Supporting Information (*SI Appendix*) for

Host Membrane Lipids Are Trafficked to Membranes of Intravacuolar Bacterium *Ehrlichia chaffeensis*

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SUPPLEMENTARY VIDEO LEGENDS

Video S1. Time-lapse images of DiI-labeled *E. chaffeensis* in infected THP-1 cells.
Host cell–free *E. chaffeensis* was purified from heavily infected THP-1 cells and incubated with 5 μM DiI for 15 min. Bacteria were washed twice with PBS and used to infect RF/6A cells seeded on a 35-mm glass-bottom culture dish. At 2 h pi, infected cells were washed twice to remove uninternalized bacteria and cultured in phenol red–free AMEM. Live cells were observed under a DeltaVision microscope at 3 d pi, and time-lapse images were captured using ~15-second intervals for 10 min. The video was constructed from single *z*-sections following time points using Softworx software.

Video S2. Keyframe image stacks of *E. chaffeensis*–infected DH82 macrophages by FIB-SEM.
E. chaffeensis–infected DH82 macrophages were embedded in resin at 2 d pi, and resin-embedded samples were subjected to an iterative process of milling (slicing) with a focused gallium ion beam and imaged by SEM. The scanning electron beam was used to record images of an entire cell (~25 μm wide) at pixel sizes of 5 nm in the *xy* plane, and a focused gallium ion beam iteratively recorded *z* slices every 15 nm to generate a voxel size of 5 × 5 × 15 nm. Sequential stacks of 2D images were converted computationally to produce the video. Red box indicates region of interest shown in Video S3.

Video S3. High-resolution keyframe image stacks of a selected region of *E. chaffeensis*–infected DH82 macrophages by FIB-SEM.
The selected region of interest (red box in Video S2) was segmented at high resolution. Data were binned by 3 in the *xy* plane to give a final voxel size of 15 × 15 × 15 nm. Sequential stacks of 2D images were converted computationally to produce the video.

Video S4. 3D reconstruction of subcellular structures of *E. chaffeensis*–infected DH82 macrophages by FIB-SEM.
Individual 2D image slices from a single inclusion shown in Video S3 were merged, cropped, and aligned. Sequential stacks of 2D images were then reconstructed to give a 3D ultrastructural volume of the sample. 3D presentation shows one *E. chaffeensis*–containing inclusion filled with bacteria (green), filaments (red), and vesicles (blue).

