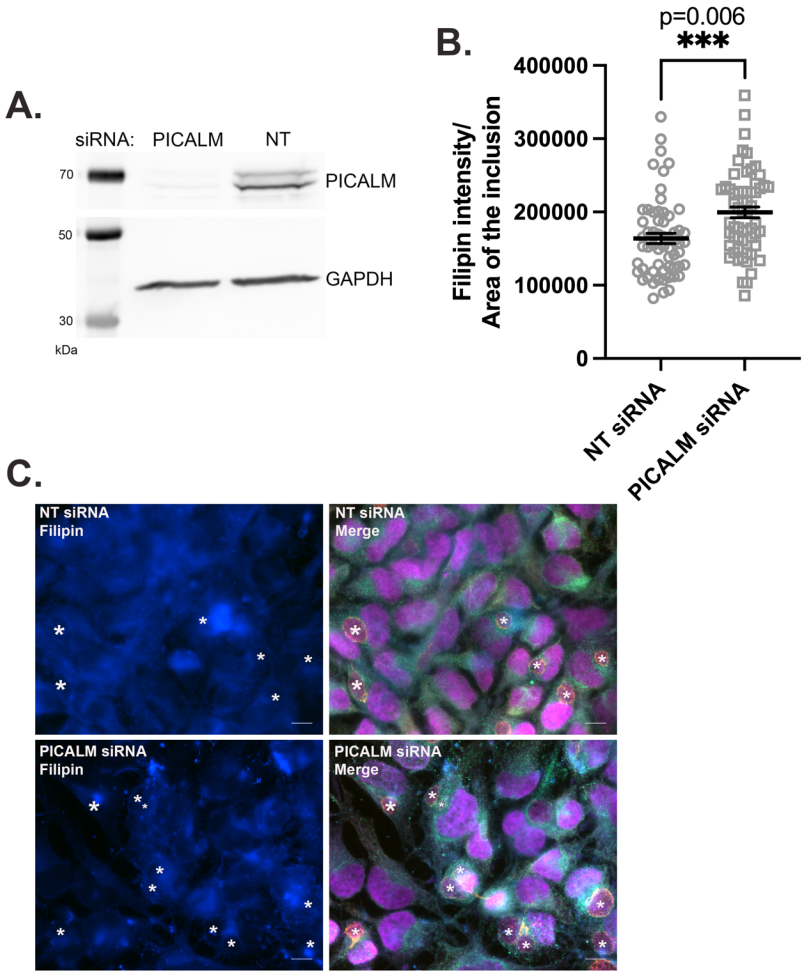



Figure 1

**Figure 1. PICALM siRNA knockdown alters cholesterol trafficking to the inclusion.** HeLa cells seeded in a 24-well plate were transfected with 20 nM of PICALM or non-targeting (NT) siRNA as described in Methods and Materials and then infected with *C. trachomatis* serovar L2 using an MOI of 0.5. (A) To confirm PICALM knockdown, cell lysates were harvested and resolved via SDS-12%PAGE and western blot to detect PICALM and GAPDH (loading control). These data are representative of knockdown of PICALM with siRNA obtained for all experiments with only samples demonstrating a 70% or greater knockdown efficiency being used for data production. (B and C) After fixation, HeLa cells were incubated with filipin as described in Methods and Materials.

Images in C were captured with a Zeiss Axio Imager without the ApoTome at 100x magnification, scale bar = 10 μm. The same exposure time was used for all imaging. To quantify the intensity of filipin, as a marker for cholesterol, an inclusion membrane marker (inclusion membrane protein IncA) was used to determine regions of interest (ROIs) in Fiji/ImageJ, which was also used to determine the integrated density of filipin and the area of the inclusions within the ROIs. 60 inclusions total (30 in 2 independent experiments) were measured. For each ROI the integrated density was divided by the area of the inclusion, then individual data points, mean, and standard error of the mean graphed in GraphPad Prism. Statistical significance was determined using an unpaired t test. Data are representative of 4 independent experiments.

