Supporting information

SI Materials and Methods

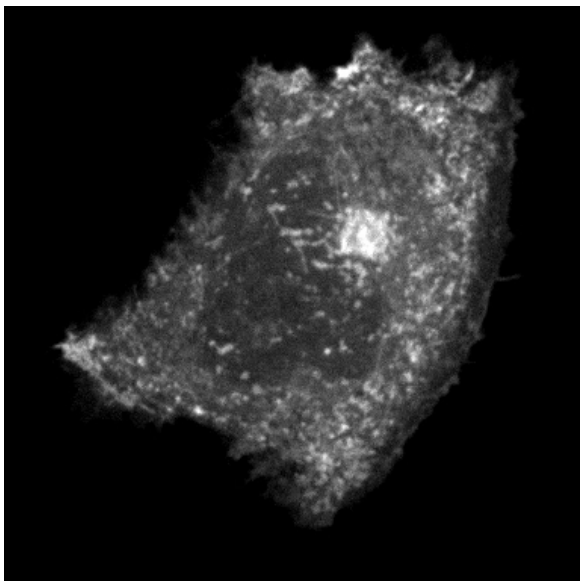
Generation of expression plasmids. Mutations in CvpA endocytic sorting motif were generated by two rounds of PCR. In the first round, pENTR/N-CvpA was used as template for pENTR/N-CvpA primer pairs that introduced LL to AA or Y to A mutations. The M13 forward sequencing primer was paired with a pENTR/N-CvpA reverse primer, and the M13 reverse sequencing primer was paired with a pENTR/N-CvpA forward primer. The two PCR products were purified, combined, and used as template for a second round of PCR with pENTR/N-CvpA-Fwd and pENTR/N-CvpA-Rev primers. The resulting amplicon was cloned into pENTR/D (Invitrogen) and moved into pT-REx-DEST30/N-mCherry using the Gateway system (Invitrogen). To generate constructs encoding GST fused to 30mer peptides containing CvpA endocytic sorting motifs or mutated endocytic sorting motifs, PCR was conducted with peptide primer sets (Table S2) using as template the pENTR/N-CvpA plasmid or pENTR/N-CvpA plasmids encoding mutated sorting motifs (Table S3). Subsequent cloning of 30mer PCR amplicons into pENTR/D and transfer to pDEST15 (Invitrogen) was accomplished as described above.

Construction of pT-RexT-DEST30/N-mCherry and pcDNA6.2/N-BioEase-DEST. The mCherry gene was amplified from pcDNA6.2/N-mCherry-DEST (Invitrogen) by PCR using primers pcDNA6.2/N-mCherry-1 and pcDNA6.2/N-mCherry-2. The mCherry PCR product was cloned by In-Fusion (Promega) into

pT-RexT-DEST30 (Invitrogen) digested with AgeI and EcoRV to create pT-RexT-DEST30/N-mCherry. The BioEase gene was amplified by PCR from pET104-DEST (Invitrogen) using primers pET104-DEST-3 and pET104-DEST-4. The pcDNA6.2 backbone was amplified from pcDNA6.2/N-mCherry-DEST (1) by inverse PCR using primers pcDNA6.2-5 and pcDNA6.2-6. The BioEase gene was cloned into the pcDNA backbone by In-Fusion to create pcDNA6.2/N-BioEase-DEST. All plasmid constructs used in this study were verified by sequencing.

1. Voth DE*, et al.* (2011) The *Coxiella burnetii* cryptic plasmid is enriched in genes encoding type IV secretion system substrates. *J Bacteriol* 193:1493-1503.

SI video



Video S1. Time-lapse video of a live HeLa cell ectopically expressing mCherry-CvpA. Images were captured every 30 second for 15 min by confocal fluorescence microscopy and are shown at 3 frames per second.

