Supporting Information (*SI Appendix*)

Ehrlichia type IV secretion system effector Etf-2 binds to active RAB5 and delays endosome maturation

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Supplemental Materials and Methods

Bacteria and cell culture.
E. chaffeensis Arkansas strain (1) was cultured in THP-1 cells (ATCC, Manassas, VA) (2) 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 DMEM (Dulbecco's minimal essential medium; Mediatech) supplemented with 5% FBS and 2 mM L-glutamine (3). Cultures were incubated at 37°C under 5% CO_2 in a humidified atmosphere.

Antibodies. Affinity-purified rabbit IgG against Etf-2 (residues 152–264) was prepared by GenScript Inc. (Piscataway, NJ). The following antibodies were also used: mouse monoclonal anti-HA, mouse monoclonal anti-Myc **(**Invitrogen, Manassas, VA**),** mouse monoclonal anti-GFP (Santa Cruz Biotechnology, Dallas, TX), Alexa Fluor (AF) 488-conjugated rat monoclonal anti-FLAG (BioLegend, San Diego, CA), AF555 or AF488-conjugated goat anti-mouse IgG and anti-rabbit IgG (Invitrogen), and horseradish peroxidase–conjugated goat anti-mouse IgG (Cell Signaling Technology, Danvers, MA).

Transformation of *E. chaffeensis* with FLAG-Etf-2C expressing Himar plasmid.
The pCis-FLAG-Etf-2C-SS-Himar A7 plasmid expressing FLAG-tagged Etf-2C (C-terminus of Etf-2 containing T4SS signal, amino acids 152-264) and the spectinomycin/streptomycin antibiotic resistance gene (*aad*) was created from the pCis-mCherry-SS-Himar A7 construct described previously (4, 5). Multiple restriction sites (KpnI, AscI, and BamHI) were first inserted into the pCis-mCherry-SS-Himar A7 plasmid between mCherry and *aad* using two-step PCR amplification with overlapping primers (Supplementary Table 1) that contained the restriction sites and removed the stop codon from the mCherry gene. mCherry tag was then replaced by FLAG using primers encoding DYKDDDDK. The gene encoding *E. chaffeensis etf-2c* was PCR amplified from *E. chaffeensis* genome and cloned into the BamHI and KpnI sites, resulting the polycistronic expression of genes encoding FLAG-Etf-2C/Aad driven by the *Anaplasma marginale* promoter (*Am-tr1*). This plasmid construct also contained the Himar1 transposase gene driven by *Am-tr1*. Plasmids were prepared from transformed *dam*$^-$*/dcm*$^-$ *E. coli* strain C2925 (New England Biolabs) using a Maxiprep plasmid DNA isolation kit (Qiagen, Valencia, CA).

For transformation using the pCis-FLAG-Etf-2C-Himar plasmid, *E. chaffeensis*–infected DH82 cells at confluency and at ~95% infectivity were harvested from a T75 flask. Host cell–free *E. chaffeensis* was obtained by sonication on ice for 8 s twice at an output setting of 2 using a W-380 Sonicator (Heat Systems, Newtown, CT). Lysed cells were centrifuged at 700 × *g* to remove unbroken cells and nuclei, filtered through 5.0- and 2.7-μm syringe filters (Millipore, Billerica, MA), and centrifuged at 10,000 × *g* to pellet host cell–free bacteria. The pellets were washed twice

with 1 ml ice-cold 0.3 M sucrose in water and resuspended in 100 µl of 0.3 M sucrose. Host cell–free *E. chaffeensis* was mixed with 10 µg plasmid, transferred to a 2-mm gap electroporation cuvette (Bio-Rad, Hercules, CA), and incubated on ice for 15 min (4). *E. chaffeensis* organisms were electroporated at 2,500 V, 25 µF, and 400 Ω using a Gene Pulser Xcell™ Electroporation System (Bio-Rad). Transformed *E. chaffeensis* were cultured in a T25 flask with a confluent monolayer of DH82 cells, incubated at 34°C overnight, and then transferred to a 37°C incubator. After 2 days, transformed *E. chaffeensis* expressing *aad* were selected in the presence of 100 µg/ml spectinomycin, 100 µg/ml streptomycin, and 0.1 µg/ml cycloheximide, and the culture medium containing antibiotics was replaced twice a week until the infectivity was ≥ 80% (~ 3 weeks).

To determine the localization of FLAG-Etf-2C, transformed *E. chaffeensis* expressing FLAG-Etf-2C were used to infect RF/6A cells, and at 2 d pi, cells were fixed and labeled with AF488 rat anti-FLAG mAb. Fluorescence images with overlay differential interference contrast (DIC) images were acquired and analyzed by a DeltaVision PersonalDV deconvolution microscope system (GE Healthcare Life Sciences, Marlborough, MA). The genomic locus of the Himar insertion site was determined by semi-random nested PCR as previously described (5).

Yeast two-hybrid assay.

Codon-optimized full-length Etf-2 was cloned into vector pGBKT7 (Clontech) at restriction sites EcoRI and PstI and transformed into yeast strain Y187 using the Quick & Easy yeast transformation kit (Clontech). Transformants were selected on minimal synthetically defined (SD) medium without Trp (SD/-Trp) agar plates for 3–5 days. Each of RAB5-WT, -CA, and -DN was cloned into vector pGADT7 and transformed into yeast strain AH109 using the same transformation kit. Transformants were selected on SD/-Leu agar plates for 3–5 days. Yeast two-hybrid assays were performed according to user manual for the Matchmaker Gold Yeast Two-Hybrid System (Clontech). Briefly, the prey strain Y187 expressing Etf-2 and bait strain AH109 expressing RAB5-WT, -CA, or -DN were mated in 45 ml of 2× YPDA yeast medium containing 50 µg/ml kanamycin in a 2-liter flask at 40 rpm at 30°C for 24 h. The zygotes were pelleted in a tabletop centrifuge and resuspended in 10 ml of 0.5× concentration of the same medium containing 50 µg/ml kanamycin. Zygotes in the medium were then diluted 1:100, 1:1,000, and 1:10,000 and plated on SD/-Leu/-Trp double-dropout agar plates and incubated at 30°C for 3–5 days. Twenty colonies were randomly picked from the 1:1,000 plates for each of the RAB5-WT, -CA, and -DN groups and cultured in 2 ml SD/-Leu/-Trp liquid medium overnight at 30°C in 270 rpm. The cultures were inoculated onto SD/-Leu/-Trp or SD/-Leu/-Trp/-His/-Ade quadruple-dropout agar plates for each of the three groups and incubated at 30°C for 3 days or 15 days, respectively. Images were taken for SD/-Leu/-Trp and SD/-Leu/-Trp/-His/-Ade quadruple-dropout agar plates of the three groups.

MST and RAB5 GAP assay.