SUPPLEMENTARY FIGURES

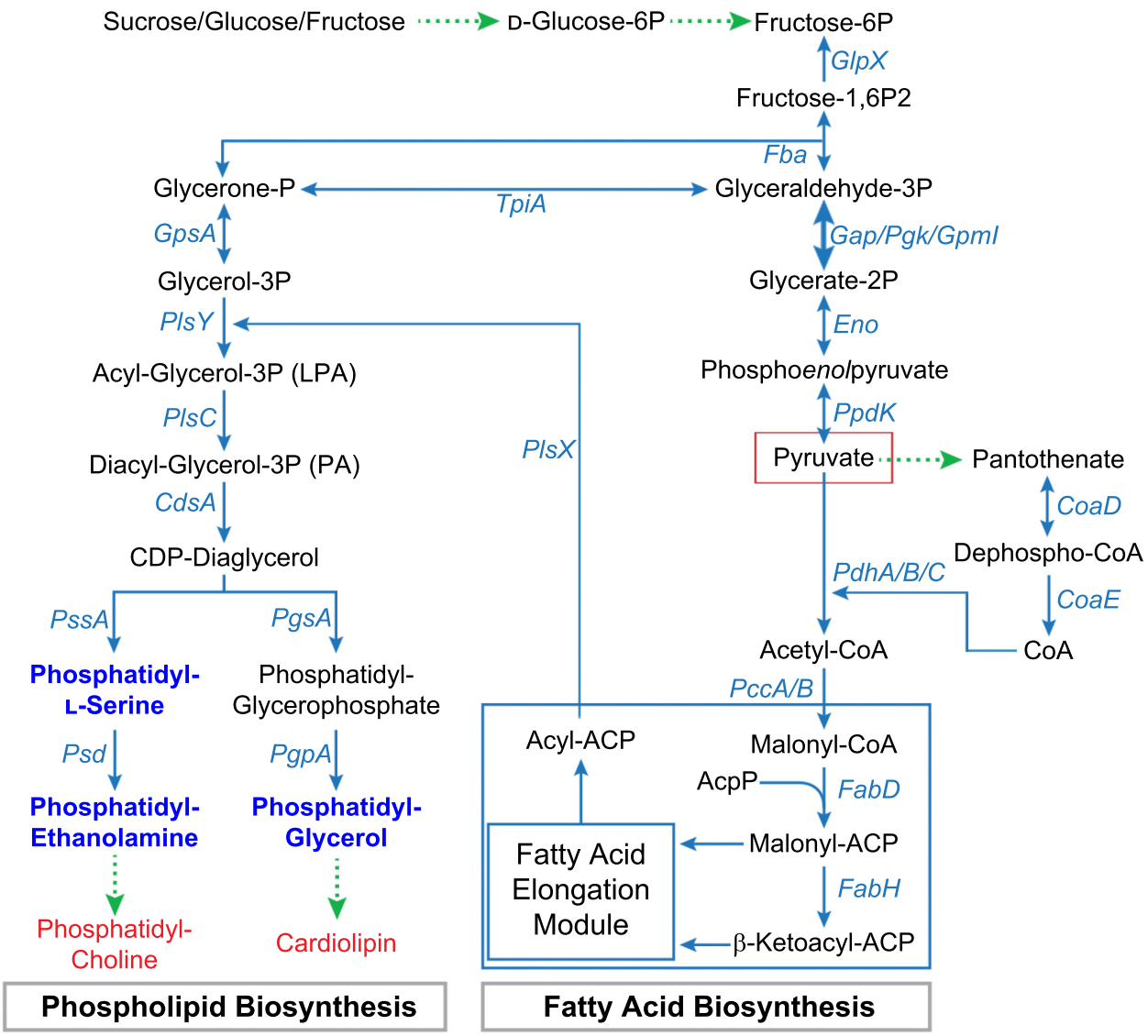

Figure S1. Fatty acids and glycerophospholipids biosynthesis pathways in *E. chaffeensis.*

E. chaffeensis lacks a complete glycolysis pathway and therefore must utilize anabolic pathways and chemical energy from pyruvate to synthesize acyl-acyl carrier protein (ACP) for fatty-acid biosynthesis (bottom right box) and to produce glycerol-3-phosphate, the precursor required for glycerophospholipid biosynthesis (left). *E. chaffeensis* encodes enzymes for biosynthesis of phosphatidylserine, phosphatidylethanolamine, and phosphatidylglycerol (via phosphatidyl-glycerophosphate, highlighted in blue fonts), but lacks enzymes to synthesize phosphatidylcholine (PC) and cardiolipin (highlighted in red fonts). *E. chaffeensis* also lacks genes for biosynthesis or modification of cholesterol (not shown in the drawing). Solid line, pathways present; dashed lines, pathways not present; thicker lines, multiple steps involved. *E. chaffeensis* enzymes responsible for catalyzing each reaction are shown in italic fonts, and the locus ID for each enzymes are:
GlpX, ECH_0356; Fba, ECH_0097; Gap, ECH_0011; Pgk, ECH_0055; GpmI, ECH_0505; Eno, ECH_0544; PpdK, ECH_0330; CoaD/E, ECH_0737/ ECH_0801; PdhA/B/C, ECH_0220/ ECH_0149/ECH_0098; PccA/B, ECH_0599/ECH_0487; FabD/H, ECH_0227/ECH_0448; TpiA, ECH_0646; GpsA, ECH_0340; PlsX/Y/C, ECH_0447/ECH_0027/ECH_0072; CdsA, ECH_0269; PssA, ECH_0780; Psd, ECH_0779; PgsA, ECH_1078; PgpA, ECH_0905.