SI Figures

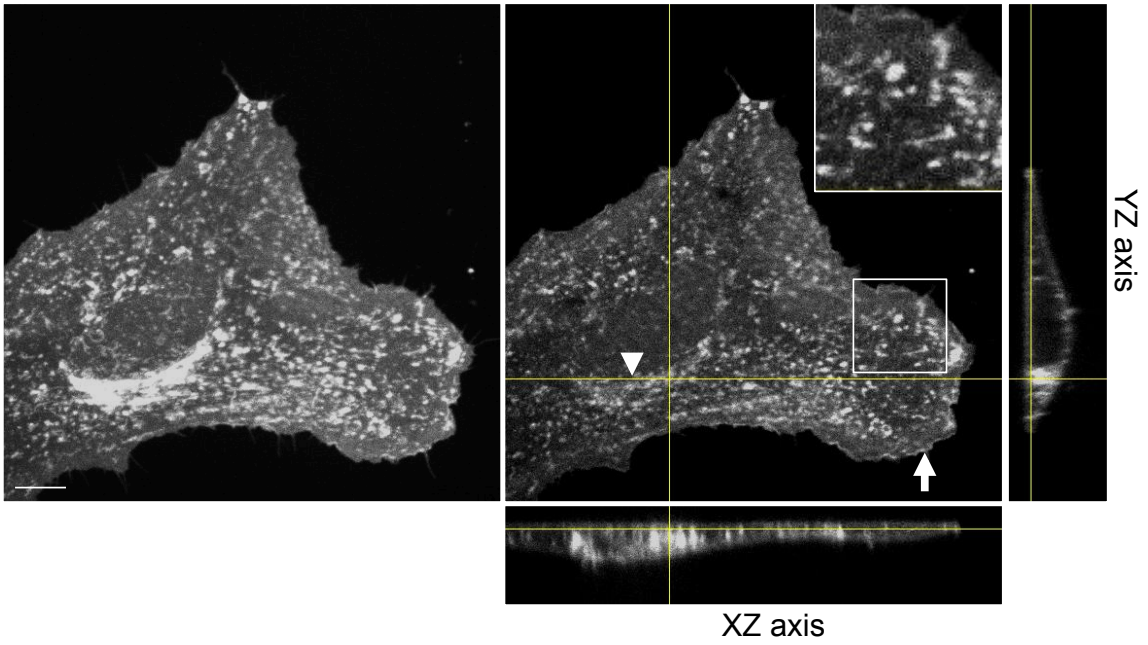


Fig. S1. Representative confocal fluorescence micrograph of a live HeLa cell ectopically expressing mCherry-CvpA. Projection of z-stacks (*left*) and single slice (*right*) with orthogonal views. MCherry-CvpA localized to pericentrosomal (arrowhead) and peripheral (box, inset) tubulovesicular structures, and to the plasma membrane (arrow). (Scale bar = 5 μm).

