Chlamydia Subversion of Host Lipid Transport: Interactions with Cytoplasmic Lipid Droplets

by

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Department of Molecular Genetics and Microbiology
Duke University

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Dr. Joseph St. Geme III

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular Genetics and Microbiology in the Graduate School of Duke University

2009
ABSTRACT

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Abstract

The *Chlamydiaceae* are Gram-negative, obligate intracellular bacteria that are significant pathogens of humans and animals. Intracellularly, the bacteria reside in a membrane-bound vacuole, called the inclusion, from which they manipulate host processes to create a niche optimal for survival and propagation. Acquisition of host-derived lipids is essential for chlamydial growth, yet the source of lipids and mechanisms of trafficking to the inclusion are not well-established. The inclusion avoids interaction with several classical membrane and lipid transport pathways. In a functional genomic screen to identify host modulating chlamydial proteins, our lab identified cytosolic lipid droplets (LDs) as potential target organelles of *Chlamydia*. LDs are postulated to function in many cellular processes, such as lipid metabolism and transport, membrane trafficking, and cell signaling; therefore, we hypothesized that LDs may be important for *Chlamydia* pathogenesis as a source of lipids or as a platform for regulating other cellular functions. Here, we characterize the interaction between eukaryotic LDs and the chlamydial inclusion.

We find that LDs are recruited to the *Chlamydia* inclusion, chlamydial infection disrupts neutral lipid homeostasis, and pharmacological prevention of LD formation inhibits chlamydial replication. *Chlamydia* produces proteins (Ldas) that localize with LDs in yeast and mammalian cells when transiently expressed and are exported out of
the inclusion to peripheral lipid-rich structures during infection. By electron microscopy and live cell imaging, we observe the translocation of intact LDs into the *Chlamydia* inclusion lumen. Biochemical and microscopic analysis of LDs from infected cells reveals that LD translocation may occur at specialized subregions of the inclusion membrane. The *Chlamydia* Lda3 protein is implicated in LD tethering to the inclusion membrane, and displacement of the protective coat protein, ADRP, from LD surfaces. This phenomenon could provide access for lipases to the LD core for utilization by the replicating bacteria. Additionally, the functional domains of Lda3 involved in binding to LD and inclusion membranes are identified.

In these studies, we identify eukaryotic lipid droplets (LDs) as a novel target organelle important for *Chlamydia* pathogenesis and describe a unique mechanism of whole organelle sequestration not previously observed for bacterial pathogens. These results represent a fundamental shift in our understanding of host interactions with the chlamydial inclusion, and may represent a new area for research in the field of cellular microbiology.
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<td>AACOCF3</td>
<td>arachidonyltrifluoromethyl ketone</td>
</tr>
<tr>
<td>ACSL</td>
<td>acyl-CoA synthetase</td>
</tr>
<tr>
<td>ADRP</td>
<td>apidocyte-related protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATGL</td>
<td>adipose triglyceride lipase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BFA</td>
<td>brefeldin A</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CADD</td>
<td><em>Chlamydia</em> associating with death domain protein</td>
</tr>
<tr>
<td>CE</td>
<td>cholesterol ester</td>
</tr>
<tr>
<td>CL</td>
<td>cardiolipin</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CPAF</td>
<td><em>Chlamydia</em> protease-like factor</td>
</tr>
<tr>
<td>cPLA2</td>
<td>cytosolic phospholipase A2</td>
</tr>
<tr>
<td>CSM</td>
<td>complete synthetic medium</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy terminal</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>EB</td>
<td>elementary body</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GEF</td>
<td>GTP exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GRP94</td>
<td>glucose-regulated protein of 94 kDa</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione sepharose transferase</td>
</tr>
<tr>
<td>HPTLC</td>
<td>high-performance thin layer chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IFU</td>
<td>infection forming units</td>
</tr>
<tr>
<td>IM</td>
<td>inclusion membrane</td>
</tr>
<tr>
<td>Inc</td>
<td>inclusion membrane protein</td>
</tr>
<tr>
<td>IncA</td>
<td>inclusion membrane protein A</td>
</tr>
<tr>
<td>IncG</td>
<td>inclusion membrane protein G</td>
</tr>
<tr>
<td>LB</td>
<td>luria bertani</td>
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<tr>
<td>LBPA</td>
<td>lysobisphosphatidic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LCFA</td>
<td>long chain fatty acid</td>
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<tr>
<td>LD</td>
<td>lipid droplet</td>
</tr>
<tr>
<td>Lda</td>
<td>lipid droplet-associated protein</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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<tr>
<td>LGV</td>
<td>lymphogranuloma venereum</td>
</tr>
<tr>
<td>LSCM</td>
<td>laser scanning confocal microscopy</td>
</tr>
<tr>
<td>LTP</td>
<td>lipid transfer protein</td>
</tr>
<tr>
<td>lyso-PL</td>
<td>lysophospholipid</td>
</tr>
<tr>
<td>MAT</td>
<td>mating type locus</td>
</tr>
<tr>
<td>MEK</td>
<td>map-erk kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MOMP</td>
<td>major outer membrane protein</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organizing center</td>
</tr>
<tr>
<td>MVB</td>
<td>multivesicular body</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NR</td>
<td>nile red</td>
</tr>
<tr>
<td>PAGFP</td>
<td>photoactivatable GFP</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
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<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PID</td>
<td>pelvic inflammatory disease</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PL</td>
<td>phospholipid</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PZ</td>
<td>plasticity zone</td>
</tr>
<tr>
<td>RB</td>
<td>reticulate body</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SL</td>
<td>scanning laser</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble NSF-sensitive attachment receptor</td>
</tr>
<tr>
<td>SPG</td>
<td>sucrose/ phosphate/ glutamic acid</td>
</tr>
<tr>
<td>T3SS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
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</table>
Acknowledgements

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Chapter 1. Introduction

1.1. Chlamydiae and human disease

The *Chlamydiales* is an order of Gram-negative bacteria unified by their obligate, intracellular lifestyle and unique biphasic developmental cycle (Horn, 2008). The diversity of this order has expanded over the last decade from one identified family, the *Chlamydiaceae*, to eight currently recognized families (Horn, 2008). This expansion was sparked by the identification of chlamydia-like organisms as symbionts of amoebae (Amann et al., 1997; Fritsche et al., 2000) and the implementation of new standards for taxonomic classification (Everett et al., 1999). The size of chlamydiae genomes is relatively small, ranging from ~1-2.4 MB (Horn et al., 2004). With the advances in methods for high-throughput sequencing, the genomes of several chlamydiae species and subtypes have recently become available (Horn et al., 2004; Kalman et al., 1999; Thomson et al., 2008; Thomson et al., 2005). *Chlamydia* genomes display a remarkably high degree of similarity (Read et al., 2000). Despite this, *Chlamydia* species and serovars vary in terms of host and tissue tropism and the modulation of host cellular processes (Carlson et al., 2004; Carlson et al., 2005; Nelson et al., 2006).

**Chlamydial diseases in human health**

Though the natural host reservoirs vary widely for different families of Chlamydiae, many species cause disease in humans and animals (Corsaro and Greub,
Two members of the Chlamydiaceae family, *Chlamydia trachomatis* and *Chlamydophila pneumoniae*, are well-known human pathogens that have a significant impact on global public health. *C. trachomatis* is subdivided into fifteen serovars which differ in their tropism for host tissues and related disease sequelae (Carlson et al., 2005; Schachter, 1999). Serovars A-C primarily infect conjunctival epithelium while D-K more commonly infect the genital epithelium. The L1, L2, and L3 genital serovars are more invasive and spread beyond mucosal epithelia to the inguinal lymph nodes causing lymphogranuloma venereum (LGV) (Ward et al., 2007).

The progression of ocular infections leading to trachoma is initiated by inflammation of the conjunctivae, followed by eyelash inversion, corneal scratching and eventual blindness if untreated (Schachter, 1999). Currently, an estimated 84 million people are afflicted with active trachoma, and trachoma accounts for 3% of global blindness, making it the leading cause of infection induced blindness worldwide (Burton, 2007; Resnikoff et al., 2004).

*C. trachomatis* is also the most common sexually transmitted pathogen in the United States, and the prevalence of reported infection continues to rise annually (CDC, 2008). Complications of acute genital infections include epididymitis, cervicitis, and male urethritis (Schachter, 1999). Initial infection is often asymptomatic which leads to lack of treatment and infection progression to serious complications such as pelvic
inflammatory disease (PID), ectopic pregnancy, and infertility (Schachter, 1999).

Treatment of sexually transmitted diseases (STDs) stemming from chlamydial infection is also a large economic burden. In 2000, the total direct medical costs of sexually acquired Chlamydia infections in men and women age 15-24 were estimated at $250 million in the U.S. alone (Chesson et al., 2004).

* C. pneumoniae is widespread respiratory tract pathogen. The prevalence of serum antibodies to *C. pneumoniae* indicates that more than 60% of adults have been exposed to this pathogen, and this estimate climbs to 80% in the elderly (Grayston et al., 1990). *C. pneumoniae* is frequently associated with community-acquired pneumoniae, potentially accounting for anywhere between 6-22% of cases depending on the conditions of the study (Hammerschlag, 2000). Infection with *C. pneumoniae* has also been implicated in the development of several chronic inflammatory diseases including atherosclerosis, multiple sclerosis, and Alzheimer’s disease (Blasi et al., 2009; Borel et al., 2008; Gerard et al., 2005). This is likely due to pathogenic consequences of *C. pneumoniae* infection. For instance, *C. pneumoniae* promotes inflammation by inducing elevated secretion of inflammatory cytokines including TNFα, IL-6, and IL-8 from infected cells (Belland et al., 2004; Campbell and Kuo, 2004).

Currently, there is no effective vaccine against chlamydial pathogens, and statistical data show that the incidence of Chlamydia infection has steadily risen over the
last decade despite the implementation of screening and antibiotic control programs (CDC, 2008). Greater understanding of basic chlamydial biology is clearly an important endeavor toward being able to develop vaccines and identify new targets for drug intervention. While progress has been slowed by the inability to genetically manipulate *Chlamydia*, great strides have been made in recent years through comparative genomics and functional analysis of chlamydial proteins.

1.2. The developmental cycle of chlamydiae

Chlamydiae are distinguished from other bacteria by their unique, biphasic developmental cycle. Successful propagation of the bacteria is dependent on proper temporal progression of the developmental cycle. During the course of infection, *Chlamydia* undergoes conversion between two structurally and functionally distinct developmental forms, the elementary body (EB) and the reticulate body (RB). Many features of the cycle, most notably the time-span, can differ between serovars and species, but the basic sequence of events is the same for all and include: I) attachment and internalization, II) EB to RB differentiation, III) bacterial replication and remodeling of the parasitophorous vacuole, or “inclusion”, IV) inclusion expansion and transition of RB into EB, and V) release of bacteria from the host cell and infection of new target cells by EBs (Figure 1).
Attachment and entry

The EB, the infectious form of the bacteria, mediates attachment and is internalized by the host cell (Abdelrahman and Belland, 2005). As the form responsible for dissemination, EBs are tailored for stability in the extracellular environment by
virtue of their extensively cross-linked outer-membrane proteins (Hatch, 1996; Newhall and Jones, 1983). The nucleoid of EBs is highly condensed by the chlamydial histone-like proteins, Hc1 and Hc2, rendering them transcriptionally inactive (Barry et al., 1993; Barry et al., 1992; Brickman et al., 1993).

Much effort has been directed at identifying the host receptors and bacterial ligands involved in attachment and uptake of *Chlamydia* by host cells; however, to date there are no consensus candidates for these factors (reviewed in (Dautry-Varsat et al., 2004; Dautry-Varsat et al., 2005). Electrostatic interactions and a variety of bacterial ligands such as glycosaminoglycan (GAG) (Menozzi et al., 2002), major outer membrane (MOMP) (Su et al., 1996), OmcB (Stephens et al., 2001), and PmpD (Wehrl et al., 2004) may play roles in attachment. Four host receptors- heparan sulfate, mannose receptor, mannose 6-phosphate receptor, and estrogen receptor- have been proposed for recognition of bacterial ligands and EB internalization (reviewed in (Campbell and Kuo, 2006). A more recent study showed that cell-surface protein disulfide isomerase (PDI) can complement the infectivity of the infection resistant CHO6 cell line, implicating it as a potential host receptor for *Chlamydia* attachment and entry (Conant and Stephens, 2007). Because entry into host cells is essential, it is likely that *Chlamydia* has evolved redundant mechanisms to ensure uptake regardless of host receptor availability. Indeed,
receptor utilization appears to vary depending on the host cell type and the species or serovar of *Chlamydia* being studied (Dautry-Varsat et al., 2005).

Following attachment, EBs are endocytosed by the host cell. The mechanisms of internalization remain a controversial topic due to numerous conflicting studies (reviewed in (Dautry-Varsat et al., 2005). A recent study using targeted knock-down of factors involved in several modes of entry showed that clathrin-dependent endocytosis are important for chlamydial internalization by non-phagocytic cells (Hybiske and Stephens, 2007a). This process is facilitated by bacteria-directed activation of the Rho-GTPase, Rac1, which causes actin recruitment and reorganization at the entry site (Carabeo et al., 2004).

**EB to RB differentiation**

Following internalization, outer membrane disulfide bonds are cleaved (Hackstadt et al., 1985), and histone-DNA interactions are disrupted by an intermediate metabolite in a pathway of isoprenoid biosynthesis (Grieshaber et al., 2004). These changes lead to nuclear decondensation, and activation of bacterial transcription occurs rapidly. Within 15 minutes, new bacterial proteins are already being produced and RNA expression can be detected as early as one hour (Belland et al., 2003; Plaunt and Hatch, 1988; Shaw et al., 2000). Little is known, however, about the developmental cues or mechanisms involved in the conversion the conversion of EBs into RBs.
Replication and inclusion remodeling

RBs replicate by binary fission within the confines of the parasitophorous vacuole. Since it is host-derived, the inclusion membrane initially contains components of the plasma membrane, but these markers are promptly lost within 30 min after bacterial entry (Scidmore et al., 2003). Early on, the inclusion is trafficked along microtubules in a dynein-dependent manner to the perinuclear region of the host cell (Grieshaber et al., 2003). Through active modification by the bacteria, the nascent inclusion disassociates from the endosomal-lysosomal pathway avoiding degradation, but retains access to specific compartments, such as Golgi-derived exocytic vesicles, that provide nutrients and metabolites essential for survival and growth (as reviewed in (Fields and Hackstadt, 2002).

RB to EB transition and host cell exit

As Chlamydia replicates, the inclusion expands until almost the entire cytoplasmic volume of the host cell is filled by the inclusion. Midway through infection, chlamydial replication becomes asynchronous as RBs start to differentiate back into EBs. Similar to the early conversion of EBs to RBs, the events that trigger RB to EB transition are unknown. Bavoil and colleagues have proposed that the dissociation of T3S apparatus from the inclusion membrane as RBs detach may initiate events involved in this process (Bavoil et al., 2000; Wilson et al., 2006). At the end of the developmental
cycle, EBs are released from the cell by either lysis or extrusion of the inclusion, and spread to infect to new host cells (Hybiske and Stephens, 2007b).

