Supplementary Information (SI Appendix) for:

Iron Robbery by Intracellular Pathogen Via Bacterial Effector–induced Ferritinophagy

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**Supplemental Materials and Methods**

**Bacteria and cell culture.** The *E. chaffeensis* Arkansas strain was cultured in THP-1 cells (ATCC, Manassas, VA) in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and 2 mM L-glutamine (GIBCO, Waltham, MA). RF/6A cells (ATCC) were cultured in advanced minimal essential medium (AMEM, GIBCO) supplemented with 8% FBS and 2 mM L-glutamine. HEK293 cells (ATCC) and DH82 cells were cultured in Dulbecco’s minimal essential medium (DMEM, Mediatech) supplemented with 5% FBS and 2 mM L-glutamine. Cultures were incubated at 37°C under 5% CO₂ in a humidified atmosphere.

**Antibodies.** The following antibodies were used in this study: mouse monoclonal anti-HA (BioLegend, San Diego, CA), rabbit monoclonal anti-FTL (Abcam, Cambridge, MA), rabbit polyclonal anti-FTH (Invitrogen, Carlsbad, CA), mouse monoclonal anti-GFP (Santa Cruz Biotechnology, Dallas, TX), anti-mouse IgG2b (Santa Cruz Biotechnology), HisProbe-HRP (ThermoFisher Scientific, Waltham, MA), rabbit polyclonal anti-LC3 (NovusBio, Littleton, CO), rabbit anti-P28 (1), rabbit anti-actin (Sigma-Aldrich, St. Louis, MO), Alexa Fluor (AF) 488–conjugated rat monoclonal anti-FLAG (BioLegend), AF555 or AF488-conjugated goat anti-mouse IgG and anti-rabbit IgG (Invitrogen, Carlsbad, CA), AF350-conjugated goat anti-rabbit IgG (Invitrogen), and horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG and anti-rabbit IgG (Cell Signaling Technology, Danvers, MA), FITC-conjugated goat anti-llama IgG (Bethyl Laboratories, Montgomery, TX), and rabbit polyclonal anti-MnSOD (Invitrogen).

**Cloning.** Genes encoding Etf-3 and FTH1 were PCR amplified from *E. chaffeensis* genomic DNA and cDNA from THP-1 cells, respectively, and subsequently cloned into pET33b(+) (Novagen, Gibbstown, NJ) at the NheI and Xhol restriction sites to create plasmids expressing 6×His-tagged
Etf-3. For expression in mammalian cells, full-length Etf-3 was codon-optimized, custom-synthesized (GenScript, Piscataway, NJ, Table S1), and recloned into pEGFP-N1 (Takara, Mountain View, CA) at the XhoI and BamHI restriction sites to create a plasmid encoding Etf-3-GFP. The HA-Etf-3 mammalian expression vector was constructed from the Etf-3-GFP plasmid, and the HA-NCOA4 mammalian expression vector was constructed from a cDNA from THP-1 cells using two-step PCR with overlapping primers and subsequently cloned into pEGFP-N1 by replacing the GFP tag at the XhoI and NotI restriction sites.

The pCis-FLAG-Etf-3-SS-Himar A7 or pCis-FLAG-Etf-3ΔC-SS-Himar A7 plasmid encoding FLAG-tagged Etf-3 or Etf-3ΔC (residues 1-588; Etf-3 for C-terminal T4SS signal deletion), respectively, and the spectinomycin/streptomycin antibiotic resistance gene (aad), were created from the pCis-FLAG-Etf-2-SS-Himar A7 plasmid (2-4) by replacing eft-2 with etf-3 or etf-3Δc at the BamHI and KpnI restriction sites. Cloning primers are listed in Table S1.

**Transformation of *E. chaffeensis* with FLAG-Etf-3 or FLAG-Etf-3ΔC Himar plasmid.**

FLAG-Etf-3 and FLAG-Etf-3ΔC Himar plasmids were amplified in dam−/dcm− Escherichia coli strain C2925 (New England Biolabs, Ipswich, MA) and purified using a Maxiprep plasmid DNA isolation kit (Qiagen, Valencia, CA). Transformation of purified *E. chaffeensis* with the pCis-FLAG-Etf-3-Himar plasmid or the pCis-FLAG-Etf-3ΔC-Himar plasmid was done as previously described (4). At 2 day post-transfection, transformed *E. chaffeensis* expressing the spectinomycin/streptomycin resistance gene were selected in the presence of 100 µg/ml spectinomycin (Millipore Sigma, Burlington, MA), 100 µg/ml streptomycin (Millipore Sigma), and 0.1 µg/ml cycloheximide (Millipore Sigma), and the culture medium containing antibiotics was replaced twice a week until ≥80% infected cells was achieved (~3 weeks). Transformed *E. chaffeensis* expressing FLAG-Etf-3 or FLAG-Etf-3ΔC was used to infect RF/6A cells, and at 2
days post-infection (dpi), cells were fixed and labeled with AF488-rat monoclonal anti-FLAG IgG. *E. chaffeensis* was labeled with DAPI (4',6'-diamidino-2-phenylindole; Invitrogen). To estimate intracellular replication of FLAG-Etf-3-transformed *E. chaffeensis*, THP-1 cells were infected with FLAG-Etf-3-transformed and no-transformed *E. chaffeensis* at MOI of 30. Synchronous culture of FLAG-Etf-3-transformed or untransformed *E. chaffeensis* was done as mentioned in the synchronous culture of *E. chaffeensis* method, but harvested at two time points (24 and 72 hpi). RNA (1 µg) extracted from cells with the RNeasy Mini kit (Qiagen, Germantown, MD) was used for cDNA synthesis using the Maxima H minus First Strand cDNA synthesis kit (ThermoFisher Scientific) with random hexamer primers. Bacterial and host gene expression was estimated by reverse transcription (RT)-qPCR using specific primers (Table S2).