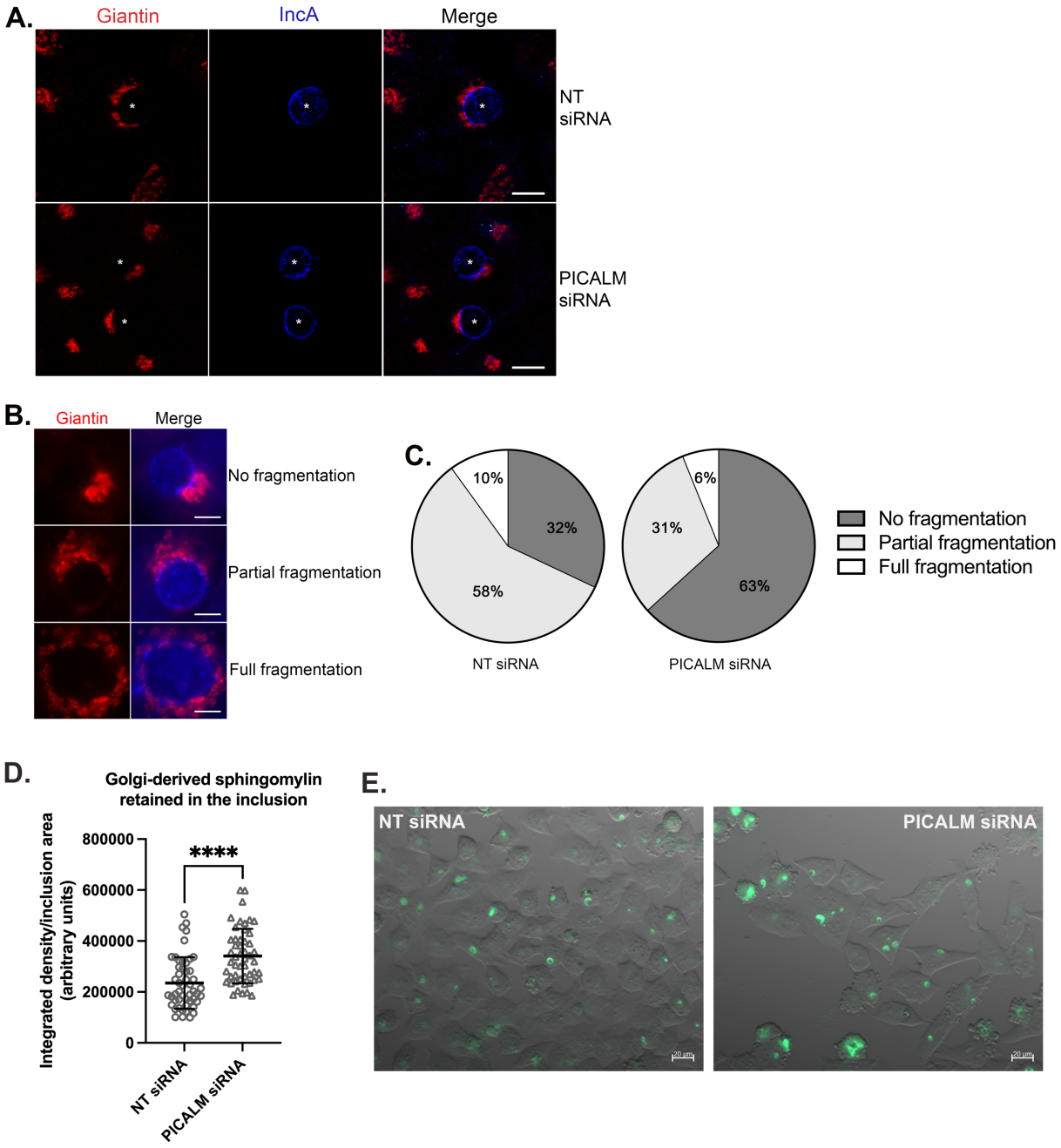



**Figure 2. PICALM siRNA knockdown prevents Golgi mini-stack formation around the inclusion.** (A) HeLa cells depleted of PICALM or not (NT) with siRNA were infected with *C. trachomatis* serovar L2 for 24 hours. Cells were fixed and processed for indirect immunofluorescence to detect the Golgi (red; giantin) and the inclusion membrane (blue, IncA). Images were taken on a Zeiss Axio Imager with ApoTome at 100x. Scale

bar = 10 μm. (B) Examples of a fully fragmented Golgi, a partially fragmented Golgi, and a Golgi with no fragmentation to manually determine (C) the percentage of Golgi fragmentation in PICALM or NT siRNA transfected cells. Golgi fragmentation phenotypes were graphed as parts of a whole in GraphPad Prism. (D and E). At 18-20 hours post infection, cells were labeled with NBD-ceramide, followed by a minimum of 3 hours of back-exchange to remove all non-incorporated fluorescent lipid from the cell (essentially as described in PMID 23798538). Labeled cells were imaged live in the brightfield and 488 fluorescent channels (set at the same exposure time for all samples) on the Zeiss Axio Imager without the ApoTome at 40x; scale bar = 20μm. To measure the intensity of NBD-lipid remaining within the inclusions, inclusions were identified and marked as ROIs in Fiji/ImageJ in the brightfield image. Then, in the 488 channel images, the integrated density and area of the inclusions were determined. Individual integrated density values were divided by the associated area of the inclusion for 50 inclusions per siRNA knockdown condition. Individual values with mean and standard deviation were graphed and statistically analyzed (Welch's t test) in GraphPad Prism (D). Representative images are provided in E. These data are representative of 4 independent experiments.

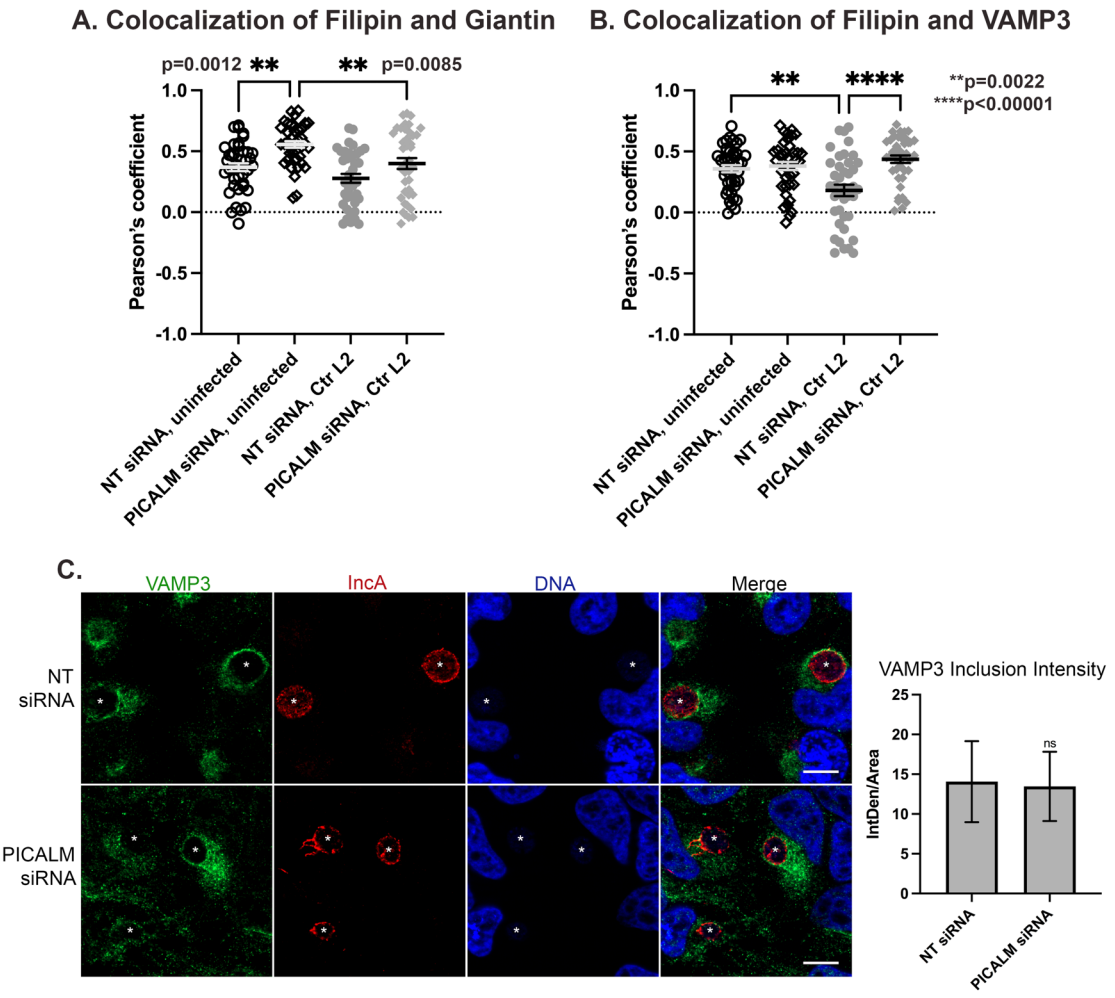



**Figure 3**

**Figure 3. PICALM siRNA knockdown increases colocalization of cholesterol in the Golgi and within VAMP3-positive structures.** HeLa cells were transfected and infected with *C. trachomatis* serovar L2 as previously described. At 24 hours post-infection, cells were fixed and labeled with filipin prior to incubation with antibodies to detect the Golgi (giantin) (A) or VAMP3 (B). The Pearson's coefficients of 40 cells/regions of interest were determined with the JACoP plugin in Fiji/ImageJ as described in Methods and Materials. For A and B, data are combined from 2 independent experiments (consistent exposure times for each set of slides) and are representative of 4 independent experiments. In GraphPad Prism, individual data points