1.3. Cell biology of Chlamydia infections

Having parasitized eukaryotic cells for more than 700 million years (Horn et al., 2004), Chlamydia is particularly adept at exploiting host cellular processes to aid its intracellular development and survival. Bacterial protein synthesis is often required for the host modifications seen during Chlamydia infection. To interfere with host processes, chlamydial proteins must first access the host cytoplasm outside the protective barrier of the inclusion membrane. Genome sequencing has revealed that Chlamydiaceae encode for several components of protein secretion systems, including both sec-dependent (type II) and -independent (type III) pathways (Cianciotto, 2005; Fields et al., 2003). Without classical genetics, identification of translocated effector proteins and their related functions has been a challenge; however, combined studies using diverse alternative approaches including expression analysis (Belland et al., 2003; Nicholson et al., 2003), heterologous expression systems (Fields et al., 2003; Subtil et al., 2005), and functional genomics (Sisko et al., 2006) have significantly expanded our knowledge of how Chlamydia controls the host cell.

The host environment and developmental requirements of the bacteria both continuously change over the course of infection. Hence, it is predicted that effector
proteins are expressed and produced in a corresponding manner when their function is needed (Valdivia, 2008). Most of our knowledge about Chlamydia-host interactions is derived from work done with C. pneumoniae and various serovars of C. trachomatis. However, these interactions can differ depending on the species or serovar of Chlamydia being studied. Cytoskeletal dynamics, vesicular traffic, apoptosis and immune signaling are cellular processes frequently modulated by Chlamydia.

**Cytoskeletal remodeling**

Attachment and entry of EBs into the cell is mediated by the recruitment of host actin at the attachment site which leads to formation of pedestal-like structures and internalization of the bacteria (Carabeo et al., 2002). The chlamydial protein Tarp is translocated early in infection by the type III secretion system (T3S), and contributes to actin remodeling (Clifton et al., 2005; Jewett et al., 2006). The N-terminal and C-terminal domains of Tarp have been implicated in separate indirect and direct mechanisms of actin nucleation, respectively. Residues in the N-terminal tyrosine-rich tandem repeats of Tarp are phosphorylated shortly after translocation by host kinases (Jewett et al., 2008). This promotes association with the guanine nucleotide exchange factors (GEFs), Sos1 and Vav2, which activate Rac1 and downstream signaling events that result in actin reorganization (Carabeo et al., 2007; Carabeo et al., 2004; Lane et al., 2008). Although Tarp phosphorylation appears to play a role in actin rearrangement by C. trachomatis,
Tarp from other *Chlamydiaceae* species is not phosphorylated suggesting that alternate mechanisms exist to control host actin (Clifton et al., 2005). Consistent with this, a WH2-like domain in the C-terminal portion of Tarp can directly bind actin subunits and promote nucleation (Jewett et al., 2006). These observations lend support to the idea that multiple, overlapping mechanisms exist to ensure *Chlamydia* entry.

*Chlamydia* also disrupts microtubule function. Around two hours after entry, the nascent inclusion moves from the host cell periphery to a perinuclear region near the microtubule-organizing center (MTOC) (Clausen et al., 1997). The minus-end directed motor protein dynein is essential for migration of the inclusion along microtubules, but the host vesicular cargo-linking machinery is not required (Grieshaber et al., 2003). Bacterial proteins are likely involved in mediating inclusion linkage to dynein, but no candidates have been identified thus far.

The heterologous expression of the *C. pneumoniae* effector protein CopN in yeast and mammalian cells causes arrest at the G2/M phase of the cell cycle (Huang et al., 2008). Arrest appears to be due to a disruption of microtubules which inhibits spindle apparatus formation. Whether endogenous CopN functions to disrupt microtubules *in vivo* during infection was not determined; however, *Chlamydia* interference with the host cell cycle has been described (Balsara et al., 2006; Greene and Zhong, 2003).
As RBs replicate, the developing inclusion must expand in order to accommodate the growing number of bacteria. One might predict that the inclusion would become unstable with the rapid increase in size; however, the structural integrity of the inclusion is maintained until late in the infection cycle. Recent work reveals that the inclusion is enveloped by a meshwork of host cytoskeletal structures primarily composed of F-actin and intermediate filaments (IFs) (e.g. vimentin) (Kumar and Valdivia, 2008). These structures act in a coordinated fashion as the recruitment of IFs is dependent on the recruitment of F-actin at the inclusion. The IFs surrounding the inclusion are cleaved by CPAF, a secreted chlamydial protease, which modifies the IF structural properties and creates a network that is simultaneously flexible and strong (Kumar and Valdivia, 2008).

**Vesicular traffic**

Throughout the developmental cycle, the inclusion is able to selectively interact with exocytic Golgi traffic to obtain essential nutrients and metabolites, but is nonfusogenic with endocytic compartments (Fields and Hackstadt, 2002; Hackstadt et al., 1996; Scidmore et al., 2003). Association of host Rab GTPases with the inclusion has been postulated to coordinate inclusion contact with host organelles (Brumell and Scidmore, 2007; Moorhead et al., 2007; Rzomp et al., 2003). Rab GTPases are essential components of eukaryotic membrane trafficking machinery which regulate vesicle transport and fusion events and maintain organelle identity (Seabra and Wasmeier,
Rab protein association with the inclusion is selective for recycling endosome and Golgi related Rabs 1, 4, and 11, while early and late endosomal markers while Rabs 5, 6 and 7 are excluded (Rzomp et al., 2003). Additionally, association of Rab6 or Rab10 with inclusions occurs in a species-specific manner implying that bacterial factors may be involved in directing Rab recruitment (Rzomp et al., 2003). Indeed, recent studies provide evidence that a family of bacterial proteins, known as Incs, contribute to this process. Incs are integral membrane proteins which share a common bi-lobal hydrophobic motif that targets them to the inclusion membrane (Bannantine et al., 2000). The C. trachomatis Inc, Ct229, was identified as a Rab4 binding partner (Rzomp et al., 2006), and the C. pneumoniae Inc, Cpn0585, was shown to interact with Rabs 1,10, and 11 (Cortes et al., 2007). In both studies, Inc binding was dependent on Rabs being in the active GTP-bound conformation suggesting that these complexes would be functional (Betts et al., 2009). Saturation of Rab11 binding by ectopically overexpressed Cpn0585 inhibited inclusion development demonstrating the importance of Rab-Inc interactions in chlamydial growth (Cortes et al., 2007).

SNARE (soluble NSF-sensitive attachment receptor) proteins, which regulate host vesicular fusion events, also appear to be targeted by Chlamydia. A recent study evaluated potential “mimicry” of host SNARE proteins by several Inc proteins including IncA, Ct223, and Ct813 which contain SNARE-like motifs (Delevoye et al., 2008). IncA
and CT813 directly bound to a group of host SNAREs in vitro, and the recruitment of these SNAREs to the inclusion during infection was reduced when IncA was absent (Delevoye et al., 2008).

**Apoptosis regulation**

The effect of *Chlamydia* infection on the host apoptotic signaling programs is complex, and mechanisms for both inhibition and induction of host cell death are described in the literature (reviewed in (Byrne and Ojcius, 2004). It has been postulated that *Chlamydia* modulates apoptosis in a temporal manner to ensure progression of a productive developmental cycle by preventing the host from dying early during in infection, and inducing host-cell death late in the cycle potentially to aid in bacterial release (proposed in review by (Ying et al., 2007).

*Chlamydia* primarily inhibits apoptosis signaling pathways that involve cytochrome c (Cyt c) release from the mitochondria (Fan et al., 1998). Cyt c release during apoptosis is regulated by the Bcl-2 family of proteins which include anti-apoptotic Bcl-2 like proteins, the pro-apoptotic BH3-only proteins, and Bax/Bak group of proteins (reviewed in (Hacker and Weber, 2007). Numerous studies have found that *Chlamydia* targets BH3-only proteins for degradation (Dong et al., 2005; Fischer et al., 2004b; Ying et al., 2005) which likely leads to the observed reduction in Bax and Bak activation (Fischer et al., 2004a) and subsequent block in Cyt c release. Recently, the
Chlamydial protease CPAF was implicated in the degradation of BH3-only proteins providing a potential mechanism for inhibition of host apoptosis (Pirbhai et al., 2006). The *Chlamydia*-induced degradation of the BH3-protein Bim has also been attributed to a non-specific proteasome-dependent mechanism (Fischer et al., 2004b).

Alternately, *Chlamydia* can promote host cell survival by preventing interaction of the pro-apoptotic host factors, phosphorylated BAD and protein kinase Cδ (PKCδ), with mitochondria (Tse et al., 2005; Verbeke et al., 2006). Sequestration of phosphorylated BAD occurs through its interaction with the 14-3-3β adapter protein which is recruited to the inclusion membrane by the bacterial IncG protein (Scidmore and Hackstadt, 2001; Verbeke et al., 2006). PKCδ is similarly recruited away from its normal site of action to the inclusion by binding to diacylglycerol which is accumulated at the periphery (Tse et al., 2005).

Other potential anti-apoptotic mechanisms by *Chlamydia* have been suggested to involve activation of the transcription factor NF-κB. Although NF-κB is mostly noted for its role in immune regulation (reviewed in (Hayden et al., 2006), some NF-κB target genes such as Bcl-Xl and the modulator of caspase 8 activation, cFLIP, have anti-apoptotic functions (Kanetaka et al., 2008). The data on NF-κB activation by *Chlamydia* infection, however, have been somewhat difficult to resolve. NF-κB activation only occurred in certain cell types, mostly innate immune effectors such as macrophages.
(Wahl et al., 2001) and dendritic cells (Prebeck et al., 2001), but was not observed in epithelial cells (Fischer et al., 2001; Xiao et al., 2005), the primary target of Chlamydia infections.

Studies characterizing the molecular details of host-cell death induction in Chlamydia-infected cells at late stages of the developmental cycle reveal that, although very similar to apoptosis, it does not involve cyt c release or caspase activation and may be a distinct mechanism (Ying et al., 2006). One chlamydial effector protein, Chlamydia protein associating with death domain or CADD, was identified bioinformatically and shown to interact with the death domains of several TNF family receptors (Stenner-Liewen et al., 2002). CADD is expressed late in the developmental cycle, and ectopic expression is sufficient to induce Fas-related apoptosis (Stenner-Liewen et al., 2002). The biological relevance of this, however, is unknown since endogenous expression of this protein during infection does not appear to be sufficient to induce apoptosis (Fan et al., 1998).

Evasion of host immune response

Similar to many of the host processes discussed, Chlamydia seems to have many different means of interfering with host innate and adaptive immune responses. The downregulation of IFNγ-inducible and constitutive MHC class I and II antigen presentation via degradation of the transcription factors USF-1 and RFX-5 during
infection is one of the most prominent examples (Zhong et al., 1999; Zhong et al., 2000). A later study showed that the secreted chlamydial protease CPAF was responsible for the degradation of both USF-1 and RFX-5 providing one of the first illustrations of direct regulation of host function by a bacterial protein (Zhong et al., 2001). Furthering this theme, CPAF was recently shown to degrade an immature glycosylated isoform of CD1d, a MHC-like molecule important in lipid antigen presentation (Kawana et al., 2007).

Interference with NF-κB and its related signaling pathways by *Chlamydia* is another emerging theme that may play a role in bacterial modulation of host immune surveillance. The NF-κB transcription factor is essential in several facets of host innate and adaptive immunity (reviewed in (Hayden et al., 2006). Recent findings present two new mechanisms by which chlamydial effectors may inhibit the function of NF-κB.

The NF-κB transcription factor member RelA (p65) and p50 are often found as a heterodimeric complex that acts as a transcriptional activator (reviewed in (Hayden et al., 2006). During *Chlamydia* infection, the RelA subunit is proteolyzed and its translocation to the nucleus is inhibited (Lad et al., 2007a). The *C. trachomatis* protein CT441 was identified as the protease responsible for RelA cleavage, and ectopic CT441 expression blocked TNF-α-induced NF-κB activation in human cells (Lad et al., 2007a; Lad et al., 2007b).
*Chlamydia* may also block NF-κB activation by regulating ubiquitination. The ubiquitin-mediated protein degradation pathway is centrally important for a broad range of host processes (reviewed in (Glickman and Ciechanover, 2002). NF-κB activation and nuclear translocation is dependent on the degradation of its inhibitor IκBα which is accomplished via ubiquitin-mediated proteolysis (Sun and Ley, 2008). Two chlamydial proteins, ChlaDub1 and ChlaDub2, were recently identified as effectors with deubiquitinating and deneddylating activity (Misaghi et al., 2006). Subsequently, it was found that ChlaDub1 specifically bound to IκBα and inhibited its ubiquitination and degradation, and expression suppressed NF-κB activation (Negrate, 2008).

**Remaining challenges**

Recent breakthroughs have provided exciting clues about how *Chlamydia* controls host cell function. However, it is becoming increasingly clear that we still have a long way to go in understanding the functions of chlamydial effectors. For instance, very few functions have been documented for Inc proteins compared to the ~50 that are believed to be encoded by the genome (Rockey et al., 2002). Also, some estimates predict that up to 10% of the *Chlamydia* genome may encode T3S effectors, but few have been identified (Subtil et al., 2005). In addition, CPAF substrates and targets of chlamydial deubiquitinating enzymes are largely unidentified.
1.4. Modulation of host lipid transport by *Chlamydia*

Defining the basic properties of the inclusion, including the source of its limiting membrane and its interactions with host organelles and metabolites, has been a major focus of research in the *Chlamydia* biology field (Hackstadt et al., 1997). *Chlamydia* inhibits interaction with the host endocytic pathway shortly after entry into the cell-failing to accumulate endocytic markers and preventing fusion of the inclusion with host lysosomes (Fields and Hackstadt, 2002; Scidmore et al., 2003). Despite apparent sequestration from host trafficking, *Chlamydia* retains access to many eukaryote-specific lipids, including sphingolipids (Hackstadt et al., 1996; Hackstadt et al., 1995; Moore et al., 2008), cholesterol (Carabeo et al., 2003; Wylie et al., 1997), phosphatidylcholine (PC), and phosphatidylinositol (PI) (Hatch and McClarty, 1998; Wylie et al., 1997). The acquisition of host lipids is essential for bacterial replication even though *Chlamydia* has the ability to synthesize many of its own membrane lipids (Su et al., 2004; van Ooij et al., 2000).

*Sphingolipid and cholesterol traffic to the inclusion*

Trafficking of sphingolipids to the inclusion was first observed by following the transport of a Golgi-specific probe, the fluorescent ceramide analog C6-NBD-cer, in L2-infected HeLa cells (Hackstadt et al., 1995). The probe and its metabolite, C6-NBD-sphingomyelin (formed by processing in the *cis*-Golgi) accumulated within the cell walls
of intra-inclusion *Chlamydia*. Uptake and incorporation of the sphingomyelin was inhibited by treatment with brefeldin A indicating that the bacterial inclusion is able to intercept Golgi-derived exocytic vesicles (Hackstadt et al., 1995). Later studies showed that this transfer came directly from the Golgi to the inclusion rather than from the plasma membrane (Hackstadt et al., 1996). A recent study confirmed that sphingomyelin trafficking to the *Chlamydia* inclusion occurs similarly between non-polarized and polarized cells; however, basolaterally directed transport pathways appears to be preferentially targeted (Moore et al., 2008). Sphingolipids are also acquired by *C. psittaci* and *C. pneumoniae* suggesting that this is a conserved feature of *Chlamydiae* infection (Wolf and Hackstadt, 2001).