**Production of llama antiserum against Etf-3 and native Etf-3 expression analysis in *E. chaffeensis*–infected RF/6A cells.** Competent *E. coli* BL21 (DE3) (New England Biolabs) was transformed with a pET33b (+) plasmid encoding Etf-3N, and expressed protein was purified with HisPur Cobalt Resin (ThermoFisher Scientific). Purified rEtf-3N (~1 mg) was mixed with immunostimulating complex in 1:1 ratio (v/v) as previously described (5) and used for immunization of a llama three times at 2-week intervals. The llama anti-Etf-3N serum was affinity-purified with rEtf-3N bound to the AminoLink® Plus Coupling Resin (ThermoFisher Scientific). The specificity of purified antibodies was tested with rEtf-3N and cell lysates from *E. chaffeensis*–infected THP-1 cells by western blotting. To determine the localization of native Etf-3, *E. chaffeensis*–infected RF/6A cells at 2 dpi were fixed with 4% paraformaldehyde (PFA) and labeled with purified llama anti-rEtf-3N (pre-adsorbed with uninfected RF/6A cells) in PGS (phosphate-
buffered saline supplemented with 0.1% gelatin [Sigma], and 0.1% saponin [Sigma]) and AF488-conjugated goat anti-llama antibody, and \textit{E. chaffeensis} was labeled with DAPI.

\textbf{PNA synthesis, biotin labeling, RNA-PNA hybridization, PNA transfection of host cell–free \textit{E. chaffeensis}, and knockdown and complementation analysis.} An antisense PNA oligomer targeting bp 30 – 45 following ATG start codon of \textit{etf-3} gene (Fig. 2b) and a scrambled control PNA (3’-CACATATCTCGG-5’) were designed based on PNABio PNA Tools as previously described (4, 6), and synthesized by PNABio (Newbury Park, CA). PNA oligomers were biotinylated using EZ-Link Sulfo-NHS-Biotin (ThermoFisher Scientific) and purified using Pierce C18 Spin Columns (ThermoFisher Scientific), and dot blots were used to confirm the biotinylation of oligomers, as described (4). To verify that Etf-3 PNA could bind the targeted region of \textit{etf-3} mRNA, a single-stranded RNA (ssRNA, Fig. 2b) was designed and synthesized by Sigma-Aldrich. An electrophoretic mobility shift assay was performed to confirm the hybridization of biotin-labeled Etf-3 PNA and the ssRNA as previously described (4).

To transfect \textit{E. chaffeensis} with PNA, 3 μg Etf-3-specific PNA or scrambled control PNA in 10 μl nuclease-free water was mixed with 100 μl host cell–free \textit{E. chaffeensis} in 300 mM sucrose, and electroporation was performed in a 2-mm gap electroporation cuvette (Bio-Rad, Hercules, CA) under the same condition as for the Himar plasmid. The PNA-transfected \textit{E. chaffeensis} was transferred to a T25 flask containing 5×10^5 HEK293 cells and incubated at 37°C for 60 min with gentle shaking every 10 min to allow \textit{Ehrlichia} internalization. The cells were washed to remove uninternalized bacteria and continuously cultured in fresh medium.

For trans-complementation analysis, HEK293 cells (1×10^6 cells per 100 μl) were transfected with 5 μg Etf-3-GFP or 1 μg pEGFP-N1 by electroporation in a 2-mm gap cuvette (Bio-Rad) at 100 V, 1000 μF using a Gene Pulser Xcell™ Electroporation System (Bio-Rad). At 1 dpt, host cell–free
E. chaffeensis freshly transfected with 3 µg Etf-3 or CTL PNA (as described above) were added
to pre-transfected HEK293 cells at a multiplicity of infection (MOI) of 100, and cells were
harvested at 2 dpi. Bacterial and host gene expression was estimated by RT-qPCR.

Effect of Etf-3-GFP and NCOA4 overexpression on E. chaffeensis infection. Exponentially
growing HEK293 cells (2×10^6 cells) were suspended in 100 µl Opti-MEM (Gibco) and
electroporated with 5 µg Etf-3-GFP or GFP plasmid. Transfected cells were infected with freshly
isolated E. chaffeensis at MOI of 50 at 12 h post-transfection. Bacteria that were not internalized
were removed at 1 dpi, and cells were harvested at 2 dpi. Bacterial and host gene expression was
estimated by RT-qPCR.