Full-length human RAB5 (RAB5A) was expressed and purified as described (6). His-tagged Etf-$2^{\Delta HY}$ was transformed into chemically competent Rosetta 2 DE3 pLysS *E. coli* cells (Millipore

Sigma, Billerica, MA) with all growths initiated from freshly transformed selection plates (Luria-Bertani [LB] medium with 50 μg/ml kanamycin) or from glycerol stocks stored at –80°C. An overnight starter culture (47 ml) was added to 1 liter of LB media (with 50 μg/ml kanamycin) in 2.8-liter Fernbach baffled culture flasks. Cells were incubated at 37°C with constant shaking (220 rpm) until the optical density (600 nm) reached 0.6. Incubator temperature was then decreased to 32°C followed by addition of 0.5 mM IPTG (final concentration; Carbosynth, Berkshire, UK) to induce transgene expression. Cells were grown for an additional 5 h and harvested by centrifugation at 4,500 × *g*. Pelleted cells were resuspended in phosphate-buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4] with 5 mM $MgCl_2$, 1 mM DNase, and 1 mM fresh phenylmethylsulfonyl fluoride (Buffer A) to a final volume of 20 ml, lysed via sonication (35 A with 30-s on/off cycles over 10 min), and then centrifuged at 27,150 × *g* for 30 min to pellet insoluble material. The resulting pellet was washed with Buffer A and recentrifuged. Protein in the insoluble fraction was solubilized in 20 mM Tris pH 7.4 at room temperature, 500 mM NaCl, and 6 M urea with gentle stirring for 3 h at 4°C. Solubilized protein was further purified using HisPur Cobalt Resin (Thermo Fisher, Waltham, MA), and unfolded His-tagged Etf-2$^{\Delta 132\text{-}151}$ was eluted from the resin with the aforementioned Tris buffer containing 200 mM imidazole. The pooled elutes were each diluted with 50 mM Tris pH 8.5 room temperature, 25 mM NaCl, and 500 mM arginine (refold buffer) to yield a net of 3 M urea. Refold buffer (100 ml) was then added dropwise to 10 ml of each protein solution with gentle stirring overnight at room temperature. Refolded protein was filtered, and 2-mercaptoethanol was added to a final concentration of 5 mM. Protein was concentrated in an Amicon stirred-cell concentrator fitted with a YM10 membrane (molecular-weight cutoff, 10 kDa; both from Millipore, Billerica, MA). Arginine was removed by overnight dialysis at 4°C with stirring with PBS.

Purified non-liganded apoRAB5 in PBS was treated with EDTA at a 4× relative molar concentration of the RAB5 for 30 min on ice, and buffer was subsequently exchanged via dialysis in PBS containing 5 mM $MgCl_2$, 1 mM DTT, and 1 mM EDTA; the resulting RAB5 in solution was either used immediately or flash frozen and stored at –20°C. GTPγS or GDPβS was added to apoRAB5 with incubation for 30 min on ice. RAB5 complexed with GTPγS (or GDPβS) was then labeled using an Alexa Fluor 647 Protein Labeling kit (Molecular Probes, Eugene, OR). After removing any remaining free dye via dialysis, the concentration of RAB5 and labeling efficiency were determined using Monolith NT.115 (NanoTemper Technologies, San Francisco, CA). The MST concentration screen and experiments were also performed with Monolith NT.115, and data were collected and analyzed using the NTControl v2.2.1 and MO Affinity Analysis v2.1.2 software as described (7).

For the RAB5 GAP assay, affinity-purified GST-RAB5 on glutathione-Sepharose resin was preloaded with [α-^{32}P]GTP (0.1 μM) for 15 min at room temperature, followed by a GTP hydrolysis reaction at 30°C in 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 1 mM DTT in the absence or presence of RABGAP5 or Etf-2$^{\Delta 132\text{-}151}$ (10 nM). Samples were taken at 0, 5, 10, 15, and 20 min and subjected to thin-layer chromatography to separate the hydrolysis product [α-

^{32}P]GDP from substrate [α-^{32}P]GTP, followed by autoradiography and quantification using a PhosphorImager (GE Healthcare Life Sciences).

Uptake of latex beads and localization time-course experiments.
DH82 cells cultured in a 24-well plate were co-transfected for 2 days with plasmids encoding Etf-2-GFP or GFP, with HA-RAB5 (WT), pcDNA3.1-MYC-RABGAP5, or HA-RAB7 with Fugene HD (Promega, Madison, WI), and latex beads (Flash Red, $\lambda_{EX}/\lambda_{EM}$ = 660 nm/690 nm, 1 μm, Bangs Laboratories, Fishers, IN) were added. After incubating at 37°C under 5% CO_2 in a humidified atmosphere for 20–80 min, cells were treated with 0.25% trypsin to remove uninternalized beads, cytocentrifuged, fixed in 4% PFA and incubated with mouse monoclonal anti-Myc or monoclonal anti-HA in PGS followed by AF555-conjugated goat anti-mouse IgG in PGS.

DH82 cells cultured in a Nunc™ Lab-Tek™ II Chambered Coverglass (ThermoFisher) were transfected with a plasmid encoding Etf-2-GFP or GFP for 2 days, then incubated with LysoTracker Red (Life Technologies, Carlsbad, CA**)** for 30 min. Latex beads were added and incubated for 20–80 min at 37°C under 5% CO_2 in a humidified atmosphere. After washing cells to remove uninternalized beads, cells were cultured in phenol red–free AMEM, and live-cell imaging was performed in an environment-controlled humidified chamber (37°C with 5% CO_2) connected to a DeltaVision deconvolution microscope system.

Internalization of EtpE-C-coated latex beads in RF/6A cells.
Flash Red latex beads at 5–6 × 10^6 beads in 180 μl of 25 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 6.0, were incubated with 200 ng of recombinant EtpE-C proteins in 20 μl of 8 M urea in sodium phosphate buffer (50 mM sodium phosphate, pH 7.4, 0.3 mM NaCl) at room temperature with rotation at 80 rpm. MES buffer (50 μl) was sequentially added to the mixture every 5 min and incubated at room temperature, rotating at 80 rpm until the total volume reach 1.5 ml. The mixture was centrifuged at 14,000 × *g* for 2 min at room temperature, and 1 ml buffer was removed from the supernatant. The remaining supernatant and beads were washed repeatedly with MES buffer as described above, and finally all buffer was aspirated from the beads. The beads were resuspended in 200 μl AMEM supplemented with 8% FBS and 2 mM L-glutamine.

RF/6A cells cultured on coverslips in a 24-well plate were co-transfected with plasmids encoding Etf-2-GFP or GFP, and HA-RAB5 (WT), pcDNA3.1-MYC-RABGAP5, or HA-RAB7 with Lipofectamine 3000 (Invitrogen) for 2 days. Freshly prepared EtpE-C-coated beads were added to wells of a 24-well plate (~5 × 10^6 beads per well). After incubating at 37°C with 5% CO_2 in a humidified atmosphere for 30–120 min, uninternalized beads were removed by washing with PBS. Cells were then fixed in 4% PFA and incubated with mouse monoclonal anti-Myc or monoclonal anti-HA followed by AF555-conjugated goat anti-mouse IgG in PGS.