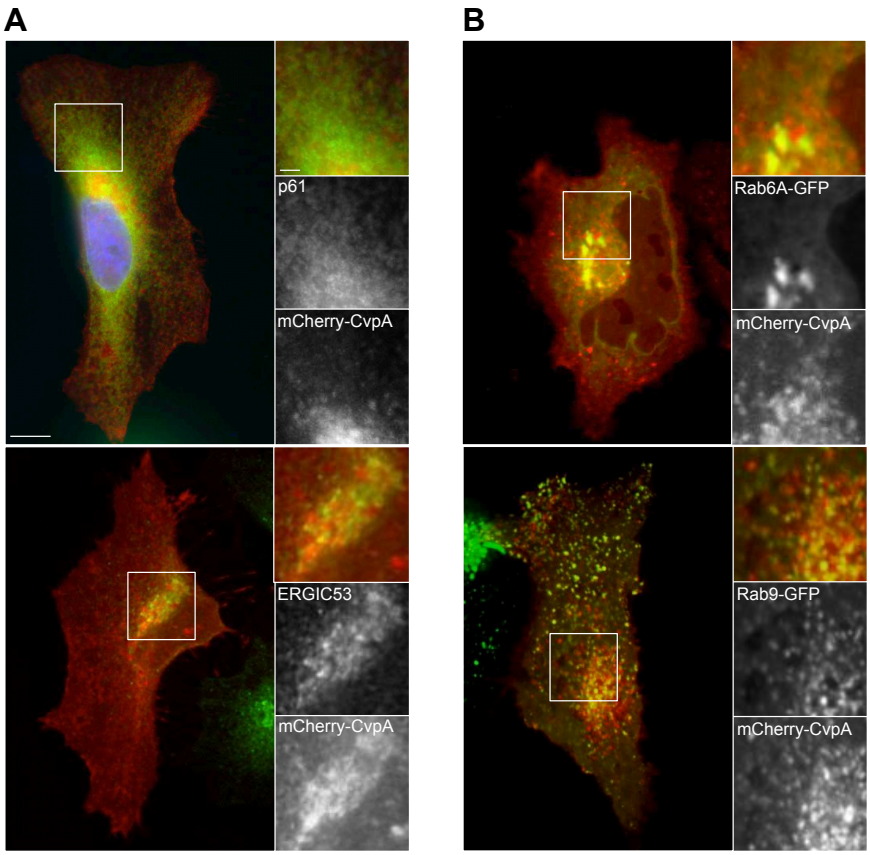


Fig. S2. Localization of secretory system proteins in HeLa cells expressing mCherry-CvpA. (*A*) Representative micrographs of fixed HeLa cells immunostained with antibodies against the endoplasmic reticulum (ER) protein p61 or the ER-Golgi intermediate compartment protein ERGIC53 (green). (*B)* Representative micrographs of live HeLa cells co-expressing mCherry-CvpA and Rab6A-GFP or Rab9-GFP (Scale bar = 10 μm).

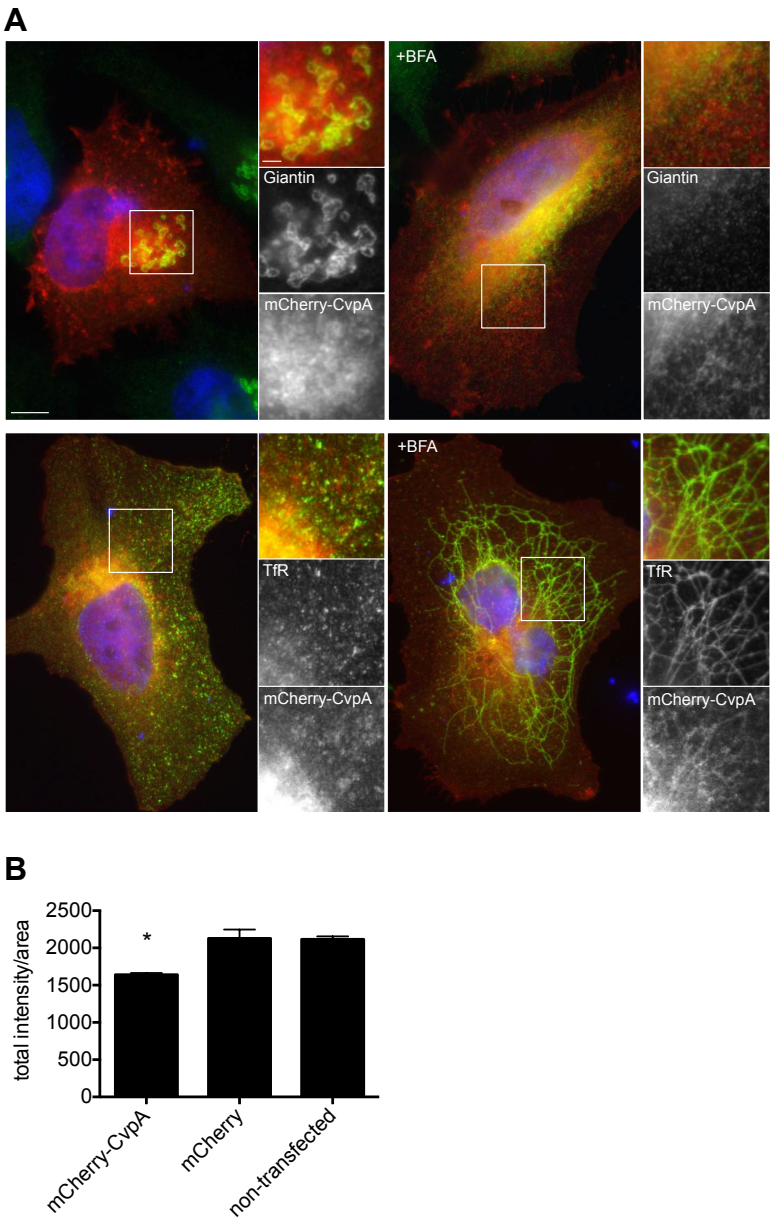


Fig. S3. Ectopically expressed mCherry-CvpA localizes to recycling endosomes (REs) and disrupts endocytosis of transferrin (Tf). (*A)* Representative images of untreated or brefeldin A (BFA)-treated cells immunostained for REs and the Golgi using antibodies against Tf receptor (TfR) and giantin, respectively (green). Cells were treated with 10 µg/mL BFA for 30 min prior to fixation to disrupt vesicle coats (Scale bar = 10 µm). (*B*) Endocytosis of transferrin Alexa Fluor 488 (Tf488)