Yeast two-hybrid assay. Codon-optimized, full-length Etf-3 was cloned into vector pGBKT7
(Takara) at restriction sites NdeI and BamHI and transformed into Saccharomyces cerevisiae Y187
using the Quick & Easy yeast transformation kit (Takara). Transformants were selected on
minimal synthetically defined (SD) medium (Takara) with amino acid dropout supplements-Trp
(SD/-Trp, Takara) agar plates for 3–5 days. Human Leukocyte Matchmaker™ cDNA Library
(Takara) was amplified and transformed into S. cerevisiae AH109. Yeast two-hybrid assays were
performed according to user manual for the Matchmaker Gold Yeast Two-Hybrid System (Takara).
Approximately 3,800 colonies obtained from SD/-Leu/-Trp double-dropout agar plates were
transferred to SD/-Leu/-Trp/-His/-Ade quadruple-dropout with X-alpha-Gal (final concentration:
40 mg/l) agar plates. In total, 55 blue colonies were picked, cultured, and plasmids were extracted
with the Yeast Plasmid Isolation kit (Takara). Plasmids were transferred to DH5α E. coli
competent cells (New England Biolab). Prey plasmid transformants were selected on Luria-Bertani
agar with 50 µg/ml ampicillin, and 55 plasmids were extracted from positive clones and sequenced.
To validate the results of the yeast two-hybrid screen using Etf-3 as bait, human FTL coding
sequence and human FTH1 coding sequence were amplified from THP-1 cell cDNA. Each sequence was cloned into vector pACT2 (Takara) at restriction sites EcoRI and XhoI and transformed into *S. cerevisiae* AH109 as described above. pGBKKT7-53 and pGADT7-T (Takara) were used as positive controls while pGADT7-RAB5S/N (4) was used as negative control.

**Far-Western blotting, co-immunoprecipitation, and WB.** Far-Western blotting was performed using 10 μg rEtf-3 and rEtf-1 (7) that were separated by SDS-PAGE, transferred to a PVDF membrane (ThermoFisher Scientific), and renatured in a graded series of serial dilutions of guanidinium-HCl as described (8). Renatured proteins were incubated with 35 μg purified human holoferritin (Bio-Rad). After stringent washing, the membrane was incubated with rabbit anti-FTL (1:1000) and HRP-conjugated goat anti-rabbit IgG (1:1000). The membrane was stripped with Restore™ Western Blot Stripping Buffer (ThermoFisher Scientific) and reprobed with HisProbe-HRP (1:2000). For the co-immunoprecipitation assay, HEK293 cells were electroporated with Etf-3-GFP (GFP as negative control), HA-NCOA4, HA-NCOA4 and Etf-3-GFP, or HA-NCOA4 and GFP. Transfected cells were harvested and lysed in RIPA buffer (ThermoFisher Scientific) with a 1% protease inhibitor cocktail (Sigma-Aldrich) at 2 dpt. Whole-cell lysates were immunoprecipitated with anti-GFP nanobody affinity gel (BioLegend) or with anti-HA-protein A agarose (ThermoFisher Scientific) (2 mg). For the native Etf-3 co-immunoprecipitation assay, cell lysates of *E. chaffeensis*-infected THP-1 cells at 2 dpi were immunoprecipitated with llama anti-Etf-3N-protein A agarose (2 mg), and subjected to western blotting.

**Open SPR.** Binding experiments were carried out with an OpenSPR system (Nicoya Lifesciences, Ontario, Canada). rEtf-3 was labeled with biotin via EZ-Link® NHS-Biotin Reagents (ThermoFisher Scientific). Then biotin-rEtf-3 was immobilized on a streptavidin sensor chip via non-covalent coupling at a final concentration of 10 μg/ml. After achieving a stable baseline, the
running buffer was injected for blank measurement. Measurements were then made as described in the user manual (Nicoya Lifesciences). Interactions of rEtf-3 with purified human ferritin (Bio-Rad) were assessed at concentrations of 50, 150 and 300 nM, or with recombinant FTH1 were assessed at concentrations of 471, 141 and 47 nM. The data were collected and analyzed using TraceDrawer program (Nicoya Lifesciences). All curves were corrected for the blank measurement.

**Cellular localization analysis.** For binding and internalization analysis of Etf-3 PNA-transfected *E. chaffeensis*, Etf-3 PNA-transfected or non-transfected *E. chaffeensis* was added at MOI of 20 to 5 × 10⁴ RF/6A cells. After co-incubation for 3 h at 37º C, cells were harvested and fixed with 4% PFA. Two steps of labeling of fixed cells with anti-P28 antibody were carried out: the first step was performed without PGS permeabilization to detect bound but not internalized bacteria using anti-P28 antibody and AF555-conjugated anti-rabbit IgG, and the second step was performed after permeabilization with PGS to detect total bacteria using AF488-conjugated anti-rabbit IgG. For colocalization analysis of Etf-3-GFP or Etf-1-GFP and endogenous ferritin, 2×10⁶ DH82 cells were electroporated with 5 µg Etf-3-GFP or GFP plasmid. Transfected cells were either left untreated or treated with 40 μM FAC (ThermoFisher Scientific) for 24 h prior to harvesting at 2 dpt. Cells were cytocentrifuged on slides and fixed with 4% PFA and incubated with rabbit anti-FTL in PGS followed by AF555-conjugated goat anti-rabbit IgG in PGS. For colocalization analysis of Etf-3-GFP and RFP-LC3 or RFP-FTL, 5×10⁴ RF/6A cells were cultured on coverslips in wells of a 24-well plate and cotransfected with plasmids encoding Etf-3-GFP/GFP and RFP-LC3 or RFP-FTL (provided by Dr. Kavita Lole) using Lipofectamine 3000 (Invitrogen). Subcellular localization was determined at 2 dpt. For colocalization analysis of RFP-LC3 and endogenous ferritin with/without Etf-3-GFP, 5×10⁴ RF/6A cells were cultured on coverslips in
wells of a 24-well plate and cotransfected with plasmids encoding RFP-LC3 and Etf-3-GFP or GFP using Lipofectamine 3000. Cells were harvested at 2 dpt. Cells were incubated with rabbit anti-FTL followed by AF350-conjugated goat anti-rabbit IgG in PGS. For colocalization analysis of Etf-3-GFP and HA-NCOA4, 5×10^4 RF/6A cells were cultured on coverslips in wells of a 24-well plate and cotransfected with plasmids encoding HA-NCOA4 and Etf-3-GFP or GFP using Lipofectamine 3000. Other HA-NCOA4 and GFP-cotransfected cells were treated with 40 μM FAC for 24 h prior to harvesting at 2 dpt. Cells were labeled with mouse anti-HA in PGS followed by AF555-conjugated goat anti-mouse IgG in PGS. Fluorescence images with overlay differential interference contrast (DIC) images were acquired and analyzed with a DeltaVision PersonalDV deconvolution microscope system (GE Healthcare Life Sciences, Marlborough, MA). Colocalization analysis was performed with Coloc 2 in the NIH Image J software package to calculate the Pearson correlation coefficient.

