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Report

The Obligate Intracellular Pathogen *Chlamydia trachomatis* Targets Host Lipid Droplets

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Summary

Lipid droplets (LDs) are ubiquitous but poorly understood neutral-lipid-rich eukaryotic organelles that may participate in functions as diverse as lipid homeostasis, membrane traffic, and signaling [1]. We report that infection with the obligate intracellular pathogen *Chlamydia trachomatis*, the causative agent of trachoma and many sexually transmitted diseases [2], leads to the accumulation of neutral-lipid-rich structures with features of LDs at the cytoplasmic surface of the bacteria-containing vacuole. To identify bacterial factors that target these organelles, we screened a collection of yeast strains expressing GFP-tagged chlamydial ORFs and identified several proteins with tropism for eukaryotic LDs. We determined that three of these *LD-a*ssociated (Lda) proteins are translocated into the mammalian host and associate with neutral-lipid-rich structures. Furthermore, the stability of one Lda protein is dependent on binding to LDs, and pharmacological inhibition of LD formation negatively impacted chlamydial replication. These results suggest that *C. trachomatis* targets LDs to enhance its survival and replication in infected cells. The co-option of mammalian LD function by a pathogenic bacterium represents a novel mechanism of eukaryotic organelle subversion and provides unique research opportunities to explore the function of these understudied organelles.

Results and Discussion

C. trachomatis replicates within a membrane bound vacuole (termed the "inclusion") that is inaccessible to endocytic traffic or secretory glycoproteins and is devoid of most known endosomal, lysosomal, or Golgi markers [3]. Despite the apparent dissociation of the inclusion from classical vesicle-mediated membrane transport, acquisition of host-derived lipids through vesicle-mediated and non-vesicle-mediated pathways is essential for *C. trachomatis* replication [4, 5].

Our understanding of how *C. trachomatis* co-opts lipid transport has been limited by its intractability to genetic manipulation and by the abundance of *Chlamydia*-specific proteins with no known functional homologs [6]. We reasoned that because the Chlamydiaceae have parasitized eukaryotic cells since early in evolution [7], lipid transport pathways targeted by *C. trachomatis* virulence factors would be conserved in the yeast *Saccharomyces cerevisiae*. Indeed, we recently identified a cytotoxic chlamydial ORF (CT163) that displayed strong tropism for yeast lipid droplets (LDs) [8]. LDs are endoplasmic reticulum (ER)-derived lipid storage organelles consisting of a hydrophobic core of neutral lipids (sterol esters and triacylglycerides) surrounded by a phospholipid monolayer [1]. Because *C. trachomatis* must acquire host-derived lipids for intracellular growth, we examined the interaction between *C. trachomatis* and mammalian LDs.

LDs and Neutral-Lipid-Rich Structures Accumulate at the Chlamydial Inclusion

To monitor the subcellular localization of LDs, we stained *C. trachomatis*-infected cells with the neutral-lipid-specific fluorescent dyes BODIPY493/503 and Nile red. Both dyes labeled LDs scattered in the cytoplasm of mammalian cells and a hazy, neutral-lipid-rich network of tubules and small droplets enveloping the chlamydial inclusion (Figures 1A and 1B and data not shown). Because these structures were morphologically distinct from larger classical LDs observed in adipocytes or lipid-loaded cells, we hypothesized that the neutral-lipid-rich structures surrounding the inclusion consisted primarily of small LDs or intermediates in LD formation. Consistent with this, the inclusions were enveloped by prominent lipid-rich coats upon oleic acid treatment (Figure S1 in the Supplemental Data available with this article online). Interestingly, BODIPY493/503 also stained bacterial membranes (Figures 1B and 1C and Figure S2), suggesting that *C. trachomatis* may acquire neutral lipids directly from LDs.