are graphed with mean and standard error of the mean. Statistical analysis included ordinary One-way Anova with a Šidák's multiple comparison test. (C). Trafficking of VAMP3 to the inclusion in NT or PICALM siRNA-transfected cells was determined after fixation at 24 hours post-infection. Cells were processed for indirect immunofluorescence to detect VAMP3 (green), IncA (inclusion membrane, red), or DNA stained with DAPI. Images were captured with a Zeiss Axio Imager without the ApoTome at 100x magnification, scale bar = 10 μm. The same exposure time was used for all imaging. White stars denote the chlamydial inclusions. Regions of interest were generated in ImageJ using IncA as a marker for the inclusion and the integrated density of VAMP3 around the inclusions was measured and divided by the area. The mean with standard deviation was graphed in GraphPad Prism. Statistical significance was determined with an unpaired t test where ns = not significant and **** $p \leq 0.0001$. These data are representative of at least 3 independent experiments.

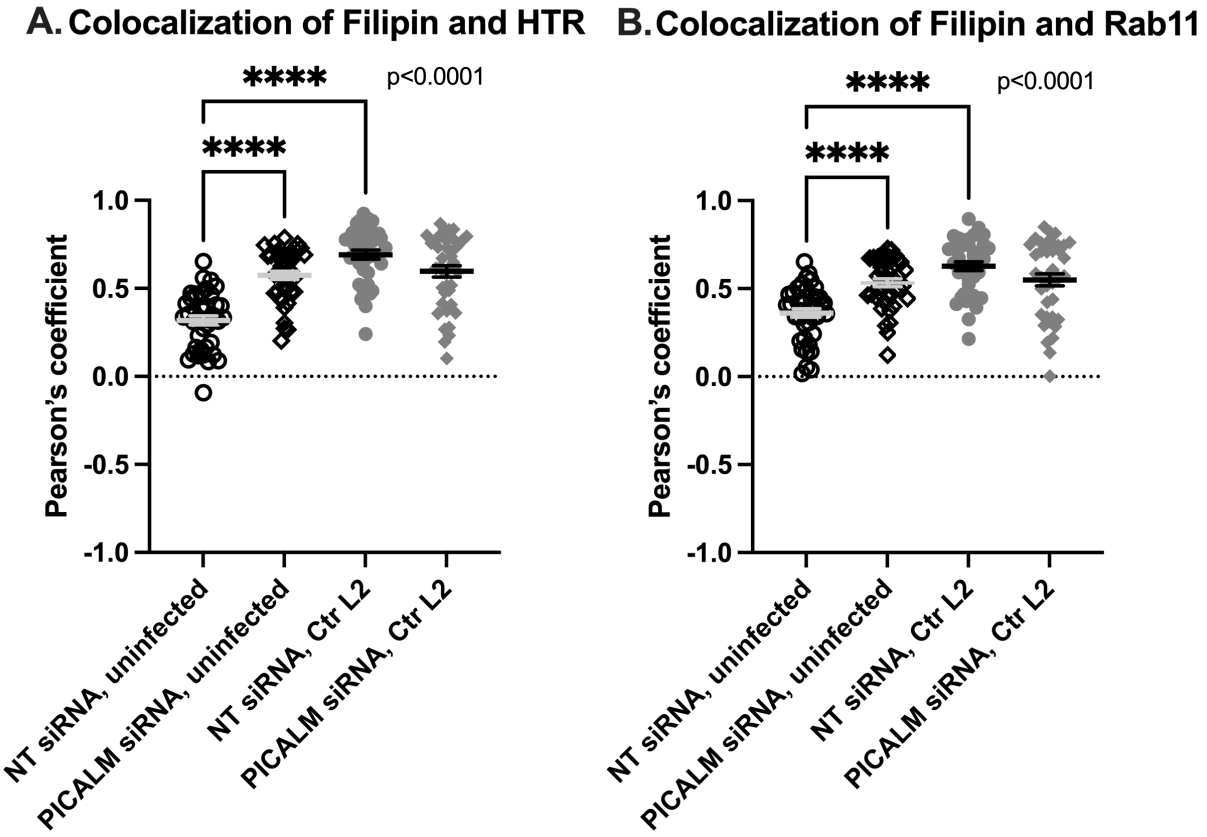



Figure 4

**Figure 4. PICALM siRNA knockdown and chlamydial infection increases the colocalization of cholesterol (filipin) with Human Transferrin Receptor (HTR) and Rab11 positive structures.** Images from experiments described Supplemental Figure 2 were used to determine the colocalization of cholesterol (labeled with filipin) with HTR(A) or Rab11 (B). Briefly, individual cells within original 8-bit images were selected as regions of interest (ROI) and exported using the multi-crop function of the ROI manager in Fiji/ImageJ for the channel associated with filipin and Rab11 or HTR. The Pearson's coefficient was determined using the JACoP plugin in Fiji for 40 cells/regions of interest. Data are combined from 2 independent experiments (consistent exposure times for each set of slides) and are representative of 4 independent experiments. In

GraphPad Prism, individual data points are graphed with mean and standard error of the mean. Statistical analysis included ordinary One-way Anova with a Šidák's multiple comparison test.