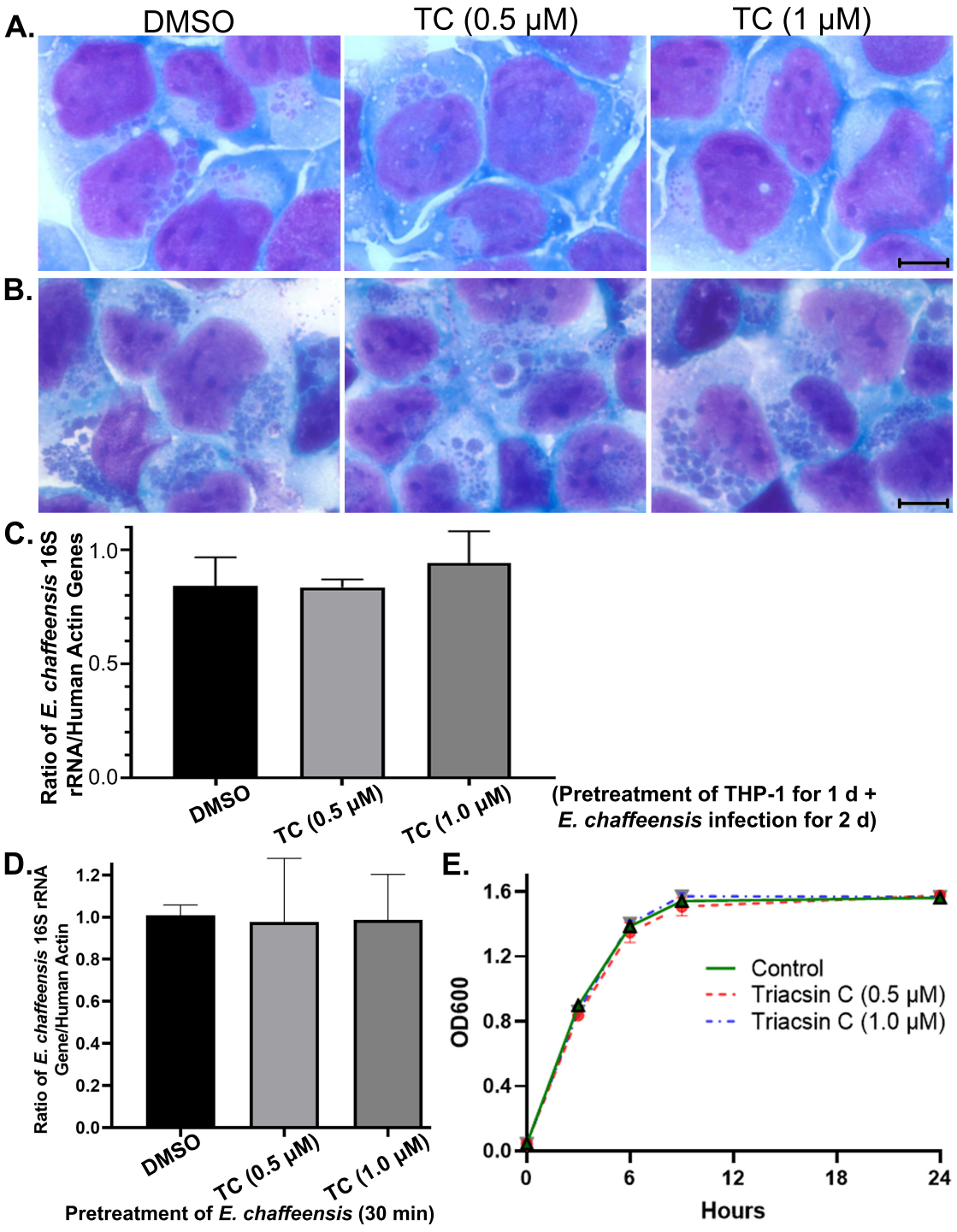

Figure S2. Effects of ACSLs inhibitor triacsin C on *E. chaffeensis* infection or internalization, and *E. coli* growth.

Figure S2. (continued from previous page)

(A) Inhibition of host-cell glycerolipid biosynthesis blocked *E. chaffeensis* infection. *E. chaffeensis*–infected THP-1 cells were seeded in a 6-well plate. At 1 hpi, cells were incubated with 0, 0.5, or 1 μM triacsin C (TC) for 2 d at 37°C. Approximately 100 μl cells were cytospun onto slides and stained using Diff-Quik to visualize bacterial infection. Bar, 5 μm.

(B-C) Pretreatment of THP-1 cells with Triacsin C had no effects on *E. chaffeensis* internalization and infection. THP-1 cells were pretreated with DMSO control or 0.5 ~ 1 μM of triacsin C for 1 d. After washing twice with RPMI medium, cells were infected with *E. chaffeensis* for 2 d. Approximately 100 μl cells were cytospun onto slides and stained by Diff-Quik (**B**). Bar, 5 μm. DNA was extracted from the remaining samples, and quantitative PCR was performed for the *E. chaffeensis* 16S rRNA gene that were normalized against human *ACTIN* (**C**). Results are shown as the mean ± SD. Triacsin C treated groups showed no significant difference compared with control (DMSO-treated) groups ($P > 0.05$, unpaired Student's *t* test).

(D) Pretreatment of *E. chaffeensis* with triacsin C had no effects on its infection. Host cell–free *E. chaffeensis* was purified from infected THP-1 cells and incubated with DMSO control or 0.5 ~ 1 μM of triacsin C for 30 min at 37°C. After washing with medium, triacsin C–treated *E. chaffeensis* was used to infect THP-1 cells seeded in a 6-well plate and cultured at 37°C for 3 d. DNA was extracted from treated samples, and quantitative PCR was performed for the *E. chaffeensis* 16S rRNA gene and normalized against human *ACTIN*. Results are shown as the mean ± SD from three independent experiments.

(E) Incubation of *E. coli* with triacsin C had no effects on its growth. Overnight culture of *E. coli* DH5α was diluted 1/100 in LB media, and aliquoted 4-ml each into 14-ml tubes in duplicate. Triacsin C (0.5 ~ 1 μM), or DMSO control were added to *E. coli* culture, and the growth curve was measured by OD_{600} following time by culturing at 37° C, 275 rpm. Triacsin C treated groups showed no significant difference compared with control (DMSO-treated) groups ($P > 0.05$, unpaired Student's *t* test).