To determine if the neutral-lipid-rich structures enveloping the inclusion constituted LDs, we monitored the localization of host LD-associated proteins. HeLa cells were infected with *C. trachomatis* and transfected with EGFP-tagged forms of LD-associated proteins, including two members of the PAT family of LD-associated proteins, Adipocyte-Differentiation-Related Protein (ADRP) and perilipin [9], the triacylglyceride lipase ATGL [10], and the GTPase Rab18 [11]. Upon treatment with oleic acid, these markers localized to classical LDs (Figure S3). In addition, LD markers labeled reticular structures (perilipin and Rab18) and hazy structures (ATGL and ADRP) at the periphery of the inclusion (Figure 1D and Figure S3). However, all LD markers localized to structures adjacent to but distinct from the inclusion membrane (IM), indicating that the IM does not mimic LD membranes (Figure S3).

Given the role of LDs in lipid storage, we hypothesized that *C. trachomatis* may exploit these organelles as a source of lipids. To assess the impact of chlamydial infection on LDs, we compared the neutral lipid content of LDs isolated from infected and uninfected cells. Surprisingly, infection with *C. trachomatis* markedly increased

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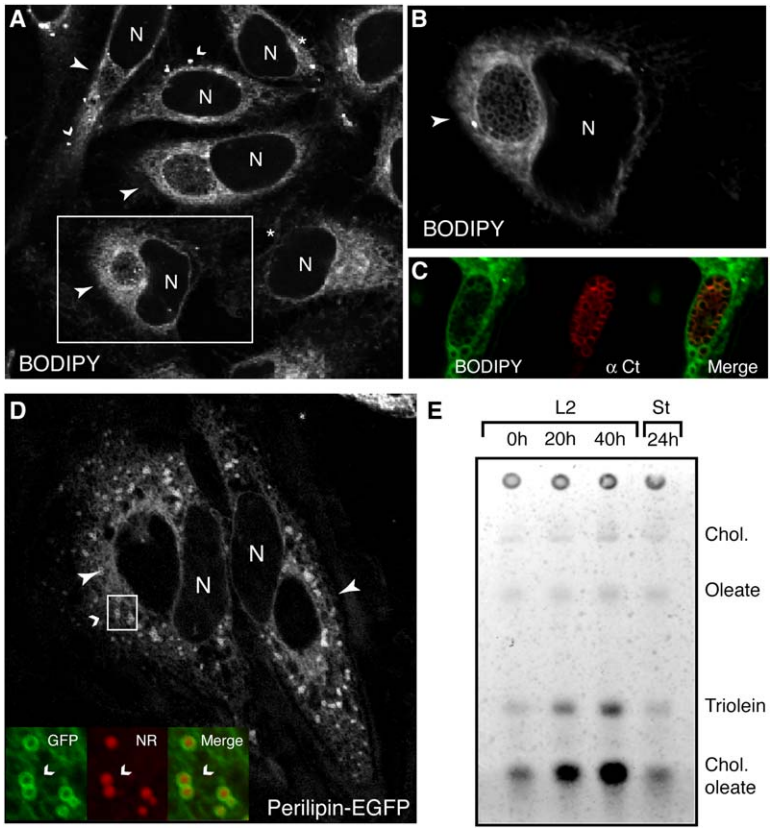

Figure 1. Neutral-Lipid-Rich Structures and Lipid Droplet Markers Are Recruited to the *C. trachomatis* Inclusion

(A and B) Accumulation of neutral lipids at the chlamydial inclusion. HeLa cells were infected with LGV L2 at an MOI of 0.5:1 for 20–24 hr. Infected cells were fixed in 3% formaldehyde/0.025% glutaraldehyde and stained with the neutral lipid dye BODIPY493/503. (B) shows a higher magnification of the cell boxed in (A). Note BODIPY 493/503-positive reticular network enveloping the inclusion (arrowheads). N, host nuclei. *Uninfected cells.

(C) Chlamydial reticulate bodies accumulate neutral lipids. LGV L2-infected HeLa cells were fixed as above, permeabilized with 0.05% saponin, and incubated with BODIPY493/503 and anti-*Chlamydia* antibodies (*Ct*).

(D) Subcellular localization of the LD marker perilipin in infected cells. LGV L2-infected HeLa cells were transfected with EGFP-perilipin 7 hr postinfection, pulsed with 50 μM oleic acid for 3 hr, and imaged at 24 hr. Note localization of perilipin to classical Nile red (NR)-positive LDs (arrows and inset) and to reticular structures enveloping the chlamydial inclusion (arrowheads).