by HeLa cells expressing mCherry-CvpA. Cells were fixed after a 15 min incubation with media containing Tf488. Total cellular Tf488 fluorescence was measured using ImageJ in non-transfected cells, and cells expressing mCherry-CvpA or mCherry alone. Results are expressed as the means of biological replicates (N=25) and are representative of two independent experiments. Error bars indicate standard error from the means and an asterisk indicates a statistically significant difference ($P < 0.05$) relative to mCherry alone and non-transfected cells.

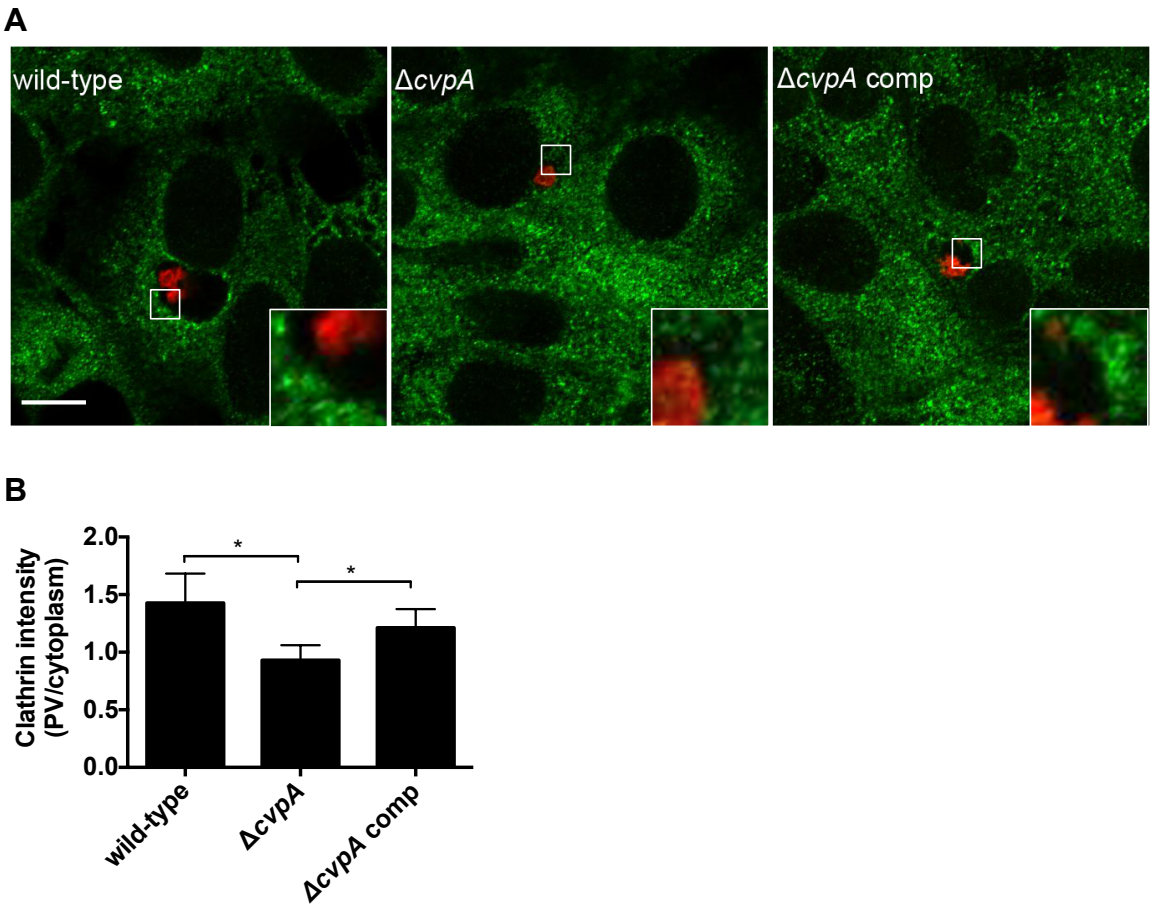


Fig. S4. Clathrin is enriched around PV harboring CvpA-expressing *C. burnetii*. (*A*) Representative confocal micrographs of Vero cells infected with wild-type *C. burnetii*, the *ΔcvpA* mutant, or the complemented *cvpA* mutant at 5 days post infection immunostained for *Coxiella* (red) and clathrin (green) (scale bar = 10 μm). (*B*) Quantification of PV-associated and cytoplasmic clathrin. Clathrin signal intensities were determined as described in the Materials and Methods. The PV-associated signal was divided by the cytoplasmic signal to yield the plotted clathrin intensities. Results are expressed as the means of two biological replicates representative of three independent experiments. Error bars indicate

the standard error from the means and an asterick indicates a statistically significant difference ($P < 0.05$).

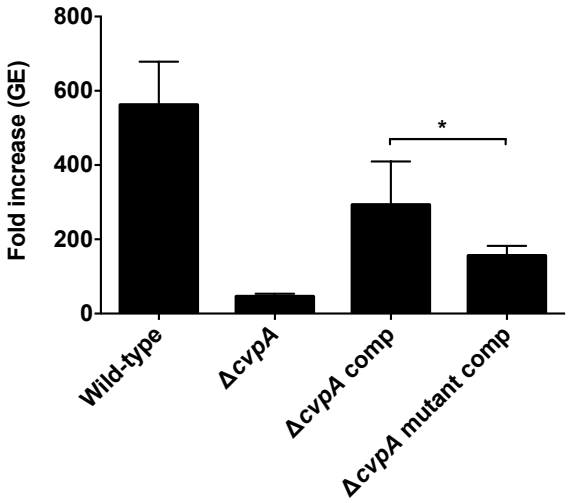


Fig. S5. Complementation with CvpA containing mutated sorting motifs is deficient. Replication of wild-type *C. burnetii*, the Δ*cvpA* mutant, and the Δ*cvpA* mutant expressing CvpA or CvpA with mutations in the sorting motifs DiLeu1, DiLeu2, Dileu3, Tyr1, and Tyr2 was assessed in THP-1 macrophages. Fold increases in genomic equivalents (GE) at 5 days post infection are depicted. Results are expressed as the means of two biological replicates representative of three independent experiments. Error bars indicate standard error from the means and an asterisk indicates a statistically significant difference ($P < 0.05$).