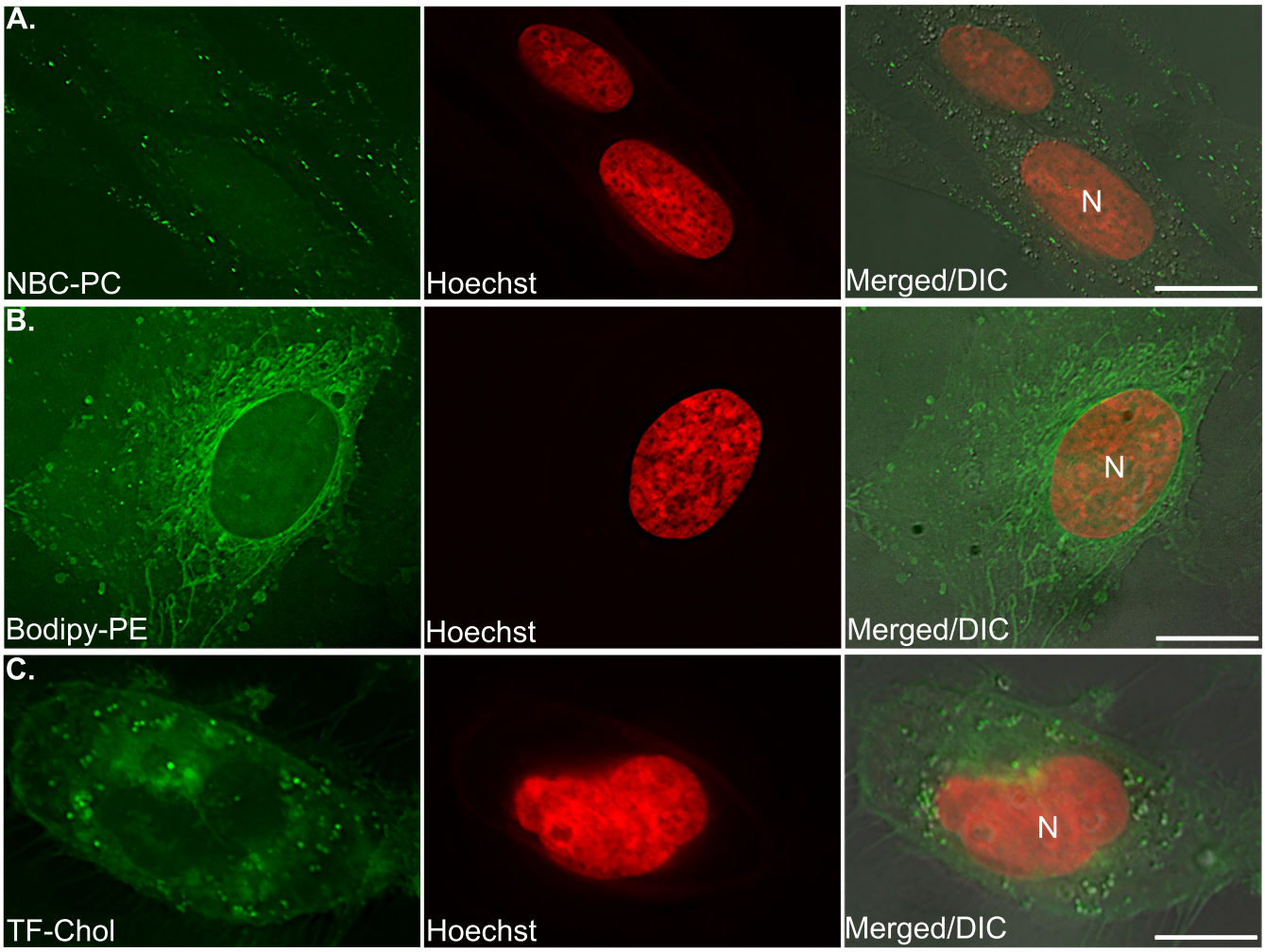

Figure S3. Incorporation of NBD-PC, Bodipy-PE, and TopFluor-cholesterol by the plasma membrane and intracellular vesicles of RF/6A cells.
RF/6A cells were seeded onto coverglasses in a 6-well plate, and incubated with 25 μM NBD-PC for 1 d (**A**), or with 5 μM Bodipy-PE for 4 h (**B**). (**C**) Alternatively, cells were washed and replaced with AMEM containing LPDS for 8 h, then incubated with 1 μM TopFluor-cholesterol (TF-Chol) for 1 d. C ells were fixed in 4% PFA for 20 min, and DNA was stained with 1 μg/ml Hoechst 33342 in PBS for 15 min (pseudocolored red). Samples were observed under a DeltaVision microscope. DIC, differential interference contrast; N, nucleus. Images are representative of three independent experiments with similar results. Bar, 10 μm.

