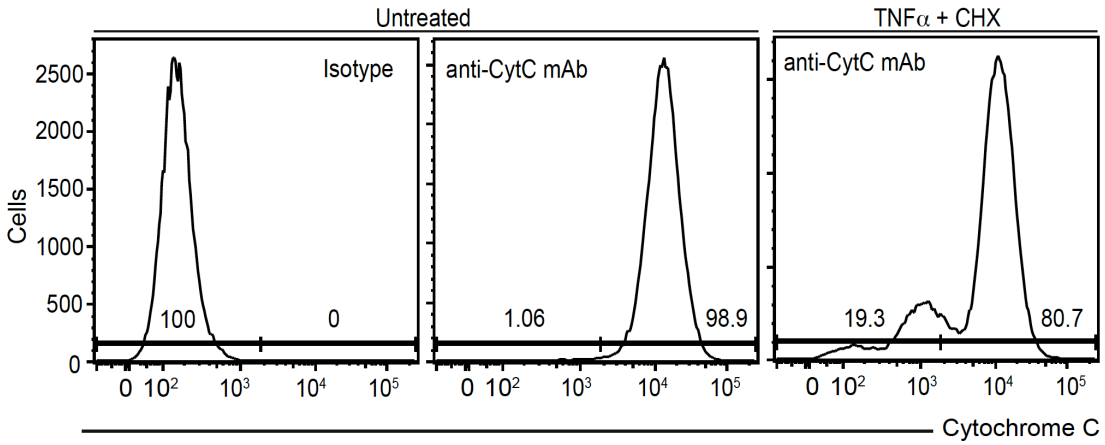

Fig. S3. Cytochrome C depletion determined by flow cytometry. Untreated macrophages (middle panel) and macrophages treated with TNFα and cycloheximide for 6 h (right panel) were used as negative and positive control, respectively, for Cytochrome C (CytC) depletion. Isotype IgG was used as a control (left panel). The histograms show fluorescence intensity for CytC staining (x axis) and cell counts (y axis).

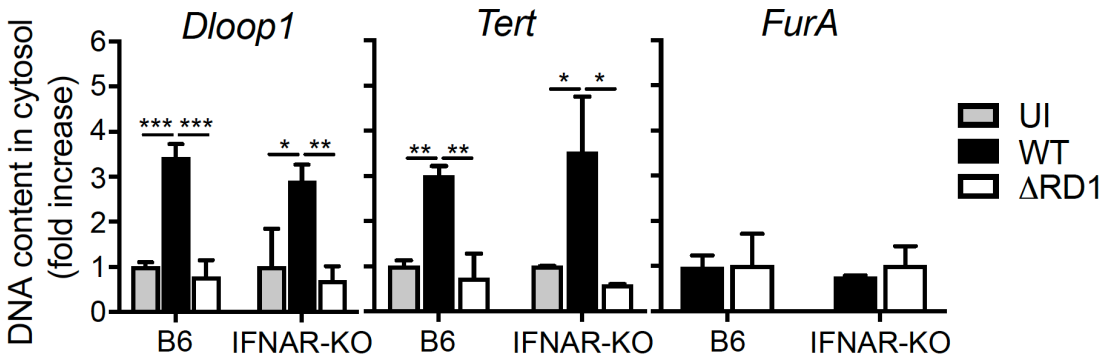

Fig. S4. Type I IFN signaling is not required for release of host DNA into the cytosol. Wild type C57BL/6 (B6) and IFNAR-KO macrophages were infected with WT or ΔRD1 *M. marinum*, or uninfected (UI), as indicated. At 24 hpi, the levels of mitochondrial (*Dloop1*), nuclear (*Tert*) and bacterial (*FurA*) DNA in the cytosol of macrophages were analyzed by qPCR. Results (mean ± SD, $n = 3$) are representative of 2 independent experiments. 1-way ANOVA (*Dloop1* and *Tert*) or unpaired t test (*FurA*), *P<0.05, **P<0.01, ***P<0.001.

SI Table

Table S1. Materials and reagents.