(E) Accumulation of cholesterol esters in LDs of *C. trachomatis*-infected cells. LDs were purified from cells infected with LGV L2 for 20 and 40 hr or with *S. typhimurium* (*St*) for 24 hr. Neutral lipids were extracted with diethyl ether, separated on TLC plates, and detected by fluorimetry after primuline staining.

levels of cholesteryl esters (CE) recovered from LD fractions (Figure 1E), suggesting that these organelles may expand during infection. This increase in LD yield does not constitute a generalized response to bacterial infection, since this increase in neutral lipids was not observed in cells infected with the intracellular pathogen *Salmonella typhimurium* (Figure 1F). To independently assess whether lipid homeostasis was disrupted in infected cells, we monitored the subcellular fractionation of Nsdhl, a sterol biosynthetic enzyme that relocalizes from the ER to LDs in response to increased levels of cellular lipids [12]. Nsdhl recovery in LD fractions increased during chlamydial infection, and this accumulation was inhibited by the bacterial protein synthesis inhibitor chloramphenicol (Figure S4). Overall, our results indicate that LD-like structures accumulate at the periphery of the inclusion and that *C. trachomatis* alters neutral lipid homeostasis.

Identification of Chlamydial LD-Associated Proteins

We predicted that *C. trachomatis* exported proteins into the host cell to target LDs. To identify these proteins, we screened a collection of yeast strains expressing GFP-tagged *Chlamydia*-specific ORFs [8] for proteins that colocalized with an RFP-tagged LD protein (Erg6p). This screen revealed four *Chlamydia*-specific ORFs (CT156, CT163, CT257, and CT473) with marked tropism for yeast LDs (Figures 2A and 2B). We focused our analysis on three of these LD-associated chlamydial proteins.

Lda1 (CT156) and Lda2 (CT163) have no known functional homologs, while Lda3 (CT473) displays limited homology to putative hemolysins. The *lda1* and *lda2* genes map to a region of high genetic diversity within the *C. trachomatis* chromosome that is termed the "plasticity zone" (PZ). The PZ encodes several proteins that expand the pathogenic properties of *C. trachomatis*, including ADP-ribosylating toxins, enzymes for the biosynthesis of tryptophan, and a family of phospholipase D (PLD)-like proteins [13]. Lda1 has been postulated to be a regulator of these PLD-like proteins [14]. Interestingly, PLD activity is necessary for the generation of LDs in cell-free systems [15], suggesting that Lda1 and chlamydial PLD-like proteins may participate directly in LD biogenesis. However, because Lda1 and Lda2 are only found in *C. trachomatis*, this chlamydial species may have specifically adapted to exploit intracellular neutral lipid stores.

To test if the tropism of Lda proteins for LDs was conserved in mammalian cells, HeLa cells were transfected with vectors expressing EGFP-tagged forms of Lda1, Lda2, and Lda3. Lda1 and Lda3 localized to ring-like structures that stained with Nile red, indicating that these proteins associated with mammalian LDs (Figure 2C). In contrast, Lda2-EGFP-expressing cells displayed low levels of cytoplasmic fluorescence with no apparent association with LDs (data not shown). We raised antibodies to synthetic peptides derived from the predicted amino acid sequences of Lda1 and Lda3 and determined by immunofluorescence (IF) microscopy that these proteins are translocated into the host cell and localize to structures adjacent to the cytoplasmic face of the IM (Figure 3A). Consistent with the described sensitivity of LD-associated proteins to detergent permeabilization [16], the localization of Lda1,