For labeling with LysoTracker Red, RF/6A cells cultured on coverslips in a 24-well plate were co-transfected with a plasmid encoding Etf-2-GFP or GFP for 2 days. Freshly prepared EtpE-C-coated beads were added to wells of a 24-well-plate (~5 ×10^6 beads per well). After incubating at 37°C with 5% CO_2 in a humidified atmosphere for 30–120 min, cells were incubated with

LysoTracker Red for 10 min. Uninternalized beads were removed by washing with PBS, and cells were fixed in 3.2% PFA and then immediately observed using a DeltaVision Deconvolution microscope.

Image acquisition and analysis. Fluorescence images with overlay DIC images were captured with a DeltaVision PersonalDV Deconvolution microscope. Colocalization analysis was performed on a single *z*-section by counting >100 inclusions, phagosomes, or vesicles per cell in 20–30 cells per experiment from three independent experiments to obtain percentage colocalization of *E. chaffeensis* inclusions and phagosomes/endosomes containing beads with various markers including several Etf-2-GFP fusions, HA-RAB5, LysoTracker Red, HA-RAB7, and Myc-RABGAP5. Colocalization of several Etf-2-GFP fusions with RAB5 (DN or CA) was also analyzed. Experiments were repeated at least three times. Statistical analysis was performed with a two-tailed Student's *t*-test, and $P < 0.05$ was considered to reflect a statistically significant difference. For experiments involving more than two groups, analysis of variance (ANOVA) was performed, and $P < 0.05$ was again the significance level. All statistical analyses were performed using Prism 7 software (GraphPad, La Jolla, CA).

Effects of Etf-2-GFP overexpression on *E. chaffeensis* infection.
Exponentially growing HEK293 cells were transfected with Etf-1-GFP or GFP control using Fugene HD, and infected with freshly isolated *E. chaffeensis* at 8 h pt as described (8). Bacteria that were not internalized were removed at 1 dpi, and cells were harvested at 2 dpi. Bacterial numbers in each sample were determined by quantitative PCR using specific primers for *E. chaffeensis 16S rDNA* and normalized against the level of human *G3PDH* as described (8).

PNA synthesis, biotin labeling, RNA-PNA hybridization, PNA transfection of host cell–free *E. chaffeensis*, and knockdown and complementation analysis.
Etf-2 PNA (Fig. 8B) and a scrambled PNA control (CTL PNA; 3'-CACATATCTCGG-5') were designed based on PNA Bio PNA Tools (https://www.pnabio.com/support/PNA_Tool.htm). To ensure the specificity of the PNA sequence, each sequence was subjected to a BLAST search against the *E. chaffeensis* whole genome (http://riki-lb2.vet.ohiostate.edu/blast/blast.php). Custom PNA oligomers were synthesized, and PNA quality and quantity were verified by HPLC and mass spectrometry (PNABio, Newbury Park, CA). PNA oligomers were biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Fisher), and purified using Pierce C18 Spin Columns (Thermo Fisher). Dot blots were used to confirm the successful biotinylation of oligomers as described (9). The dot blot membrane was developed using the Pierce Chemiluminescent Nucleic Acid Detection Module kit (Thermo Fisher).

To verify that Etf-2 PNA binds to the targeted region near the translation start site of *etf-2* mRNA, a single-stranded RNA (ssRNA; 5'-GUAAAA**AA<u>AUG</u>CCAAC**AACACUAGGGA-3': bold, RNA sequence corresponding to the designed PNA; underlined, translation start codon [Fig. 8B]) was designed and synthesized by Sigma-Aldrich (St. Louis, MO). An electrophoretic mobility

shift assay was performed. In 15-µl reactions, PNA-ssRNA was hybridized with 10 µM biotin-labeled Etf-2 PNA with or without 2.0 µl (200 µM) ssRNA in 250 mM Tris buffer (pH 7.2) with 1 µl of RNaseOUT (Thermo Fisher) at room temperature for 30 min. The samples were subjected to agarose gel electrophoresis (2% agarose) in TBE buffer (40 mM Tris, pH 8.3, 45 mM boric acid, and 1 mM EDTA) and transferred to an Amersham HyBond N+ membrane (GE Healthcare Life Sciences) using a semi-dry blotting apparatus (WEP, Seattle, WA). Each membranes was UV cross-linked in a Stratalinker1800 (Stratagene) and developed using the Pierce chemiluminescence kit as noted above.

To transfect *E. chaffeensis* with PNA, 3 µg of Etf-2-specific PNA or CTL PNA in 10 µl nuclease-free water was mixed with 100 µl host cell–free *E. chaffeensis* in 300 mM sucrose in a sterile electroporation cuvette (2-mm gap, Bio-Rad) on ice as described (10). *E. chaffeensis* were electroporated using a Gene PulserXcell Microbial System (Bio-Rad) at 2,000 V, 25 µF, 400 Ω. Immediately after electroporation, 400 µl of prewarmed RPMI 1640 medium containing 5% FBS was added to the electroporated bacteria. The PNA- *E. chaffeensis* suspension was transferred to a T25 flask containing 5×10^5 HEK293 cells for internalization at 37°C for 90 min. 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 90 µl) were transfected with 1 µg pEGFP-N1 or 5 µg Etf-2-GFP by electroporation as described (10). At 1 d pt, host cell–free *E. chaffeensis* were transfected with 6 µg CTL or Etf-2 PNA as described above and then added to pre-transfected HEK293 cells at a multiplicity of infection of ~100:1 as described (10). mRNA was extracted at 2 dpi, and reverse transcription quantitative PCR was performed as described (10). Primer sequences for bacterial Etf-2 are shown in Table S1, and those for *E. chaffeensis* 16S rRNA and human G3PDH were reported previously (11).

