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Supplementary Information for:

Coxiella co-opts the Glutathione Peroxidase 4 (GPX4) to protect the host cell from oxidative stress-induced cell death

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Supplementary text
Figure S1 to S2
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39 **Supplementary Information Text**
40 **Extended Methods:**
41
42 **Bacterial culture and preparation**
43 *Escherichia coli* DH5α (Clontech) was grown in Luria–Bertani medium
44 supplemented with 50 μg/mL kanamycin (Sigma–Aldrich), with 100 μg/mL
45 ampicillin (A. G. Scientific) or with 10 μg/mL chloramphenicol (Boehringer
46 Mannheim) when required.
47
48 *L. pneumophila* JR32 *ΔflaA* strains were used in this study as previously described
49 [32,77]. Bacteria were cultured on ACES buffered charcoal-Yeast Extract Agar [32] at
50 37°C, for 4 days from frozen stocks. Single colonies were streaked on fresh plates
51 containing Isopropyl β- d-1-thiogalactopyranoside (IPTG) and allowed to grow for
52 another 2 days. For *in vitro* and *in vivo* infections, bacteria grown on BCYE agar
53 plates were resuspended in sterile distilled water and diluted in RPMI to the
54 required density based on OD600 nm.
55
56 *C. burnetii* Nine Mile Phase II RSA439 (Ted Hackstadt, NIH), referred to as wild
57 type (WT), *ΔmceF*, *ΔmceF* pFLAG:*mceF* [pJB-CAT:3xFLAG_*mceF*] and the
58 T4BSS mutant *IcmL* were routinely cultivated in ACCM-2 as previously described
59 [78,79]. The media was supplemented with 350 μg/ml kanamycin or 3 μg/ml
60 chloramphenicol when required and cultures incubated at 37°C, 5% CO_2, 2.5% O_2.
61
62 **Construction of *L. pneumophila* expressing *C. burnetii* effectors**
63 *L. pneumophila* JR32 Δ*flaA* were electroporated with pJB-CAT:3xFLAG
64 constructs, cloned individually with Dot/Icm effectors from *C. burnetii*. Briefly, a 2-
65 day patch of *L. pneumophila* was resuspended in 10 mL of ice-cold 10% glycerol
66 and centrifuged at 3,220 x g for 5 minutes at 4°C, then washed twice with 10 mL
67 ice-cold 10% glycerol. Bacterial pellets were resuspended in 50 μL ice-cold 10%
68 glycerol and stored at -80°C long term. Cells were electroporated with 100-500 ng
69 of plasmid DNA at 2.5 kV, 25 μF and 200 Ω in a BioRad Gene Pulse XCell and
70 immediately resuspended in 950 μL of ACES-buffered Yeast Extract. Effector

71 expression of transformed *L. pneumophila* was amplified with the addition of 1 mM
72 of isopropyl b-D-1-thiogalactopyranoside (IPTG, Invitrogen) to agar plates and
73 confirmed by immunoblot using an anti-FLAG antibody (Sigma-Aldrich).
74
75 **BlaM:CBU1543 plasmid construction**
76 Plasmids pJB-CAT:BlaM-MCS and pcDNA4/TO:3xFLAG:CBU1543 were digested
77 with BamHI and XhoI, CIP treated, and gel purified. The *cbu1543* fragment was
78 ligated into the digested vector and transformed into PIR2 *E. coli*. Plasmids were
79 confirmed by sequencing. The newly created plasmid, pJB-CAT:BlaM:CBU1543,
80 was then transformed into WT and *IcmL* mutant *C. burnetii* NMII and clonally
81 isolated. Expression of the fusion protein was confirmed by western blot. WT and
82 *IcmL* mutant *C. burnetii* NMII expressing pJB-CAT:BlaM:CBU0077(MceA) were
83 used as a positive control.
84
85 **Translocation assay**
86 Translocation assays were performed using the LiveBLAzer FRET loading kit
87 (Thermo Fisher Scientific) as previously described [1] and according to
88 manufacturer's instructions. Briefly, HeLa CCL2 cells were seeded in black-walled
89 clear bottom plates and infected with the relevant *C. burnetii* strains at an MOI of
90 500 the next day. After 20 hours, cells were treated with 2 mM $MnCl_2$ for 2 hours
91 before loading with CCF2-AM and incubating at room temperature for a further 2
92 hours. Plates were read on a Clariostar plate reader. Ratios of fluorescence at 520
93 and 450 were calculated after subtracting background fluorescence from cell-free
94 wells, with an increase in ratio corresponding to an increase in translocation.
95
96 **Recombinant DNA and molecular biology**
97 See Table S2 for oligonucleotides used in this study. Pfu Ultra was used to amplify
98 DNA for cloning and GoTaq® Green (Promega) or MyTaq™ Red (Bioline) were
99 used to screen *E. coli* colonies with inserts >1 kb or <1 kb, respectively, according
100 to the manufacturer's protocol. Samples were sequenced using the Sanger
101 method by AGRF (Australian Genome Research Facility). Analysis of the results

102 was conducted using Sequencher (Gene Codes Corporation) and compared to
103 known GenBank sequences using BLAST [2].
104
105 The library of *C. burnetii* effector expression plasmids was constructed using
106 oligonucleotides listed in Table S1 and In-Fusion cloning protocols (BD Clontech)
107 or traditional restriction enzyme cloning. pJB-CAT3xFLAG and genomic DNA for
108 *C. burnetii* Dugway 5J108-111, G Q212 and K Q154 were kindly provided by Prof
109 Bob Heinzen.
110

111 **Stable cell lines**

112 Stable doxycycline-inducible HeLa cells were generated using a lentiviral system
113 to introduce pTRE_3G or pTRE_3G:3xFLAG:*mceF* (plasmids are listed in Table
114 S1). Briefly, 10 cm dishes (Corning) containing HEK293T cells in DMEM + 10%
115 FCS were co-transfected with pTRE_3G:3xFLAG:*mceF*, pLV-iVSV-G, and pCMV-
116 R8.2 using FUGENE6 (Promega), and incubated at 37°C, 5% CO_2 for 24 hours.
117 The media was replaced and the plates were incubated for a further 48 hours. The
118 supernatant containing virus was harvested, filtered (0.45 µm) and polybrene (5
119 mg/mL) was added. Supernatant were added to 2 x 10^5 cells/well of HeLa CCL2
120 cells in 6 well plates with DMEM + 10% FCS. Following selection with 5 µg/mL of
121 puromycin (Gibco), cells were single sorted at The Doherty Institute Flow
122 Cytometry Facility into 96 well plates (Corning) and incubated with DMEM + 20%
123 FCS at 37°C, 5% CO_2 to expand. Clones were selected and screened for protein
124 expression by the addition of doxycycline at 20 ng/mL for 48 hours. Doxycycline
125 induction time was adjusted as required.
126