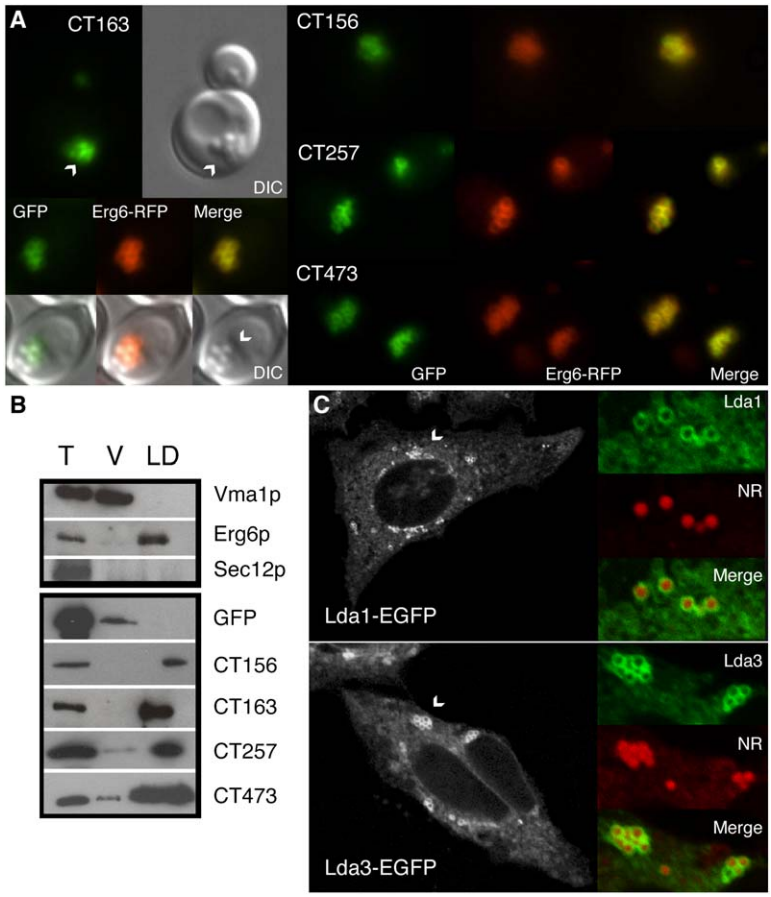

Figure 2. Identification of Chlamydial LD-Associated Proteins

(A) Identification of Lda proteins by subcellular localization screens in yeast. Chlamydial LD-tropic proteins were identified in yeast by screening an expression library [8] for GFP-tagged bacterial proteins that colocalized with an RFP-tagged form of the yeast LD protein Erg6p. Tropism of CT163-GFP to cytoplasmic LDs (arrows) was readily apparent by DIC microscopy and by colocalization with Erg6-RFP.

(B) Biochemical fractionation of chlamydial LD-tropic proteins expressed in yeast. LDs were purified from yeast strains expressing GFP or GFP-tagged forms of chlamydial LD-associated proteins (Lda1/CT156, Lda2/CT163, Lda4/CT257, and Lda3/CT473). The distribution of these chlamydial proteins was assessed in total lysates (T), vacuolar fractions (V), and purified LDs (LD) by immunoblot analysis. The purity of fractions was confirmed by monitoring the fractionation of vacuolar (Vma1p), LD (Erg6p), and ER (Sec12p) proteins.

(C) Ectopically expressed Lda proteins localize to mammalian LDs. HeLa cells were transfected with EGFP-tagged forms of Lda1 and Lda3 and stained with Nile red (NR). Note NR-positive ring-like structures enveloped by Lda proteins (right panels).

and to a lesser extent Lda3, was sensitive to solubilization with 0.1% Triton X-100 (Figure 3B and data not shown). Furthermore, Lda1 and BODIPY 493/503-labeled structures partially overlapped at the inclusion periphery (Figure 3C). These results strongly suggest that *C. trachomatis* translocates proteins into the host cytoplasm to target LD-like structures enveloping the inclusion.