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Supplemental Figures

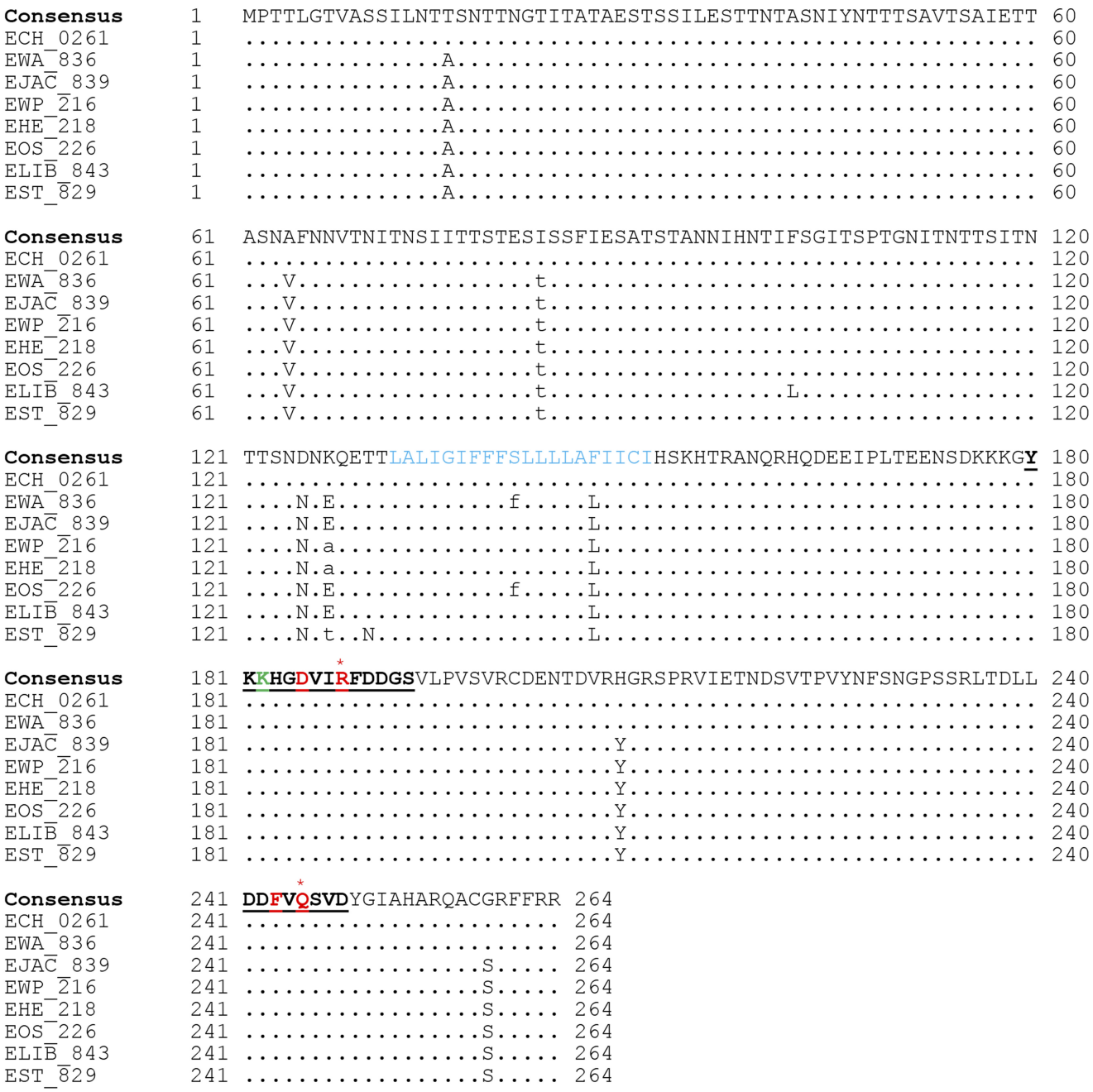

Fig. S1. Alignment of Etf-2 among eight *E. chaffeensis* strains.
The GAP TBC-like Dual-Catalytic-Finger Motif (Fig. 4A) is shown in boldface type and is underlined. Blue type denotes the HY domain (amino acids 132–151). Locus ID abbreviations and GenBank accession numbers for *E. chaffeensis* strains that were culture-isolated during 1991–1998 and genomic sequences were determined in 2013 (12) are: ECH, Arkansas (type strain), NC_007799.1; EHE, Heartland (13), NZ_CP007473.1; EJAC, Jacksonville (14), NZ_CP007475.1; ELIB, Liberty (15), NZ_CP007476.1; EOS, Osceola (15), NZ_CP007477.1; EST, St. Vincent (14), NZ_CP007478.1; EWA, Wakulla (15), NZ_CP007479.1; EWP, West Paces (13), NZ_CP007480.1.

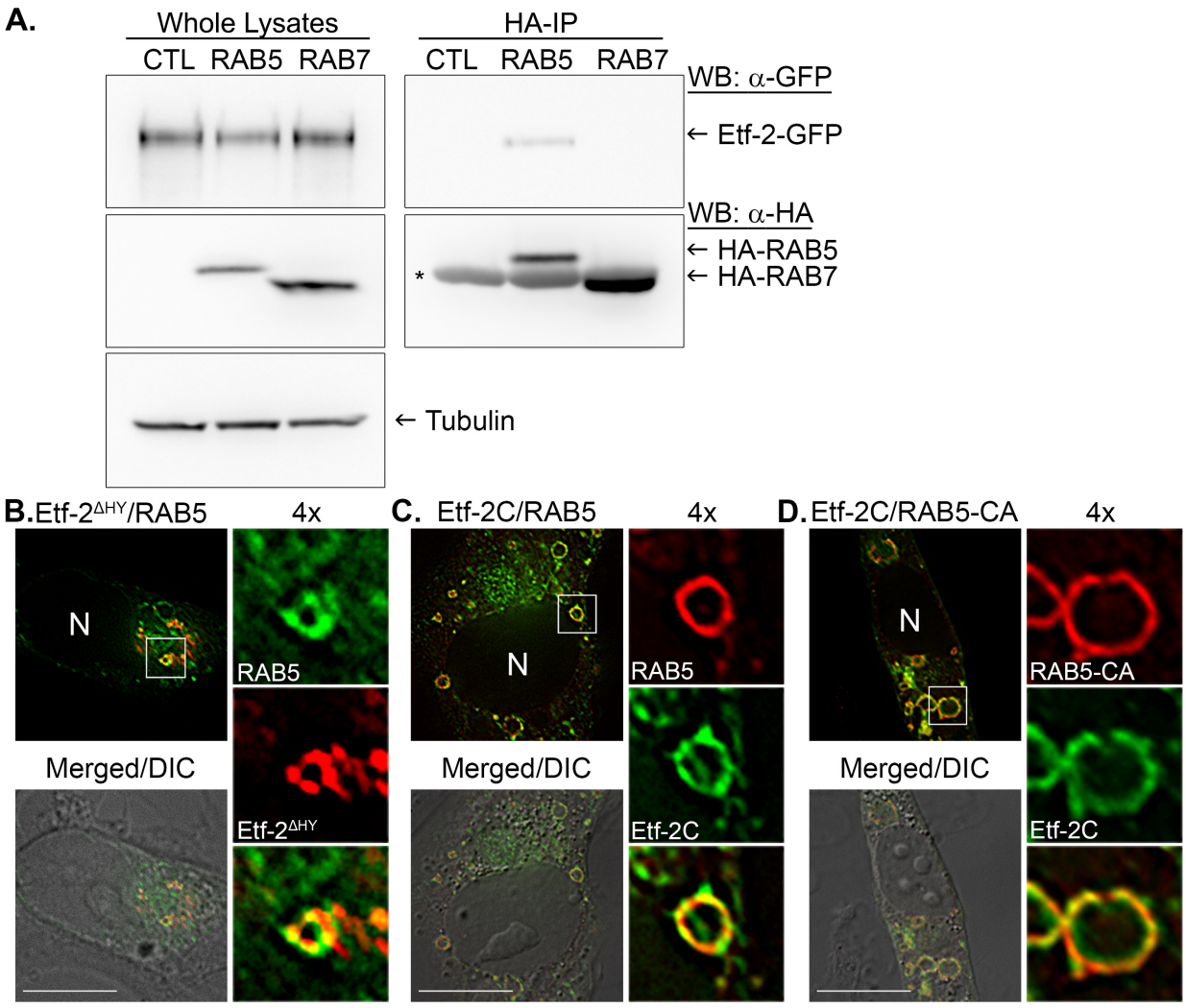

Fig. S2. Etf-2 interacts with RAB5, but not RAB7, and Etf-2C-GFP lacking the hydrophobic domain localizes to the early-endosome membrane labeled with RAB5-GTP.