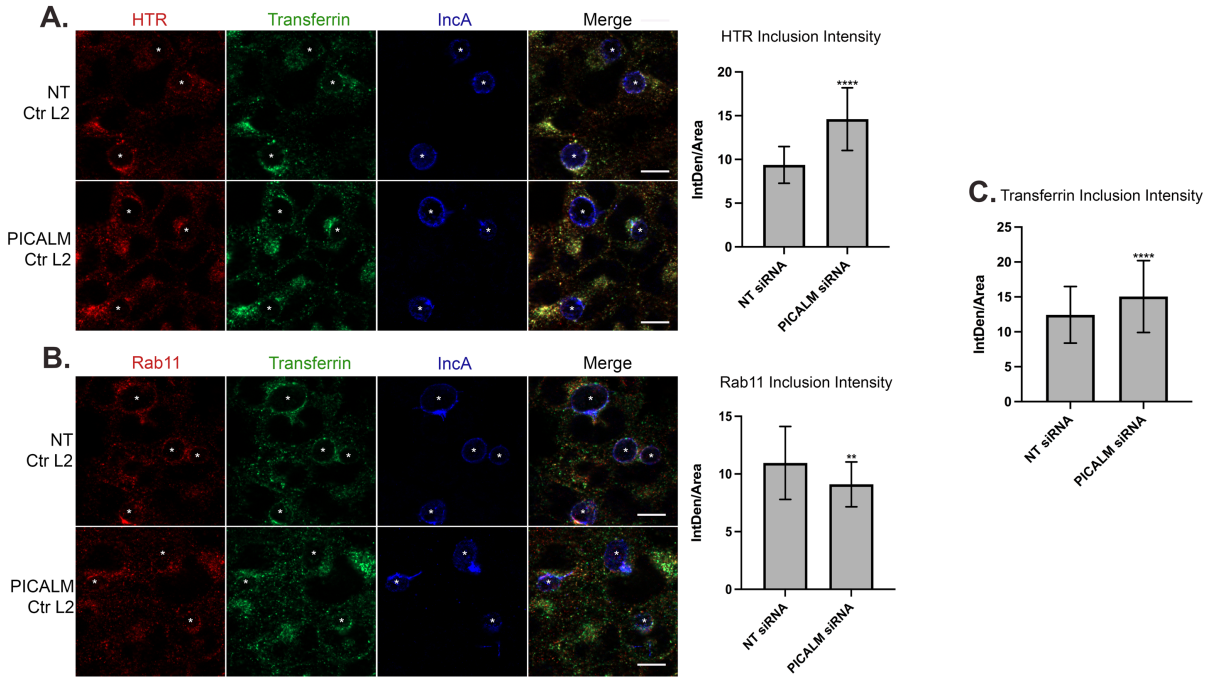



**Figure 5**

**Figure 5. Transferrin trafficking is altered in PICALM deficient cells.** HeLa cells seeded in a 24-well plate were transfected with 20 nM of PICALM or non-targeting (NT) siRNA as described in Methods and Materials and then infected with *C. trachomatis* serovar L2 using an MOI of 0.5. (A and B) Infected cells were fed with 25 µg/mL Transferrin-488 (green; Life Technologies) at 24 hours post infection and incubated for an additional hour at 37°C 5% $CO_2$ to allow for transferrin internalization and trafficking. Cells were fixed and processed for indirect immunofluorescence to detect Human Transferrin Receptor (HTR) (A; red) or Rab11 (B; red) and the inclusion membrane (IncA, blue). Images were acquired with the same exposure time on a Zeiss Axio

Imager without ApoTome at 100x magnification, scale bar = 10 μm. In Fiji/ImageJ, regions of interest were generated using IncA as a marker for the inclusion membrane and the the area of the inclusion and the integrated density of HTR (A), Rab11 (B), or transferrin-488 (C) was measured. Integrated density was divided by the area of the inclusion with the mean and standard deviation graphed in GraphPad Prism. Statistical significance was determined with an unpaired t test, **** $p \leq 0.0001$. These data are representative of at least 3 independent experiments.