127 **SDS-PAGE and Immunoblot analysis**

128 All protein samples were lysed using SDS loading dye by incubating at 95°C for
129 10 minutes unless otherwise stated. Lysed proteins were separated on NuPAGE
130 Bis-Tris gels (Life Technologies) with MES running buffer (Life Technologies).
131 Protein gels were transferred to a nitrocellulose membrane using the iBlot2 system
132 (Life Technologies). Membrane was blocked in either TBS + 0.1% Tween-20

133 (TBST)+ 5% (w/v) skim milk powder or TBST + 5% (w/v) BSA to match primary
134 antibody solutions, for 1 hour at RT. Relevant primary antibodies (Mouse anti-
135 FLAG, Sigma-Aldrich, 1:2000; Rabbit anti-NDUFAF2, Michael Ryan laboratory,
136 Monash University, 1:1000; Rabbit anti-Mfn2, Michael Ryan laboratory, Monash
137 University, 1:200; Rabbit anti-TIM29, Sigma-Aldrich, 1:1000; Rabbit anti-CoxIV,
138 Cell Signaling, 1:1000; Mouse anti-Cytochrome *c*, BD Biosciences, 1:300; Rabbit
139 anti-GPX4, Sigma-Aldrich, 1:1000) were incubated with the membrane overnight
140 at 4°C with mild rotation. Relevant secondary antibodies (Goat anti-Mouse IgG –
141 HRP, Perkin Elmer, 1:3000; Goat anti-Rabbit IgG – HRP, Perkin Elmer, 1:3000)
142 were added to the membrane for 1 hour at RT. The membrane was developed
143 using Clarity Western ECL Reagents (BioRad) and detected with MF-ChemiBIS,
144 version 3.2 (DNR Bio-Imaging Systems, Ltd.) or Amersham Imager (AI) 600
145 imager (GE Healthcare). Images were processed using Fiji software.
146

147 **Measurement of Mitochondrial Respiration**

148 Oxygen consumption rate (OCR) was measured using the XF96 analyzer with XF
149 Cell Mito Stress Test kit according to manufacturer instructions (Seahorse
150 Bioscience, MA, USA). Briefly, 2 x 10^4 cells/well of ^{3xFLAG}MceF or empty vector
151 stable cell lines were plated in Seahorse XF Cell Culture Microplate (96 wells) and
152 incubated in DMEM + 10% FCS at 37°C, 5% CO_2. Induced expression occurred
153 by the addition of 20 nM/mL of doxycycline for 24 and 2 hours before the addition
154 of the assay medium. Prior to the treatments, the assay medium (Seahorse XF
155 DMEM, pH 7.4) was supplemented with 1 mM pyruvate (Gibco), 2 mM glutamine
156 (Sigma-Aldrich), and 10 mM glucose (Sigma-Aldrich). The sensor cartridge was
157 loaded with Oligomycin (Sigma-Aldrich), Carbonyl cyanide-4 (trifluoromethoxy)
158 phenylhydrazone (FCCP, Sigma-Aldrich), and Rotenone/Antimycin A (Sigma-
159 Aldrich). The oxygen consumption ratio (OCR, pmol/min) was monitored in real-
160 time, with sequential treatments of 1 µM Oligomycin (ATP synthase inhibitor), 1
161 µM FCCP (mitochondrial uncoupler), and 1 µM Rotenone/Antimycin A (Complex I
162 and III inhibitors) that were auto-injected into the experimental wells, followed by
163 three measurement cycles. Data from Wave (Seahorse Wave Desktop Software)

164 were exported to Excel for further calculations. Basal respiration (OCR1 – OCR4),
165 maximum respiration (OCR3 – OCR4), the percentage of the spare respiratory
166 capacity ((OCR3/OCR1) x 100), proton leak (OCR2 – OCR4), ATP production
167 (OCR1 – OCR2), non-mitochondrial respiration (OCR4) and coupling efficiency
168 ((ATP production rate/Basal respiration rate) x 100) were calculated.

169

170 **Cytometric measurement of mitochondrial ROS**

171 To detect mitochondrial superoxide generation, a specific mitochondrial
172 superoxide indicator, MitoSOX™ Red (Molecular Invitrogen Probe), was used
173 according to the manufacturer's instructions. Briefly, 1 x 10^6 ^{3xFLAG}MceF cells/mL
174 were seeded in 10 cm dishes (Corning). Cells were induced with 20 nM/mL
175 doxycycline 24 hours prior to ROS induction. Media was then replaced with 1 μM
176 rotenone for a further 20 hours or with 0.1 uM Antimycin A (AA) for 4 hours. Next,
177 1 x 10^6 cells/mL were harvested and incubated with 5 μM MitoSOX for 30 minutes
178 at 37°C with mild agitation. Additionally, cells were incubated with Ghost Dye™
179 Red 780 (Tonbo Bioscience) for 20 minutes on ice and washed twice. Flow
180 cytometry analysis (BD FACS Canto II) was performed at The Doherty Institute
181 Flow Cytometry Facility. MitoSOX Red dye was laser excited at 488 nm and data
182 were collected at 585/42 nm, while Ghost Dye™ Red 780 was laser excited at 640
183 nm and data were collected at 780-60 nm.