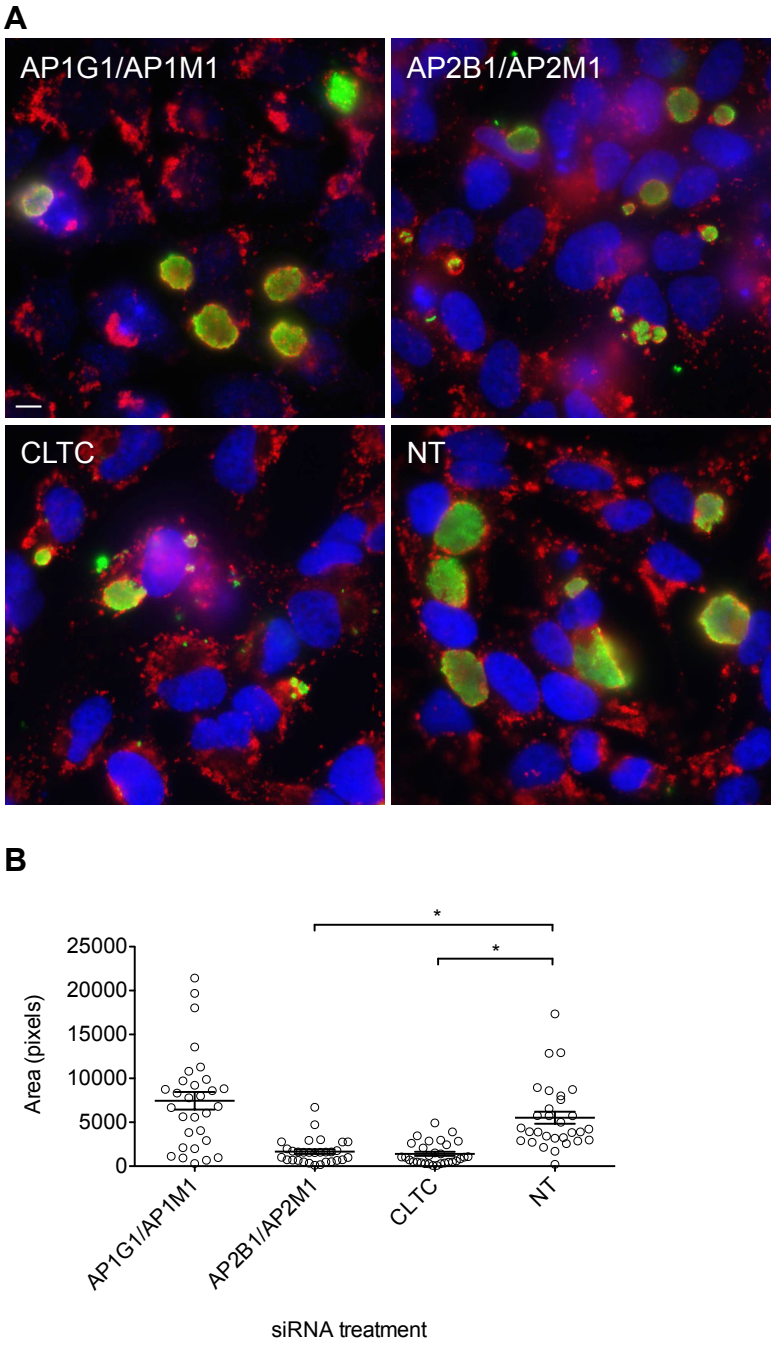


Fig. S6. Depletion of AP2 or clathrin inhibits PV expansion. (*A*) Representative fluorescence micrographs showing PV morphology in siRNA treated cells immunostained for LAMP1 (red), *Coxiella* (green) and DNA (blue) at 5 days post infection (scale bar = 10 μm). HeLa cells were treated with siRNA to deplete the

γ and μ subunits of AP1 (AP1G1 and AP1M1 siRNA), the β and μ subunits of AP2 (AP2B1 and AP2M1 siRNA) or clathrin (CLTC siRNA). Two days later, cells were infected with *C. burnetii*. (*B*) Size of PV in siRNA treated cells at 5 days post infection. The size of *C. burnetii* containing vacuoles was measured using ImageJ. Error bars indicate standard error from the means (N=30). Results are representative of two independent experiments and an asterisk indicates a statistically significant difference ($P < 0.05$).

Table S1. *C. burnetii* genomic equivalents from HeLa cells treated with siRNA

siRNA	Day 0	Day 1	Day 3	Day 5
AP1G1/AP1M1	$7.81 \pm 0.12 \times 10^4$	$1.41 \pm 0.24 \times 10^5$	$9.13 \pm 0.24 \times 10^6$	$2.86 \pm 0.46 \times 10^7$
AP2B1/AP2M1	$7.79 \pm 0.10 \times 10^4$	$2.36 \pm 0.18 \times 10^5$	$9.86 \pm 1.44 \times 10^6$	$9.19 \pm 0.77 \times 10^6$*
CLTC	$7.40 \pm 1.71 \times 10^4$	$2.07 \pm 0.15 \times 10^5$	$5.64 \pm 1.01 \times 10^6$	$5.90 \pm 1.55 \times 10^6$*
NT	$6.81 \pm 2.54 \times 10^4$	$1.86 \pm 0.19 \times 10^5$	$6.91 \pm 0.98 \times 10^6$	$2.92 \pm 0.05 \times 10^7$

* Asterisk indicates a statistically significant difference ($P < 0.01$) with respect to cells treated with non-targeting (NT) siRNA.