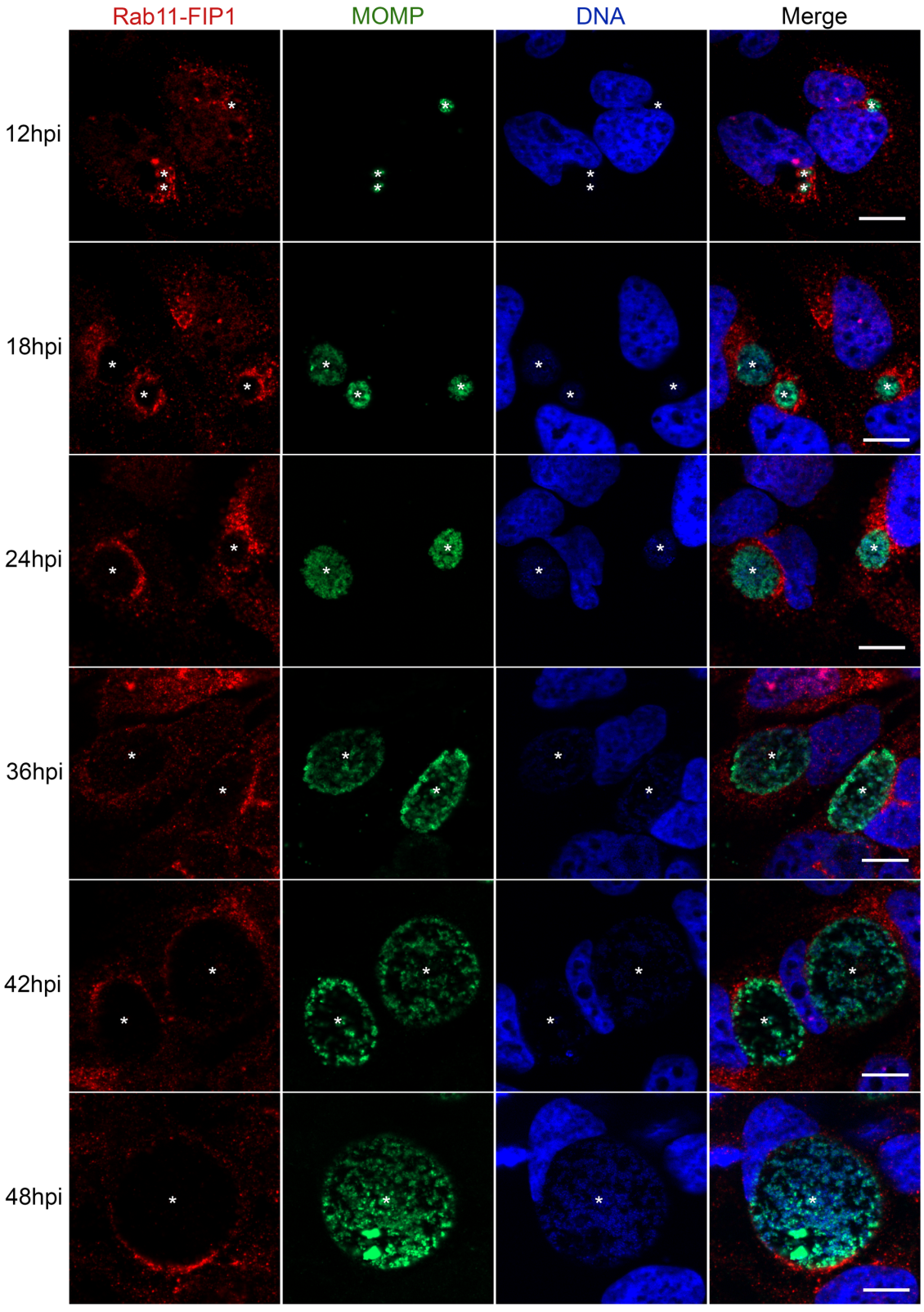
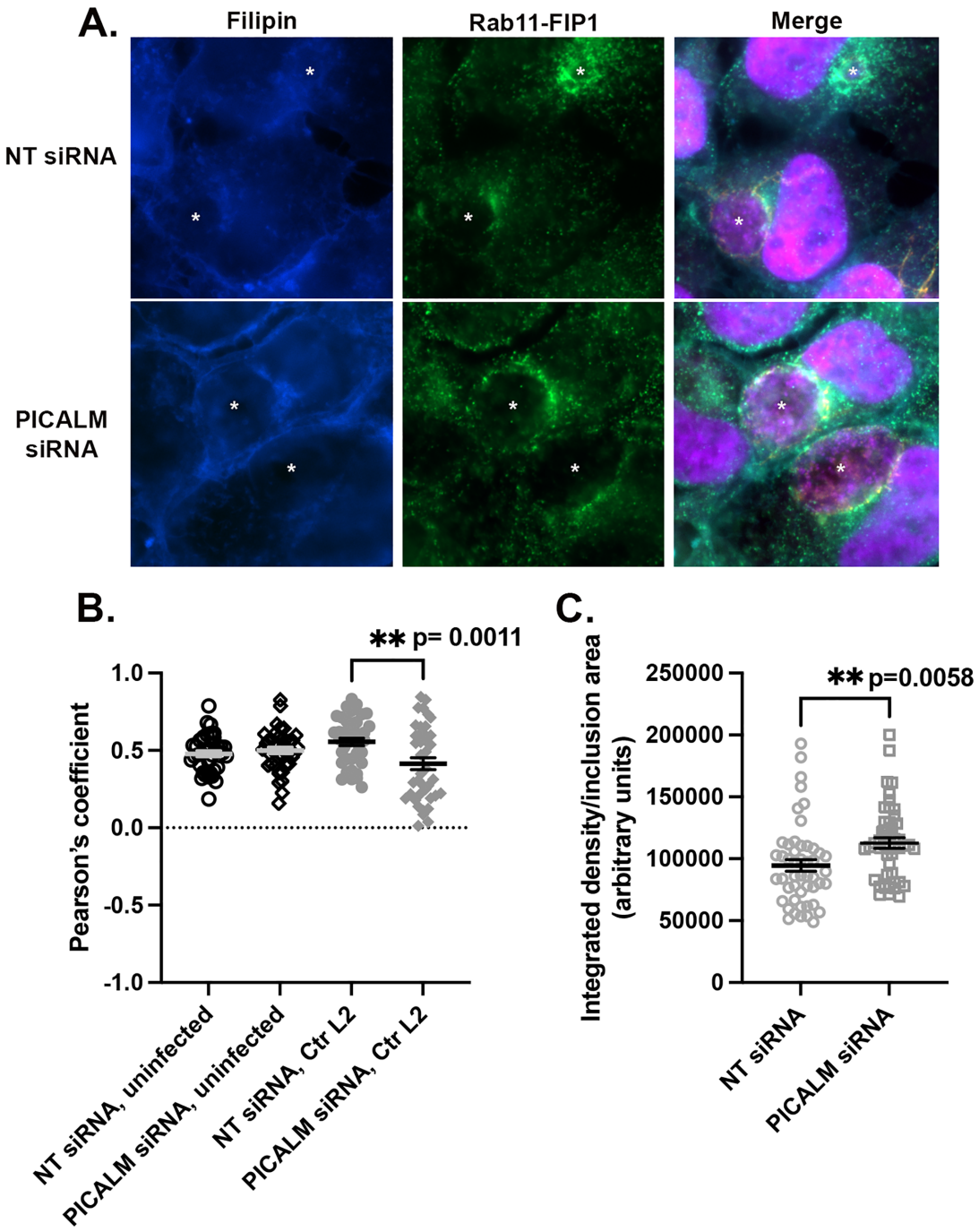