184

185 ***C. burnetii* infection of tissue culture cells**

186 Infections of THP-1 and HeLa cells with *C. burnetii* were performed as previously
187 described [31]. Briefly, for THP-1 cells, cells were seeded at 5 x 10^5 cells/well in 24-
188 well plates and were differentiated with 10 nM phorbol 12-myristate 13-acetate
189 (PMA, Adipogen Life Sciences) for 3 days before being infected at a multiplicity of
190 infection (MOI) of 5. In comparison, HeLa cells were seeded at 2 x 10^4 cells/well
191 in 24-well plates the day before infection at an MOI of 5. On the day of infection,
192 axenic cultures of *C. burnetii* were harvested and genome equivalents were
193 quantified by qPCR using oligonucleotides specific for *ompA* [83,84]. Following the
194 addition of *C. burnetii*, cells were centrifuged at 500 x g for 30 minutes at 30°C,

195 before being washed twice with PBS and given fresh media + 5% FCS. At desired
196 times post-infection, samples were lysed with distilled water and centrifuged to
197 obtain the cell pellet. gDNA was extracted using the Quick DNA MiniPrep Kit (Zymo
198 Research) according to the manufacturer's protocol. qPCR reactions were set up
199 in MicroAmp Fast Optical 96-well reaction plates using the SensiFast SYBR No-
200 ROX kit (Bioline) and *C. burnetii ompA* specific oligonucleotides. Serial dilutions of
201 *C. burnetii* gDNA were used as standards. Genomes were quantified in real-time
202 using the QuantStudio7 Flex RT-PCR system (Thermo Fisher Scientific).
203
204 **Infection of *Galleria mellonella***
205 *G. mellonella* larvae were cultured in-house and kept at 30°C. Infections were
206 conducted as described previously [26,28,85,86]. Briefly, 10 uL containing 10^6 GE of
207 relevant *C. burnetii* strains or 10^5 *L. pneumophila* JR32 Δ*flaA* were injected in the
208 right proleg of *G. mellonella* larvae. Larvae were incubated at 37°C, and survival
209 was monitored every 24 hours across 11 days for *C. burnetii* strains and every 12
210 hours across 10 days for *L. pneumophila*. PBS controls were included with each
211 experiment and each condition consisted of 10-12 larvae. For bacterial load, live
212 and dead larvae were collected at the indicated time points and frozen at -20°C.
213 Larvae were homogenized by bead beating with 5x volume of PBS and *C. burnetii*
214 GE/mL was determined by qPCR as mentioned above.
215
216 **Immunoprecipitation and LC-MS analysis of ^{3xFLAG}MceF interactome**
217 Following a 24 hours induction period with doxycycline, whole-cell lysates were
218 solubilized in a buffer containing 100 mM Tris pH 7.4, 250 mM NaCl, 50% glycerol,
219 0.5 mM EDTA and 1% digitonin. Lysates were applied to pre-washed anti-FLAG-
220 M2 magnetic beads and incubated at 4°C for 2 hours under mild agitation. Bound
221 proteins were washed 3 times with cold PBS and prepared for mass spectrometry
222 analysis.
223
224 S-Trap™ Micro spin column digestion protocol (Protifi™) was used for sample
225 processing. Briefly, beads were resuspended in 50 µL of SDS solubilization buffer

226 (5% SDS, Sigma-Aldrich), 50 mM triethylammonium bicarbonate (TEAB, Sigma-
227 Aldrich, pH 8). Samples were reduced by adding 20 mM of dithiothreitol (Sigma-
228 Aldrich, for 10 minutes at 95°C and alkylated by the addition of 40 mM of
229 iodoacetamide (Sigma-Aldrich) for 30 minutes in the dark at RT. Samples were
230 acidified with 1.2% (v/v) of phosphoric acid (Sigma-Aldrich) and the undissolved
231 matter was removed by centrifugation for 8 min at 13,000 x *g* and beads were
232 separated using magnetic racks. A volume of 165 µL of S-Trap binding buffer (90%
233 aqueous methanol (Sigma-Aldrich), 100 mM final TEAB, pH 7.1) was added to the
234 acidified lysate. Sample mixes were added into the spin columns and centrifuged
235 at 4000 x *g* until all solution had passed through. Columns were washed 3 times
236 by centrifuging through 150 µL S-Trap binding buffer. Twenty µL of trypsin at 1:25
237 enzyme:protein (ug:ug) in digestion buffer (50 mM TEAB) were added into the
238 columns and incubated for 1 hour at 47 °C in a thermomixer without agitation.
239 Peptides were eluted with 40 µL each of 50 mM TEAB and then 0.2% aqueous
240 formic acid. Hydrophobic peptides were eluted with 35 µL of 50% acetonitrile and
241 0.2% formic acid. All centrifugations occurred as mentioned above and elutions
242 were dried down with a spin vacuum before proceeded to stage tip clean up as
243 mentioned above.
244
245 Dried digests were resuspended in Buffer A and separated using a two-column
246 chromatography setup composed of a PepMap100 C18 20 mm x 75 µm trap and
247 a PepMap C18 500 mm x 75 µm analytical column (Thermo Fisher Scientific).
248 Samples were concentrated onto the trap column at 5 µL/min for 5 minutes with
249 Buffer A (0.1% formic acid) and then infused into a Orbitrap Exploris 480 Mass
250 Spectrometer (Thermo Fisher Scientific) at 300 nL/minute via the analytical column
251 using a Dionex Ultimate 3000 UPLC (Thermo Fisher Scientific). 125-minute
252 analytical runs were undertaken by altering the buffer composition from 2% Buffer
253 B (0.1% formic acid, 80% acetonitrile) to 23% B over 95 minutes, then from 23%
254 B to 40% B over 10 minutes, then from 40% B to 80% B over 5 minutes. The
255 composition was held at 80% B for 5 minutes, and then dropped to 2% B over 0.1
256 of a minute before being held at 2% B for another 9.9 minutes. The Exploris 480

257 Mass Spectrometer was operated in a data-dependent mode automatically
258 switching between the acquisition of a single Orbitrap MS scan (300-1600 m/z,
259 maximal injection time of 25 ms, an Automated Gain Control (AGC) set to 300%
260 and a resolution of 120k) and up to 3 seconds of HCD MS/MS scans of precursors
261 (Stepped NCE of 27;32 and 36%, a maximal injection time of 65 ms, an AGC set
262 to 400% and a resolution of 30k).