LDs Are Required for Lda2 Stability and for Chlamydial Replication

Lda2 and Lda3 were independently identified in a screen for chlamydial proteins that induce cytotoxic effects in yeast [8]. To determine if the cytotoxicity of these proteins is linked to LDs, we assessed if yeast mutants lacking the triacylglycerol synthases and sterol acyltransferases required for LD generation [17, 18] were resistant to Lda-mediated cytotoxicity. Interestingly, disruption of all neutral lipid biosynthesis in *lro1 dga1 are1 are2* mutants suppressed Lda2, but not Lda3, toxicity (Figure 4A). Because Lda2 was rapidly degraded in these LD-deficient mutants (Figure 4B), the stability, and by extension the function, of Lda2 is likely linked to LD binding or to a function performed by LDs. We mapped the minimal LD binding domain ($Lda2_{LD}$) of Lda2 to aa 1–99 and determined that this domain is essential for protein stability (data not shown). We hypothesize that Lda2 did not appear tropic for LDs when ectopically expressed in HeLa cells in our earlier experiments because degradation signals encoded at the carboxyl terminus of Lda2, combined with the low levels of LDs in epithelial cells, make this protein inherently unstable. In contrast, an $Lda2_{LD}$-EGFP chimera was stable and prominently localized to HeLa LDs (Figure 4C). Thus, as with Lda1 and Lda3, the LD binding properties of Lda2 are conserved from yeast to mammals.

We raised antibodies against Lda2 and determined its subcellular localization in infected cells. Like Lda1 and Lda3, Lda2 localized to structures enveloping the inclusion, and its detection by IF was detergent sensitive (Figure 4D and data not shown). To determine if translocated Lda2 associated with LDs, we purified LDs from infected and uninfected cells and detected Lda2 by immunoblots. A protein of Lda2 predicted size (~64 kDa) was detected only in LD fractions from infected cells (Figure 4E). These findings lead us to conclude that *C. trachomatis* secretes at least three proteins into the host cells to target LD-like structures enveloping the bacterial inclusion. Interestingly, Lda proteins ectopically expressed in infected cells also localize to the inclusion periphery (Figure S5), suggesting that these proteins, while capable of localizing to classical LDs, also bind to neutral-lipid-rich structures enveloping the inclusion.

Because our results suggested a role for LDs in chlamydial infection, we tested if these organelles were required for bacterial replication. The inhibitor triacsin C prevents LD formation by specifically inhibiting the activity of a subset of long chain acyl-coA synthetases (ACSL) required for triacylglyceride and cholesterol