Rab11-FIP1
MOMP
DNA
Merge
12hpi
18hpi
24hpi
36hpi
42hpi
48hpi

**Figure 6**

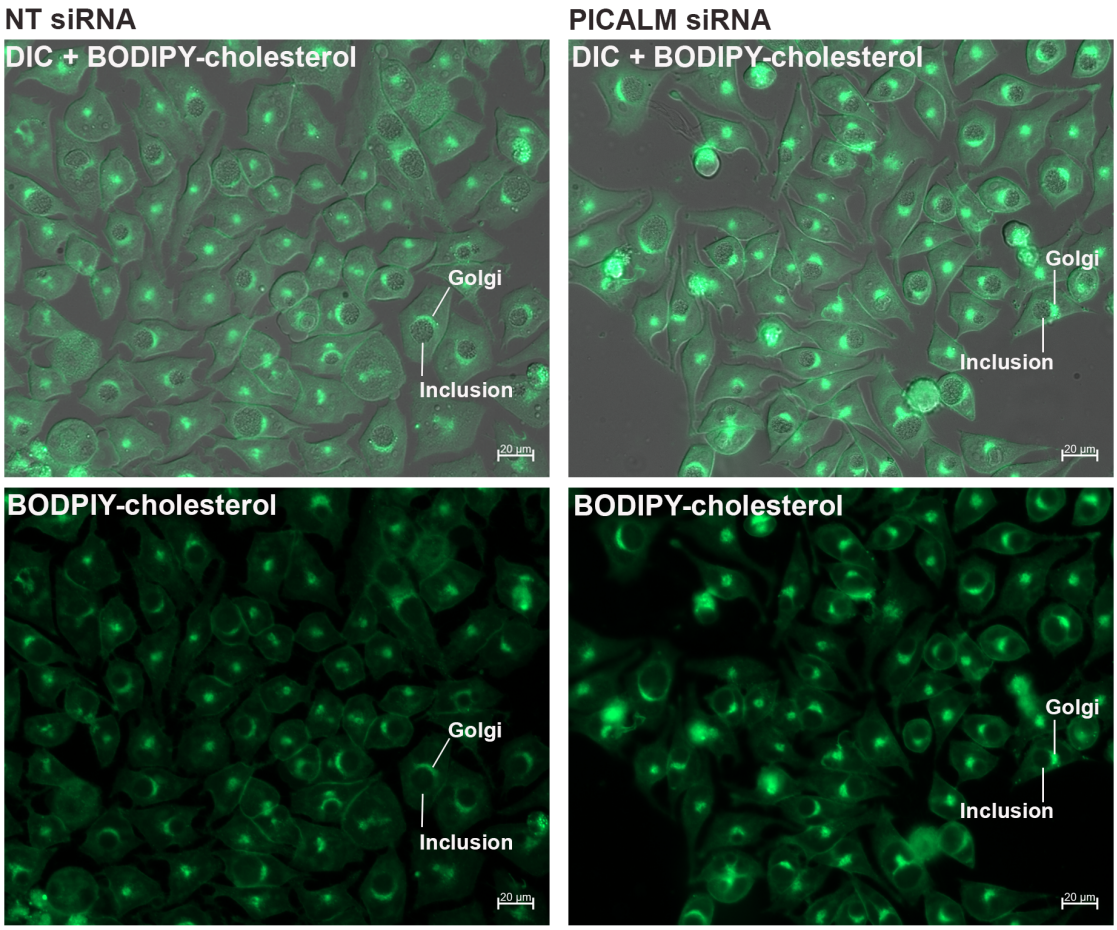
**Figure 6. Endogenous Rab11-FIP1 accumulates around *C. trachomatis* L2 inclusions throughout the developmental cycle**. HeLa cells seeded in a 24-well plate were infected with *C. trachomatis* serovar L2. At indicated timepoints post-infection cells were fixed and processed for indirect immunofluorescence to detect endogenous Rab11-FIP1 (red), chlamydial organisms (green), and DNA in blue (DAPI Life Technologies). Images were taken at 100x on a Zeiss Axio Imager with ApoTome, scale bar = 10 μm. These images are representative of at least 3 independent experiments.




## Figure 7

**Figure 7. Effect of PICALM siRNA knockdown on Rab11-FIP1 trafficking.** HeLa cells were transfected, infected for 24 hours, fixed, and labeled with filipin as described previously. Images were acquired with the Zeiss Axio Imager without the ApoTome at

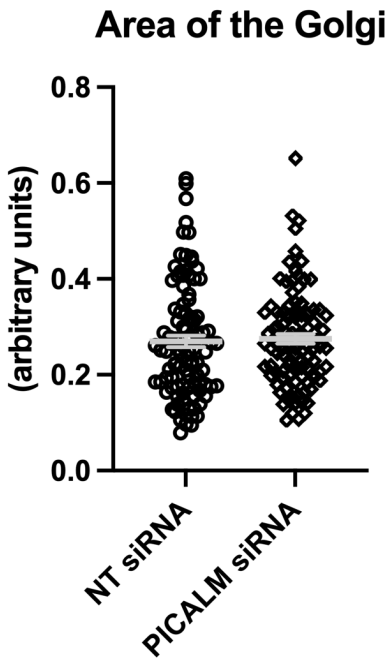
100x magnification, scale bar = 10 μm. Images in A are representative of 4 independent experiments with filipin (blue), Rab11-FIP1 (green), inclusion membrane (red, IncA), organisms and DNA (purple). White stars indicate chlamydial inclusions. For data in B, the original 8-bit images generated with the Zeiss software were used. Individual cells were selected as regions of interest (ROI) and exported with the multi-crop function of the ROI manager in Fiji/ImageJ for the channel associated with filipin or Rab11-FIP1. The Pearson's coefficient was determined using the JACoP plugin in Fiji for 40 cells/regions of interest. These same images were used to determine the intensity of Rab11-FIP1 at the inclusion in cells transfected with NT or PICALM siRNA (C). For these data, the inclusions, as demarcated with an inclusion membrane marker IncA, were identified as regions of interest in Fiji/ImageJ. Then the intensity of Rab11-FIP1 at the inclusion was determined in the green channel. Data are combined from 2 independent experiments (consistent exposure times for each set of slides) and are representative of 4 independent experiments. In GraphPad Prism, individual data points are graphed with mean and standard error of the mean. Statistical analysis included ordinary One-way Anova with a Šidák's multiple comparison test (B), and a Welch's t test (C).




## Supplemental Figure 1

**Supplemental Figure 1. BODIPY-cholesterol remains within the Golgi.** TopFluor Cholesterol (aka BODIPY-cholesterol; Avanti Polar Lipids, Alabaster Alabama) was resuspended in 2mg/100 λ. Then, 20λ of the solution was added to serum-free DMEM containing 10% methyl-β cyclodextrin (MβCD). A 400μg/ml stock of MβCD-TopFluor Cholesterol complexes were made by 2 minutes of sonication. To remove large particles, the complexes were then centrifuged for 2 minutes at 17,000 x g. Then 25 μg/ml MβCD-TopFluor Cholesterol complexes were added to each well and incubated for 4 hours, then cells were imaged live using the DIC and 488 fluorescent channels on the Zeiss Axio Imager without the ApoTome at 40x magnification. These are