**Ferritinophagy analysis.** To analyze the effects of Etf-3 on ferritinophagy, HEK293 cells (2×10^6 cells) were transfected with 5 μg Etf-3-GFP, or 1 μg GFP plasmid by electroporation. At 12 h prior to harvesting, transfected HEK293 cells were treated with 0.1 μM rapamycin (Sigma-Aldrich), 5 μM E64D (Sigma-Aldrich), or 5 μM 3-MA (Sigma-Aldrich). Cell lysates were prepared as described above and subjected to WB with anti-actin and anti-FTL. Cells were harvested at 2 dpt and lysed in RIPA buffer with a 1% protease inhibitor cocktail and subjected to western blotting.

**Estimating LCI in live cells.** Etf-3-GFP- or GFP-transfected RF/6A cells were seeded and grown in a 35-mm chambered glass-bottom culture dish (WillCo Wells, Amsterdam, Netherlands) with AMEM supplemented with 8% FBS and 2 mM L-glutamine. Cells were infected with *E. chaffeensis* at MOI of 20 at 6 h post-seeding or 20 h post-transfection, treated with 40 μM FAC at 1 day prior to testing, or treated with 2 mM 3-MA 12 h prior to testing. For live-cell imaging, cells
were incubated with 5 μM BioTracker 575 Red Fe^{2+} dye (Sigma-Aldrich) in AMEM supplemented with 8% FBS and 2 mM L-glutamine at 37°C for 1 h. Nuclei and \textit{E. chaffeensis} were labeled with Hoechst 33342 (Sigma-Aldrich) at 37°C for 15 min. Then, cells were washed with Hanks’ balanced salt solution (Sigma-Aldrich) three times and incubated with the same solution. Live cells were observed under a DeltaVision microscope at 37°C. The red fluorescence intensity was analyzed with ImageJ software.

**Synchronous culture of \textit{E. chaffeensis}**. Human monocyte cell line was synchronously infected with \textit{E. chaffeensis} as described (7). Briefly, to disrupt host cells and liberate \textit{E. chaffeensis}, infected THP-1 cells on ice were sonicated twice for 10 s at setting 2 with a W-380 sonicator (Heat System-Ultrasonics, Farmington, NY). After centrifugation at 700 × g for 10 min, the supernatant was collected and passed through 5-μm and 2.7-μm syringe filters (Millipore) to remove cell debris. The bacterial suspension on ice was further sonicated for 30 s twice at setting 4.5 to disrupt reticulate cells of \textit{E. chaffeensis}. \textit{E. chaffeensis} was centrifuged at 10,000 × g for 10 min at 4°C and used to infect 1×10^8 THP-1 cells in 5 ml culture medium in a T-25 flask at MOI of 30. After incubating at 37°C for 1 h with gentle shaking every 10 min, the cells were washed three times with fresh medium to remove unbound or un-internalized bacteria and resuspended at a density of 3 to 4×10^5 cells/ml. After unbound or un-internalized bacteria were removed, this time point was designated as 0 hpi. Infected THP-1 cells were harvested at 0, 6, 12, 24, 48, 72, and 96 hpi. \textit{E. chaffeensis} growth and expression of \textit{etf-3}, \textit{etf-1}, \textit{sodb}, \textit{fil}, \textit{fth}, and \textit{tfr} were determined by RT-qPCR analysis using specific primers (Table S2).

**Analysis of intracellular levels of ROS, and MnSOD and SODB mRNA.** HEK293 cells (1×10^4 cells) were seeded in a 96-well plate (Tecan, Morrisville, NC). Four groups were included in this assay: HEK293 cells only, HEK293 cells infected with \textit{E. chaffeensis} (MOI of 30), HEK293 cells
treated with 40 μM FAC for 24 h before ROS assay, and HEK293 cells infected with *E. chaffeensis* at MOI of 30 with 40 μM FAC treatment for 24 h before ROS assay. In another assay, a total of 1×10⁴ HEK293 cells were transfected with HA-Etf-3, HA-Etf-1 and HA-Etf-3, HA-Etf-1, or HA-RAB5S/N (control) with Lipofectamine 3000 and seeded in a 96-well plate (Tecan). Each transfected group was either left untreated or treated with 40 μM FAC for 24 h before ROS assay. Intracellular ROS were determined at 2 dpi by using the fluorescent dye H₂DCFDA (Invitrogen) as described (7). The fluorescence intensity of DCF, corresponding to the ROS level, was measured with a Tecan Microplate Reader (Infinite® 200 PRO, Tecan) at excitation and emission wavelengths of 492 nm and 520 nm, respectively. The amount of MnSOD was estimated by western blotting and SODB mRNAs were determined by RT-qPCR.