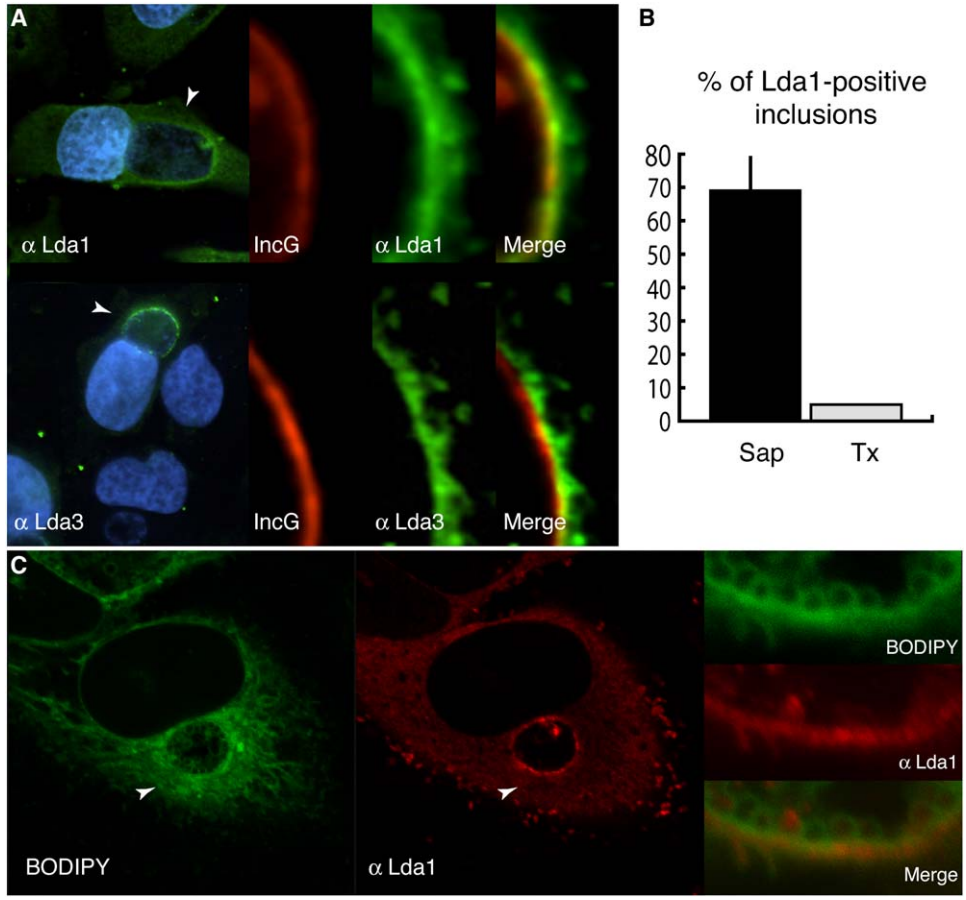

Figure 3. Lda Proteins Are Translocated into the Host Cell Cytoplasm and Associate with Neutral-Lipid-Rich Structures

(A) Immunolocalization of Lda1 and Lda3. LGV-L2-infected cells were immunostained with antibodies specific to Lda1 and Lda3. Bacterial and host DNA was detected by staining with Hoechst 33352 (blue). Lda1 and Lda3 (green) were found predominantly in close association with the inclusion (arrowheads). Deconvolution microscopy of LGV-L2-infected cells labeled with anti-Lda and anti-IncG antibodies indicated that Lda1 and Lda3 localized to structures adjacent but distinct from the IM (right panels).

(B) Lda1 localization to the inclusion periphery is sensitive to detergent permeabilization. LGV-L2-infected HeLa cells were fixed and permeabilized with 0.05% saponin or 0.1% Triton X-100 and immunostained with anti-Lda1 and -IncG antibodies. The percentage of Lda1-positive IncG-stained inclusions (n = 150) was measured. The pixel intensity of Lda1 staining around the inclusion is represented as the average of three independent experiments with standard errors shown.

(C) Lda1 colocalizes with neutral-lipid-rich structures enveloping the inclusion. HeLa cells were infected, labeled with BODIPY 493/503, and stained with anti-Lda1 antibodies. Note partial colocalization between Lda1- and BODIPY-positive structures (inset).

ester biosynthesis [19]. We examined the effect of triacsin C in cells infected with *C. trachomatis* (Figure 4F) and determined that this inhibitor greatly reduced chlamydial proliferation and this effect was comparable to that of AACOCF3, a $cPLA_2$ inhibitor that blocks chlamydial phospholipid uptake [5]. In triacsin C-treated cells, inclusions were small and misshapen, although expression of IncG, which is expressed shortly after chlamydial entry, indicated that the block in chlamydial replication occurred postinvasion (Figure S6).

Interestingly, triacsin C inhibited chlamydial growth in Hep2 cells 20 ± 1.5-fold more potently than *S. typhimurium* (Figure 4F, inset), suggesting that this inhibitor is not causing a general cellular lipid imbalance that would nonspecifically limit replication of intracellular bacteria. Although we cannot presently exclude the possibility of a nonspecific effect of triacsin on chlamydial metabolism, the absence of an ACSL homolog in *C. trachomatis* and the high in vivo specificity of triacsin C for specific subclasses of highly related mammalian ACSLs render it unlikely that the impact of triacsin C on chlamydial growth is due to the inhibition of a bacterial enzyme.

LD Function and Chlamydial Biology

Why does *C. trachomatis* target LDs? The simplest interpretation is that LDs act as conduits for the transport of neutral lipids to the inclusion. More complex possibilities are suggested by recent proteomic analysis of LDs, which revealed that mammalian LDs are enriched for proteins associated with vesicular transport (e.g., Rab proteins), signaling, and the cytoskeleton [20–22]. The presence of these proteins in LDs challenges the notion that these organelles are passive lipid storage depots and raises the intriguing possibility that LDs may regulate cellular functions ranging from signaling to membrane transport [1]. Therefore, by harnessing LD functions *C. trachomatis* may also co-opt non-vesicle-mediated phospholipids and cholesterol transport [23],