Table S2. Primers used for generation of *cvpA* (*cbu0665*) constructs

Name	Sequence
Full-length CvpA constructs	
pJB-CAT-nCyaA-CvpA-F	TTCCGGCTATGTCGACATGCTTTTAAGAGGAATAATAGTGTCACG
pJB-CAT-nCyaA-CvpA-R	GCATGCCTCAGTCGACTCATTGAAGTCTGGTTAAACCCATTTG
pENTR/N-CvpA-F	CACCATGCTTTTAAGAGGAATAATAGTGTCACG
pENTR/N-CvpA-R	TCATTGAAGTCTGGTTAAACCCATTTG
pENTR/N-CvpA-Tyr1-F	CGTTTTGGGAGACTGCTAGGGTCTTAAATTACAAAGATCTATTTG
pENTR/N-CvpA-Tyr1-R	CAAATAGATCTTTGTAATTTAAGACCCTAGCAGTCTCCCAAAACG
pENTR/N-CvpA-Tyr2-F	CGTTTTGGGAGACTTATAGGGTCTTAAATGCCAAAGATCTATTTG
pENTR/N-CvpA-Tyr2-R	CAAATAGATCTTTGGCATTTAAGACCCTATAAGTCTCCCAAAACG
pENTR/N-CvpA-Tyr1&2-F	CGTTTTGGGAGACTGCTAGGGTCTTAAATGCCAAAGATCTATTTG
pENTR/N-CvpA-Tyr1&2-R	CAAATAGATCTTTGGCATTTAAGACCCTAGCAGTCTCCCAAAACG
pENTR/N-CvpA-DiLeu1-F	CGAATCAAAAGAAGAATCTAAAGCAGCATCAGAAGAACAAAC
pENTR/N-CvpA-DiLeu1-R	GTTTGTTCTTCTGATGCTGCTTTAGATTCTTCTTTTGATTCG
pENTR/N-CvpA-DiLeu2-F	CCTGTATTCGTCATATAAATGCAGCTTTCATGAATTTTCGCTATGG
pENTR/N-CvpA-DiLeu2-R	CCATAGCGAAAATTCATGAAAGCTGCATTTATATGACGAATACAGG
pENTR/N-CvpA-DiLeu3-F	GCTGAAATACAGCAAGCAGCATCAATGGGGGC
pENTR/N-CvpA-DiLeu3-R	GCCCCCATTGATGCTGCTTGCTGTATTTCAGC
Sorting motif peptides	
pENTR/N-CvpA-P1-F	CACCCTTTTAAGAGGAATAATAGTGTCACG
pENTR/N-CvpA-P1-R	TCAGGAATGAAATACTTTCGTTTCAG
pENTR/N-CvpA-P2-F	CACCTTTTGGGAGACTTATAGGGTC
pENTR/N-CvpA-P2-Tyr1-F	CACCTTTTGGGAGACTGCTAGGGTC
pENTR/N-CvpA-P2-R	TCACTCTTTTATTCTGTTTTGCTCATTCC
pENTR/N-CvpA-P3-F	CACCGAACAACTTCCCTGTATTCGTC
pENTR/N-CvpA-P3-R	TCACACGATATGTTGTTTGCTTGAG
pENTR/N-CvpA-P4-F	CACCGATTCTGAGACTTGTGAACACTTG
pENTR/N-CvpA-P4-R	TCATTGAAGTCTGGTTAAACCCATTTG
Miscellaneous	
pcDNA6.2/N-mCherry-1	GAGGATCCCTACCGGTACCATGGTCTCTAAGGGCGAGGAAGAC
pcDNA6.2/N-mCherry-2	GATGGGCTCGAGGATATCCCTTTGTACAGCTCATCCATGCCACC
pET104-DEST-3	GGACTCTAGAGGATCCGATATCACCATGGGCGCCGGCACCCCGGTG

pET104-DEST-4	TGATGGGCTCGAGCCAAGCTTTCCCTTATCGTCATCGTCG
pcDNA6.2-5	GGATCCTCTAGAGTCCGGAG
pcDNA6.2-6	GGCTCGAGCCCATCAACAAG

Table S3. Plasmids used for cloning and expression of *cvpA* (*cbu0665*).