(**A**) Etf-2 interacts with RAB5, but not RAB7. HEK-293 cells were co-transfected with plasmids expressing Etf-2-GFP and HA-RAB5, HA-RAB7, or none (CTL). At 2 d pt, cells were lyzed, immunoprecipitated with mouse anti-HA-linked protein G-Sepharose beads, and analyzed by western blotting with anti-GFP and anti-HA antibodies. * IgG light chain.

(**B-D**) RF/6A cells were co-transfected with Etf-2$^{\Delta HY}$-GFP (B) or Etf-2C-GFP (C) and HA-RAB5-WT, or Etf-2C-GFP and HA-RAB5-CA (D). Cells were subjected to immunofluorescence labeling with mouse anti-HA (red; AF555) at 2 d pt, and observed with a DeltaVision Deconvolution microscope. Merged/DIC: fluorescence image merged with a DIC image. Boxed areas are enlarged 4× on the right. Scale bar: 10 μm.

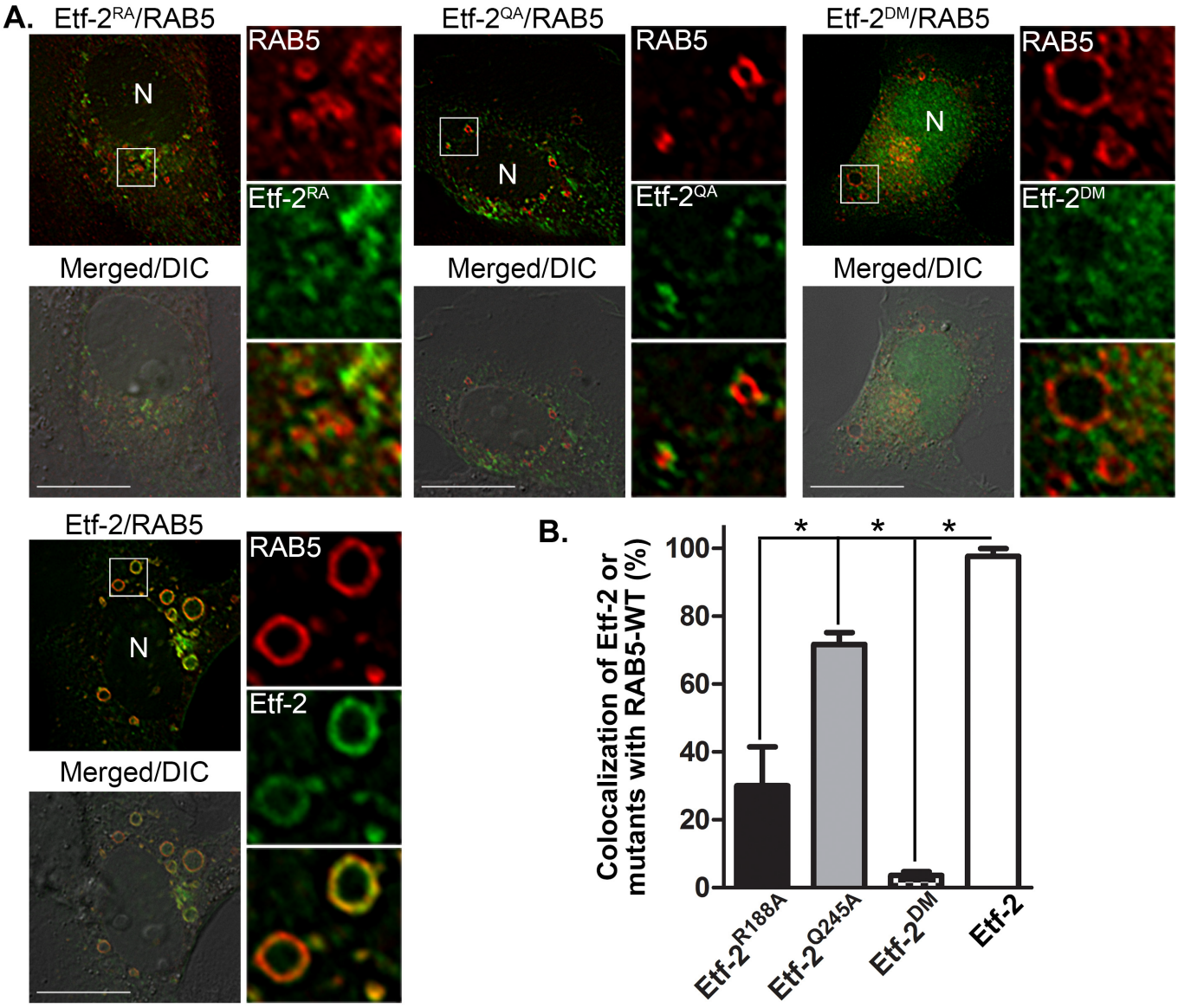

Fig. S3. Mutation of the Arg and Gln fingers of the TBC-like motif in Etf-2 impairs Etf-2 localization to endosomes marked with RAB5.

A. RF/6A cells were co-transfected with HA-RAB5-WT and one of Etf-2^{R188A} (Etf-2RA), Etf-2^{Q245A} (Etf-2^{QA}), Etf-2$^{R188A/Q245A}$ (Etf-2^{DM}), or Etf-2-GFP. At 2 d pt, cells were subjected to immunofluorescence labeling with mouse anti-HA (red; AF555). Merged/DIC: fluorescence image merged with a DIC image. Boxed areas are enlarged 4× in the right panels. Scale bar: 10 µm.

B. Quantification of 100–120 RAB5 endosomes in 20–30 co-transfected RF/6A cells from three independent experiments. * Significantly different (ANOVA, $P < 0.05$).