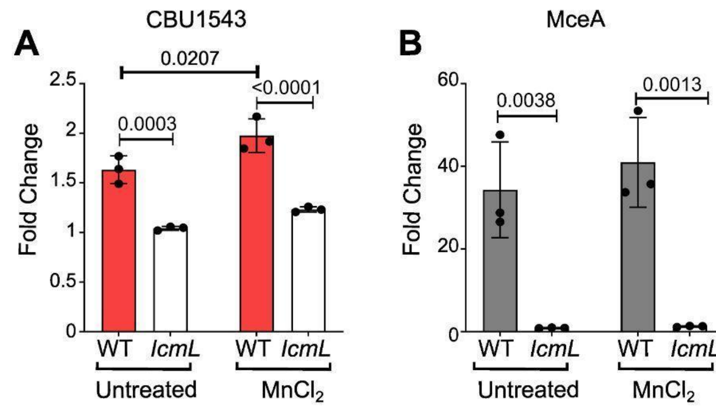
263

264 **Proteomic analysis**

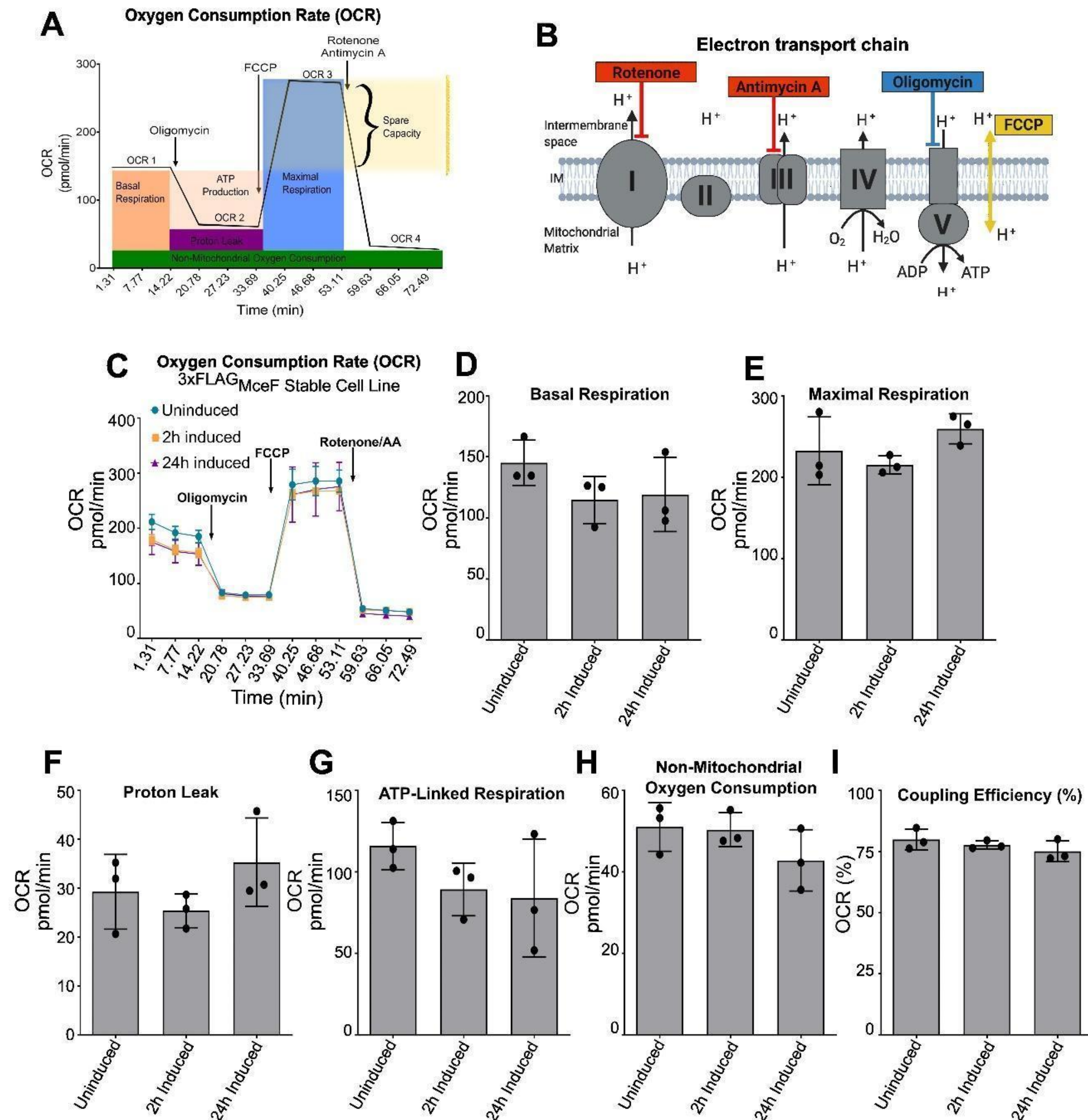
265 Immunoprecipitation samples were searched using MaxQuant (v1.6.17.0) [3] and
266 against the Human proteome (Uniprot accession: UP000005640) and CBU1543
267 protein sequence (NCBI accession: NP_820526.1). Searches were undertaken
268 using "Trypsin" enzyme specificity with carbamidomethylation of cysteine as a
269 fixed modification. Oxidation of methionine and acetylation of protein N-termini
270 were included as variable modifications and a maximum of 2 missed cleavages
271 allowed. To enhance the identification of peptides between samples, the Match
272 between Runs option was enabled with a precursor match window set to 2 minutes
273 and an alignment window of 20 minutes with the label free quantitation (LFQ)
274 option enabled [4]. The resulting outputs were processed within the Perseus
275 (v1.6.0.7) analysis environment [5] to remove reverse matches and common protein
276 contaminants and non-mitochondrial host proteins prior to further analysis. For
277 LFQ comparisons biological replicates were grouped and missing values were
278 then imputed based on the observed total peptide intensities with a range of 0.3σ
279 and a downshift of 2.5σ using Perseus. Student t-tests were undertaken to
280 compare the proteomes between groups with the resulting data exported and
281 visualized using ggplot2 [6] within R. The resulting MS data and search results have
282 been deposited into the PRIDE ProteomeXchange Consortium repository [7,8] and
283 can be accessed with the identifier: PXD030191 using the Username:
284 reviewer_pxd030191@ebi.ac.uk and Password: Hr6uHdoK.