**Statistical Analysis.** Analyses of significant differences were performed using the two-tailed Student *t* test or analysis of variance (ANOVA). Data represent the mean ± SD from three independent experiments. Data analysis and figure drawing were performed with Prism 7 software (GraphPad, San Diego, CA).

References

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Supplemental Table S1. Primer sequences for cloning useful genes into appropriate plasmids

| Target | Primer sequence | Restriction enzymes and target plasmid |
|--------|-----------------|----------------------------------------|
| **Mammalian expression** | | |
| Etf-3-GFP (codon optimized)² | F: GTAC CTGGAG CGCCACCATGGTG TGCAATAACGCAATGCTGACA R: TACG GGATCC TCGGCCATGTTGTCAGAGC | F, XhoI; R, BamHI. For cloning codon-optimized Etf-3 (aa¹⁻⁶²¹) into XhoI (F) and BamHI (R) sites in plasmid pEGFP-N1. |
| HA-Etf-3 (codon optimized) | F: GTTCCAGATTACGCT TGCAATAACGCAATGCTGACA R: GTAC GCGGCCGC TCACCTGGCGATGGTTCGGGTCAGAGC F-overlap: GTAC CTGGAGA CGCCACCATGGTG | F, XhoI; R, NotI. For cloning HA-codon-optimized Etf-3 (aa¹⁻⁶²¹) into XhoI (F) and NotI (R) sites in plasmid pEGFP-N1 (GFP was replaced with HA tag). |
| HA-NCOA4 (CDS) | F: GTTCCAGATTACGCT AATACCTTCCAAGACCAGAGTGGC R: GTAC GCGGCCGC TCACATCTGTAGAGGAGTTCGATATAACC F-overlap: GTAC CTGGAGA CGCCACCATGGTG | F, XhoI; R, NotI. For cloning HA-NCOA4 into XhoI (F) and NotI (R) sites in plasmid pEGFP-N1 (GFP was replaced with HA tag). |
| **pCis-FLAG-SS-Himar A7 plasmid construction** | | |
| Etf-3 | F: GTAC GGATCC TGTAATAATGCTATGCTAACAAAAGTT R: TACG GGTACC TTATCTTGCTATTGTTCTTGTAAGTGC | F, BamHI; R, KpnI. For cloning *Ehrlichia* Etf-3 into BamHI (F) and KpnI (R) sites in plasmid pCis-FLAG-Etf-2-SS-Himar A7. |
| Etf-3ΔC (residues ¹⁻⁵⁸⁸) | F: GTAC GGATCC TGTAATAATGCTATGCTAACAAAAGTT R: TACG GGTACC TTAATTACTATCTATATAAGATTGGAGC | F, BamHI; R, KpnI. For cloning *Ehrlichia* Etf-3 C terminus (aa³⁷²⁻⁶²¹) into BamHI (F) and KpnI (R) sites in plasmid pCis-FLAG-Etf-2-SS-Himar A7. |
### E. coli expression

| Protein                        | Forward Primer | Reverse Primer | Restriction Enzymes | Notes |
|-------------------------------|----------------|----------------|---------------------|-------|
| **His-Etf-3 (codon optimized)** | F: GTAC **GCTAGC** TGCAATAACGCAATGCTGACAAAGTC  
G: TACG **CTCGAG** CCTGGCCCAGTGTTCCGGTACAGG | R: TACG **CTCGAG** CCTGGCCCAGTGTTCCGGTACAGG | F, NheI; R, XhoI. For cloning codon-optimized Etf-3 into NheI (F) and XhoI (R) sites in plasmid pET-33b(+) |
| **His-FTH1**                  | F: GTAC **GCTAGC** ATACGACCGCGTCCACCTCGCAG  
G: TACG **CTCGAG** TTAGCTTTGCATTACACTTGCTCC | R: TACG **CTCGAG** TTAGCTTTGCATTACACTTGCTCC | F, NheI; R, XhoI. For cloning human ferritin heavy chain into NheI (F) and XhoI (R) sites in plasmid pET-33b(+) |