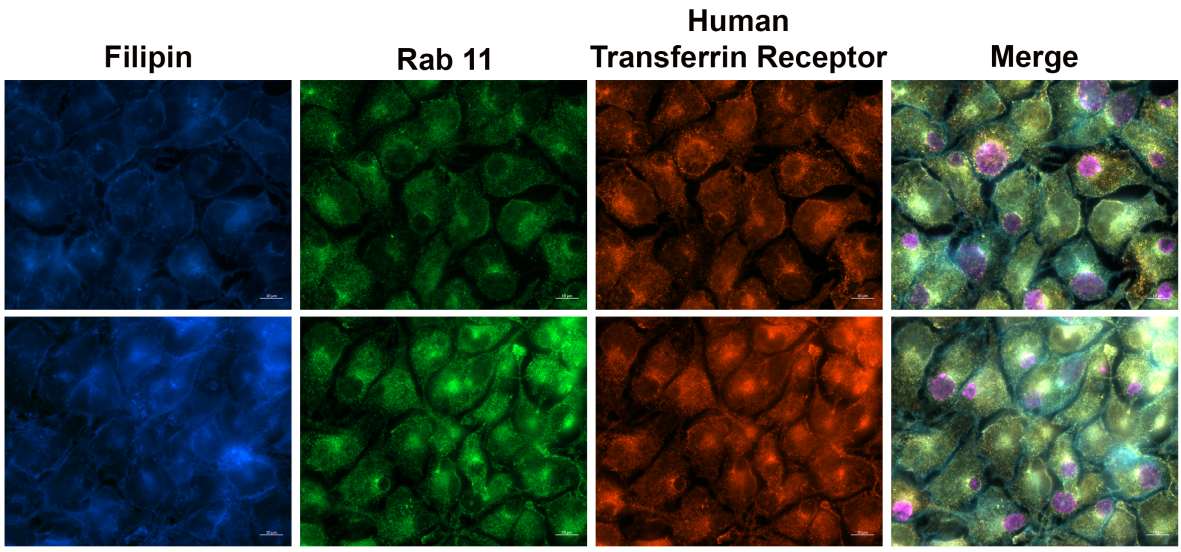
representative images from 4 independent experiments. Example inclusions and Golgi are indicated in each image.

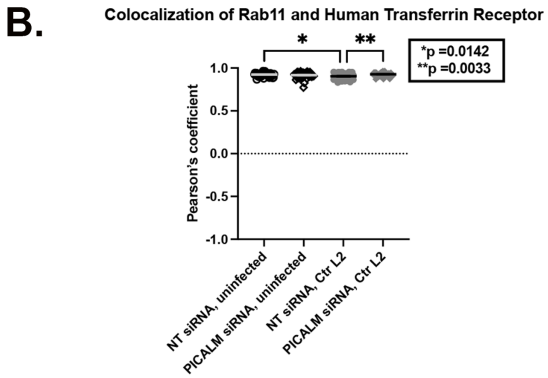



| | NT siRNA | PICALM siRNA |
|---|---|---|
| Mean | 0.2696 | 0.2748 |
| Std. Deviation | 0.1190 | 0.1001 |
| Std. Error of Mean | 0.01190 | 0.01001 |

**Supplemental Figure 2**

**Supplemental Figure 2. PICALM siRNA knockdown does not affect the size of the Golgi.** Area of the Golgi was determined by selecting Golgi, using giantin as a subcellular marker for the Golgi, in Fiji/ImageJ using the region of interest tool. The area for 100 Golgi in HeLa cells transfected with NT or PICALM siRNA were determined in Fiji and graphed and statistically analyzed with a Welch's t test in GraphPad Prism.