285

286



287 **Figure Supplementary 1. Translocation of CBU1543 is Dot/Icm-dependent.** HeLa
288 CCL2 cells were infected with WT or *IcmL* mutant *C. burnetii* NMII expressing CBU1543
289 (A) or MceA (B) fused to TEM1 at an MOI 500 for 24 hours. After 20 hours, cells were
290 treated with $MnCl_2$ as indicated for 2 hours before loading with CCF2-AM and incubating
291 at RT for a further 2 hours. 'Fold change' is the increase in corrected 450/520 ratio (see
292 methods) compared to uninfected cells. Bar graphics represent the averages of results of
293 three independent experiments and error bars are standard deviation (SD). Statistical
294 analyses were performed by two-way ANOVA with the Tukey test for multiple
295 comparisons. When *p* value was considered statistically significant ($p<0.05$), its value was
296 stated in the figure.
297



298
299 **Figure Supplementary 2: MceF-cytoprotective effects enhance mitochondrial Spare**
300 **Respiratory Capacity**. (A) Schematic representation of Oxygen Consumption Rate
301 (OCR) levels (y-axis) versus time (x-axis). Oligomycin, FCCP, and rotenone/antimycin A
302 (AA) were sequentially injected as indicated (black arrows). (B) Targets of each compound
303 in the electron transport chain. (A-B) Created with BioRender.com. (C-I) ^{3xFLAG}MceF stable
304 cell line was induced for effector expression for 2 and 24 hours with doxycycline. (C)
305 Experimental OCR in ^{3xFLAG}MceF stable cell lines, (D) Basal respiration (OCR1-OCR4),
306 (E) Maximal respiration (OCR3-OCR4), (F) Proton leak (OCR2-OCR4), (G) ATP-linked
307 production (OCR1-OCR2), (H) Non-mitochondrial respiration (OCR4) and (I) Percentage
308 of the coupling efficiency ((ATP rate/Basal rate)x100)). Curve and bar graphics represent
309 the mean result of three independent experiments with individual data points shown. Error
310 bars represent standard deviation (SD). Statistical analyses were performed by one-way
311 ANOVA with the multiple comparison test of Bonferroni. When *p* value was considered
312 statistically significant ($p<0.05$), its value was stated in the figure.

313 **Table S1. Plasmids used in this study**.

Plasmid	Features	Antibiotic selection	References
pcDNA4/TO:3xFLAG	Eukaryotic expression vector encoding 5' 3xFLAG tag and multiple cloning site (MCS)	AmpR	Clontech, Roy Laboratory, Yale University
pcDNA4/TO:3xFLAG:CBU1543	Encodes 5' 3xFLAG tag and full length of *cbu1543* with 5'BamHI and 3' XhoI restriction endonuclease (RE) sites	AmpR	Newton Laboratory
pGEM-T Easy	High copy cloning vector	AmpR	Promega
pJB-CAT:BlaM-MCS	*C. burnetii* expression vector encoding 5' BLAM under constitutive expression by the *C. burnetii* P1169 promoter. The multiple cloning site (MCS) from pcDNA4/TO:3xFLAG was inserted within the SalI site.	CmR	Newton Laboratory
pJB-CAT:BlaM-MCS:CBU1543	*C. burnetii* expression vector encoding 5' BLAM:CBU1543 under constitutive expression by the *C. burnetii* P1169 promoter.	CmR	This study
pJB-CAT:BlaM:CBU0077	*C. burnetii* expression vector encoding 5' BLAM:CBU0077 under constitutive expression by the *C. burnetii* P1169 promoter. *cbu0077* gene was inserted within SalI site.	CmR	Newton Laboratory
pJB-Kan:3xFLAG	*C. burnetii* expression vector encoding 5' 3xFLAG tag under constitutive expression by the *C. burnetii* P1169 promoter	KanR	[9]
pJB-CAT:3xFLAG	*C. burnetii* expression vector encoding 5' 3xFLAG tag under constitutive expression by the *C. burnetii* P1169 promoter	CmR	[9]
pJB-CAT:3xFLAG:CBU1543	Full length of *cbu1543* with 5' SalI and 3' SalI restriction RE sites	CmR	Newton Laboratory
pF_TRE3G_Puro	Eukaryotic expression vector under inducible expression by doxycycline	AmpR	Silke Laboratory, WEHI
pF_TRE3G_Puro:3xFLAG:CBU1543	Encodes 5' 3xFLAG tag and full length of *cbu1543*	AmpR /Puromycin	This study

	with 5'BamHI and 3' NheI RE sites		
pLV-iVSV-G	Lentiviral expression plasmid designed to expresses a viral envelope gene	AmpR	Silke Laboratory, WEHI
pCMV-R8.2	lentiviral expression plasmid for packaging	AmpR	Silke Laboratory, WEHI
pJC-CAT	*C. burnetii* vector for gene inactivation	CmR	[10]
pJC-CAT:CBU1543	Upstream (1.5 kb) and downstream (1,5 kb) of *cbu1543* with 5' BamHI and 3' SalI restriction RE sites	CmR	This study
pJC-CAT-Kan:CBU1543	Upstream (1.5 kb) and downstream (1,5 kb) regions of *cbu1543* with 5' BamHI and 3' SalI restriction RE sites	CmR/KanR	This study
lentiGuide-puro:*gpx4*	Expresses *Streptococcus pyogenes* CRISPR chimeric RNA element with customizable (*gpx4*) sgRNA from U6 promoter and puromycin resistance from EF-1a promoter. Third generation lentiviral backbone.	AmpR /Puromycin	This study
psPAX2	(Empty Backbone) 2nd generation lentiviral packaging plasmid. Can be used with 2nd or 3rd generation lentiviral vectors and envelope expressing plasmid.	AmpR	Zamboni Laboratory
pMD2.G	VSV-G envelope expressing plasmid	AmpR	Zamboni Laboratory
pENTR223-GPX4	Glutathione peroxidase 4 cDNA clone	$Spec^R$	The ORFeome collaboration, DNASU [11]
pLJM1-GFP-GPX4	GPX4 cDNA cloned downstream of GFP		This study