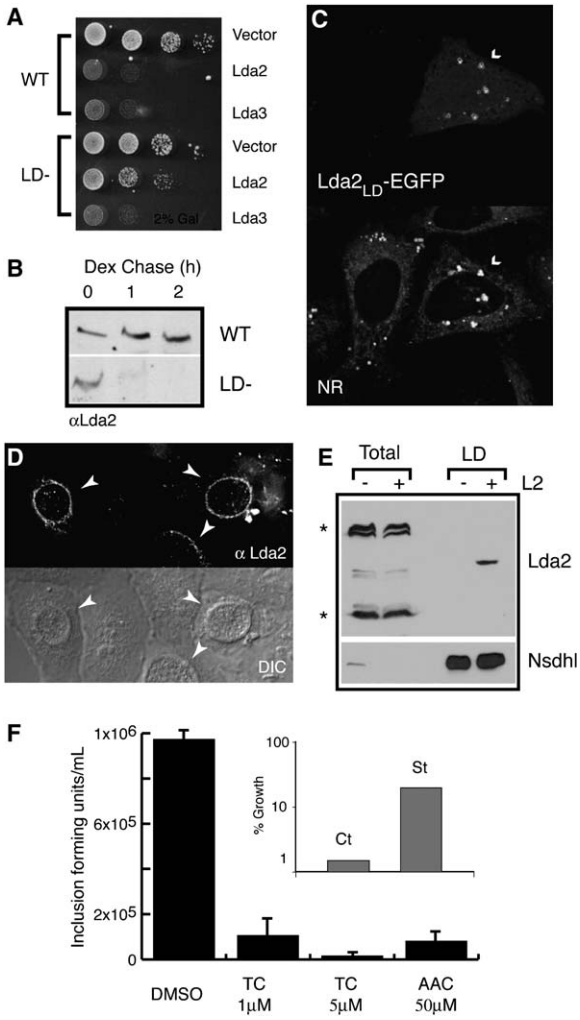

Figure 4. LDs Regulate Lda2 Stability, and Host Neutral Lipid Biosynthesis Is Required for Chlamydial Replication

(A) Lda2 cytotoxicity in yeast is suppressed in mutants lacking LDs. Ten-fold serial dilutions of wild-type (WT) or LD-deficient (LD−) yeast strains expressing Lda2 or Lda3 under the control of the *GAL1* promoter were spotted on 2% galactose plates to assess Lda-dependent toxicity.

(B) Lda2 is unstable in the absence of LDs. Lda2 stability in wild-type and LD-deficient yeast strains was determined by expressing Lda2 for 7 hr in CSM-Uracil + 0.5% galactose, followed by replacing the media with CSM-Uracil + 2% dextrose ("Dex Chase"). Levels of Lda2 proteins were assessed by Western blots using anti-Lda2 antibodies.

(C) The N terminus of Lda2 mediates LD binding. Amino acids 1–99 of Lda2 ($Lda2_{LD}$) containing the minimum LD binding domain were fused to EGFP and expressed in HeLa cells. $Lda2_{LD}$ localized to NR-positive LDs (arrows).

(D) Lda2 localizes to the periphery of the chlamydial inclusion. Infected HeLa cells were fixed, permeabilized with 0.05% saponin, and immunostained with anti-Lda2-specific antibodies. Arrows in corresponding DIC images show localization of inclusions.

(E) Lda2 is enriched in LD fractions from infected cells. LDs were isolated from infected cells, normalized to protein content, and resolved by SDS PAGE. Immunoblots with anti-Lda2 antibodies show a single immunoreactive band specifically enriched in LDs from infected cells. Nsdhl is shown as a LD fractionation control. *Crossreactive bands.

(F) Host neutral lipid biosynthesis is required for *C. trachomatis* replication. Hep2 cells were infected with LGV L2 in the presence of the indicated concentrations of triacsin C or the $cPLA_2$ inhibitor AACOCF3 for 40 hr. Bacterial replication was assessed by the generation of infectious units as outlined in the Supplemental Data. Inset: Growth inhibition of *S. typhimurium* (St) and LGV L2 (Ct) in triacsin-treated cells represented as a percent growth in untreated cells.