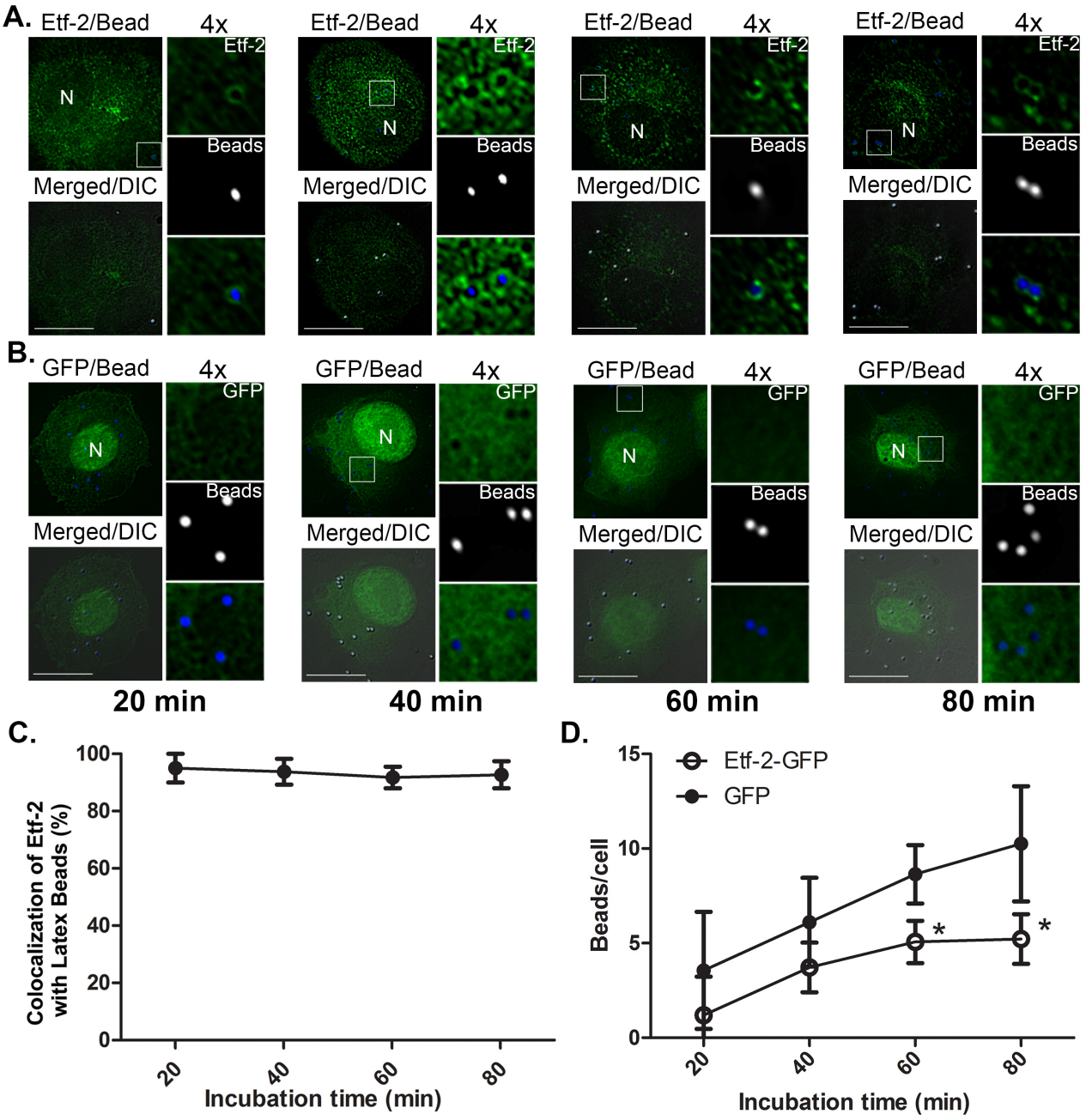

Fig. S4. Etf-2 localizes to phagosomes that have taken up latex beads and reduces phagocytosis of latex beads by DH82 cells.

(**A-B**) Etf-2-GFP- or GFP-transfected DH82 cells were incubated with FlashRed latex beads (1 μm) for 20–80 min, and the number of beads colocalized with Etf-2-GFP (**C**) and the number of beads taken up by cells (**D**) was scored in 100 cells. Data are presented as the mean ± standard deviation from three independent experiments. * Significantly different (two-tailed *t*-test, $P < 0.05$).

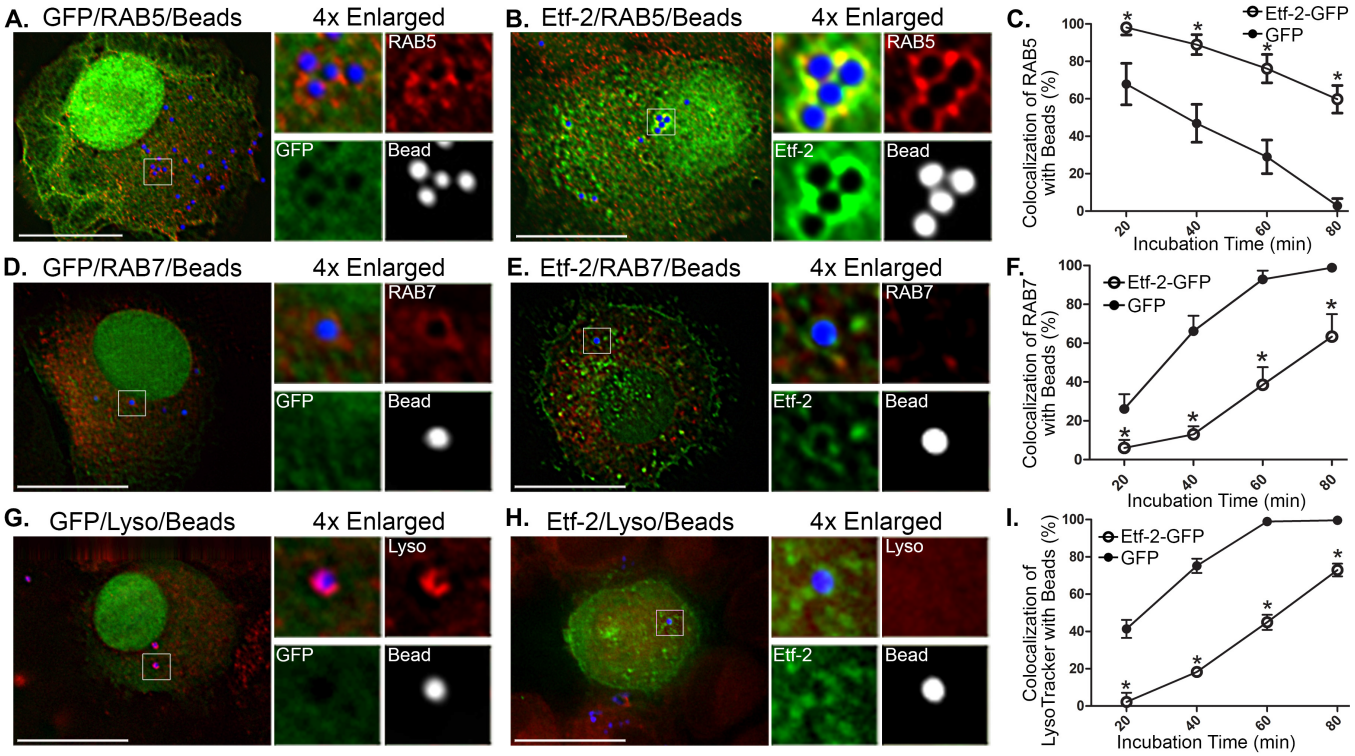

Fig. S5. Etf-2 localizes to phagosomes that have taken up latex beads and delays maturation of phagosomes to phagolysosomes in DH82 macrophages.