### Yeast expression

| Protein                        | Forward Primer | Reverse Primer | Restriction Enzymes | Notes |
|-------------------------------|----------------|----------------|---------------------|-------|
| **Etf-3 (codon optimized)**    | F: GTAC **CATATG** TGCAATAACGCAATGCTGACA  
G: TACG **GGATCC** TTACCTGGCCAGTGTTCCGGTCAGAGG | R: TACG **GGATCC** TTACCTGGCCAGTGTTCCGGTCAGAGG | F, NdeI; R, BamHI. For cloning codon-optimized Etf-3 into NdeI (F) and BamHI (R) sites in plasmid pGBK7 |
| **Ferritin light chain (CDS)** | F: GTAC **GAATTC** ATAGCTCCCAGATCGTCAGAATTATTCC  
G: TACG **GAATTC** ATAGCTCCCAGATCGTCAGAATTATTCC | R: TACG **GAATTC** ATAGCTCCCAGATCGTCAGAATTATTCC | F, EcoRI; R, BamHI. For cloning human ferritin light chain into EcoRI (F) and BamHI (R) sites in plasmid pACT2 |
| **Ferritin heavy chain (CDS)** | F: GTAC **GAATTC** ATACGACCGCGTCCACCTCGCAG  
G: TACG **GAATTC** ATACGACCGCGTCCACCTCGCAG | R: TACG **GAATTC** ATACGACCGCGTCCACCTCGCAG | F, EcoRI; R, BamHI. For cloning human ferritin heavy chain into EcoRI (F) and BamHI (R) sites in plasmid pACT2 |

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1. F, forward primer; R, reverse complement primer; underlined, restriction enzyme sites; italicized, Kozak sequences for expression from plasmid pEGFP-N1; bold, modified HA sequence; CDS, coding sequence.

2. Nucleotide sequence encoding full-length Etf-3 (residues 1–621) for codon-optimized expression in mammalian cells:

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ATGTGCAATAACGCAATGCTGACAAAGTCACACACTCAAAACACTCAACCTGAAAACGAGTATATCCGACATCACCCTGGAGAAGATCGACGAAGCTCTGATTGACATCAGCAACGTGATCCCACTGTGCATTGCACTGATGCTGGACATCACTCCCGATTTCACCTCATTTCGCAATGAGGTCACCAGCATGCTGGAGGAATACAAAGAGTGGTTCATCGCCAACGGGGAAGCTAAGATGCCAGATCGACGATCGAACCAGTGACACACTGTGGAACAGCATCAAGGAGATTCATACTCTGTATCAGCTCCTGAGAAGATCGACGAAGCTCTGATTGACATCAGCAACGTGATCCCACTGTGCATTGCACTGATGCTGGACATCACTCCCGATTTCACCTCATTTCGCAATGAGGTCACCAGCATGCTGGAGGAATACAAAGAGTGGTTCATCGCCAACGGGGAAGCTAAGATGCCAGATCGACGATCGAACCAGTGACACACTGTGGAACAGCATCAAGGAGATTCATACTCT
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### Supplemental Table S2. Primer sequences for RT-qPCR

| Target gene                        | Primer sequence                                      | Product size (bp) |
|-----------------------------------|-------------------------------------------------------|-------------------|
| *E. chaffeensis* 16S rRNA          | F: CGGGGAAAGATTTATCGCTATTA                           | 323               |
|                                   | R: CGCTTGCCCCCTCCGTATTA                              |                   |
| *E. chaffeensis* etf-3             | F: GCCCCTAATTGATGATAGAACCCTC                        | 150               |
|                                   | R: TGCCTGTTTGGTGACAGAGTAA                           |                   |
| *E. chaffeensis* etf-1             | F: GCCTAGTTTGAATACCTTGGAGGCA                        | 107               |
|                                   | R: CAGTTTGTGGGTACCGTTGTCAT                          |                   |
| *E. chaffeensis* sodB              | F: AAGCATTACATAGGGTTAT                              | 158               |
|                                   | R: TGATTCCATACTTGACCA                                |                   |
| Human transferrin receptor (*TFR*)| F: TGTGGAGATGAAACTTTC                              | 159               |
|                                   | R: TAGCCCAAGTAGCCAAC                                 |                   |
| Human ferritin light chain (*FTL*) | F: GCCTCTCTTCAGGACATC                               | 121               |
|                                   | R: GATGGAAGATCCAAAGGAC                              |                   |
| Human ferritin heavy chain (*FTH1*)| F: CTTTGACCAGTGATGAGG                               | 131               |
|                                   | R: CCTGAAAGAGATCGGCCA                               |                   |
| Human β-actin (*ACTB*)             | F: AGAGCTACGAGCTGCCTGAC                             | 184               |
|                                   | R: AGCACTGTGTGCGCTGAC                               |                   |
Fig. S1. Alignment of T4SS substrate Etf-3 among eight *E. chaffeensis* strains.

Eight *E. chaffeensis* strains were culture-isolated during 1991–1998 and genomic sequences determined in 2013 (9). Alignment of Etf-3 was performed using NCBI BLASTP algorithm, and locus IDs of Etf-3 in each strain were shown at the left of the alignment. Locus ID abbreviations and GenBank accession numbers are: ECH, Arkansas (type strain), NC_007799.1; HL, Heartland (10), NZ_CP007473.1; JAX, Jax (11), NZ_CP007475.1; LIB, Liberty (12), NZ_CP007476.1; OSC, Osceola (12), NZ_CP007477.1; STV, St. Vincent (11), NZ_CP007478.1; WAK, Wakulla (12), NZ_CP007479.1; WP, West Paces (10), NZ_CP007480.1.