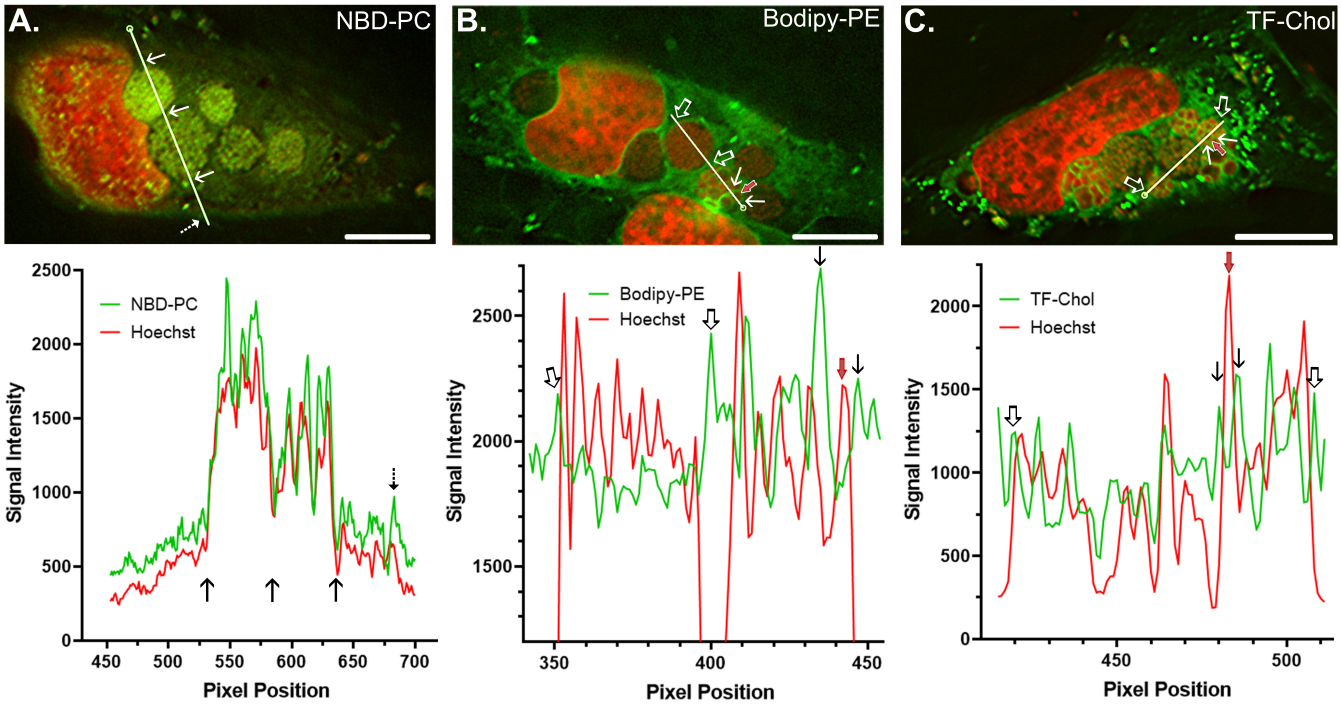

Figure S4. Line profile analysis of fluorescence intensity signals of lipids and *E. chaffeensis*.

Line profile analysis of fluorescence intensity signals of (**A**) NBD-PC (Fig. 1B), (**B**) Bodipy-PE (Fig. 1C), or (**C**) TF-Chol (Fig. 1D) (green), and Hoechst 33342 (*Ehrlichia* DNA, pseudocolored in red) along the slanted white line (starting points from the left). (**A**) *E. chaffeensis* membrane was more strongly labeled than the plasma membrane (~2-fold, indicated by dashed arrows) or other cytoplasmic membrane vesicles (~3-fold). Interestingly, inclusion membrane (solid arrows) was not labeled by NBD-PC. (**B-C**) *E. chaffeensis* membrane (indicated by solid arrows) was strongly labeled by Bodipy-PE and TF-Chol (green), which encircled *E. chaffeensis* bacteria (an individual bacterium was indicated by red arrows as shown by Hoechst 33342 staining). However, unlike NBD-PC, inclusion membranes (open arrows) were also strongly labeled by Bodipy-PE and TF-Chol. Bar, 10 μm.