DH82 cells were co-transfected with GFP control (**A, D**) or Etf-2-GFP (**B, E**) and HA-RAB5 (**A, B**) or HA-RAB7 (**D, E**) for 2 days. Alternatively, DH82 cells cultured in coverglass chambers were transfected with GFP control (**G**) or Etf-2-GFP (**H**) for 2 days, then incubated with LysoTracker Red (Lyso) for 30 min. Cells were incubated for 20–80 min with FlashRed latex beads (1 μm, pseudocolored blue for merged images or white for enlarged single-channel panels). Each boxed area is enlarged 4× on the right. Scale bars: 10 μm. Representative images at 40 min post-beads uptake are shown.

C, F, I. Quantification of localization of RAB5 (C), RAB7 (F), and LysoTracker Red (I) in 100 bead-containing phagosomes in Etf-2-GFP- or GFP-transfected DH82 cells. Data are presented as the mean ± standard deviation from three independent experiments. * Significantly different (two-tailed *t*-test, $P < 0.05$).

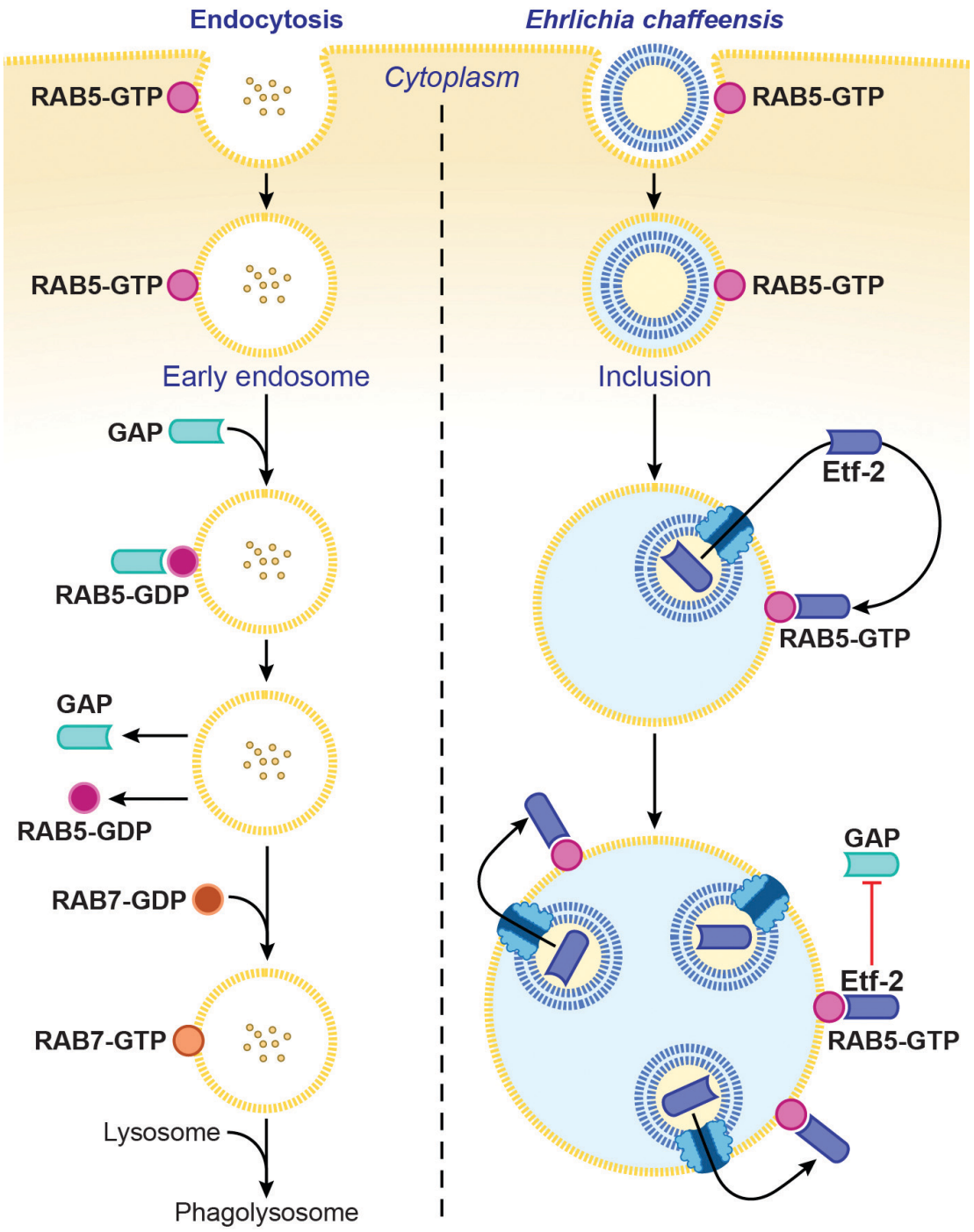

Fig. S6. Model of Etf-2 function in *E. chaffeensis*-infected cells.
RAB5-GTP hydrolysis is required for endosome maturation and lysosomal fusion (left). The T4SS effector Etf-2 is responsible for blocking lysosomal fusion with *E. chaffeensis* inclusions by 1) localizing to *E. chaffeensis* inclusions via binding to RAB5-GTP, and 2) competitively blocking RABGAP5 from acting on RAB5 on the inclusion surface (right). See the text for details.