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ECH_0767       1   MCNNAMLTKVQRAAINTNKLQYIRDITLEKIDEALIDISNVIPLCIALMLDITPDFTS 60
STV_0364       1   ............................................................ 60
WP_0364        1   ............................................................ 60
OSC_0689       1   ............................................................ 60
JAX_0374       1   ............................................................ 60
HL_0679        1   ............................................................ 60
WAK_0371       1   ............................................................ 60
LIB_0371       1   ............................................................ 60

ECH_0767       61  FRNEHTSMLEEYKWEFIANGEAKMPLIDDRTSDTLWNIKLYHQQMSISSNDGIN 120
STV_0364       61  ............................................................ 120
WP_0364        61  ............................................................ 120
OSC_0689       61  ............................................................ 120
JAX_0374       61  ............................................................ 120
HL_0679        61  ............................................................ 120
WAK_0371       61  ............................................................ 120
LIB_0371       61  ............................................................ 120

ECH_0767       121 ILDRIAFNSAHNTQDIIIDNKLYKQYIQILSSIFIDQDFDTNPTTYKTEILOPTTSEI 180
STV_0364       121 ............................................................ 180
WP_0364        121 ............................................................ 180
OSC_0689       121 ............................................................ 180
JAX_0374       121 ............................................................ 180
HL_0679        121 ............................................................ 180
WAK_0371       121 ............................................................ 180
LIB_0371       121 ............................................................ 180