Cholesterol has also been found within purified EBs and the inclusion membrane (Carabeo et al., 2003; Hatch and McClarty, 1998; Wylie et al., 1997). Cholesterol in eukaryotic cells can be synthesized *de novo* in the ER, or internalized from the extracellular milieu through uptake of LDL (Ikonen, 2008). Radiolabeled precursors squalene and cholesteryl linoleate/LDL were both incorporated into EBs when added to *Chlamydia*-infected cells, and inhibition of lysosomal LDL hydrolysis decreased the amount of radioactive label found in EBs. These data indicated that cholesterol is transported to the chlamydial inclusion by both cholesterol uptake pathways, albeit with different kinetics (Carabeo et al., 2003).
Evidence indicates that sphingomyelin and cholesterol transport to the inclusion is a vesicle-mediated event (Hackstadt et al., 1996). Delivery of these lipids is ATP- and temperature-dependent and sensitive to BFA-treatment. Nocodazole treatment only minimally decreases *Chlamydia* incorporation of fluorescent sphingomyelin indicating that microtubules are not involved in transport (Hackstadt et al., 1996). Microtubule-independence of this process has been disputed by studies that follow incorporation of radiolabeled cholesterol and sphingomyelin into EBs which is inhibited by nocodazole treatment (Carabeo et al., 2003). Since cholesterol and sphingomyelin are often found together in membrane microdomains (Ikonen, 2008), it has been hypothesized that these lipids may be transported together through the same pathway to the inclusion (Carabeo et al., 2003). Interestingly, *Chlamydia* appears to only intercept lipid from exocytic vesicles as glycoprotein processing and trafficking to the plasma membrane is not disrupted, and glycoproteins are not transported to the inclusion (Scidmore et al., 1996a).

Sphingomyelin and cholesterol acquisition are dependent on chlamydial gene expression and protein biosynthesis indicating active bacterial re-routing of lipid transport rather than passive transfer to the inclusion (Carabeo et al., 2003; Scidmore et al., 2003; Scidmore et al., 1996b).

**Glycerophospholipid transport to the inclusion**
In addition to sterols and sphingolipids, biological membranes are primarily composed of phospholipids including: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylinositol (PI) and cardiolipin (CL) (van Meer et al., 2008). Biochemical analysis of Chlamydia EBs revealed that, in addition to PE, PG, and PS which are generally found in bacteria, EBs contain PC and PI which are rarely synthesized by prokaryotes (Wylie et al., 1997). Infection does not cause a general disruption of glycerophospholipid composition; however, some lipids such as PE and PG are found at higher levels in purified RBs and EBs compared with mock-infected cells suggesting synthesis of these lipids by the bacteria. In contrast, bacterial PI and PC levels are similar to the control implying trafficking from the host cell (Wylie et al., 1997).

Phospholipids travel through eukaryotic cells from synthesis sites to intended destinations in a variety of ways including vesicular transport, soluble lipid transport proteins (LTP), and direct membrane contact sites (Holthuis and Levine, 2005; Sprong et al., 2001; Voelker, 1991). Chlamydial phospholipid acquisition is unaffected in cell lines lacking a nonspecific LTP, or by treatment with BFA and monesin, inhibitors of exocytic traffic from the Golgi (Wylie et al., 1997). Therefore, direct membrane contact is currently thought to be the main conduit for transfer of phospholipids to the inclusion from the host cell (Scidmore, 2006; Wylie et al., 1997). In support of this idea, purified
bacteria adopt a phospholipid composition similar to their host cells suggesting that *Chlamydia* can accept lipids from the host organelles it associates with during the course of infection with little restriction (Hatch and McClarty, 1998).

While sphingolipids and cholesterol isolated from bacteria are not modified (Hackstadt et al., 1995; Wylie et al., 1997), fatty acids in the Sn2-position of host glycerophospholipids are selectively exchanged with *Chlamydia*-derived branched chain fatty acids (Su et al., 2004; Wylie et al., 1997). Deacylation of glycerophospholipids is catalyzed by host Ca2+-dependent cytosolic phospholipase A2 (cPLA2) and the resultant lysophospholipid (lyso-PL) is transported into the inclusion for modification and incorporation into bacterial membranes. Activation of cPLA2 and the upstream kinases Raf, MEK, and ERK are increased during *Chlamydia* infection. Pharmacological inhibition of these enzymes led to decreased host phospholipid transport to the inclusion, as well as, reduced bacterial replication (Su et al., 2004).

At the time this project began, this was the state of the field in terms of our knowledge about how *Chlamydia* interacts with host lipid metabolism and transport. However, evidence existed that alternate transport pathways must also play a role. More recently, a pathway targeting multivesicular bodies (MVBs) has been described. MVBs are late endocytic organelles where proteins and lipids that are destined for degradation or recycling to the Golgi are sorted and processed (Woodman and Futter,
2008). Internal vesicles of MVBs are rich in lysobisphosphatidic acid (LBPA) and have been implicated in regulation of intracellular cholesterol transport and sphingolipid hydrolysis (Kobayashi and Hirabayashi, 2000). MVB protein components including CD63, LBPA, and MLN64 localize to the lumen of the *Chlamydia* inclusion, and CD63-positive vesicles are observed within the inclusion (Beatty, 2006). Inhibitors of MVB maturation result in decreased sphingolipid transport to the inclusion and reduced bacterial development. This work provided an alternative source for lipid acquisition by *Chlamydia* (Beatty, 2006).

**Significance of studying *Chlamydia* modulation of host lipid metabolism**

Many potential sources for sphingolipid and cholesterol have been described, but the source of host precursors for glycerophospholipid uptake still remain a mystery. Sphingolipid and cholesterol uptake by the inclusion is only partially sensitive to BFA-treatment, suggesting that non-vesicular transport is important in these transport events. Many pathways for lipid transport in eukaryotic cells are not well-characterized. Therefore, new insights may be gained by studying how *Chlamydia* co-opts host lipid transport. Since lipid acquisition is essential for the development and survival of *Chlamydia*, bacterial proteins that regulate this process may also be good candidates for drug and vaccine intervention.
Additionally, disregulation of lipid metabolism leads to the initiation and progression of diseases such as atherosclerosis and Alzheimer’s disease (reviewed in (Adibhatla and Hatcher, 2008; Martins et al., 2006). Many of these diseases have been associated with Chlamydia infection; therefore, a better understanding of how Chlamydia interferes with host lipid transport and metabolism will be invaluable to understanding its influence on the progression of these diseases.

Identifying chlamydial proteins that intersect host lipid metabolism

Since the standard genetic tools are not available to study Chlamydia, many of the factors involved in the basic processes of inclusion modification and acquisition of host lipids are not well-understood. At the initiation of this project, our lab was in the process of finishing a screen to identify bacterial factors that could disrupt basic cellular functions. Since many of these events are well-conserved between yeast and higher eukaryotes, we modeled the functions of chlamydial proteins by their subcellular localization and phenotypic effects in the yeast S. cerevisiae (Sisko et al., 2006). This screen revealed chlamydial proteins which localized to eukaryotic cytoplasmic lipid droplets (LDs) suggesting that these organelles may be modulated by Chlamydia. This interesting phenomenon was the basis for this PhD thesis.
Chapter 2. *Chlamydia* infection modulates host cell lipid droplet trafficking and function

2.1. Introduction

The import of host-derived membrane lipids to the *Chlamydia* inclusion is necessary for growth and development of the enclosed bacteria; however, the sources and pathways of lipid transport to the inclusion are not well-delineated. Exocytic Golgi vesicles and MVBs have been postulated to supply sphingolipids and cholesterol to the inclusion (Beatty, 2006; Carabeo et al., 2003; Hackstadt et al., 1996). In contrast, glycerophospholipid acquisition appears occur through non-vesicular pathways that do not require lipid transport proteins (Wylie et al., 1997). The acquisition of host lipids is unique to *Chlamydia* among intracellular pathogens and is essential for bacterial survival and development. We wanted to identify bacterial proteins involved in redirecting host lipid transport.

Since there are no tools for genetic disruption of protein function in *Chlamydia*, our lab employed the yeast *S. cerevisiae* for functional screening of individual *Chlamydia* proteins (Sisko et al., 2006). Yeast is a good model system in this situation since the basic mechanisms of protein and lipid trafficking are highly conserved among eukaryotes (Valdivia, 2004). Furthermore, the small genome has a high proportion of *Chlamydia*-specific genes (Stephens et al., 1998). We speculated that these proteins would be enriched in candidate proteins that impart *Chlamydia* with its unique pathogenic
characteristics. The results of this screen revealed a potential targeting of eukaryotic cytoplasmic lipid droplets (LDs) for modulation by *Chlamydia* (Sisko et al., 2006).

LDs are neutral lipid storage organelles found in all eukaryotic cells (Murphy, 2001). A hydrophobic core of neutral lipids, primarily triacylglycerols (TAG) and cholesterol esters (CE), enclosed by a phospholipid monolayer studded with proteins compose the basic LD structure (Martin and Parton, 2006; Thiele and Spandl, 2008). LDs were long considered passive lipid containers due to their discovery in cell types specialized for lipid storage, such as adipocytes and hepatocytes (Murphy, 2001). However, the roles of LDs in other cell types are starting to be explored and are revealing that these organelles potentially play roles in many cellular functions including lipid homeostasis, membrane trafficking, protein storage and degradation, and innate immune signaling (Bozza et al., 2007; Martin and Parton, 2006; Welte, 2007; Zehmer et al., 2009).

The process of LD biogenesis is still poorly understood. The predominant theory states that neutral lipids are synthesized between the leaflets of the ER bilayer, accumulate, and eventually bud off as an independent organelle contained within a portion of the cytosolic ER leaflet (Martin and Parton, 2006; Murphy and Vance, 1999). Although several reports claim support for this model by biochemical and ultrastructural methods, direct evidence of this is still considered to be lacking (Blanchette-Mackie et al., 1995; Martin and Parton, 2005). Alternative mechanisms have
been proposed for LD expansion, including direct synthesis of neutral lipids at LD membranes by diacylglycerol acyltransferase 2 (DGAT2) (Kuerschner et al., 2008). However, these models rely on already formed LDs and do not resolve the issue of initial LD formation (Thiele and Spandl, 2008).

LDs vary widely in size and in the composition of associated proteins and lipids between cell types. The complex protein composition is believed to dictate LD functions within the cell. The PAT (Perilipin, Adipocyte differentiation related protein (ADRP), and Tail-Interacting protein of 47kDa (TIP47)) family of structural proteins are the best characterized components of LDs. The coordinated association of these proteins with the LD surface controls access of lipases to the core lipids (Wolins et al., 2006). Enzymes involved in lipid synthesis (e.g. acyl-CoA synthetase) and degradation (e.g. adipose tissue triacylglycerol lipase (ATGL)) can be isolated with LDs (Brasaemle et al., 2004). More recently proteins that direct membrane and vesicular traffic, such as caveolins, Rab proteins (Rab5 and Rab18), SNAREs and coat proteins (Arf1), have been detected in LDs (Martin and Parton, 2005; Thiele and Spandl, 2008; Zehmer et al., 2009).

LDs can also travel rapidly along microtubules and are often closely associated with several organelles including the ER, mitochondria, peroxisomes, and endosomes (Goodman, 2008; Murphy et al., 2008). Together, these features suggest that LDs may function in transporting lipids and proteins to the appropriate cellular destinations (Zehmer et al., 2009).
Given the central role of LDs in lipid homeostasis, we postulated that LDs might be an alternative source of host lipids for the *Chlamydia* inclusion. Therefore, we examined the impact of *Chlamydia* infection on LD function. In particular, we explored the dynamics of LD distribution, lipid and protein composition, and association with host organelles during infection. Additionally, we tested chlamydial growth under conditions where neutral lipid synthesis was inhibited.

### 2.2. Materials and Methods

**Reagents and Antibodies**

Triacsin C and AACOCF3 were purchased from Biomol International L.P. (PA) and Calbiochem (CA), respectively. Western blots were performed with the following antibodies: anti-Nsdhl (Masato Ohashi, Okazaki, Japan), anti-IncG (T. Hackstadt, NIAID/NIH), anti-GRP94 (C. Nicchita, Duke University), and anti-caveolin (BD Biosciences). HRP-conjugated secondary antibodies (Amersham Biosciences) and Supersignal chemiluminescence kits (Pierce) were used for immunoblots.

**Bacterial Strains and Infections**

*Chlamydia trachomatis* LGV serovar L2 (gift of R.S. Stephens, University of California, Berkeley) was propagated in Hep2 cells, and chlamydial elementary bodies (EBs) were purified as previously described (Caldwell et al., 1981) and stored in SPG (0.25 M sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid) at -80°C. For all
microscopy applications, HeLa cells were seeded on glass coverslips at ~70% confluency and infected with LGV L2 at a multiplicity of infection (MOI) of 0.5:1 for 20–24 hr. To assess the effect of inhibitors on LGV L2 replication, $2 \times 10^5$ Hep2 cells were seeded in 12-well plates and infected at a MOI of 5:1 for 40 hr. Infected wells were washed with SPG buffer, scraped into 0.4 ml of SPG buffer, lysed by sonication, and 10-fold serial dilutions were used to infect Hep2 monolayers for 36 hr. Inclusions were detected by IF with anti-IncG antibodies. Nuclei and bacterial DNA were stained with Hoechst 33258 (Molecular Probes, OR). The total yield of EBs was expressed as inclusion-forming units (IFU) per ml. For *S. typhimurium* (SL1344) infections, Hep2 monolayers were infected at an MOI of 20:1 for 30 min. The cells were placed in media containing 100 μg/ml gentamicin to kill extracellular bacteria for 2 hr and subsequently incubated for 20 hr in 10 μg/ml gentamicin. Intracellular replication was determined by plating serial dilutions of infected lysates solubilized in 1% Triton X-100 on LB agar plates.

**Neutral Lipid Stains and Immunofluorescence Microscopy**

Neutral lipid stains were performed on cells fixed in 3% formaldehyde/0.025% glutaraldehyde with 1:1000-fold dilution of a saturated ethanol stock of Nile red (Sigma) or BODIPY493/503 (Molecular Probes). Lipid loading with oleic acid (Sigma) was achieved by treating cells with 50–100 μM oleic acid complexed to delipidated BSA (Sigma) in PBS. Images were acquired with a Leica TCS SL confocal microscope or a
Zeiss Axioskop 2 mot plus epifluorescence microscope equipped with an ORCA ER Hamamatsu CCD camera.

**Wet Scanning Electron Microscopy**

For wet SEM, cells were fixed with 2% glutaraldehyde (Electron Microscopy Sciences, PA) in PBS, and lipids were stained with 2% OsO4 (Electron Microscopy Sciences) in PBS. The cells were analyzed with a Philips XL 30 ESEM TMP scanning electron microscope. This microscopy methodology preserved lipid-rich structures by bypassing organic solvent-mediated dehydration steps common in standard electron microscopy.