314
315

316 **Table S2. Oligonucleotides used in this study.**
317

Description of purpose	Sequence (5' to 3')
Sequencing primers for pcDNA4/TO derivatives	
pcDNA4_F	CGCAAATGGGCGGTAGGCGTG
pcDNA4_R	CTAGAAGGCACAGTCGAGG
Sequencing primers for pJB-CAT:BLAM derivatives	
pJB-CAT:BLAM_F	CCTTCATGAAGGAGGCTG
pJB-CAT:BLAM_R	CCCTGGGTGAGTTTCACC
Sequencing primers for pJB-CAT derivatives	
pJB-CAT_F	CCTTCATGAAGGAGGCTG
pJB-CAT_R	CCCTGGGTGAGTTTCACC
Sequencing primers for pJC-CAT derivatives	
pJC-CAT_F	GTGAGCGAGGAAGCGGAAG
pJC-CAT_R	CAGGAGATAAGAACGCATCA
Sequencing primers for Kan^R gene	
Kan[1]	GATGGAAGCCGGTCTTGTCGAT
Kan[2]	CGGACAGGTCGGTCTTGACAAAA
qPCR quantification of *C. burnetii* genomes	
*ompA*_F	CAGAGCCGGGAGTCAAGCT
*ompA*_R	CTGAGTAGGAGATTTGAATCGC
Eukaryotic expression vector constructs - pFTRE	
3xFLAG_F (BglII)	AAAGATCTATGGACTACAAAGACCATGAC
*cbu1543*_R (NheI)	ATTGCTAGCTCATTTTCCAAGGGACTC
cbu1543* primers for gene inactivation in *C. burnetii	
Forward primer to amplify 1.5 kb region upstream to cbu*1543* *(*BamHI)	AAGGATCCATATTTAAATAATGAGGA
Reverse primer to amplify 1.5 kb region upstream *cbu1543* (NotI)	TTTCCAAAAGTTTTAGCGGCGGCCGCTATCTTGCCTCGTTGCTT
Forward primer to amplify 1.5 kb region downstream *cbu1543* *(*NotI)	AAGCAACGAGGCAAGATAGCGGCCGCCGCTAAAACTTTTGGAAA
Reverse primer to amplify 1.5 kb region downstream *cbu1543* *(*SalI)	AAGTCGACTTCTCGGGGTCTCGTTGT
Forward primer to amplify Kan from pJB-Kan with P1169 (NotI)	GACGCGGCCGCAGCTTATGGCTTCGTTTCGCAG
Reverse primer to amplify Kan from pJB-Kan (NotI)	GACGCGGCCGCTCAGAAGAACTCGTCAAGAAGGCG
Forward internal primer to sequence pJC-CAT-15435'3'	CGGTTGTTTAAAAGATATTCG
Reverse internal primer to sequence pJC-CAT-15435'3'	GGTTTAATTCACATGGCCG

Double-stranded guide RNA (gRNA) targeting *gpx4*	
sgRNA1 5'3'	CGTGTGCATCGTCACCAACG
sgRNA2 5'3'	CATGCCCGATATGCTGAGTG
pJB-CAT *C. burnetii* effector expression constructs	
CBU0041_F	CGATGACAAGGTCGACATGAGGGATGGCACTACACG
CBU0041_R	GCATGCCTCAGTCGACTTATCCGCTCAACGAATGTGG
CBU0077_F	CGATGACAAGGTCGACATGAGACAACTCGTTTCAATTAAA
CBU0077_R	GCATGCCTCAGTCGACTTACATAATAGAACACCCACGA
CBU0080_F	CGATGACAAGGTCGACTTGGGTCATCGTGAGAAAGAA
CBU0080_R	GCATGCCTCAGTCGACTTATTGAAATGCTCTCGTCTGG
CBUK1976_F	CGATGACAAGGTCGACATGAGGCACGAAAATCCCCA
CBUK1976_R	Used CBU0080_R
CBU0113_F	GACGGATCCATGTCGGCAACCCAACTTTTAA
CBU0113_R	GACGAATTCCTAGCCCCCTGGAGCTGGA
CBU0175_F	CGATGACAAGGTCGACATGTTAATGGCTTATATGAGGC
CBU0175_R	GCATGCCTCAGTCGACTTAATCCCATTCAATATTTTCTAAA
CBU0295_F	CGATGACAAGGTCGACTTGAGGTACAAGCGACACATG
CBU0295_R	GCATGCCTCAGTCGACTTAAAAAGTAAAGGATTGTTTAGAG
CBU0590_F	GACGGATCCATGAACACAAGAGAGACACTTGCC
CBU0590_R	GACGAATTCTTAGACCGTTTTAGGCTCTGTCAT
CBU0635_F	CGATGACAAGGTCGACATGCGAGAGGAAAAAGAGGA
CBU0635_R	GCATGCCTCAGTCGACCTAAACTAATGTCATTAAACGGT
CBU0781_F	CGATGACAAGGTCGACATGAGTAGACGTGAGACTCC
CBU0781_R	GCATGCCTCAGTCGACTCACCGAGGACTAGACAGAC
CBU0794_F	CGATGACAAGGTCGACATGAAAATTATTAAATTAGTGGAAAA
CBU0794_R	GCATGCCTCAGTCGACTTATCTAAATCTGGCTTTTTGC
CBU0801_F	CGATGACAAGGTCGACGTGAAAATTCGAAACTGGATTAA
CBU0801_R	GCATGCCTCAGTCGACCTAAAAATTTAAATTAAATACCAATG
CBU0881_F	CGATGACAAGGTCGACATGCCAATAATGAGCCCCA
CBU0881_R	GCATGCCTCAGTCGACCTACCTGAGGGACTTATACC
CBU0937_F	CGATGACAAGGTCGACGTGACTTGTGACAGGGTTGT
CBU0937_R	GCATGCCTCAGTCGACTTAAAAATAAAGATCGAACTGTG
CBU1213_F	CGATGACAAGGTCGACATGAGAGAATCATCAGAAAATCA
CBU1213_R	GCATGCCTCAGTCGACCTAAATTCCAAAAGAACCCGG
CBU1217_F	CGATGACAAGGTCGACATGAGAACTTCGCATCAAAATC
CBU1217_R	GCATGCCTCAGTCGACTCACTTTCTGGAAAAAGGGGC
CBU1253_F	CGATGACAAGGTCGACATGGCGAAATTTACTATACGTTTAG
CBU1253_R	GCATGCCTCAGTCGACTTACGCAGCGCGCATGGTT
CBU1314_F	CGATGACAAGGTCGACGTGTATAAAAATGGAGGTTACC
CBU1314_R	GCATGCCTCAGTCGACTCACGATCGCTTGGCAGGC
CBU1379_F	CGATGACAAGGTCGACATGTCGTCGTCAATCCCAAC
CBU1379_R	GCATGCCTCAGTCGACCTAGGCTGCTTTCATTCCAG
CBU1387_F	GACGGATCCATGCCCAATAAGGAACCCGAATC
CBU1387_R	GACGAATTCTCACTTTGGAGTCAACCTTGTGG
CBU1425_F	CGATGACAAGGTCGACATGAAAAAAATCGCAACTATTGG
CBU1425_R	GCATGCCTCAGTCGACTTATCGAATGATCTTCCATTGT
CBU1457_F	CGATGACAAGGTCGACATGCCTTACCCTTACGAAGC
CBU1457_R	GCATGCCTCAGTCGACTCATGTTTTTACCCTATCATTAC
CBU1460_F	CGATGACAAGGTCGACATGTCTAGTTTTGGCGATGCT
CBU1460_R	GCATGCCTCAGTCGACTTACATTTGCTTACAACACGGA
CBU1485_F	CGATGACAAGGTCGACATGCCTGGAGGTTGCATTATG
CBU1485_R	GCATGCCTCAGTCGACTTAGAATCGAGGGCGATGTAA
CBU1493_F	CGATGACAAGGTCGACATGAGTCGCAAAAACATACCAG