Chlamydial growth in the presence of indicated inhibitors was measured as described in the Supplemental Data. Values shown are an average of three independent experiments with standard error shown.

membrane dynamics, and/or lipid-mediated signaling pathways. Alternatively, the proliferation of LDs at the chlamydial inclusion may constitute an example of "organelle mimicry," whereby the inclusion is cloaked in LD-like structures and thus excluded from fusion with degradative compartments.

In summary, our observations indicate that LD-like structures accumulate at the chlamydial inclusion, and at least three LD-tropic chlamydial proteins are secreted across the IM. Furthermore, LDs are required for Lda2 stability, and inhibition of neutral lipid biosynthesis impairs chlamydial replication. These results strongly suggest that *C. trachomatis* targets LDs as a pathogenic strategy. These findings provide a unique opportunity to explore the function of these understudied organelles and unravel the molecular mechanisms of organelle subversion by *Chlamydiae*.

Experimental Procedures

Cell Lines, Yeast Strains, Reagents, and Antibodies

Hep2 and HeLa cells were obtained from ATCC. Yeast growth conditions and screens for Lda proteins are described in the Supplemental Data. For Lda suppression analysis, the congenic yeast strains G175 (MATα *ADE2 his3 leu2 ura3 trp1*) and H1246 (MATα *ADE2 ura3 dga1::KanMX4 lro1::TRP1 are1::HIS3 are2::LEU2*) [24] were used. Triacsin C, AACOCF3, and nocodazole were purchased from Biomol International L.P., (PA), Calbiochem (CA), and Sigma-Aldrich (MO), respectively. Antibodies against synthetic peptides derived from Lda1 (RNTNRENREFHHHDQDRT) and Lda3 (ESPDDRTVPHTQETS) were generated in hens (Aves Labs Inc., OR). Antibodies against GST-Lda2 were generated in rabbits (Covance Immunology Services, PA). The specificity of anti-Lda antibodies was tested against recombinant proteins expressed in yeast. Additional antibodies and methods used are described in the Supplemental Data.

Microscopy

Images were acquired with a Leica TCS SL confocal microscope and a Zeiss Axioskop 2 mot plus epifluorescence microscope equipped with an ORCA ER Hamamatsu CCD camera. Detailed IF methods are described in the Supplemental Data.

LD Analysis

LDs were isolated from yeast cells as described earlier [25]. LDs were isolated from infected and uninfected Hep2 cells by a modification of previously described protocol [22]. Briefly, cells were washed with PBS and harvested by scraping in TNE (20 mM Tris.Cl [pH 8.0], 120 mM NaCl, 2 mM EDTA) containing a protease inhibitor cocktail (Roche Diagnostics, Germany). The cells were lysed on ice with a Dounce homogenizer, and cell lysates were adjusted to 0.45 M sucrose. The lysate was overlaid with 2 ml each of 0.25 M, 0.15 M, and 0 M sucrose/TNE and centrifuged at 30,000 rpm for 90 min in a SW41 rotor (Beckman Coulter Inc., CA). The floating lipid layer was collected and centrifuged at 45,000 rpm for 45 min in a TLA55 rotor on a tabletop ultracentrifuge (Beckman Coulter, CA). For protein analysis, pure LDs were delipidated twice with 10 volumes of diethylether, and the proteins were precipitated from the aqueous layer with ice-cold acetone and dried before dissolving in 0.1% SDS/0.1 N NaOH. For neutral lipid analysis, lipids were extracted from

LDs with diethylether and dried under a stream of N_2. Lipids were redissolved in a small volume of ether and analyzed by thin-layer chromatography on glass-backed HPTLC silica plates (Merck, USA). Neutral lipids were separated in a solvent mix of 70:30:1 v/v/v (hexane: diethylether: acetic acid) and stained with 0.1% primuline (Sigma). Fluorescence was captured on a Typhoon laser scanner (GE Healthcare).

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/16/1646/DC1/.

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