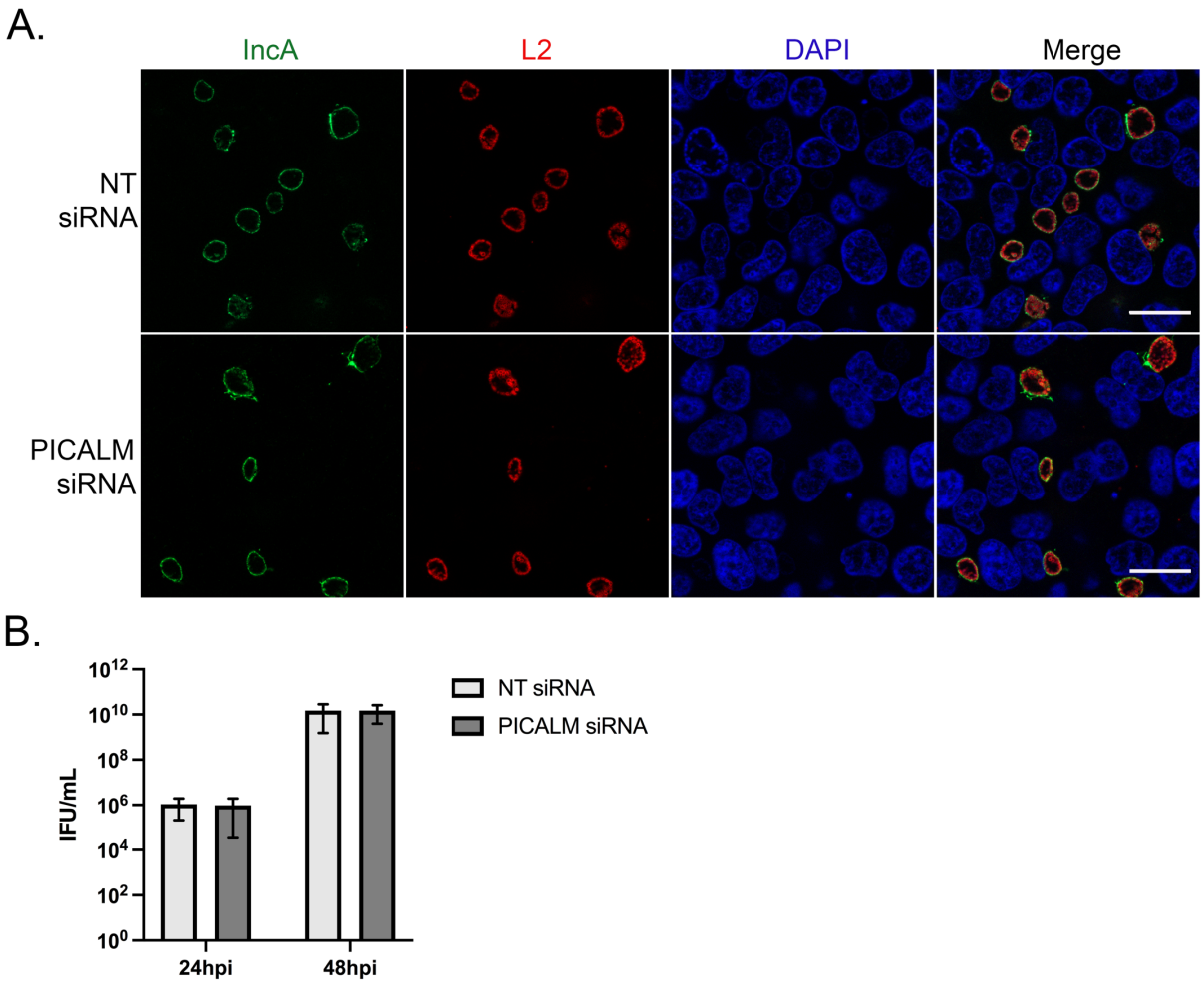




| | NT siRNA, uninfected | PICALM siRNA, uninfected | NT siRNA, Ctr L2 | PICALM siRNA, Ctr L2 |
|---|---|---|---|---|
| Mean | 0.9244 | 0.9169 | 0.9036 | 0.9276 |
| Std. Deviation | 0.02242 | 0.03735 | 0.03802 | 0.02487 |
| Std. Error of Mean | 0.003545 | 0.005906 | 0.006011 | 0.003932 |

## Supplemental Figure 3

**Supplemental Figure 3. Cholesterol trafficking in PICALM siRNA treated cells.** HeLa cells were transfected with non-targeting (NT) or PICALM siRNA as described in Methods and Materials, then infected with *C. trachomatis* serovar L2 (moi 0.7) for 24 hours. Cells were fixed in 4% paraformaldehyde and labeled with 50 μg/ml filipin in DPBS + 1% BSA for 1 hour at room temperature. Then samples were incubated with the appropriate primary and secondary antibodies to detect Rab11 (green) and Human Transferrin Receptor (red). Images were acquired with the Zeiss Axio Imager without

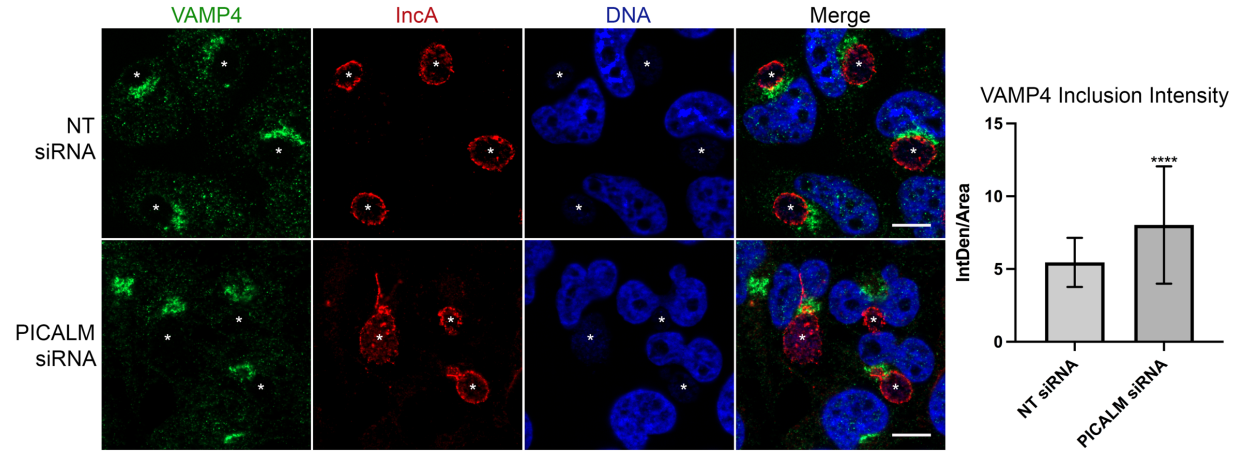
the ApoTome at 100x magnification, scale bar = 10 μm. Images in A are representative of 4 independent experiments and correspond with data in Figure 4. For data in B, the original 8-bit images generated with the Zeiss software were used. Individual cells were selected as regions of interest (ROI) and exported with the multi-crop function of the ROI manager in Fiji/ImageJ for the channel associated with Rab11 or Human Transferrin Receptor. The Pearson's coefficient was determined using the JACoP plugin in Fiji for 40 cells/regions of interest. Data are combined from 2 independent experiments (consistent exposure times for each set of slides) and are representative of 4 independent experiments. In GraphPad Prism, individual data points are graphed with mean and standard error of the mean. Statistical analysis included ordinary One-way Anova with a Šidák's multiple comparison test.




**Supplemental Figure 4**

**Supplemental Figure 4. Effect of PICALM knockdown on the generation of chlamydial infectious progeny.** (A) HeLa cells depleted of PICALM or not using siRNA were infected with *C. trachomatis* serovar L2 for 24 hours. Cells were fixed and processed for indirect immunofluorescence to detect the inclusion membrane in green (sheep anti-IncA Seramun Diagnostica GmbH), chlamydial organisms in red (Guinea pig anti-L2 Rocky Mountain Labs), and DNA in blue (DAPI Life Technologies). Images were acquired at 100x using a Zeiss Axio Imager with ApoTome. Scale bar = 10 µm. (B) Inclusion forming units (IFU) per mL were determined by collecting a primary infection in

either PICALM or NT siRNA knockdown cells at 24 and 48 hpi. Bacteria were re-infected onto fresh monolayers of untransfected HeLa cells and IFU/mL were enumerated as described in materials and methods. The mean with standard deviation was graphed in GraphPad Prism.




Suppmental Figure 5

**Supplemental Figure 5. siRNA knockdown of PICALM increases VAMP4 trafficking to the inclusion.** HeLa cells seeded in 24-well plates were transfected with either PICALM or NT siRNA for 18 hours. Media was removed and replaced, and cells were allowed to recover for 24 hours. Then cells were infected with *C. trachomatis* serovar L2. At 24hpi cells were fixed and processed for indirect immunofluorescence to detect VAMP4 in green (rabbit anti-VAMP4 Sigma) and IncA in red (sheep anti-IncA Seramun Diagnostica GmbH), and DNA in blue (DAPI Life Technologies). Images were acquired on a Zeiss Axio Imager with ApoTome at 100x, scale bar = 10 µm. White stars denote chlamydial inclusions. VAMP4 inclusion intensity was determined by acquiring images at the same exposure time without the ApoTome at 100x. Regions of interest were generated in ImageJ using IncA as a marker for the inclusion and the integrated density of VAMP4 around the inclusions was measured and divided by the area. The mean with standard deviation was graphed in GraphPad Prism. Statistical significance was determined with an unpaired t test where ns = not significant and **** $p \leq 0.0001$.