CBU1493_R	GCATGCCTCAGTCGACTTAGGAGCAAGTACAGGTTGT
CBU1524_F	CGATGACAAGGTCGACATGAACACAAGTCCTACATCAA
CBU1524_R	GCATGCCTCAGTCGACCTATGTCCTTTTGGGAGCGT
CBUD0462_F	CGATGACAAGGTCGACATGAGAAAAAAACCTAACGTCG
CBUD0462_R	Used CBU1524_R
CBU1532_F	CGATGACAAGGTCGACTTGGCAGGCATAGCTGCAAC
CBU1532_R	GCATGCCTCAGTCGACTTACTTATTAAATTCGGGTATGTA
CBUD0454_F	CGATGACAAGGTCGACTTGCGAGACGAGAACGATCC
CBUD0454_R	Used CBU1532_R
CBU1543_F	CGATGACAAGGTCGACATGCAACCCACCGCAGAGA
CBU1543_R	GCATGCCTCAGTCGACTCATTTTCCAAGGGACTCGC
CBU1556_F	CGATGACAAGGTCGACATGCCTTCTGATAGAAACGG
CBU1556_R	GCATGCCTCAGTCGACCTAAACGGGAGCATAATTAACC
CBU1566_F	AAGGATCCATGCCTTCTGATAGAAACGG
CBU1566_R	AAGAATTCCTAAACGGGAGCATAATTAACC
CBU1569_F	CGATGACAAGGTCGACATGCCTATTACCAGCTTAGAAT
CBU1569_R	GCATGCCTCAGTCGACTTAATTGTTATTTCGAGGAGAG
CBU1636_F	CGATGACAAGGTCGACATGACCTGGAAATTAAATGAGAT
CBU1636_R	GCATGCCTCAGTCGACCTAAAGAACCAGCCCGCTT
CBU1751_F	CGATGACAAGGTCGACATGGGTAGAGTTTTTTCGTACA
CBU1751_R	GCATGCCTCAGTCGACTTAAGGCCTTGCGGGTCG
CBU1752_F	AAGTCGACATGAGAGATCCAGATCAAGAAA
CBU1752_R	AAGTCGACTTATGAAGGGCCGAATGCCG
CBU1769_F	CGATGACAAGGTCGACATGACGAATGAAGATTTTTTAATC
CBU1769_R	GCATGCCTCAGTCGACCTATGCTAAATTTCGGACCAAT
CBU1823_F	CGATGACAAGGTCGACATGCCTAAACTCAGTAACCGT
CBU1823_R	GCATGCCTCAGTCGACTTATGGCCTCTTATTTGTTGGC
CBU1863_F	CGATGACAAGGTCGACATGCGAAATGATGATGATACTC
CBU1863_R	GCATGCCTCAGTCGACTTACGCAGTAAGTGCAGAAGG
CBU1963_F	CGATGACAAGGTCGACATGGAGTTTCTAATAAAGTTTTCT
CBU1963_R	GCATGCCTCAGTCGACTCAAGGATGATGGTGGCGAG
CBUD2063_F	CGATGACAAGGTCGACATGCGAGTTGAGCTGTGGC
CBUD2063_R	Used CBU1963_R
CBU2013_F	AAGGATCCATGCCATCGATAAATTTGACGA
CBU2013_R	AACTCGAGTTAAGAAACTAGCTGAAGATGAG
CBU2016_F	AAGGATCCATGGTGGTTATGCTAGAAGAC
CBU2016_R	AACTCGAGTTAGGGATCGAAGCCGGAG
CBU2052_F	CGATGACAAGGTCGACATGCCTAAAAACACAAATCCAG
CBU2052_R	GCATGCCTCAGTCGACTTATTTCAAAAAAGCATTTACAAGA
CBU2056_F	CGATGACAAGGTCGACGTGGTGAGTTTAATTTTCATAAAG
CBU2056_R	GCATGCCTCAGTCGACCTAAGGTGCGGGTGCGC
CBU2078_F	CGATGACAAGGTCGACATGTTCAAACCCCTATTCCAAA
CBU2078_R	GCATGCCTCAGTCGACCTACTTAGCTGATTTCTCTTTTA
CBUA0006_F	CGATGACAAGGTCGACATGCGTATGTTCAGAGAAAGC
CBUA0006_R	GCATGCCTCAGTCGACTTATGGATTCTTACTTCTCAAAG
CBUA0013_F	CGATGACAAGGTCGACATGCCATATTTTTTACACTACC
CBUA0013_R	GCATGCCTCAGTCGACCTATCTGGAACAGAAGGGAAA
CBUA0015_F	CGATGACAAGGTCGACGTGGTAAAAGCCAAGGACTTA
CBUA0015_R	GCATGCCTCAGTCGACTTAAGTTAATTTTATTTGATTTTTAGT
CBUA0016_F	CGATGACAAGGTCGACATGAGATTAGAACAACCAAGAAA
CBUA0016_R	GCATGCCTCAGTCGACTTAGCAAAGTCTGAAAGAAGGA
CBUA0020_F	ACGTCGACATGAATGATGATGAGAAAAAAAAGC
CBUA0020_R	GGGTCGACTTACTTATTAAATTCGGGTA