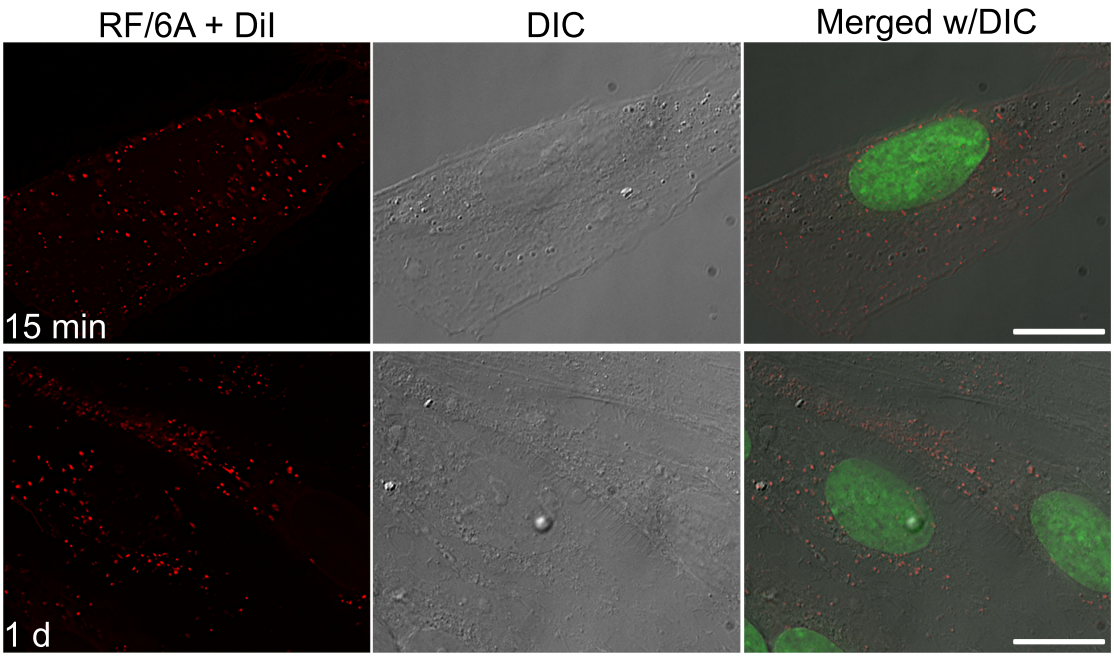

Figure S5. Time course of DiI labeling in uninfected RF/6A cells. RF/6A cells were seeded onto coverglasses in a 12-well plate for 1 or 2 d and incubated with 5 μM DiI for 1 d or 15 min, respectively. Cells were washed three times with PBS and fixed in 4% PFA for 20 min. DNA was stained with 1 μg/ml Hoechst 33342 in PBS for 15 min (pseudocolored green). Images were captured using a DeltaVision microscope. Bar, 10 μm.

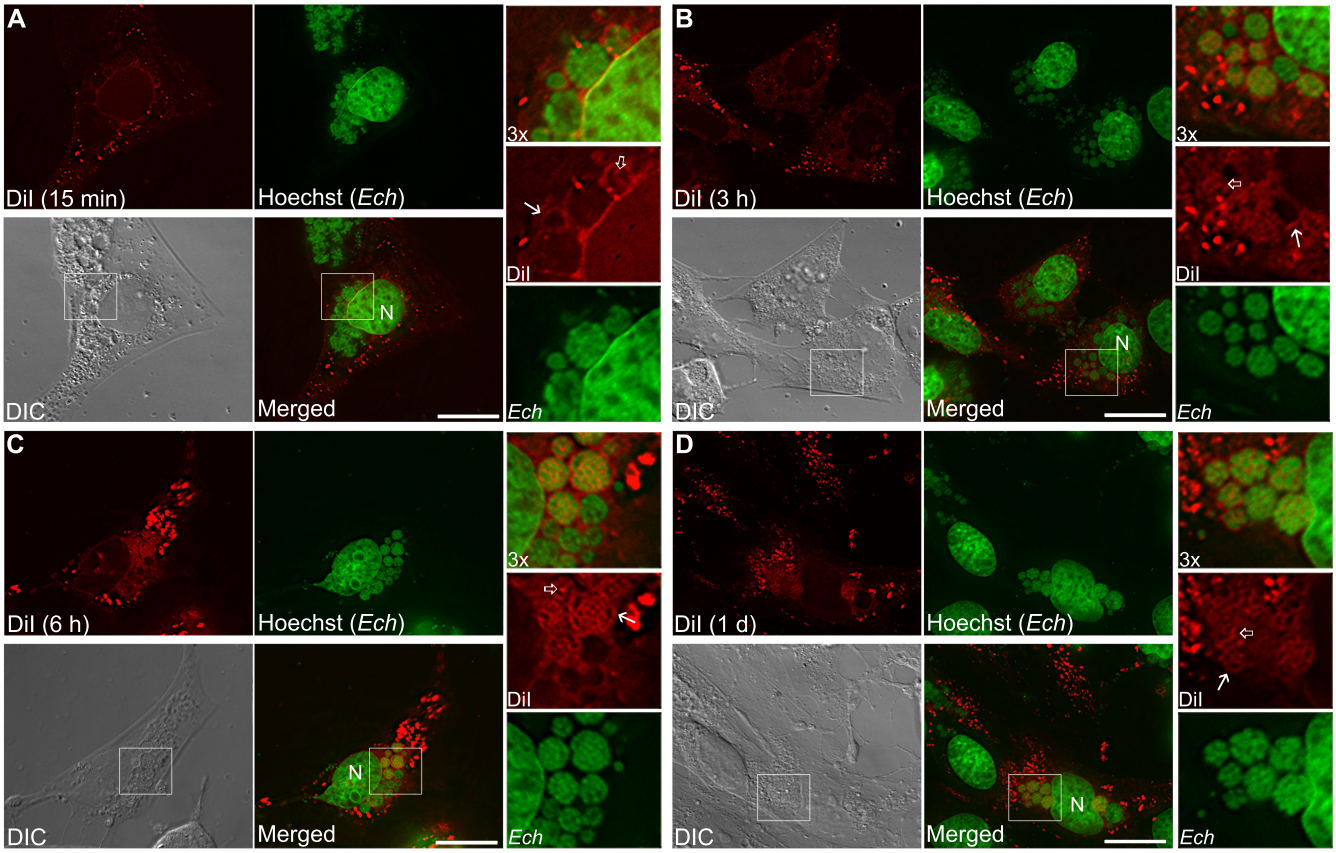

Figure S6. Time course of DiI labeling in *E. chaffeensis*–infected RF/6A cells.
RF/6A cells were seeded onto coverglasses in a 12-well plate and infected with *E. chaffeensis* for 2 d. Cells were incubated with 5 µM DiI at 2 dpi for 15 min (**A**), 3 h (**B**), or 6 h (**C**), or at 1 dpi for 1 d (**D**). Cells were washed, fixed, and DNA was stained with Hoechst 33342 (pseudocolored green). Images were captured using a DeltaVision microscope. For each panel, the boxed area in the merged image is enlarged 3× on the right. Solid arrows, inclusion membranes; open arrows, ILVs or bacterial membranes. Images are representative of at least three independent experiments. Bar, 10 µm.