**Mammalian Expression Vectors and Transfections**

EGFP-tagged forms of ATGL (C. Jackson, NIH), ADRP and perilipin (C. Landos, NIH), and Rab18 (S. Martin, University of Queensland) were transiently transfected into infected HeLa cells seeded on glass coverslips and analyzed 14–16 hr post-transfection (20–24hr post-LGV L2 infection). RFP-KDEL was obtained from Erik Snapp (Albert Einstein). All transfections were performed with FuGene (Roche) as detailed by the manufacturer. Active mitochondria were detected by pre-incubation of infected cells with a 400nM solution of MitoTracker Red CM-H:XRos (Molecular Probes) prior to fixation as directed by the manufacturer. Fluorescence images were acquired by Leica LSCM.
LD Analysis

LDs were isolated from infected and uninfected Hep2 cells by a modification of previously described protocol (Umlauf et al., 2004). 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). 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). 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). For protein analysis, pure LDs were delipidated twice with 10 volumes of diethyl ether, 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 diethyl ether and dried under a stream of N2. Lipids were redissolved in a small volume of ether and analyzed by thin-layer chromatography on glass-backed HPTLC silica plates (Merck). Neutral lipids were separated in a solvent mix of 70:30:1 v/v/v (hexane: diethyl ether: acetic acid) and stained with 0.1% primuline (Sigma). Fluorescence was captured on a Typhoon laser scanner (GE Healthcare).
2.3. Results

2.3.1. Neutral-lipid rich structures and lipid droplet-associated proteins accumulate at the inclusion periphery

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 (Figure 2).

![Figure 2. Neutral lipid-rich structures are recruited to the *C. trachomatis* inclusion](image)

**Figure 2. Neutral lipid-rich structures 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).
Figure 3. LD formation at inclusion periphery and neutral lipid staining of cell-free EBs and RBs

(A) Accumulation of lipid-rich coats on the chlamydial inclusion. Hep2 cells were infected with C. trachomatis (L2) for 36 hr, and oleic acid (100 μM) was added during the last 12 hr to infected (right panel) and uninfected (left panel) cells to stimulate LD formation. (B) Magnified images of lipid-loaded infected cells. Distinct droplets (arrows) can be seen among the electron-dense structures surrounding the inclusion (arrowheads). (C) Accumulation of neutral lipids in C. trachomatis density gradient purified EBs and RBs. Bacteria were incubated with BODIPY493/503 for 10 min and imaged live by fluorescence microscopy. As a control for non-specific staining of Gram-negative membranes, log-phase E. coli were also stained. Images were acquired with identical exposure times.
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 3). Interestingly, BODIPY 493/503 also stained bacterial membranes, suggesting that C. trachomatis may acquire neutral lipids directly from LDs (Figure 3).

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 (Miura et al., 2002), the triacylglyceride lipase ATGL (Smirnova et al., 2006), and the GTPase Rab18 (Martin et al., 2005). Upon treatment with oleic acid, these markers localized to classical LDs (Figure 4C and 4D). In addition, LD markers labeled reticular structures (perilipin and Rab18) and hazy structures (ATGL and ADRP) at the periphery of the inclusion (Figure 4A). 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 4B).
Figure 4. LD marker proteins are recruited to the chlamydial inclusion

(A) Expression of EGFP-tagged LD proteins in C. trachomatis-infected cells. HeLa cells were infected with LGV-L2, transfected with EGFP-tagged forms of ADRP, perilipin, ATGL, and Rab18 at 7 hr post-infection, and imaged at 24 hr. Images represent fixed average projections of 40 to 50 SL confocal sections. Note accumulation of LD markers to the periphery of the chlamydial inclusion (arrowheads).

(B) LD markers localized to structures adjacent to the IM. Infected HeLa cells were transfected with Rab18-EGFP stained with rabbit anti-IncG antibodies and secondary goat anti-rabbit Alexa555-conjugated antibodies. Note that reticular structures closely envelope the IM but do not colocalize to the IM itself.

(C and D) Distinct patterns of association of LD markers with the surface of the chlamydial inclusion. HeLa cells were infected with LGV-L2 and transfected with Rab18-EGFP and ADRP-EGFP. Three hours prior to analysis, cells were treated with 50 μM oleic acid. Both Rab18 and ADRP labeled Nile red-positive LDs (arrowheads and bottom panel of (C). In addition to localizing to classical oleate-induced LDs, both LD markers label neutral-lipid-rich structures enveloping the chlamydial inclusion. N: nuclei.
2.3.2. Neutral-lipid rich organelles including mitochondria and ER envelop the inclusion

LDs are often observed apposed to other host organelles, particularly those with a function link to neutral lipid biosynthesis or utilization (Murphy et al., 2008). We hypothesized that these organelles might also associate with the inclusion. To assess this, we examined the colocalization of the BODIPY 493/503-positives structures enveloping the inclusion with ER, mitochondria, peroxisomes and lysosomes. Organelle-specific stains or transfected organelle-specific fluorescent reporter proteins were used to avoid fixation and membrane permeabilization artifacts that can impact the stability of LDs and other lipid rich organelles (Ohsaki et al., 2005). Red fluorescent protein (RFP) tagged with the ER retrieval signal, KDEL, closely associated with the inclusion membrane and colocalized with BODIPY 493/503-positive structures (Figure 5). Active mitochondria were also observed at the periphery of the inclusion through co-staining of BODIPY 493/503-positive tubular structures with the mitochondrial tracer, MitoTracker Red CM-H2XRos (Figure 5). The degree of colocalization between RFP-KDEL and BODIPY 493/503 was reduced at sites further away from the inclusion edge. In contrast, lysosomes and peroxisomes failed to show any preferential association with the inclusion periphery (not shown). These results indicate an intimate association between the ER, mitochondria, and the chlamydial inclusion. Since ER is the proposed site or LD formation, we hypothesize that the ER is a likely source for the LDs that surround the inclusion.
Figure 5. Mitochondria, Endoplasmic Reticulum accumulate at the periphery of the inclusion membrane and overlap with BODIPY-positive structures

A-B. Close association of mitochondria and ER with the inclusion. HeLa cells were infected and transfected 6 hr later with a Red Fluorescent Protein (RFP)-KDEL expression construct to detect the ER (A) or treated with MitoTracker Red to detect mitochondria for 2hr prior to analysis (B). At 24hr post infection, infected cells were fixed and stained with BODIPY 493/503. A close up of the inclusion-cytoplasm interface revealed extensive overlap between BODIPY 493/503-positive material enveloping the inclusion and ER and mitochondria. Arrows: inclusions, Red Borders: inclusion membrane, N: HeLa nuclei.

2.3.3. Chlamydia infection alters the composition of host lipid droplets

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.

Figure 6. Changes in LD neutral lipid and protein content induced by *Chlamydia*-infection

(A) 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. (B) Accumulation of Nsdhl in LDs from *C. trachomatis*-infected cells. LDs were purified from LGV-L2-infected cells (24 hr, 44 hr, or 44 hr with 60 mg/ml chloramphenicol (Cm) added at 20 hpi). Proteins in total (1%) and LD (25%) fractions were separated by SDS-PAGE. Immunoblot analysis reveals a Cm-sensitive accumulation of Nsdhl in LDs of infected cells. Fractionation markers for plasma membrane (caveolin), ER (GRP94), and the IM (IncA) indicate that LD samples were devoid of contaminating membranes.

Surprisingly, infection with *C. trachomatis* markedly increased levels of cholesteryl esters (CE) recovered from LD fractions (Figure 6A), 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 6A). To independently assess whether lipid homeostasis was disrupted in infected cells, we monitored the subcellular fractionation of Nsdhl, a sterol biosynthetic enzyme that relocates from the ER to LDs in response to increased levels of cellular lipids (Ohashi et al., 2003). Nsdhl recovery in LD fractions increased during chlamydial infection, and this accumulation was inhibited by the bacterial protein synthesis inhibitor chloramphenicol (Figure 6B). Overall, our results indicate that LD-like structures accumulate at the periphery of the inclusion and that *C. trachomatis* alters neutral lipid homeostasis.

### 2.3.4. Inhibition of host neutral lipid biosynthesis restricts chlamydial replication

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 ester biosynthesis (Igal et al., 1997). We examined the effect of triacsin C in cells infected with *C. trachomatis* (Figure 7B) and determined that this inhibitor greatly reduced chlamydial proliferation and this effect was comparable to that of AACOCF3, a cPLA2 inhibitor that blocks chlamydial phospholipid uptake (Su et al., 2004). 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 post-
vasion (Figure 7A).

Figure 7. Host neutral lipid biosynthesis is required for chlamydial growth and replication

(A) Effects of LD inhibitors on inclusion morphology and expression of early inclusion proteins. Hep2 cells were infected with LGV-L2 for 36 hr in the presence or absence of Triacin C (0.5 μM). Inclusions were detected with anti-IncG polyclonal antibodies. Note small aborted inclusions formed in the presence of the neutral lipid synthesis inhibitor. (B) 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 Trias C or the cPLA2 inhibitor AACOCF3 for 40 hr. Bacterial replication was assessed by the generation of infectious units. Inset: Growth inhibition of S. typhimurium (St) and LGV L2 (Ct) in Trias-in-treated cells represented as a percent growth in untreated cells.
Interestingly, triacsin C inhibited chlamydial growth in Hep2 cells 20 ± 1.5-fold more potently than *Salmonella typhimurium* (Figure 7B, 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.

### 2.4. Conclusions

In this chapter, we investigated the effect of *Chlamydia* infection on the cellular dynamics of LDs, and the role of neutral lipids in chlamydial replication.

We find that neutral lipid-rich structures accumulate at the periphery of the chlamydial inclusion and neutral lipids are incorporated into the membranes of the bacteria (RBs) inside. LD-associated proteins also display recruitment to the inclusion periphery. We hypothesize that the neutral-lipid structures at the inclusion periphery may represent sites of nascent LD assembly. This is supported by the fact that oleic acid treatment induces the formation of lipid coats around the inclusion. Alternatively, this observation may indicate recruitment and aggregation of existing LDs from dispersed cellular sites. At this point, these two mechanisms are not distinguished.
Evaluation of the neutral lipid structures surrounding the inclusion revealed that ER and mitochondria, organelles involved in neutral lipid metabolism, intimately associate with the inclusion. The ER is presumed to be the site of LD formation, and the mitochondria generate energy through β-oxidation of fatty acids that are released from the degradation of neutral lipids (Murphy et al., 2008). Interactions between LDs are postulated to function in the transfer of lipids and other small molecules through transient membrane contact sites (Zehmer et al., 2009). Similar mechanisms have been postulated for the transport of host phospholipid precursors to the inclusion (Wylie et al., 1997).

*Chlamydia* infection also increased in the LD levels of neutral lipids and induced redistribution of the Nsdhl protein to LDs. The increase in LD lipids was somewhat unexpected since we hypothesized that *Chlamydia* might utilize or degrade these organelles which would predict a decrease in the amount of neutral lipid. It is unclear whether this effect is due to enhanced packaging of neutral lipids within LDs or an increase in the overall number of LDs within the cell.

The general accumulation of LDs could be more of an immunopathologic response due to the induction of inflammatory cytokines or other signals being produced during infection. Similar responses are seen in macrophages that are infected by *Mycobacterium tuberculosis* or *C. pneumoniae*, and are believed to be regulated through a TLR2-dependent pathway (Cao et al., 2007; D’Avila et al., 2008; D’Avila et al., 2006).
LDs in leukocytes are also noted for their unique role as sites of eicosanoid generation, linking LDs to functions in innate immune responses (Bozza and Bandeira-Melo, 2005).

While this theory would explain the unexpected increase in cellular neutral lipid during infection, it does not exclude the earlier hypothesis that Chlamydia may utilize or recruit LDs as a source of lipids for energy production or membrane lipid precursors. In fact, since epithelial cells contain relatively few LDs unless stimulated by excess lipid, the induction of LD formation or recruitment of LDs to the surface of the inclusion would provide Chlamydia with easier access and more availability to these components for utilization than would otherwise be available in these cell types.

We hypothesized that lipids contained within LDs or functions performed by LDs are important for Chlamydia growth and survival. Supporting this idea, chlamydial replication is restricted when host neutral lipid biosynthesis is inhibited by triacsin C. We do not believe that this decrease in replication is due non-specific effects of triacsin C on general cellular lipid balance since growth of the facultative intracellular pathogen, S. typhimurium, was not as severely impaired by triacsin C treatment.

In summary, we identified eukaryotic lipid droplets as a novel target of modulation by Chlamydia. Our results suggest that Chlamydia targets LDs as a source for the acquisition of lipids. Given the many predicted functions of LDs within the cell, however, this interaction could also provide the means to redirect the transport of other
essential lipids to the inclusion, to interfere with the host immune signaling, or intercept membrane and vesicle traffic (Martin and Parton, 2006).

All methods and data sections of this chapter with the exception of section 2.3.2 and Fig.5 were published in:


Relative contributions:
This publication was the result of collaborative effort by the authors. For the data displayed in this chapter, the Wet SEM, Bodipy staining of E. coli versus Chlamydia EBs and RBs, biochemical analysis of LDs, and Triacsin C inhibition experiments were performed by Y. Kumar. Analysis of LDs and neutral lipid-rich structures in infected cells, characterization of host LD marker protein association with the inclusion membrane, and identification of ER and mitochondria as neutral lipid-rich structures at the inclusion periphery was done by J. Cocchiaro.
Chapter 3. Chlamydial proteins target to host lipid droplets

3.1. Introduction

The acquisition and incorporation of host-derived lipids into bacterial membranes is a unique feature of *Chlamydia* pathogenesis (Hackstadt, 2000; Su et al., 2004; Wylie et al., 1997). *Chlamydia* can intercept vesicle-mediated traffic from the Golgi apparatus and multivesicular endosomes (MVBs) to gain access to sphingolipid and cholesterol reservoirs (Beatty, 2006; Hackstadt et al., 1996); however, phospholipid precursors are not transported by these pathways and the source of these phospholipids is still unknown (Hatch and McClarty, 1998). In addition, the factors produced by *Chlamydia* which modulate host lipid trafficking have not yet been identified.

A T3S system has been described in *Chlamydia*, and several studies have utilized surrogate T3S systems to identify chlamydial proteins with the potential to modulate host functions (Fields and Hackstadt, 2000; Ho and Starnbach, 2005; Stephens et al., 1998; Subtil et al., 2005). One set of *Chlamydia* proteins that have been widely studied due to their localization at the interface between bacteria and the host cytoplasm are the integral Inclusion membrane proteins (Inc). Incs are theorized to regulate cell signaling and membrane fusion events, and as many as 50 of these proteins are predicted to be encoded by the *Chlamydia* genome (Bannantine et al., 2000; Delevoye et al., 2008; Rockey et al., 2002). Although many candidate effectors have been identified through these
methods, the direct confirmation of functions for these proteins still remains a challenge due to the lack of genetic tools to study this organism.

In our lab, we developed a system in the yeast *S. cerevisiae* to express chlamydial proteins and identify their functions based on cellular localization and phenotypic outcomes of expression (Sisko et al., 2006). This system is particularly useful for studying the effect of bacterial proteins on basic host cellular functions, such as protein and lipid trafficking, since these processes are fairly conserved in eukaryotes (Lesser and Miller, 2001; Valdivia, 2004). In the original screen, two proteins were identified with novel localization to eukaryotic lipid droplets (Sisko et al., 2006).

In chapter 2, we found that Chlamydia-infection alters the cell biology of host lipid droplets (LDs), and identified neutral lipids as another class of host lipid that is required for growth and survival of Chlamydia. Neutral lipid-rich membranes and host LD-marker proteins accumulated at the inclusion periphery. Mitochondria and ER were also found in close association. Neutral lipid and Nsdhl protein levels were altered in cells infected with *Chlamydia* which indicated the expansion of LDs in the host cell. The inhibition of bacterial protein synthesis blocked this amplification, suggesting the action of bacterial proteins was involved in regulating the interaction of *Chlamydia* with LDs. We predicted that the bacterial LD-associated (Lda) proteins we identified through functional genomics were likely candidates for regulators of the interaction between
Chlamydia and host LDs. In this chapter, we further identified and characterized Chlamydia LD-targeting proteins.