CBUA0023_F	CGATGACAAGGTCGACATGAAAGATTATGTTAAAGAAATTC
CBUA0023_R	GCATGCCTCAGTCGACCTATCCGATACCAGGCCC
CBUD0054_F	CGATGACAAGGTCGACGTGTATGCAAACCATTTGATAC
CBUD0054_R	GCATGCCTCAGTCGACCTATTTTTTTGTCATTTCCAGATT
CBUD0231_F	CGATGACAAGGTCGACATGCCATTAAAAGACTTACGC
CBUD0231_R	GCATGCCTCAGTCGACTTACTTTATAAAAGTCCGTGTATA
CBUD0461_F	CGATGACAAGGTCGACATGTCCCGTCGCGAGCCT
CBUD0461_R	GCATGCCTCAGTCGACTTAAATCGTAAGGCGCTTTGTT
CBUD0588_F	AAGGATCCGTGACTCTCATGTTTCGAGC
CBUD0588_R	AAGAATTCTCATTGAAGGTTGAACGGTTC
CBUD0997_F	AAGGATCCATGAAAATGAAGGCAATGGAAG
CBUD0997_R	AACTCGAGTTAGATTTTAGTTACTGGACGC
CBUD1019_F	CGATGACAAGGTCGACTTGTCAGAATTAGGAGGGGAT
CBUD1019_R	GCATGCCTCAGTCGACTTAGATTAACGTGCGCGTATAA
CBUD1627_F	CGATGACAAGGTCGACATGAGACAGCGTGAAATTAATG
CBUD1627_R	GCATGCCTCAGTCGACCTACCGCTGGAAGCCGCG
CBUD1656_F	CGATGACAAGGTCGACATGAGGAGAAATATCATGGCAA
CBUD1656_R	GCATGCCTCAGTCGACTTATCGACCGGGAAACGGA
CBUD1750_F	CGATGACAAGGTCGACATGGTTGCAAAAAAAGAGTGTG
CBUD1750_R	GCATGCCTCAGTCGACTTATCTGCGTTGCAATCGGG
CBUD2034_F	CGATGACAAGGTCGACTTGCTTAGCTTAATGGCCAGC
CBUD2034_R	GCATGCCTCAGTCGACTTAAAACAGTCCGGGGCCTG
CBUD2035_F	CGATGACAAGGTCGACGTGGGACAAAATACAACGAGG
CBUD2035_R	GCATGCCTCAGTCGACTTAAGCTAAGCAAGGGGTATTA
CBUD2154_F	CGATGACAAGGTCGACATGACAAGACAAACAACTGAAA
CBUD2154_R	GCATGCCTCAGTCGACTCATTTGATTGTTAAGGAAGAAG
CBUG1587_F	CGATGACAAGGTCGACATGAATCAACAACAACGAATACA
CBUG1587_R	GCATGCCTCAGTCGACTTATGTGGATGCGCTGGGC
CBUG1886_F	CGATGACAAGGTCGACTTGTTGGAGATAGTGATGAGAA
CBUG1886_R	GCATGCCTCAGTCGACTCACTTTGGCCGATGATGTTT
CBUK0684_F	CGATGACAAGGTCGACATGAGTCGCCAACAACGTAAC
CBUK0684_R	GCATGCCTCAGTCGACTTAAAGCAGCGTGCAGCTG
CBUK1330_F	CGATGACAAGGTCGACATGAGCCGGACCTACAACG
CBUK1330_R	GCATGCCTCAGTCGACTTAAATGCAAACCACCCGATTC
CBUK1690_F	CGATGACAAGGTCGACTTGGAGGCTATGATGCGTTC
CBUK1690_R	GCATGCCTCAGTCGACCTAATTTTTAAATTCAGGCCCAT
CBUK1907_F	CGATGACAAGGTCGACATGAGACATCCCATAATTCACA
CBUK1907_R	GCATGCCTCAGTCGACTCAAAAAATTAGGACGCCATCC

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320 **SI References**

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1. Newton, P., Latomanski, E. A. & Newton, H. J. Applying fluorescence resonance energy transfer (FRET) to examine effector translocation efficiency by Coxiella burnetii during siRNA silencing. *Journal of Visualized Experiments* **113**, 1–12 (2016).
2. Altschul, S. F. *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402 (1997).
3. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* **26**, 1367–1372 (2008).
4. Cox, J. *et al.* Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular and Cellular Proteomics* **13**, 2513–2526 (2014).
5. Tyanova, S. & Cox, J. Perseus: A Bioinformatics Platform for Integrative Analysis of Proteomics Data in Cancer Research. *Cancer Systems Biology: Methods and Protocols, Methods in Molecular Biology* **1711**, 133–148 (2018).
6. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis*. *Springer-Verlag New York* (2016).
7. Vizcaíno, J. A. *et al.* 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res* **44**, D447–D456 (2016).
8. Perez-Riverol, Y. *et al.* The PRIDE database and related tools and resources in 2019: Improving support for quantification data. *Nucleic Acids Res* **47**, D442–D450 (2019).
9. Beare, P. A., Sandoz, K. M., Omsland, A., Rockey, D. D. & Heinzen, R. A. Advances in genetic manipulation of obligate intracellular bacterial pathogens. *Front Microbiol* **2**, 1–13 (2011).
10. Beare, P. A., Larson, C. L., Gilk, S. D. & Heinzen, R. A. Two Systems for Targeted Gene Deletion in Coxiella burnetii. *Appl Environ Microbiol* **78**, 4580–4589 (2012).
11. Seiler, C. Y. *et al.* DNASU plasmid and PSI:Biology-Materials repositories: Resources to accelerate biological research. *Nucleic Acids Res* **42**, (2014).

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