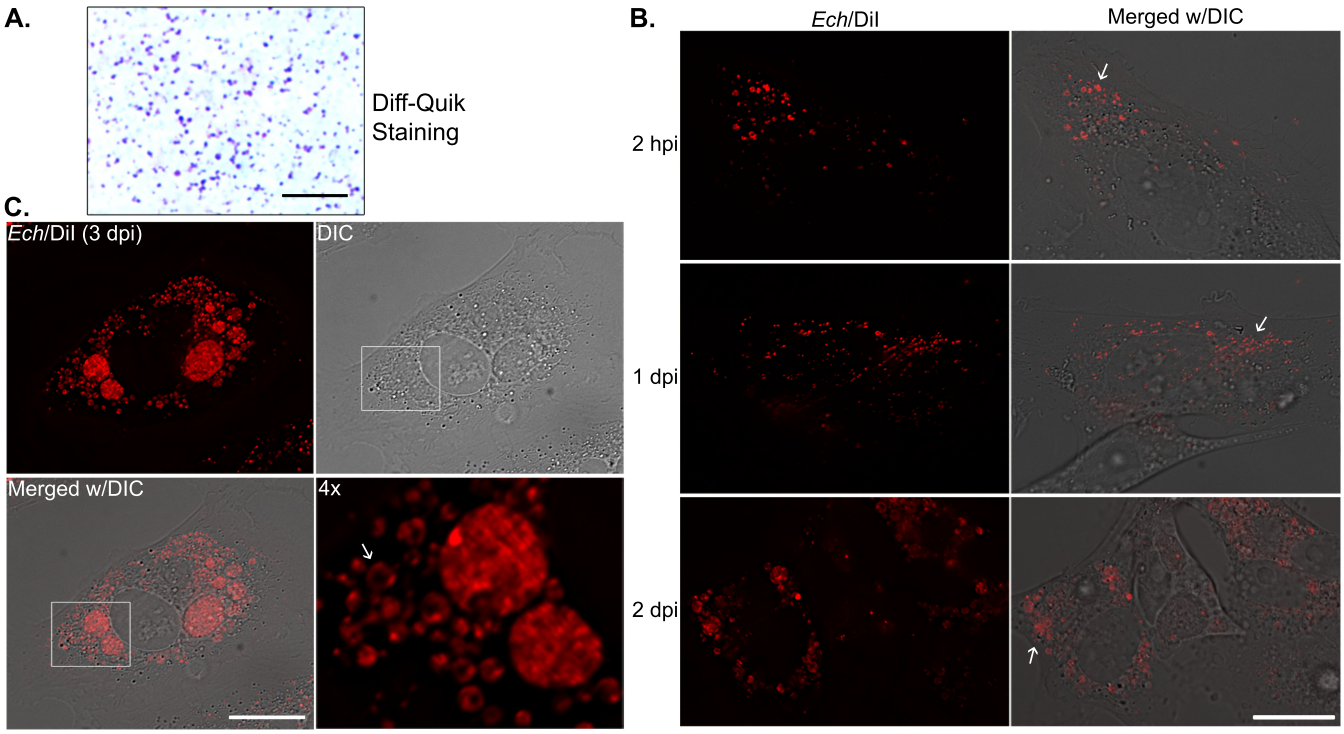

Figure S7. DiI-prelabeled *E. chaffeensis* membranes do not traffic to host-cell membranes. Host cell–free *E. chaffeensis* was purified from heavily infected THP-1 cells and the purity was determined by Diff-Quik staining (**A**). After incubation with 5 μM DiI for 15 min, *E. chaffeensis* was washed twice with PBS and used to infect RF/6A cells seeded on a 35-mm glass-bottom culture dish. After 2 h incubation, cells were washed to remove uninternalized bacteria (with time point set as 0 h pi), and cultured in phenol red–free AMEM containing 5% FBS and 2 mM L-glutamine. Live-cell images were obtained under a DeltaVision microscope in a heated environment (37°C with humidified air containing 5% CO_2) at 2 h, 1 d, 2 d (**B**), and 3 d pi (**C**). The boxed area in image is enlarged 4× and shown at bottom right panel (**C**, arrow indicates DiI-labeled bacterial membrane). Culture dish were returned to the incubator after each live-cell imaging, and different regions of cells were chosen for live-cell imaging following time courses to minimize the effects of photo-bleaching. DIC, differential interference contrast; Bar, 10 μm.