Antibodies	**Source**	**reference**
Polyclonal rabbit anti-ESAT-6	BEI Resources	Cat# NR-13803
Polyclonal rabbit anti-CFP-10	BEI Resources	Cat# NR-13801
Polyclonal rabbit anti-Ag85 complex	BEI Resources	Cat# NR-13800
Polyclonal rabbit anti-*E. coli* GroEL	ENZO LifeSciences	Cat# ADI-SPS-875-F
Polyclonal rabbit anti-mouse STAT1	Cell Signaling Technologies	Cat# 9172
Monoclonal rabbit anti-mouse phospho STAT1 (p-Tyr701)	Cell Signaling Technologies	Cat# 9167
Polyclonal rabbit anti-mouse STAT2	Millipore	Cat# 06-502
Polyclonal rabbit anti-mouse phospho STAT2 (p-Tyr689)	Millipore	Cat# 07-224
Polyclonal rabbit anti-mouse GAPDH	Sigma-Aldrich	Cat# 9545
Monoclonal mouse anti-human Pyruvate deshydrogenase E1 alpha subunit	Abcam	Cat# ab110330
Monoclonal mouse anti-rat Cytochrome C	BD Biosciences	Cat# 556432
Polyclonal goat anti-rabbit IgG conjugated with Horseradish Peroxidase	Jackson ImmunoResearch	Cat# 111-036-003
Polyclonal donkey anti-mouse IgG conjugated with Horseradish Peroxidase	Jackson ImmunoResearch	Cat# 715-036-150
Polyclonal donkey gamma globulin unconjugated	Jackson ImmunoResearch	Cat# 017-000-002
Rat anti-mouse IgG1 conjugated with Phycoerythrin (PE)	Biolegend	Cat# 406607
Mouse IgG1 isotype	Biolegend	Cat# 400101
***Mycobacterium marinum* strains**	**Reference**	
Wild type (WT)	Volkman *et al.* PLoS Biol 2004	
ΔRD1	Volkman *et al.* PLoS Biol 2004	
EspE::tn	Carlsson *et al.* PLoS Pathog 2009	
EspG1::tn	Gao *et al.* Mol Microbiol 2004	
EspH::tn	Gao *et al.* Mol Microbiol 2004	
EccA1::tn	Gao *et al.* Mol Microbiol 2004	
EccCb1::tn	McLaughlin *et al.* PLoS Pathog 2007	
EspI::tn	Gao *et al.* Mol Microbiol 2004	
EspJ::tn	Gao *et al.* Mol Microbiol 2004	
EspK::tn	Gao *et al.* Mol Microbiol 2004	
EspB::tn	Gao *et al.* Mol Microbiol 2004	
Commercial Assays	**Source**	**reference**
Mouse IFN-β ELISA Kit	Biolegend	Cat# 439407
Mouse IL-1β ELISA kit	RnD Systems	Cat# DY401-05
SSoFast EvaGreen qPCR supermix	Biorad	Cat#1725204
Cytotoxicity Assay (LDH release)	Promega	Cat# G1780
Luminescent cell viability assay (ATP detection)	Promega	Cat# G7570
RNA purification system	Promega	Cat# Z3100
RT-PCR Reverse transcription kit	Promega	Cat# A5000
GeneJET PCR Purification kit	ThermoFischer	Cat# K0701
Chemicals	**Source**	**reference**
Fibronectin	Sigma-Aldrich	Cat# F1141
Mowiol 4-88	Sigma-Aldrich	Cat# 81381
NP-40	Sigma-Aldrich	Cat# I8896

cOmplete™, EDTA-free Protease Inhibitor Cocktail	Roche	Cat# 000000011873580001
PhosSTOP, phosphatase inhibitor cocktail	Roche	Cat# 000000004906845001
Digitonin	Sigma-Aldrich	Cat# D141
Saponin	Sigma-Aldrich	Cat# 84510
TNFα	MACS Miltenyi Biotec	Cat# 130-101-689
Cycloheximide	Sigma-Aldrich	Cat# C7698
Alexa Fluor 594 Phalloidin	Invitrogen	Cat #A12381
Zombie aqua viability kit	Biolegend	Cat#4231102
Hygromycin B	Invitrogen	Cat# 10687-010
Kanamycin	LifeTechnologies	Cat# 11815-024
Gentamycin	Sigma-Aldrich	Cat# G1397
Amikacin	Sigma-Aldrich	Cat# A3650
Middlebrook 7H9	BD Biosciences	Cat# 271310
Middlebrook 7H10	BD Biosciences	Cat# 262710
ACD enrichment for 7H9	BD Biosciences	Cat# 212352
OADC enrichment for 7H10	Conda Lab	Cat# 6037
Sauton's defined broth	Teknova	Cat #S0351
Primers and plasmid		
ifnb (forward)	ATGAGTGGTGGTTGCAGGC	
ifnb (reverse)	TGACCTTTCAAATGCAGTAGATTCA	
reep5 (forward)	GCCATCGAGAGTCCCAACAA	
reep5 (reverse)	GCATCTCAGCCCCATTAGC	
dloop1 (forward)	ATTCTA CCATCCTCCGTGAAACC	
dloop1 (reverse)	TCAGTTTAGCTACCCCCAAGTTTAA	
tert (forward)	CTACGTCATGTGTCAAGACCCTCTT	
tert (reverse)	GCCAGCACGTTTCTCTCGTT	
furA (forward)	CGACACCGAAAC GATCTACT	
furA (reverse)	GCCCACCGAGGTAAGTG	
pTEC15 plasmid (Wasabi)	Addgene	Cat# 30174
Equipment	**Source**	
Flow cytometer LSRII	BD Biosciences	
iQ5 Real-Time PCR Detection System	Biorad	
Trans-blot Turbo transfer system	Biorad	
ChemiDoc Imaging systems	Biorad	
Epifluroescence microscope	Zeiss	

SI References

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