ECH_0767       241 ILNQRTFRKINLLLDSGCEINVKCDIPTYEIFLEISDITFGTVYGSIFNYGSIIT 300
STV_0364       241 ............................................................ 300
WP_0364        241 ............................................................ 300
OSC_0689       241 ............................................................ 300
JAX_0374       241 ............................................................ 300
HL_0679        241 ............................................................ 300
WAK_0371       241 ............................................................ 300
LIB_0371       241 ............................................................ 300
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ECH_0767  301  AKEECNLSIFGTVSINGKIIKNLPDFIPTANFCRNKHKTIIITRDQCQAEKVI  360
STV_0364  301 ............................................................  360
WP_0364   301 ............................................................  360
OSC_0689  301 ............................................................  360
JAX_0374  301 ............................................................  360
HL_0679   301 ............................................................  360
WAK_0371  301 ............................................................  360
LIB_0371  301 ............................................................  360
ECH_0767  361  TSFHPCDVINQKDPNGIRALNIELFHPNFQYHTVIAIGWPINKQNNTSTENAELFHNI  420
STV_0364  361 ............................................................  420
WP_0364   361 ............................................................  420
OSC_0689  361 ............................................................  420
JAX_0374  361 ............................................................  420
HL_0679   361 ............................................................  420
WAK_0371  361 ............................................................  420
LIB_0371  361 ............................................................  420
ECH_0767  421  IHNANHLNIDNISKNQRLPNTFFNDSIPMFIHNIRITNKLIENLSNALYLYTH  480
STV_0364  421 ............................................................  480
WP_0364   421 ............................................................  480
OSC_0689  421 ............................................................  480
JAX_0374  421 ............................................................  480
HL_0679   421 ............................................................  480
WAK_0371  421 ............................................................  480
LIB_0371  421 ............................................................  480
ECH_0767  481  SGTNLTSKFGVNEVIIHSKAVDFSILHDTVDVFSGKFLHHTKGNITIVGQRKSK  540
STV_0364  481 ............................................................  540
WP_0364   481 ............................................................  540
OSC_0689  481 ............................................................  540
JAX_0374  481 ............................................................  540
HL_0679   481 ............................................................  540
WAK_0371  481 ............................................................  540
LIB_0371  481 ............................................................  540
ECH_0767  541  IISNTGLIKIENNSLNATIPIVIDSSIAQYGAVEITGCSNSYIDSNKTRPLNNST  600
STV_0364  541 ............................................................  600
WP_0364   541 ............................................................  600
OSC_0689  541 ............................................................  600
JAX_0374  541 ............................................................  600
HL_0679   541 ............................................................  600
WAK_0371  541 ............................................................  600
LIB_0371  541 ............................................................  600
ECH_0767  601  PPSYLTRLSEIFSALTRTIAR  621
STV_0364  601 ............................................................  621
WP_0364   601 ............................................................  621
OSC_0689  601 ............................................................  621
JAX_0374  601 ............................................................  621
HL_0679   601 ............................................................  621
WAK_0371  601 ............................................................  621
LIB_0371  601 ............................................................  621
Fig. S2. FLAG-Etf-3 promotes intracellular replication of *Ehrlichia*. THP-1 cells were infected with pCis-FLAG-Etf-3-SS-Himar-A7-transformed *E. chaffeensis*. Samples were collected at 24 and 72 hpi. Bacterial replication was determined by RT-qPCR using primers specific to *Ehrlichia* 16S rRNA and were normalized against the level of human β-actin mRNA. Data are presented as the mean ± SD from three independent experiments. *Significantly different by Student's t test (P < 0.05).
Fig. S3. *etf-3*-specific antisense PNA transfection does not affect *E. chaffeensis* binding or entry into host cells. RF/6A cells were infected with WT *E. chaffeensis* (*Ech*) or *Etf-3* PNA-transfected *Ech* at MOI of 20. Cells were harvested and fixed at 3 hpi. External WT *Ech* (A) or *Etf-3* PNA transfected *Ech* (B) bound to RF/6A cells (External *Ech*) were immunofluorescence labeled with anti-P28 and AF555-conjugated goat anti-rabbit IgG without cell permeabilization. Total WT *Ech* (A) or *Etf-3* PNA transfected *Ech* (B) were immunofluorescence labeled with anti-P28 and AF488-conjugated goat anti-rabbit IgG after cell permeabilization. Merge/DIC, merge of the fluorescence and DIC images; N, nucleus; *Ech*, *E. chaffeensis*. Each boxed area is enlarged 4× on the right. Scale bars: 10 µm. (C) The ratios of internalized (total - bound) bacteria were not significantly different between WT *Ech* and *Etf-3* PNA-transfected *Ech*. Bacteria in 30 cells were scored. Data indicate the mean ± SD from three independent experiments.
FIG. S4. Etf-3 does not bind to FTH1. Analysis of the binding kinetics of rEtf-3 with purified rHis-FTH1 via OpenSPR. Each curve corresponds to the responses of different rHis-FTH1 concentrations (471, 141, or 47 nM). No interaction was observed.
Fig. S5. Ectopically expressed Etf-1 does not induce ferritinophagy. Endogenous ferritin remains diffuse in the cytoplasm in RF/6A cells transfected with Etf-1-GFP. Whereas Etf-1-GFP produces distinctive puncta of autophagosomes. Ferritin was detected with anti-FTL. Merge/DIC, merge of the fluorescence and DIC images; N, nucleus; Ftn, ferritin. Each boxed area is enlarged 4× on the right. Scale bars: 10 µm.
Fig. S6. Etf-3 induces ferritinophagy by facilitating the interaction between FTL and NCOA4. (A) HEK293 cells were cotransfected with plasmids encoding HA-NCOA4 and Etf-3-GFP, GFP, or none (CTL). At 2 dpt, cell lysates were immunoprecipitated (IP) with anti-HA and protein A-Sepharose beads, then analyzed by western blotting (WB) with mice anti-GFP, mice anti-HA, rabbit anti-actin and rabbit anti-FTL. The asterisk (*) indicates the IgG light chain. (B) The relative ratio of FTL to NCOA4 reflects the mean ± SD from three independent experiments that had three replicates per sample. CTL group was arbitrarily set to 1. * Significantly different by ANOVA (P < 0.05).
FIG. S7. Temporal Etf-3, Etf-1, SODB, FTL, FTH, and TfR mRNA expression during synchronous *E. chaffeensis* infection. (A) *E. chaffeensis* developmental cycle in synchronously infected THP-1 cells was examined by Diff-Quik staining. Scale bar: 10 µm. (B–D) Total RNA extracted from infected THP-1 cells at indicated hpi was subjected to RT-qPCR analysis. (B) The relative bacteria number is represented by *E. chaffeensis* 16S rRNA normalized against human actin mRNA. (C) Temporal expression of Etf-3, Etf-1 and SODB mRNAs by *E. chaffeensis* in THP-1 cells. The mRNA levels were normalized to that of the *E. chaffeensis* 16S rRNA. (D) FTL, FTH, and TfR expression by THP-1 cells during *E. chaffeensis* infection. The mRNA levels were normalized to that of human actin mRNA. Data indicate the mean ± SD from three independent experiments that had three replicates per sample. * Significantly different from 0 hpi by ANOVA (P < 0.05).
FIG. S8. Etf-1 enhances Etf-3-induced ferritinophagy and attenuates ROS production in response to Etf-3-induced ferritinophagy. (A) HEK293 cells were transfected with a plasmid encoding Etf-1-GFP, Etf-3-GFP, or GFP, or were cotransfected with plasmids encoding Etf-1-GFP and Etf-3-GFP. Cells were harvested at 2 dpt for western blotting using anti-GFP, anti-actin, anti-FTL, and anti-LC3. (B, C) The relative band densities of FTL was normalized against human actin (B), and the conversion of LC3-I to LC3-II (C) was compared among different groups. Data indicate the mean ± SD from three independent experiments that had three replicates per sample. * Significantly different by ANOVA (P < 0.05). (D) HEK293 cells were transfected with a plasmid encoding HA-Etf-3 or HA-Etf-1 or were cotransfected with plasmids encoding HA-Etf-1 and Etf-3 (HA-RAB5S/N as negative control). At 1 dpt, cells were either not treated or treated with 40 μM FAC for 1 day. ROS production was analyzed with the fluorescent indicator H2DCFDA. Data are presented as the mean ± SD from three independent experiments that had three replicates per sample. * Significantly different by ANOVA (P < 0.05).
FIG. S9. Model for Etf-3 function in *E. chaffeensis*-infected cells. Iron hemostasis is tightly regulated by cells to maintain the LCI pool (left). The T4SS effector Etf-3 is responsible for enhancing ferritinophagy and thereby providing enough Fe\(^{2+}\) for *E. chaffeensis* proliferation. Etf-3 directly binds ferritin via ferritin light chain, then enhances ferritinophagy by increasing the affinity of NCOA4 for Etf-3-ferritin complexes. The Fe\(^{2+}\) level in cells is increased by ferritinophagy. Secreted Etf-1 induces autophagy and delivers extra Fe\(^{2+}\) to *Ehrlichia* inclusions (right).

Tf: transferrin, which binds Fe\(^{3+}\) atoms and transports them into cells.
TfR: transferrin receptor, which binds and delivers iron-saturated transferrin via endocytosis.
STEAP2: Six-transmembrane epithelial antigen of prostate-2, a metalloreductase that chemically reduces Fe\(^{3+}\) to Fe\(^{2+}\).
DMT1: Divalent metal transporter 1 that transports Fe\(^{2+}\) from endosomes to the cytoplasm.