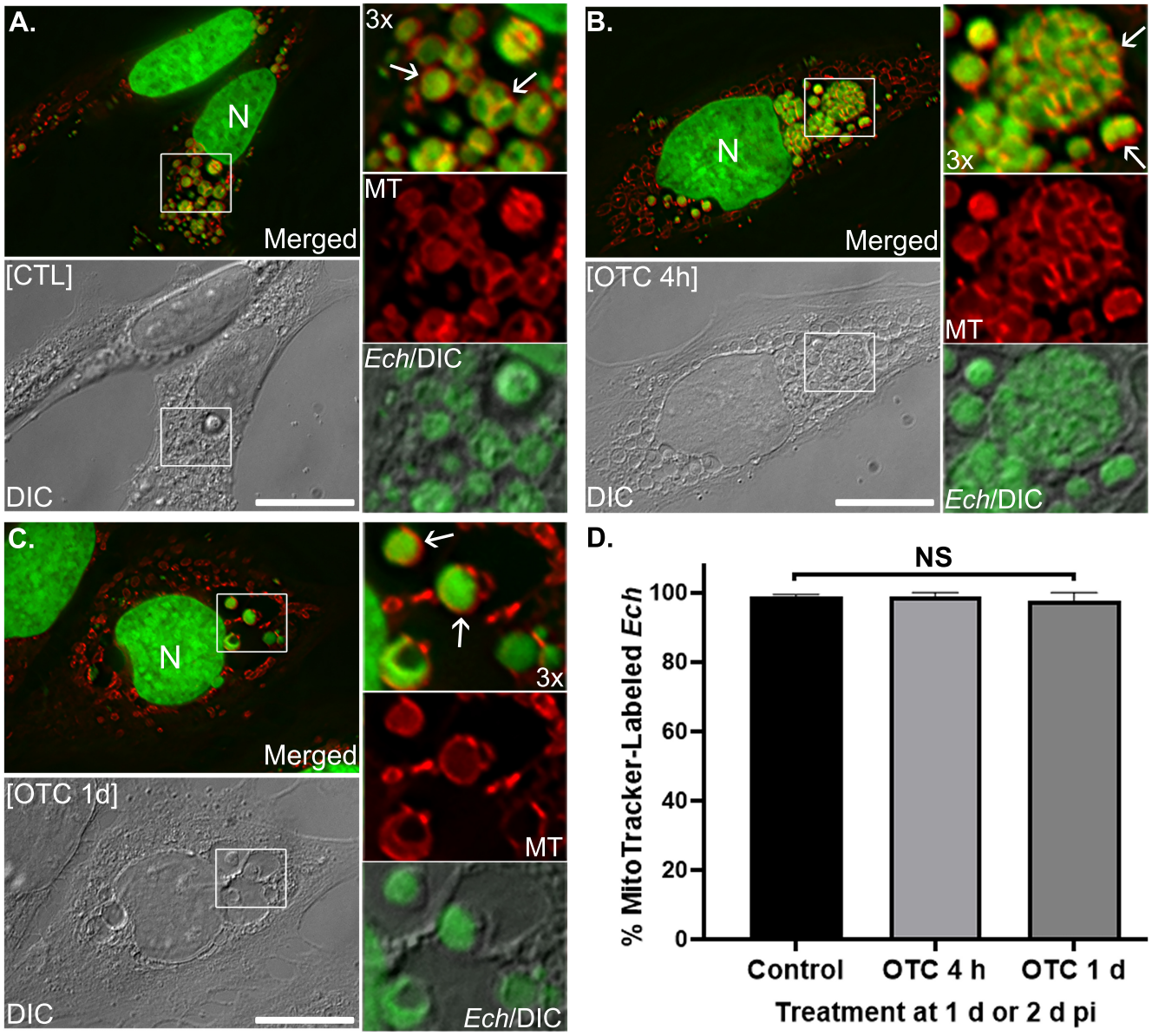

Figure S8. Examination of *E. chaffeensis* viability by MitoTracker labeling following oxytetracycline treatment.

RF/6A cells were seeded on coverglasses and infected with *E. chaffeensis* (*Ech*). Cells were treated with medium control (CTL, **A**), 5 μg/ml of oxytetracycline (OTC) at 2 d pi for 4 h (**B**), or at 1 d pi for 1 d (**C**), then incubated with 500 nM MitoTracker Deep Red FM (MT) in growth media for 30 min at 37°C. After PBS wash, cells were fixed in ice-cold, 100% methanol for 15 min at -20°C, and washed 3 times with PBS for 5 min each. DNA was stained with 300 μM DAPI for 5 min (pseudocolored in green) during the first PBS wash. DeltaVision microscope. Solid arrows, *E. chaffeensis* bacteria. Bar, 10 μm.

(**D**) Percentage of MitoTracker-labeled *E. chaffeensis* among DAPI-stained bacteria was quantified by counting at least 10 cells per group, and results were shown as mean ± SD. Although DNA staining of individual bacteria became less compact, and bacterial infection was significantly reduced with OTC treatment for 1 d, MitoTracker labeling showed the DAPI-stained *E. chaffeensis* bacteria in the inclusions were still viable (NS, not significant by analysis of variance).