Supplemental Table S1. Primer sequences for cloning *E. chaffeensis etf-2* and its mutants into plasmids

Target	Primer sequence [1]	Restriction enzymes and target plasmid
***E. coli* and yeast Expression:**		
Etf-2$^{\Delta HY}$*-His*$_6$	F: CTAG CCATGG GCCCAACAACACTAGGGACTGTAGC R: TACG CTCGAG TCTGCGAAAAAATCTACCAC F-overlap: CAGGAGACCACC CACTCAAAACATACAAGA R-overlap: ATGTTTTGAGTG GGTGGTCTCCTGTTTGTTATCAT	F, NcoI; R, XhoI. For cloning Etf-2 with internal deletion of hydrophobic regions (aa$^{132\text{-}151}$) into NcoI (F) and XhoI (R) sites on pET33b(+) plasmid.
Gal4DNA-BD-Etf-2 (codon optimized) [2]	F: GCTA CATATG CCTACTACACTGGGGACTGTCG R: AGCT CTGCAG TTATCTCCTGAAGAATCTTCCG	F, NdeI; R, PstI. For cloning codon optimized *Etf-2* (aa$^{1\text{-}264}$) into NdeI and PstI sites on pGBKT7 yeast expression plasmid.
Mammalian Expression:		
Etf-2-GFP (codon optimized)	F: GTAC CTCGAG *CGCCACCATGGTG* CCTACTACACTGGGGACTGTCGCC R: TACG GGATCC CTCCTGAAGAATCTTCCGCAAGCCTG	F, XhoI; R, BamHI. For cloning codon optimized *Etf-2* (aa$^{1\text{-}264}$) into XhoI (F) and BamHI (R) sites on pEGFP-N1 plasmid.
Etf-2N-GFP (codon optimized aa$^{1\text{-}134}$)	F: GTAC CTCGAG *CGCCACCATGGTG* CCTACTACACTGGGGACTGTCGCC R: TACG GGATCC AGGGCCAGAGTTGTCTCCTGCTTGTT	F, XhoI site; R, BamHI. For cloning codon optimized Etf-2 N terminus (aa$^{1\text{-}134}$) into XhoI (F) and BamHI (R) sites on pEGFP-N1 plasmid.
Etf-2C-GFP (codon optimized aa$^{152\text{-}264}$)	F: GTAC CTCGAG *CGCCACCATGGTG* CACTCCAAGCATACCCGCGCTAAC R: TACG GGATCC CTCCTGAAGAATCTTCCGCAAGCCTG	F, XhoI site; R, BamHI. For cloning Etf-2 C terminus (aa$^{152\text{-}264}$) into XhoI (F) and BamHI (R) sites on pEGFP-N1 plasmid.
Etf-2C1-GFP (codon optimized aa$^{135\text{-}264}$)	F: GTAC CTCGAG *CGCCACCATGGTG* ATCGGCATTTTCTTTTTCAGCCTG R: TACG GGATCC CTCCTGAAGAATCTTCCGCAAGCCTG	F, XhoI site; R, BamHI. For cloning Etf-2 C terminus (aa$^{135\text{-}264}$) into XhoI (F) and BamHI (R) sites on pEGFP-N1 plasmid.
Etf-2^{R188A}*-GFP* (codon optimized)	F: AAAAAGCATGGGGATGTGATCGCATTCGACGATGGGTCAGTCCTG R: CAGGACTGACCCATCGTCGAATGCGATCACATCCCCATGCTTTTT	For site-direct mutation of the Arginine finger in TBC-like domain in Etf-*2*.
Etf-2^{Q245A}*-GFP* (codon optimized)	F: GATCTGCTGGACGATTTCGTGGCGTCAGTGGATTACGGGATTGCT R: AGCAATCCCGTAATCCACTGACGCCACGAAATCGTCCAGCAGATC	For site-direct mutation of the Glutamine finger in TBC-like domain in *Etf-2*.

Etf-2^{DM}-GFP (codon optimized)	F: GATCTGCTGGACGATTTCGTGGCGTCAGTGGATTACGGGATTGCT R: AGCAATCCCGTAATCCACTGACGCCACGAAATCGTCCAGCAGATC	For site-directed mutation of Gln finger in TBC-like domain in codon optimized *Etf-2^{R188A}-GFP* (DM: dual mutations of R188A and Q245A).
pCis-FLAG-Etf-2C-SS-Himar A7 plasmid construction:		
aad	F: TACG AAGCTT GCATGCCTGCAGGTCGACTCTAG R: *TACAAA* GGATCC GGCGCGCC GGTACC ATTAAATTATGAGGGAAGCG	F, HindIII; R, KpnI+AscI+BamHI sites with complemental sequence against *mCherry* gene (stop codon removed, italicized).
mCherry	F: *ATAATTTAAT* GGTACC GGCGCGCC GGATCC TTTGTATAATTCGTCCATTCCA R: GATC GAATTC GGCTCCTCCTAGAACGAT	F, KpnI+AscI+BamHI sites with complemental sequences against *aad* gene (italicized); R, EcoRI site.
FLAG	F: GTAC GGATCC *CTTGTCGTCATCGTCTTTGTAGTC* GACCATTATAATATCCCTTATGTTACTC R: CGAGT GCGGCCGC AAGCTC	Replace mCherry with FLAG tag (italicized) at BamHI and NotI sites.
Etf-2C (aa$^{152-264}$)	F: CGAT GGATCC CACTCAAAACATACAAGAGCCAATCAAC R: GCTA GGTACC TTATCTGCGAAAAAATCTACCACATG	For cloning *Etf-2C* (aa$^{152-264}$) into BamHI (F) and KpnI (R) sites on modified Himar plasmid.
***E. chaffeensis etf-2* qRT-PCR:**		
etf-2	F: 5'-CAGCAACAAGTACCGCAAAC-3' R: 5'-AGGTGGTCTCCTGTTTGTTATC-3'	Primers for detecting *E. chaffeensis etf-2* mRNA expression by qRT-PCR.

[1] F, forward; R, reverse complement primers; underlined, restriction enzyme sites; italicized, Kozak sequences for expression in pEGFP-N1 plasmid.

[2] Nucleotide sequences encoding full length Etf-2 (aa$^{1-264}$) codon-optimized for mammalian expression are:

ATGCCTACTACACTGGGGACTGTCGCCTCCTCCATTCTGAACACCACATCCAATACTACCAACGGGACTATCACCGCAACCGCCGAGTCCACTAGCTCCATTCTGGAGT
CCACCACAAACACTGCCTCTAACATCTACAATACTACCACAAGCGCAGTGACCTCCGCCATTGAAACTACCGCCTCTAATGCTTTTAACAATGTGACTAACATCACCAA
TAGTATCATTACAACTTCCACTGAGTCTATCTCTAGTTTTATTGAAAGTGCTACCTCAACAGCAAACAATATCCACAACACCATTTTCTCTGGAATCACAAGTCCTACT
GGCAACATTACTAATACCACAAGTATCACCAATACTACCTCAAACGACAACAAGCAGGAGACAACTCTGGCCCTGATCGGCATTTTCTTTTTCAGCCTGCTGCTGCTGG
CCTTTATCATTTGCATTCACTCCAAGCATACCCGCGCTAACCAGCGACACCAGGATGAGGAAATCCCCCTGACAGAGGAAAATTCTGACAAGAAAAAGGGCTACAAAAA
GCATGGGGATGTGATCAGATTCGACGATGGGTCAGTCCTGCCAGTGAGCGTCCGGTGTGACGAGAATACGGATGTGCGGCATGGAAGAAGCCCCAGGGTCATCGAAACC
AATGACAGCGTGACACCCGTCTATAACTTTTCCAATGGGCCTTCAAGCCGGCTGACCGATCTGCTGGACGATTTCGTGCAGTCAGTGGATTACGGGATTGCTCACGCTC
GGCAGGCTTGCGGAAGATTCTTCAGGAGATGA