Name	Features	Reference or source
pJB-CAT-CyaA	*C. burnetii* expression vector, N-term fusion with CyaA; Cm^r	(1)
pJB-CAT-CyaA-CvpA	*cvpA* inserted into pJB-CAT-CyaA	This study
pMiniTn7T-CAT-CvpA	*cvpA* inserted into pMiniTn7T-CAT	This study
pMiniTn7T-CAT-CvpAmut	*cvpA* with LL to AA substitutions in DiLeu1, DiLeu2, DiLeu3 and Y to A substitutions in Tyr1 and Tyr2 inserted into pMiniTn7T-CAT	This study
pENTR/D-TOPO	Entry cloning vector; Kan^r	Invitrogen
pENTR/N-CvpA	*cvpA* inserted into pENTR/D-TOPO	This study
pENTR/N-CvpA-DiLeu1	*cvpA* with LL to AA substitution in DiLeu1 inserted into pENTR/D-TOPO	This study
pENTR/N-CvpA-DiLeu2	*cvpA* with LL to AA substitution in DiLeu2 inserted into pENTR/D-TOPO	This study
pENTR/N-CvpA-DiLeu3	*cvpA* with LL to AA substitution in DiLeu3 inserted into pENTR/D-TOPO	This study
pENTR/N-CvpA-Tyr1	*cvpA* with Y to A substitution in Tyr1 inserted into pENTR/D-TOPO	This study
pENTR/N-CvpA-Tyr2	*cvpA* with Y to A substitution in Tyr2 inserted into pENTR/D-TOPO	This study
pENTR/N-CvpA-Tyr1/Tyr2	*cvpA* with Y to A substitution in Tyr1 and Tyr2 inserted into pENTR/D-TOPO	This study
pDEST15	*E.coli* expression vector, N-term GST tag; Amp^r	Invitrogen
pDEST15-P1	*cvpA* bp 6-93, contains DiLeu1	This study
pDEST15-P2	*cvpA* bp 153-243, contains Tyr1 and Tyr2	This study
pDEST15-P3	*cvpA* bp 516-603, contains DiLeu2	This study
pDEST15-P4	*cvpA* bp 897-984, contains DiLeu3	This study
pDEST15-P1-DiLeu1	pDEST15-P1 with LL to AA substitution within DiLeu1	This study
pDEST15-P2-Tyr1/Tyr2	pDEST15-P2 with Y to A substitutions within Tyr1&2	This study
pDEST15-P3-DiLeu2	pDEST15-P3 with LL to AA substitution within DiLeu2	This study
pDEST15-P4-DiLeu3	pDEST15-P4 with LL to AA substitution within DiLeu3	This study
pET104-DEST	N-terminal BioEase fusion vector; Amp^r	Invitrogen

pT-RexT-DEST30	anhydrotetracycline-inducible expression; Amp^r	This study
pT-RexT-DEST30/N-mCherry	anhydrotetracycline-inducible expression of N-terminal mCherry fusions; Amp^r	This study
pT-RexT-DEST30/N-mCherry-CvpA	*cvpA* inserted into pT-RexT-DEST30/N-mCherry	This study
pT-RexT-DEST30/N-mCherry-CvpA-DiLeu1	*cvpA* with LL to AA substitution in DiLeu1 inserted into pT-RexT-DEST30/N-mCherry	This study
pT-RexT-DEST30/N-mCherry-CvpA-DiLeu2	*cvpA* with LL to AA substitution in DiLeu2 inserted into pT-RexT-DEST30/N-mCherry	This study
pT-RexT-DEST30/N-mCherry-CvpA-DiLeu3	*cvpA* with LL to AA substitutions in DiLeu3 inserted into pT-RexT-DEST30/N-mCherry	This study
pT-RexT-DEST30/N-mCherry-CvpA-Tyr1	*cvpA* with Y to A substitution in Tyr1 inserted into pT-RexT-DEST30/N-mCherry	This study
pT-RexT-DEST30/N-mCherry-CvpA-Tyr2	*cvpA* with Y to A substitution in Tyr2 inserted into pT-RexT-DEST30/N-mCherry	This study
pT-RexT-DEST30/N-mCherry-CvpA-Tyr1/Tyr2	*cvpA* with Y to A substitutions in Tyr1 and Tyr2 inserted into pT-RexT-DEST30/N-mCherry	This study
pcDNA6.2/N-mCherry-DEST	N-terminal mCherry fusion vector; Amp^r	(1)
pcDNA6.2/N-BioEase-DEST	N-terminal BioEase and C-terminal V5 fusions vector; Amp^r	This study
pcDNA6.2/N-BioEase-DEST-CvpA	*cvpA* inserted into pcDNA6.2/N-BioEase-DEST	This study