3.2. Materials and Methods

Cell Lines, strains, antibodies, reagents and vectors

Hep2 and HeLa cells were obtained from ATCC. For Lda suppression analysis, the congeneric yeast strains G175 (MATα ADE2 his3 leu2 ura3 trp1) and H1246 (MATα ADE2 ura3 dga1::KanMX4 lro1::TRP1 are1::HIS3 are2::LEU2) (Sandager et al., 2002) were used. Western blots were performed with the following antibodies: anti-Sec12p (R. Schekman, University of California, Berkeley), anti-Vma1p (Molecular Probes), anti-Erg6p (G. Daum, Basel, Switzerland), mouse monoclonal anti-GFP (Stressgen Biotechnologies), HRP-conjugated secondary antibodies (Amersham Biosciences) and Supersignal chemiluminescence kits (Pierce) were used for immunoblots. RFP-KDEL was obtained from Erik Snapp (Albert Einstein).

Identification of Lda proteins in yeast

Yeast cells (DLY5214 [MATa ura3 leu2 his3 lys2]) were grown in complete synthetic medium (CSM) dropout mixes (Bio 101 Labs) supplemented with dextrose or galactose as indicated. All yeast methods were based on protocols outlined in (Brown and Tuite, 1998). To identify LD-associated chlamydial proteins, MATα yeast cells expressing an RFP-tagged LD marker, Erg6p (Huh et al., 2003), were mated with a collection of MATα yeast strains expressing galactose inducible GFP-tagged chlamydial
proteins (Sisko et al., 2006) and imaged live 4 hr after induction in 2% galactose. Clones displaying extensive overlap between RFP and GFP signal were isolated for further analysis.

**Yeast lipid droplet purification**

LDs were isolated from yeast cells a modified version of the protocol described in (Leber et al., 1994). Spheroplasts were prepared from ~100ml of overnight cultures of yeast containing the inducible GFP-fusion proteins grown in CSM-ura media with 2% galactose for protein induction. Cultures were centrifuged at 800xg for 10 min. The pellet was washed once with dH2O and once with PBS then resuspended in 10 ml of 1.2M sorbitol/PBS containing 5mM DTT. A 100μl aliquot of the suspension was saved as a no lysis control. Zymolyase (ICN Biomedicals) was added to 100μg/ml final concentration and the suspension was incubated at 30°C for 1h. OD 680 was checked every 20 min until OD was ~10% of the control. Spheroplasts were pelleted at 250xg for 4 min. All subsequent steps were performed on ice or at 4°C. The spheroplast pellet was resuspended in 5ml of breaking buffer (10mM HEPES pH 7.0/12% Ficoll 400/0.2mM EDTA). Using a dounce homogenizer, the cells were lysed with ~20 strokes using a loose fitting pestle on ice. The homogenate was transferred to a SW41 tube and overlaid with equal volume of breaking buffer then centrifuged at 30,000 rpm at for 30 min in a Beckman L8-70M ultracentrifuge with a SW41 rotor. The floating lipid layer was collected, resuspended in 5ml of breaking buffer by gentle strokes in the homogenizer.
on ice. Homogenate was transferred to a SW41 tube, overlaid with an equal volume of 8% buffer (10mM HEPES pH 7.0/8% Ficoll 400/0.2mM EDTA), and re-centrifuged as above. The floating lipid layer consisting of both vacuoles and lipid droplets was resuspended in 5 ml of 8% buffer with 0.6M sorbitol (10mM HEPES pH 7.0/8% Ficoll 400/0.6M sorbitol/0.2mM EDTA) in the dounce homogenizer and overlaid with 0.25M sorbitol buffer (10mM HEPES pH 7.0/0.25M sorbitol/0.2mM EDTA). After another round of centrifugation at 30,000 rpm for 30 min, the purified lipid droplets floated while vacuoles pelleted. The floating lipid droplet layer was collected carefully and delipidated twice with 10 volumes of diethylether. Proteins were precipitated from the aqueous layer with ice-cold acetone and dried before reconstituting in 0.1% SDS/0.1 N NaOH. Proteins were analyzed by Western blot.

**Lda-specific Antibody Production**

Antibodies generated against synthetic peptides derived from Lda1 (RNTNRENREFHHHDQDRT) and Lda3 (ESPDDRTVPHTQETS) were generated in hens (Aves Labs Inc.). Antibodies against GST-Lda2 were generated in rabbits (Covance Immunology Services). The specificity of anti-Lda antibodies was tested against recombinant proteins expressed in yeast. Lda1 and Lda3 chicken antibodies were column affinity-purified against the immunizing peptide crosslinked to SulfoLink coupling resin (Thermo Scientific/Pierce).
Indirect immunofluorescence of Lda proteins

Infected cells were fixed and permeabilized with 0.05% saponin in 0.2%BSA/ PBS for 30 min. Affinity-purified anti-Lda1 and -Lda3 antibodies (5 mg/ml) were added in the presence of BSA, and antibodies were detected with fluorescently labeled secondary goat anti-chicken antibodies (Molecular Probes). The labeling pattern was specific to each protein because their detection was blocked by pre-incubation with the peptides used for immunization. Images were acquired with a Leica TCS SL confocal microscope or a Zeiss Axioskop 2 mot plus epifluorescence microscope equipped with an ORCA ER Hamamatsu CCD camera. Images on Figure 3A were acquired as a z series of 60 stacks and deconvolved with constrained iterative algorithms (Axiovision v3.1 imaging software). To assess the detergent sensitivity of Lda1 localization, the signal intensity of Lda1 around IncG-positive inclusions (n = 150) was measured. Inclusions with Lda1 staining at least 2-fold over background were considered positive for Lda1. The pixel intensity of Lda1 staining around the inclusion is represented as the average of three independent experiments with standard errors shown.

Analysis of Lda Protein Association with Host Organelles

For expression of Lda proteins in mammalian cells, Lda genes were subcloned into a modified pCDNA5-EGFP mammalian expression vector (Invitrogen) to generate C-terminal fusions to EGFP. For ER labeling, cells were co-transfected with individual pCDNA5-EGFP-Lda constructs and RFP-KDEL using Fugene (Roche) as instructed by
the manufacturer. Active mitochondria were detected by pre-incubation of infected cells with a 400nM solution of MitoTracker Red CM-H2XRos (Molecular Probes) prior to fixation as directed by the manufacturer. Cells were fixed in 3% formaldehyde/0.25% glutaraldehyde in PBS, and fluorescence images were acquired with a Leica LSCM.

**Lda2 Deletion and Suppression Analysis**

For deletion analysis of Lda2, oligonucleotide primers were designed to amplify segments of Lda2 spanning the coding sequences for aa 1–99, 1–160, 84–548, and 144–548 by PCR. The PCR products were spliced into the yeast expression vector pSDY10 (PGAL-GFP 2μ LEU2) by homologous recombination to create galactose-inducible C-terminal GFP-fusion proteins as described in (Sisko et al., 2006). pSDY10 plasmids expressing Lda2 fragments were transformed into yeast and tested for growth defects, protein stability, and association with 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 media with CSM-Uracil + 2% dextrose (‘Dex Chase’). Levels of Lda2 proteins were assessed by Western blots using anti-Lda2 antibodies (1:1000).

**3.3. Results**

**3.3.1 Identification of chlamydial proteins that localize to lipid droplets**

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 (Sisko et al., 2006) for proteins that colocalized with an RFP-tagged LD protein (Erg6p).

Figure 8. Identification of chlamydial LD-Associated (Lda) 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 (Sisko et al., 2006) 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 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).

This screen revealed four Chlamydia-specific ORFs (CT156, CT163, CT257, and CT473) with marked tropism for yeast LDs (Figure 8A and 8B). We focused our analysis on three of these LD-associated chlamydial proteins.

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 8C). In contrast, Lda2-EGFP-expressing cells displayed low levels of cytoplasmic fluorescence with no apparent association with LDs (data not shown).

3.3.2. Chlamydial Lda1 and Lda3 associate with neutral lipid-rich structures at the periphery

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 9A).

![Image of Figure 9 with immunolocalization of Lda1 and Lda3]

**Figure 9.** 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). 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 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).

Consistent with the described sensitivity of LD-associated proteins to detergent permeabilization (Ohsaki et al., 2005), the localization of Lda1, and to a lesser extent Lda3, was sensitive to solubilization with 0.1% Triton X-100 (Figure 9B and data not shown). Furthermore, Lda1 and BODIPY 493/503-labeled structures partially overlapped at the inclusion periphery (Figure 9C). These results strongly suggest that C. trachomatis translocates proteins into the host cytoplasm to target LD-like structures enveloping the inclusion.

3.3.3. Lda2 stability and function requires lipid droplets

Lda2 and Lda3 were independently identified in a screen for chlamydial proteins that induce cytotoxic effects in yeast (Sisko et al., 2006). To determine if the cytotoxicity of these proteins was linked to LDs, we assessed if yeast mutants lacking the triacylglycerol synthases and sterol acyltransferases required for LD generation (Mullner and Daum, 2004) and (Sorger et al., 2004) were resistant to Lda-mediated cytotoxicity.
Figure 10. LDs are required for Lda2 stability

(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 in galactose, followed by a dextrose 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. Lda2LD 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.

Interestingly, disruption of all neutral lipid biosynthesis in lro1 dga1 are1 are2 mutants suppressed Lda2, but not Lda3, toxicity (Figure 10A). Because Lda2 was rapidly degraded in these LD-deficient mutants (Figure 10B), 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 (Lda2LD) 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 Lda2LD-EGFP chimera was stable and prominently localized to HeLa LDs (Figure 10C). 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 10D 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 10E). 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.

3.3.4. Ectopically expressed Chlamydia Lda proteins target to LDs, ER, and mitochondria

Antibody staining for anti-Lda1 and Lda3 revealed labeling of reticular structures at the inclusion membrane rather than the distinct, ring-like structures observed by ectopic expression. These structures were, however, rich in neutral lipids and sensitive to detergent permeabilization. Since mature LDs are not abundant in epithelial cells without lipid-loading, we hypothesized that Lda proteins may bind to the ER and mitochondria which surround the inclusion. To test the organelle targeting capabilities of the Lda proteins, HeLa cells were transfected with GFP fused Lda proteins, and either co-expressed with ER-RFP, a construct containing the KDEL localization signal, or probed for mitochondria using MitoTracker Red (Figure 11A and 11B). Analysis was first done in uninfected cells to exclude interference by endogenous proteins produced during infection. The lipid-droplet binding portion of Lda2, Lda2LD, was used as a comparison for strong LD-specific association.
Figure 11. Chlamydial Lda proteins display unique patterns of localization with host organelles

(A-B). Patterns of *Chlamydia* Lda protein localization with mitochondria and ER in uninfected cells. Localization of Lda proteins was observed in the absence of lipid-loading. HeLa cells were transfected with GFP-tagged Lda1, Lda3, or Lda2<sub>LD</sub> and either co-expressed with ER-RFP (A) or treated with MitoTracker (B) at 2hr prior to fixation. Close-ups of peripheral regions of the cell are shown to see better separation of host components. (A) Lda localization with ER. Lda1 labeled structures are in close apposition to the ER but not overlapping. Lda3 and Lda2<sub>LD</sub> signals display partial colocalization with ER. (B) Lda labeling of mitochondria. Lda1 colocalizes heavily with mitochondria and labels small LDs. Lda3 also binds to mitochondria, appearing more membranous, and LDs. Lda2<sub>LD</sub> mostly labeled the surface of mature LDs in a ring-like pattern and did not bind mitochondria.

Lda1 and Lda3 exhibited differential labeling of ER and mitochondria. Lda1 displayed a preference for mitochondria and small LDs, while Lda3 appeared to localize with the ER network in addition to mitochondrial membranes and LDs (Figure 11A and 11B). Lda1-labeled tubules appeared to be encased by the ER reticular network despite the lack of colocalization. Lda2<sub>LD</sub> mostly labeled the surface of mature LDs in a ring-like pattern,
and had partial overlap with the ER. Lda2\textsubscript{LD} was not detected within mitochondria, but Lda2\textsubscript{LD}–positive LD clusters were often seen intimately associated with the ends of mitochondrial tubules (Figure 11B).

![Figure 11B](image)

**Figure 12. Tropism of ectopically expressed Lda proteins for Inclusion-associated LDs**

HeLa cells were infected with *C. trachomatis* L2, transfected 6 hr later with EGFP-tagged Lda1, Lda3, and Lda2\textsubscript{LD}, and imaged by SL confocal microscopy. Ectopically expressed Lda proteins localized to both classical LDs (arrows) as well as to the periphery of inclusions (arrowheads). *Uninfected cells.

Ectopically expressed Lda proteins also localize to the inclusion periphery in infected cells (Figure 12), suggesting that these proteins, while capable of localizing to classical LDs, also bind to neutral-lipid-rich structures enveloping the inclusion. These results indicate that the Lda proteins likely have distinctive activities and may play different roles in the interaction between the inclusion and host organelles that lie at the periphery. Interestingly, Lda3 also exhibited localization to the inclusion membrane and was the only Lda protein with this property. Lda3 targeting to LDs and the inclusion membrane will be discussed further in Ch.4 and Ch.5.
3.4. Conclusions

We identify four chlamydial proteins that localize to yeast LDs and confirm this targeting biochemically. We demonstrate by ectopic expression that for three of these LD-associated (Lda) proteins, Lda1, Lda2, and Lda3, targeting to LDs is conserved in mammalian cells. Moreover, using specific antibodies to detect these proteins during infection, we find that Lda proteins are able to access the host cytoplasm and associate with neutral lipid-rich reticular structures at the periphery of the inclusion.

Based on our previous observations, we hypothesized that the reticular structures stained by the Lda antibodies were likely host mitochondria, ER, and nascent LDs that surround the inclusion. Indeed, when ectopically expressed, Lda1, Lda2LD, and Lda3, also displayed localization to ER, mitochondria, and LDs in both uninfected and infected cells. The patterns of organelle association varied between the Lda proteins suggesting that the determinants which guide their targeting to host structures are different.

Together these data indicate the Lda proteins display all the characteristics that would be necessary for them to target to and interfere with LD-related functions during infection. Lda2 interaction with LDs appears to be important for its function since this protein is unstable in the absence of LD formation.

In comparing the Lda proteins, there is little to suggest that these proteins might have a common function. They are not homologous with each other or with other...
proteins of known function. The only unifying theme is that are all basic proteins with pIs in the range of 8.5-9.5. The Lda3 protein is conserved between Chlamydiae species, whereas the genes that encode Lda1 and Lda2 are contained within the Plasticity Zone (PZ).

The PZ is a hypervariable region of chlamydial genomes that contains several features that correlate with differences in host tropism and disease outcomes of C. trachomatis serovars (Caldwell et al., 2003; Carlson et al., 2005; Read et al., 2003). Interestingly, the gene encoding Lda1 is in the center of a cluster of genes that encode phospholipase D (PLD)-like proteins and are postulated to function in the acquisition and processing of host lipids by C. trachomatis (Nelson et al., 2006). Since Lda1 is truncated and is expressed before the surrounding genes, it is proposed to regulate the other PLD-like proteins (Nelson et al., 2006). Providing a connection to LDs, PLD activity together with ERK2 is required for LD biogenesis (Andersson et al., 2006). Suggesting that Lda1 or the chlamydial PLD-like proteins could potentially regulate LD biogenesis.

Identifying the features of Lda proteins that control their targeting to LDs and other organelles, the host interacting proteins of these proteins, and their functions in LD regulation will be an important goal for future studies.
All data sections of this chapter with the **exception of section 3.3.4 and Fig.11** were published in:


**Relative contributions:**
This publication was the result of collaborative effort by the authors. For the specific data displayed in this chapter- The original yeast expression screen for LD localization and pictures were done by R. Valdivia (later repeated and confirmed by J. Cocciaro). Biochemical confirmation of Lda purification from yeast LDs was done by Y. Kumar. Analysis of *Chlamydia* Lda protein localization to mammalian LDs was done by J. Cocciaro. Lda1 and Lda3 peptide antibodies were affinity purified by J. Cocciaro, and IFs and detergent sensitivity in infected cells were performed by Y. Kumar. Lda2 antibody production and IFs were by Y. Kumar. Evaluation of Lda protein cytotoxicity and stability in LD- yeast, and Lda2 deletion analysis and LD-binding domain identification were done by J. Cocciaro.
Chapter 4. Host lipid droplets are translocated into the lumen of the *Chlamydia* inclusion

4.1. Introduction

As an obligate, intracellular pathogen, *Chlamydia* has economized its genome to maximize the efficient uptake and utilization of essential nutrients and metabolic intermediates from the host cell (Stephens et al., 1998). Some of the factors acquired from host sources include essential amino acids, ribonucleotides, and lipids (Hackstadt et al., 1996; McClarty, 1994; McClarty et al., 1993; Moulder, 1991; Su et al., 2004; Wylie et al., 1997). How these nutrients are transported to the bacteria across the inclusion membrane, however, is not well-understood and remains a major question in the field of chlamydial biology.

Many intracellular parasites can acquire nutrients by directly accessing the host cytoplasm or interacting with host endocytic traffic (Hackstadt, 1998, 2000). However, *Chlamydia* growth and replication is contained entirely within the inclusion, and studies characterizing the inclusion membrane reveal that the inclusion is largely non-fusogenic with endocytic and lysosomal compartments even at early time points after entry (Fields and Hackstadt, 2002; Scidmore et al., 2003). Passive diffusion of cytosolic components, which has been described for other non-fusogenic parasitophorous vacuoles, is also unlikely to be a mechanism involved in the transport of nutrients to *Chlamydia* since the inclusion membrane restricts uptake of fluorescent tracers as small as 520 Da (Hackstadt et al., 1997; Heinzen and Hackstadt, 1997).
Apart from avoiding host degradative machinery, *Chlamydia* also re-routes cellular lipid transport pathways to acquire host-derived sphingomyelin, cholesterol, and glycerophospholipids (Carabeo et al., 2003; Hackstadt et al., 1996; Hatch and McClarty, 1998; Wylie et al., 1997). Vesicles from the Golgi and multivesicular bodies (MVBs) are thought to be the primary source of sphingomyelin and cholesterol (Beatty, 2006; Hackstadt et al., 1996); however, inhibition of Golgi function with brefeldin A (BFA) does not impact the uptake of host phospholipids and only partially blocks sphingomyelin delivery to the inclusion (Hackstadt et al., 1996; Hatch and McClarty, 1998; Wolf and Hackstadt, 2001). These results suggest that lipid transport to *Chlamydia* also requires non-vesicular processes. Although these pathways have not been identified, transfer of these lipids by direct membrane contact with host organelles, such as the ER and mitochondria, which closely appose the inclusion has been postulated (Wylie et al., 1997).

The observations from our work indicate that LDs may represent one previously unrecognized source of lipid transport to the *Chlamydia* inclusion. Within cells, LDs are often found closely apposed with other cellular organelles including ER, mitochondria, peroxisomes, and endosomes (Goodman, 2008). LDs also move along microtubules and contain several associated Rab proteins (Murphy et al., 2008). Combined, these features suggest that LDs may also play a role in membrane trafficking processes (Zehmer et al., 2009).

We find that *Chlamydia* infection redirects the normal trafficking of host LDs accumulating them at the inclusion periphery. Additionally, we show that *Chlamydia*
produces several proteins (Ldas) that are able to access the host cytosol and target to neutral lipid structures just outside the inclusion. These proteins localize to the LD surface and other organelles when ectopically expressed. The stability and function of at least one of these proteins, Lda2, appears to be dependent on LDs biogenesis. We speculate that these proteins are involved in the regulation of interactions between the inclusion and LDs. The bacterial requirement for host neutral lipid synthesis implies that interactions with LDs are important in for Chlamydia pathogenesis.

The underlying molecular events that guide the recruitment and utilization of LDs by the inclusion are unknown. Therefore, we decided to explore the physical interaction between the inclusion and LDs in finer detail, and we investigated the potential contribution of LD-associated host and chlamydial proteins to this phenomenon.

4.2. Materials and Methods

Strains, Infections, and Cell Culture Reagents

HeLa cells were obtained from ATCC and grown in DMEM supplemented with 10% Fetal Bovine Serum (Invitrogen). C. trachomatis LGV-L2 was propagated and stored as EBs in SPG (0.25 M sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid) as described (Caldwell et al., 1981). C. trachomatis serovar C and MoPn EBs were obtained from H. Caldwell (Rocky Mountain Laboratories, National Institutes of Health). For infections, EBs were diluted in DMEM, added to HeLa monolayers at an MOI of 0.5–1 and centrifuged at 1,600 × g for 30 min at 4°C. Cells were incubated at 37°C/5% CO₂ for
30 min. Then, cells were washed with PBS, media was replaced, and plates were returned to 5% CO₂ at 37°C for the indicated times. For lipid loading experiments, oleic acid (Sigma) was precomplexed with fatty acid-free BSA (Sigma) in PBS and emulsified by sonication. Oleic acid was added to growth media at final concentration of 100 μM.

**Expression Constructs and Antibodies**

HeLa cells stably expressing EGFP-ADRP were obtained from P. Targett-Adams and J. McLauchlan (Medical Research Council Virology Unit, Institute of Virology, Glasgow, U.K.) and were derived as described (Targett-Adams et al., 2003). Lda3-EGFP and Lda3-DsRed were generated by inserting Lda3 (CT473) coding sequence (Stephens et al., 1998) into pEGFP-N1 and pDsRed-N1 (Clontech). Transfections were performed with FuGene6 reagent (Roche) as detailed by the manufacturer. Antibodies were from the following sources: ADRP (ProGen Biotechnik), Nsdhl (M. Ohashi, Okasaki Institute, Okasaki, Japan), Rab1 and 14–3-3β (Santa Cruz Biotechnology), Rab11 (BD Biosciences), Omp2 (RDI) and Cap1 (M. Starnbach, Harvard Medical School, Boston, MA). Mouse monoclonal anti-IncA and anti-CT223 antibodies were obtained from D. Rockey (Oregon State University, Corvallis, OR). Anti-IncG antibodies have been described (Scidmore-Carlson et al., 1999). Polyclonal antibodies to IncA and CT229 were generated by immunizing rabbits with purified GST-IncA (80–324 aa) and GST-CT229 (92–215 aa), respectively.

**Transmission Electron Microscopy**

Samples were fixed for 2 h at room temperature with 2.5% glutaraldehyde and 0.05% malachite green (EMS) in 0.1 M sodium cacodylate buffer, pH 6.8. Samples were
post-fixed for 30 min with 0.5% osmium tetroxide and 0.8% potassium ferricyanide in 0.1 M sodium cacodylate, for 1 h in 1% tannic acid, and for 1 h in 1% uranyl acetate at room temperature. Specimens were dehydrated with a graded ethanol series, and embedded in Spurr's resin. Thin sections were cut with an RMC MT-7000 ultramicrotome (Ventana) stained with 1% uranyl acetate and Reynold’s lead citrate before viewing at 80 kV on a Philips CM-10 transmission electron microscope (FEI). Digital images were acquired with an AMT digital camera system (AMT).

**LD Analysis**

LDs were isolated from HeLa cells as described (Umlauf et al., 2004). Briefly, two T-175 flasks were either infected with LGV L2 (MOI≈5) or left uninfected. Gentamicin (100 μg/ml) and oleic acid (100 μM) were added to cells 12–14 h before harvesting LDs at the end of the infectious cycle (40 h). Cells were washed with PBS and harvested in 5 ml of TNE [20 mM Tris-Cl (pH 8.0), 120 mM NaCl, and 2 mM EDTA] containing protease inhibitors (Roche Diagnostics). The cells were lysed on ice with ≈40 strokes in a Dounce homogenizer, cell lysates were adjusted to 0.45 M sucrose; overlaid with 2 ml of each of 0.25 M, 0.15 M, and 0 M Sucrose/TNE; and centrifuged at 30,000 rpm for 90 min in an SW41 rotor (Beckman Coulter). The floating LD-enriched fat cake was collected, diluted in TNE, and refloated at 47,000 rpm for 45 min in a TLA55 rotor (Beckman Coulter). LDs were collected, and lipids were extracted with 4 vol of diethyl ether. De-lipidated proteins were precipitated with ice-cold acetone, solubilized in 0.1%SDS and 0.1N NaOH, and normalized for total protein content before SDS/PAGE and immunoblot analysis.
**Fluorescence Microscopy**

To evaluate LD association with the inclusion, L2-infected HeLa cells or EGFP-ADRP stable transfectants were fixed in 3% formaldehyde and 0.025% glutaraldehyde in PBS for 20 min at room temperature. Cells were permeabilized and blocked in 0.05% saponin and 0.2% BSA/PBS (SBP) then incubated with primary antibodies to LD and bacterial proteins, followed by Alexa Fluor-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Invitrogen). For assessing intra-inclusion accumulation of IncA and IncG, fixed cells were permeabilized in 0.1% Triton X-100 for 10 min and blocked in 5% BSA/PBS. For neutral lipid stains, fixed cells were incubated with a 1:1,000 dilution of a saturated acetone solution of BODIPY 493/503 (Invitrogen) in PBS. Nuclei and bacterial DNA were stained with Topro3 (Invitrogen). Fluorescence images were acquired with a Leica TCS Scanning Laser Confocal Microscope. *P* values shown in Figs. 13D and 18C were determined by one-way ANOVA Tukey–Kramer multiple comparisons test.

**Image Acquisition and Quantification**

Samples were imaged with a Leica TCS LSCM with the 488 and 633nm lasers lines to detect EGFP and Alexa-647, respectively. Stacks of images were taken for each field (63X objective, zoomed to 1.19X) to include ~30 inclusions per field. Approximately 20 confocal sections that covered the entire depth of infected cells were acquired and fixed maximum projections of stacks were generated with Leica software. LD association with inclusions was determined manually. For cells that had more than one inclusion, each inclusion was counted separately.
Live cell imaging of LD translocation

HeLa cells were seeded on 3cm tissue culture dishes with glass bottom coverslips (MatTek) and transfected with Lda3-EGFP expression plasmids for 6h. Four hours post-transfection, the media was replaced and LGV-L2 EBs were added at an MOI of 2:1 and incubated for 18h and 28h. Infected cells were placed on a heated stage (37oC) and imaged live at 45 sec intervals for 30-40min with a Leica TCS SL Confocal Microscope after excitation with the 488nm laser line. Serial confocal sections (20-25 slices) were acquired, and fixed average projections for each time point were generated with Leica Confocal Software.

4.3. Results

4.3.1. Mature lipid droplets preferentially associate with the bacterial inclusion in infected cells

The fluorescent neutral lipid dye BODIPY 493/503 labels reticular structures and scattered “classical” LDs in HeLa cells grown in standard tissue culture media. In contrast, when HeLa cells are infected with C. trachomatis genital serovars, ocular serovars, or the mouse pneumonitis strain MoPn, the periphery and lumen of inclusions are prominently labeled with BODIPY (Figure 13A). Since HeLa cells do not accumulate the large LDs commonly observed in adipocytes and lipid-loaded cells, we enhanced LD formation by adding 100μM oleic acid to growth media or overexpressing ADRP and assessed LD interaction with the inclusion. Neither of these treatments adversely affected chlamydial replication (not shown).
Figure 13. Neutral lipid-rich reticular structures and Lipid Droplets (LDs) accumulate at the periphery of the \textit{C. trachomatis} inclusion

(A) The neutral lipid dye BODIPY 493/503 labels \textit{C. trachomatis} inclusions. HeLa cells were infected with \textit{C. trachomatis} serovars L2, C and the mouse pneumonitis strain, MoPn, for 24–32 hr and stained with the neutral lipid dye BODIPY 493/503. Note
extensive BODIPY-positive reticular structures enveloping inclusions (arrowheads) and scattered bright lipid droplets (arrows). (B–D) LDs associate with the inclusion periphery. The formation of LDs was enhanced by addition of 100 μM oleic acid (B) or overexpression of EGFP-ADRP (C) and the degree of LD association with inclusions was assessed at various stages in the infectious cycle (D). Inclusion membranes were detected with anti-IncG antibodies. (B–C) Shown are fixed average projections of confocal stacks. Note the accumulation of distinct mature LDs at the periphery of the inclusions. (D) Data represent the mean ± SD from three independent experiments. N, nuclei

Both BODIPY- and EGFP-ADRP positive LDs preferentially associated with the periphery of inclusions as early as 18h post infection (P<0.01, Student’s t test, for combined association vs. non-association phenotypes at 18 and 24h) (Figure 13B-D). Based on these results, we postulate that the BODIPY-positive reticular structures enveloping the inclusion constitute sites of neutral lipid biosynthesis and, by extension, LD assembly.

4.3.2. Ultrastructural analysis reveals intact lipid droplets within the inclusion lumen

We also observed BODIPY-positive droplets in close association with inclusion membranes and within the inclusion lumen, especially when LD formation was enhanced by oleic acid treatment (e.g. Figure 13A&B arrows). To confirm that these neutral lipid-rich droplets were within the inclusion lumen, even in the absence of lipid-loading, we co-stained the inclusion membrane with antibodies against Inclusion membrane protein G (IncG) and assessed the subcellular localization of BODIPY-positive structures by laser scanning confocal microscopy (L SCM). Although the detergent permeabilization led to a reduction in BODIPY staining from reticular structures and RBs, droplet-like material was readily apparent in the inclusion lumen
(Figure 14A). Intra-inclusion and inclusion membrane-associated BODIPY-positive droplets of various sizes were present in >70% of inclusions (not shown).

Figure 14. Intact LDs are present in the chlamydial inclusion lumen

(A) Neutral lipid-rich droplets are found within inclusions. Non lipid-loaded HeLa cells were infected with L2 for 20 hr and the interaction between inclusions and neutral lipid-rich droplets assessed. Note the presence of distinct droplets (arrowheads) within IncG-positive membranes in xy (Top) and zy (Lower) laser scanning confocal sections. (B and C) Ultrastructural analysis of inclusions reveals intact LDs in the inclusion lumen. HeLa cells were infected with L2 for 18 hr, fixed in the presence of malachite
green to preserve lipid structures, and processed for electron microscopy. LDs, internal membrane structures, and LD-like structures (black arrows) accumulated inside the inclusion. Note membrane blebs associated with intrainclusion LDs (arrowheads) and contacts between LDs, RBs, and the inclusion membrane (C). N, nuclei; M, mitochondria.

Because we could not distinguish between bona fide LDs and aggregates of neutral lipid-rich membranes by light microscopy, we performed ultrastructural analysis of infected cells to determine the nature of these structures. LDs are disrupted by fixatives and organic solvents commonly used for electron microscopy applications. Therefore, we specifically preserved lipid-rich structures by fixing infected cells in the presence of malachite green (Teichman et al., 1972) before processing the samples for transmission electron microscopy (TEM). This fixation methodology revealed previously unappreciated structural complexity inside the inclusion lumen, including intact LDs (Figure 14B-C). Intra-inclusion LDs displayed all the features of cytoplasmic LDs, including a thin phospholipid monolayer and weak staining of the lipid core. Intra-inclusion LDs were often associated with the inclusion membranes and bacterial outer membranes (Figure 14B). LDs entering the inclusion were occasionally surrounded by membrane blebs and vesicles (Figure 14C), presumably originating from the inclusion membrane. In addition, the inclusion lumen displayed a significant amount of debris including electron dense material and membranous structures of unknown origin (Figure 14B). Overall, our LSCM and TEM observations established the presence of LDs in the inclusion lumen and led us to hypothesize that these organelles are translocated from the cytoplasm of the infected cell.
4.3.4. The inclusion membrane protein IncA specifically co-purifies with LDs from infected cells and accumulates in the inclusion lumen

TEM analysis revealed LDs at different stages of translocation into the inclusion lumen, with LDs occasionally remaining associated with inclusion membranes (Figure 15). Therefore, we predicted that a portion of inclusion membranes would co-fractionate with LDs.

Figure 15. Translocation of LDs across the inclusion membrane

(A) Representative electron micrographs of LGV-L2 inclusions show LDs at various stages of crossing the inclusion membrane. Note membranes and blebs (arrowheads) associated with translocating LDs. CYT, cytoplasm.

To test this, we isolated LDs from infected and uninfected HeLa cells grown in 100μM oleic acid for 12 hr to obtain enough material for reliable biochemical analysis. Because Rab and 14-3-3 proteins have been reported to bind to inclusion membranes (Rzomp et al., 2003; Scidmore and Hackstadt, 2001) and co-purify with LDs (Liu et al., 2004), we tested if these proteins displayed differential association with LDs during infection. Rab1 and Rab11 co-fractionated with LDs, but this association was independent of Chlamydia infection (Figure 16A). In contrast, we were unable to detect 14-3-3β in LDs. Next, we assessed a variety of Inc proteins including IncG, IncA, CT223, CT229, and the non-classical Inc protein, Cap1 for presence in LDs. Interestingly, only IncA significantly
associated with purified LDs (Figure 16A), suggesting that inclusion membranes do not co-fractionate in bulk with translocating LDs.

Figure 16. The inclusion membrane protein IncA co-purifies with LDs and accumulates in the inclusion lumen.

(A) IncA co-fractionates with LDs. HeLa cells were infected with LGV-L2 for 40 hr and treated with 100 μM OA 12–14 hr before purification of LDs by density gradient ultracentrifugation. The fractionation of a host LD protein (Nsdhl), chlamydial outer membrane protein (Omp2), host proteins associated with the inclusion membrane (Rab11 and 14–3-3β), and Inc proteins (CT223, CT229, IncA, IncG and Cap1) were assessed by immunoblots. Note cofractionation of IncA with purified LDs and lack of other Inc proteins. (B-C) IncA-positive structures accumulate in the inclusion lumen. HeLa cells were infected with LGV-L2 for 18 and 24 hr and immunostained with polyclonal anti-IncG or anti-IncA antibodies. Note the accumulation of IncA-positive material in the inclusion lumens (B). The frequency of IncA and IncG-positive intra-inclusion vesicles (C) was determined by LSCM as in (B), except that an anti-IncA mAb was used. Inclusions (150–300 per time point per experiment) were binned in categories according to the number of vesicles per inclusion. Data represent the mean ± SD of three independent experiments. The number of inclusions with intralumenal IncA-positive vesicles was significantly higher than IncG-positive (P < 0.001). (D) Partial colocalization of IncA-positive membranes with intra-inclusion LDs. HeLa cells were infected with L2 and processed with anti-IncA antibodies and BODIPY.
The relative enrichment of IncA with LDs suggested that IncA may mark sites on the inclusion membrane permissive for LD translocation. As such, we predicted that IncA-positive membranes should accumulate in the inclusion lumen. To test this, HeLa cells were infected for 18 and 24h, processed for immunofluorescence with anti-IncA and anti-IncG antibodies, and analyzed by LSCM. IncA localized to the inclusion membrane as previously described (Rockey et al., 1997), and also to distinct intra-inclusion aggregates (Figure 16B-D). These aggregates did not colocalize with chlamydial LPS (data not shown) suggesting that IncA in the lumen was not associated with bacterial membranes. Since these intra-inclusion structures were recognized by two different sources of anti-IncA polyclonal antibodies (Figure 16B and not shown) and an anti-IncA monoclonal antibody (Figure 16C), it is unlikely that these structures are artifacts of immunostaining procedures. In contrast, IncG was primarily restricted to the inclusion membrane (Figure 16B). The association of IncA-positive membranes and LDs is likely transitory as intra-inclusion BODIPY-positive droplets only show partial colocalization with lumenal IncA by LSCM (Figure 16D). Based on these observations, we propose that LD-associated IncA represents segments of inclusion membrane that invaginate into the lumen and remain transiently associated with intra-inclusion LDs.

4.3.5. Lda3 binds to the inclusion membrane and the LD surface during LD translocation into the lumen

We hypothesized that the chlamydial Lda proteins might participate in the capture, translocation and eventual processing of LDs in the inclusion lumen. Lda3, in
particular, is an attractive candidate as a mediator of LD recognition at the inclusion surface. Lda3 is conserved among Chlamydiae, anti-Lda3 antibodies label reticular structures closely associated with the cytoplasmic face of the inclusion membrane, and Lda3-EGFP expressed in mammalian cells localizes to LDs. To begin to evaluate the role of Lda3 in co-opting LDs, we followed the fate of ectopically expressed Lda3 in Chlamydia-infected cells. HeLa cells were transfected with an Lda3-EGFP expression vector, infected for 18h and 32h, and imaged by LSCM. Surprisingly, Lda3-EGFP prominently labeled the inclusion membrane in addition to LDs, especially at later stages in infection (Figure 17A).

These results indicated that Lda3 could potentially provide a physical link between LDs and the inclusion. We also detected Lda3-EGFP within the inclusion lumen (Figure 17B), suggesting that cytoplasmic Lda3 had translocated across the inclusion membrane. When Lda3-DsRed was expressed in oleic acid-treated cells, LDs that were positive for both Lda3-DsRed and BODIPY were observed in the cytoplasm and inclusion lumen suggesting that the intra-inclusion Lda3-positive structures are likely LDs (Figure 17C).
Figure 17. Ectopically expressed Lda3 binds to LDs and inclusion membranes

(A–C) Lda3-EGFP localizes to inclusion membranes and LDs. HeLa cells expressing Lda3-EGFP were infected with L2 for 20 hr (A and B) and imaged by LSCM. Lda3-EGFP localized prominently to LDs at the periphery of the inclusion and inclusion membranes (A). Intra-inclusion Lda3-positive material (arrows) was apparent in xz confocal sections (B). These intra-inclusion structures are likely LDs as Lda3-DsRed positive structures in OA-treated cells also stain with BODIPY. (Inset) Magnification of Lda3-positive LDs (C).

(D) Live cell analysis of LD translocation into the inclusion lumen. HeLa cells expressing Lda3-EGFP were infected with L2 for 30 hr and imaged for 30 min. Representative frames show Lda3-EGFP tagged LDs (arrows) docked at the inclusion lumen in the process of translocation. (Images rotated counterclockwise 90° from movie orientation.)

We took advantage of the dual tropism of Lda3-EGFP to monitor the interaction of LDs with the inclusion in real time. Selected frames from a time-lapse series show the translocation of Lda3-tagged LDs docked on the inclusion membrane into the lumen (Figure 17D, Suppl.movie 1, http://www.pnas.org/content/early/2008/06/27/0712241105/suppl/DCSupplemental). Lda3-labeled LDs were present close to the inclusion and in
contact with the inclusion membrane. Furthermore, time-lapse microscopy revealed that the association of Lda3-tagged LDs with inclusion membranes is dynamic, with docking and partial penetration stages that are reversible, especially in early- to mid-cycle inclusions (<24h) (Suppl. movie 2, http://www.pnas.org/content/early/2008/06/27/0712241105/suppl/DCSupplemental). However, LDs bound to the inclusion membrane in mature inclusions (>30h) either ceased to move or displayed restricted movement on the plane of the membrane (Suppl.movie 1).

**4.3.6. Lda3 overexpression promotes loss of the LD coat protein, ADRP**

LDs are heterogenous organelles with distinct protein compositions which may reflect different cellular functions (Welte, 2007). To determine if Lda3 labels all LDs, we expressed EGFP and Lda3-EGFP in lipid-loaded HeLa cells and monitored the co-localization of Lda3 with ADRP, a LD marker found in all cell types. Untransfected and EGFP-expressing cells displayed abundant ADRP-positive LDs. In contrast, Lda3-EGFP positive transfectants had a marked decrease in overall ADRP staining (Figure 18A). The loss of ADRP was more prominent in Lda3 transfected cells compared with Lda1, the LD-binding domain of Lda2 (Lda2_{LD}), and a catalytic mutant of the LD-associated lipase ATGL (Smirnova et al., 2006) (Figure 18C). Closer examination of LDs in transfected cells revealed that any remaining endogenous ADRP was restricted to distinct puncta on the surface of Lda3-EGFP positive LDs (Figure 18B).
Figure 18. Overexpression of Lda3 induces loss of ADRP from LDs

(A) Lda3-EGFP expressing cells display reduced levels of ADRP. HeLa cells were transiently transfected with Lda3-EGFP, treated with 100 μM OA for 12 hr, and fixed. ADRP on LDs was detected by IF. Prominent localization of ADRP to LDs was only observed in untransfected cells (*).(B) Close-up of Lda3-EGFP (green) positive LDs. Displacement of endogenous ADRP (red) to distinct puncta on the surface of LDs (arrowheads). (C) Loss of ADRP from LDs. The loss of ADRP from LDs was most pronounced in Lda3 compared with Lda1, Lda2, Lda1LD, and a catalytically inactive ATGL (ATGL*). Overexpression of wild-type ATGL led to a loss ADRP. Data represent the mean ± SD of triplicates. IM, inclusion membrane.
Overall, these results demonstrate that exogenously expressed Lda3 remains associated with LDs during the initial translocation into the inclusion lumen, and that the association of ADRP with LDs decreases upon Lda3 overexpression.

4.4. Conclusions

The *Chlamydia* inclusion is frequently noted for its abilities to restrict fusion with endolysosomal traffic, and its impermeability to small molecules (Heinzen and Hackstadt, 1997; Scidmore et al., 2003). For this reason, most research on the subject of lipid metabolism has concentrated on identifying vesicle and membrane transport pathways that can fuse with the inclusion and deliver essential lipids. Unexpectedly, we observed the translocation of entire, intact LDs into the lumen of the *Chlamydia* inclusion. This is the first description of such an event by a bacterial pathogen, and it reshapes our view of how *Chlamydia* interacts with the host cell.

Although the molecular basis for the capture and translocation of LDs into the inclusion lumen remains to be elucidated, our findings suggest a potential role for the bacterial protein Lda3. When ectopically expressed, Lda3 has tropism for both LDs and the inclusion membrane indicating its potential to act as molecular bridge between them. Overexpression of Lda3 also leads to the redistribution and loss of ADRP from the surface of LDs (Figure 18). Based on our live cell observations and TEM analysis, we propose a model for events during LD capture and translocation into the inclusion lumen (Figure 19). Secreted Lda3 binds to LDs in the vicinity of the inclusion. The Lda3-tagged LDs then dock with the inclusion membrane by binding to a hypothetical
chlamydial protein (IncX). Lastly, the inclusion membrane invaginates to deliver an intact LD into the inclusion lumen where it is engaged by RBs. Lda3 may also participate in this process by aiding in the localized displacement of ADRP from the LD surface, presumably to promote lipolysis. Although Lda3 has properties compatible with a role in LD entry, limitations of the experimental system preclude us from excluding additional factors or conducting direct tests by mutational analysis.

Interestingly, the membranes associated with intralumenal LDs appear to be distinct from the bulk of inclusion membranes as IncA was the only Inc tested that cofractionated with LDs and accumulated in the inclusion lumen. These findings suggest that IncA may mark inclusion membrane sites permissive for LD entry, although we have no evidence that IncA is required for this process.

![Figure 19. A model for LD interaction with the Chlamydia inclusion](image)

(I) LDs are engaged by secreted Lda3 at the surface of the inclusion. (II) Lda3-tagged LDs are captured at the inclusion membrane by an unidentified inclusion membrane protein(s) (IncX). (III) The inclusion membrane invaginates to deliver the LD to the inclusion lumen. (IV) RBs intimately bind to the intrainclusion LD and associated inclusion membranes. Lda3 may participate in initiating LD lipolysis by promoting the removal of ADRP.
In summary, we have found that cytoplasmic LDs are translocated into the lumen of the *Chlamydia trachomatis* inclusion, providing an alternative to Golgi-derived vesicles and MVBs for lipid acquisition. This pathogenic strategy would allow *Chlamydia* to remain hidden from innate immune surveillance in the cytoplasm while directly obtaining nutrients. Furthermore, by sequestering lipolysis in the inclusion lumen, the bacteria can limit the release of toxic byproducts that activate inflammatory responses. Whether organelle translocation into the inclusion is restricted to LDs or it represents a more generalized strategy for nutrient acquisition remains to be determined.

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**Relative contributions:**
For this work, our lab partnered with Dr. Ted Hackstadt’s group at the Host–Parasite Interactions Section, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT. The fixation method for LD preservation and excellent EM images were done by E.R. Fischer. Y. Kumar helped in some sample preparation for EM imaging. Final live cell movies were taken by R. Valdivia. Western blots of LDs isolated from Chlamydia-infected cells were done by Y. Kumar and J. Cocchiaro. Y. Kumar helped J. Cocchiaro as second, blinded investigator for quantitation of ADRP loss in Lda3-transfected cells. All remaining experiments were performed by J. Cocchiaro.
Chapter 5. Functional analysis of the chlamydial Lda3 protein

5.1. Introduction

Protein targeting to LDs

The general mechanisms of LD protein targeting in eukaryotic cells are not well-understood. Proteomics studies suggest that cellular LDs can associate with as many as 50 different proteins, and the protein complement varies depending on the cell type and nutritional state of the cell (Brasemle et al., 2004; Liu et al., 2004; Thiele and Spandl, 2008). So far, however, no common primary sequence motifs that are universally required for LD-targeting have been identified, and most research suggests that protein structural characteristics are more influential in regulating LD association (Hickenbottom et al., 2004; Targett-Adams et al., 2003; Thiele and Spandl, 2008).

Many LD proteins are peripherally associated with the LD surface. Hydrophobic regions and amphipathic helices are emerging as common features of these LD-binding domains (Boulant et al., 2006; Mullner et al., 2004; Zehmer et al., 2008). The PAT family proteins (e.g. ADRP, TIP47) are among this category. Some proteins are anchored by a lipid moiety, such as the Rab GTPases (e.g. Rab18). Others, such as Nsdhl (Caldas and Herman, 2003), caveolins (Ostermeyer et al., 2004) and diacylglycerol acyltransferase 2 (DGAT2) (Stone et al., 2006), are “monotopic” integral membrane proteins that have a hairpin loop which extends into the LD lumen (reviewed in (Thiele and Spandl, 2008)). LD structure, with very hydrophobic core and phospholipid monolayer, generally
excludes the incorporation of integral membrane proteins with multiple transmembrane segments (Thiele and Spandl, 2008).

Understanding the determinants that govern LD protein localization is important since associated proteins are believed to govern many aspects of LD function. This is especially notable in the case of PAT family proteins which regulate lipid storage and mobilization from LDs through exchangeable localization with the LD surface (Wolins et al., 2006). Additionally, association with motor proteins, such as dynein, and Rab GTPases are implicated in control of LD traffic and interaction with other cellular organelles (reviewed in (Murphy et al., 2008; Zehmer et al., 2009). While chlamydial Ldas are the only bacterial proteins known to target LDs, several viral proteins are noted to have this capability including HCV core protein (Hope and McLauchlan, 2000), HCV NS5A (Shi et al., 2002), reovirus capsid protein μ1 (Coffey et al., 2006). Association of HCV core with LDs is essential for the production of infection-competent viral particles, and it is suggested that some steps of virus assembly may actually occur at LD membranes (Miyanari et al., 2007).

**Chlamydia proteins that target LDs**

*Chlamydia* infection disrupts the normal functioning of LDs in host cells. One of our main goals is to understand how the bacteria is able harness these functions. The best candidates to be involved in this process are the chlamydial Lda proteins, which we identified by their tropism for LDs in both yeast and mammalian cells. When natively expressed during infection, LD-associated (Lda) proteins are able to access to the cytoplasmic face of the inclusion and associate with lipid-rich structures at the
periphery. Colocalization studies showed that the neutral lipid-coat at the inclusion contains closely apposed ER, mitochondria, and LDs, and ectopically expressed Lda proteins can target to these organelles with different affinities. This suggests that they may have distinct roles in maintaining the interaction between Chlamydia, LDs, and other host compartments involved in lipid metabolism.

Of particular interest, Lda3 is unique in its ability to bind both the inclusion membrane and LD surface representing an unparalleled opportunity to examine how components may be exchanged between the two compartments. Also, displacement of the coat protein, ADRP, from LD surfaces is more pronounced during overexpression of Lda3 compared with other Lda proteins. These features suggest a possible role for Lda3 in the capture of LDs for translocation, and in providing access to the LD core for lipolysis. Although this is an appealing hypothesis, we have not been able to directly test whether Lda3 is an essential factor in these processes. In addition to LD and inclusion membrane binding, Lda3 interacts with itself by yeast 2-hybrid and is toxic when expressed in yeast.

Elucidation of Lda3 functional domains will allow us to compare these features among the Lda proteins and gain a better understanding of how Chlamydia modulates LD function. Additionally, separating out the regions with bacterial-specific functions from with those in their eukaryotic counterparts will help to define consensus signals that lead to LD association.
5.2. Materials and Methods

Cell lines, yeast strains, reagents

HeLa cells were acquired from ATCC and grown in DMEM supplemented with 10% FBS unless noted. Wild-type yeast cells [DLY1554 (MATa ura3Δ leu2Δ his3Δ lys2Δ)] were used for phenotypic assays. For yeast 2-hybrid (Y2H) analysis, GAL4 activation domain (AD) vector was transformed into pJ69-4A (MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ) and binding domain (BD) vector was in AH109 (MATα, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1uas-GAL1tata-HIS3, GAL2uas-GAL2tata-ADE2, URA3::MEL1uas-MEL1tata-lacZ, MEL1). Yeast cultures were grown in complete synthetic medium (CSM) dropout mixes (Sunrise Science Products) supplemented with 2% dextrose (dex) or galactose (gal) as indicated. All yeast methods were based on protocols outlined in (Brown and Tuite, 1998).

Lda protein primary and secondary structure analysis

Lda3 protein primary sequence and homology searches were done using the Comprehensive Microbial Resource (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi) and STGEN (http://stdgen.northwestern.edu/) databases. Secondary structure of Lda3 was analyzed with a variety of prediction programs. Most of these programs were freely available at the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) (http://expasy.org/). Jpred3 (Cole et al., 2008) and Macvector were used to identify helices, β-sheets, and coiled-coil regions. Sites for
truncation of Lda3 were based on these predictions. Prediction of amphipathic helices and in-plane membrane anchors was done using the Amphipaseek program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_amphipaseek.html) (Combet et al., 2000; Sapay et al., 2006). Helical wheel diagrams were generated using the WheelApp applet (http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html).

**Construction of Lda3 truncation mutants**

Lda3 deletion mutant sets for yeast phenotypic assays and Y2H analysis were made using the yeast homologous recombination system as previously described (Sisko et al., 2006). Briefly, to generate constructs with C-terminal GFP fusions, long-tail oligonucleotide primers were designed to amplify segments of *lda3* from the already sequenced and confirmed pSDY8 library vector (Sisko et al., 2006). PCR products were amplified using Expand High Fidelity DNA polymerase (Roche) and transformed into yeast with pSDY10 vector (P*GAL*-GFP, 2μ *LEU2*) linearized with SpeI/HindIII digestion and CIAP-treatment (Roche). Plasmids from Leu+ transformants were recovered and sequenced to confirm in-frame splicing with the GFP and lack of nonsynonymous polymorphisms.

For Y2H deletion libraries, *lda3* fragments were amplified from the sequenced pSDY10 constructs using primers homologous to the GAL4 activation domain (AD) vector, pGAD424 (2μ *LEU2*, Amp®), and binding domain (BD) vector, pGBT9 (2μ *TRP2*, Amp®) (Matchmaker system, Clontech) as described. Plasmids were recovered, sequenced, and retransformed into AH109 and pJ69-4A reporter yeast strains. For
analysis of Lda3 truncation targeting in mammalian cells, Lda3 fragments were excised out of pSDY10 sequenced vectors using SpeI/HindIII and cloned into the Nhel/HindIII sites of pEGFP-N1 (Clontech).

Yeast growth toxicity assay

The pSDY10 plasmids containing Lda3 coding fragments were transformed into MATα and MATa yeast. Isolated transformants were grown overnight in CSM-leu/dex liquid cultures at 30°C. The next day, 10-fold serial dilutions were made in CSM-leu lacking sugar and 10μl aliquots were spotted to CSM-leu/gal to induce protein expression. Plates were grown at 30°C for at least 3 days to allow for appropriate growth. Truncation mutants were evaluated for loss or enhancement of yeast growth defects compared to the full-length Lda3 protein or vector alone.

Yeast 2-hybrid assay

To identify Lda3 domains responsible for self-interaction, Y2H assay was performed for all Lda3 truncation mutant s against the full-length Lda3. PJ69-4A with pGAD424-Lda3 full-length and AH109 containing pGBT9-Lda3 truncation mutants were grown at 30°C overnight in 3 ml cultures in selective media with 2% dex. Yeast transformants were reciprocally mated to obtain diploids containing both an AD and BD plasmid. Mating was done in 96-well tray format where 5μl each of AD and BD cultures were mixed together in 200μl rich liquid growth media (YPD), and incubated at 30°C for 2-3 days. Cultures were pinned to CSM-leu-trp/dex agar plates and incubated at 30°C for 2-3 days to select for yeast containing both plasmids. Mated cultures were re-pinned into CSM-trp-leu/dex liquid media and incubated for 3 days at 30°C. Mated yeast
cultures were pinned to CSM-trp-leu/dex, CSM-histidine/dex, and CSM-adenine/dex and grown for 3 days at 30°C to assess expression of the reporter constructs. Prototrophy on CSM-histidine/dex was scored as a positive interaction. Vectors lacking Lda3 fragments were used as a positive control for mating and negative control for protein interaction.

**LD and inclusion membrane localization of Lda3**

To identify LD-associated chlamydial proteins in yeast, MATα yeast cells expressing an RFP-tagged LD marker, Erg6p (Huh et al., 2003), were transformed directly with the GFP-tagged Lda3 truncation constructs in pSDY10. Transformants were grown overnight in CSM-leu/dex liquid culture, and cultures were spotted to CSM-leu/gal for expression of truncation constructs, and incubated at 30°C overnight. Yeast were resuspended in small volumes of sterile water and imaged live for colocalization of RFP and GFP signals. Yeast cells were imaged on a Zeiss Axioskop microscope.

For analysis of Lda3 localization in mammalian cells, pEGFP-N1 vectors expressing Lda3 fragments were transfected into HeLa cells using Fugene (Roche) as instructed by the manufacturer. Expression was assessed between 24-36 hrs of transfection both with and without oleic acid treatment (200μM for 12 hr). Coverslips were fixed with 3% formaldehyde/0.025% glutaraldehyde in PBS. For experiments examining inclusion membrane binding, HeLa cells were infected with *C. trachomatis* L2 at an MOI ~2 at around 4 hr post-transfection. Cells were fixed and imaged at 24 hr post-infection. Samples were imaged by LSCM on a Leica SP5 confocal microscope.
Live cell analysis of Lda protein mobility

Lda protein mobility was analyzed using fluorescence recovery after photobleaching (FRAP) and GFP photoactivation (PA). Photoactivatable GFP (PAGFP) was excised from the mito-PAGFP vector (Karbowski et al., 2004) by BamHI/NotI and ligated into pCDNA5-Lda3 generating a C-terminal fusion protein.

For all live cell imaging, HeLa cells were seeded at 3x10^5 in 35mm tissue culture dishes with glass coverslips (MatTek) and grown in DMEM with 10% FBS. For FRAP, cDNA5-Lda3-EGFP was transfected into cells using Fugene (Roche) as instructed, and ER-RFP was co-transfected as a marker of cellular architecture. Cells were infected with C. trachomatis serovar L2 between 2-4 hr post-transfection. Media was replaced 12 hr prior to imaging with fresh medium containing 50μm oleic acid to induce the accumulation of LDs. Cells were imaged live at around 20 hr post-transfection.

Immediately prior to imaging, cells were rinsed and media was replaced with 3 mL phenol-free DMEM plus 50mM HEPES to maintain pH of the media.

For photoactivation, experimental details were the same except that cDNA5-Lda3-PAGFP and soluble mCherry-C1 were co-transfected into HeLa cells. Imaging was done at 37°C with a 63X/1.2 Leica Plan Apochromat water objective on a Leica SP5 confocal microscope outfitted with a temperature controlled Ludin cube. Photobleaching was done using a 488 nm argon laser at 100% transmittance for 3-5 iterations at 1.314 sec (512x512 format and 400Hz scan speed). Lda3-PAGFP photoactivation was induced with a 405nm Diode laser at 100%T for 2 iterations at
1.314sec or 8 iterations at 0.222sec. Images pre- and post-activation were acquired at 488nm and 561nm with low intensity light.

5.3. Results

5.3.1. Primary and secondary structure characteristics of Lda3

![DUF37](image)

**Figure 20. Secondary structure features of the C. trachomatis Lda3 protein**

Graphical representation of the *C. trachomatis* serovar D Lda3 protein. Predicted secondary structures and locations are noted. The coding sequence above the drawing highlights the amino acid residues that correspond to predicted helices. Helical wheel projections were created with the WheelApp applet (http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html). Note the amphipathic organization of helix1. Residue color key: yellow = nonpolar; green = uncharged, polar; blue = basic; pink = acidic.

Lda3 is a small, 114 amino acid protein, and is relatively basic with a pI of ~9.4. Although the sequence does not display a highly complex secondary structure, three main alpha-helices are predicted and lay toward the N-terminus of the protein (Figure 20). Because amphipathic helices are increasingly being recognized as features of LD-
tropic proteins (Boulant et al., 2006; Hickenbottom et al., 2004), Lda3 protein sequence was run through the AmphipaSeek prediction program to identify regions with potential amphipathic character (Sapay et al., 2006). The first N-terminal helix displays a high degree of amphipathy with perfectly equal polar and non-polar faces within the predicted in-plane membrane anchor (IPM) region (Figure 19). Helix3 is also moderately amphipathic, but helix 2 is not (Figure 20). Lda3 is conserved between C. trachomatis serovars and has orthologs in other chlamydial species including, C. muridarum, C. pneumoniae, C. felis, C. abortus, and C. caviae (Read et al., 2000; Stephens et al., 1998). Lda3 also contains a domain of unknown function (DUF37) from aa 6-73 that is conserved among bacteria but has no known function (NCBI BLAST).

5.3.2. The N-terminus of Lda3 is necessary and sufficient for LD targeting

We reasoned that LD protein targeting is likely controlled by similar mechanisms in both eukaryotic and bacterial proteins. Therefore, one of our main goals was to identify features of Lda3 that control its ability to target to the LD surface. Since Lda3 is a Chlamydia-specific protein and does not have sequence homology to known proteins, our only clue to the potential functional regions of Lda3 is the structure. Because helix 1 has amphipathic character, we suspected that this domain may be involved. To identify regions of Lda3 responsible for LD binding, a set of Lda3 truncation mutants fused with GFP at the C-terminus was constructed based on helix positions. The localization properties of these mutants were examined in yeast and mammalian cells.
Figure 21. Targeting of Lda3 truncation mutants to lipid droplets in yeast

Lda3 deletion mutant association with LDs. Inducible expression vectors containing Lda3 truncation fragments were transformed into yeast with a chromosomally encoded erg6:RFP protein as a marker for LD surfaces. Lda3 truncation expression was induced by growth on agar plates with 2% galactose. LD association was scored by colocalization with erg6:RFP and refractile bodies in DIC by fluorescence microscopy.

In yeast, all Lda3 truncation fragments containing the N-terminal portion of the protein correctly target to the surface of LDs as shown by the colocalization with erg6-positive ring-like structures (Figure 21). Removal of the N-terminal 26 residues from Lda3 results in the mistargeting of GFP to punctuate structures at the periphery of the yeast cell which do not match up with Erg6-RFP. C-terminal fragments of Lda3 lacking all helices do not localize with LDs and are soluble in the cytoplasm.
Lda3 deletion mutants in the pEGFP-N1 mammalian expression vector were ectopically expressed in HeLa cells. At 26-30 hours, post-transfection cells were fixed, and targeting of truncated fragments to LD surfaces was assessed by fluorescence microscopy. Insets at top left are magnified images of the regions indicated in yellow. N: nuclei.

Next, the targeting of ectopically expressed Lda3 deletion constructs was examined in mammalian cells. Similar to the results seen in yeast, the N-terminal fragment of Lda3 containing amino acids 1-26 is sufficient for targeting GFP to the surface of mammalian LDs (Figure 22). Removal of this region results in a loss of Lda3 localization to LDs in HeLa cells, and GFP is redistributed to tubular structures that resemble mitochondria. Interestingly, targeting to the LD surface appears to be enhanced in the 1-65 fragment containing helix 3 (Figure 22).

These data identify helix1 as the primary determinant of LD binding in Lda3. The more prominent labeling of LD surfaces when helix3 is included suggests that this domain may help to stabilize the interaction with LDs but is not essential.
5.3.3. The Lda3 N-terminal helix mediates inclusion membrane binding

The inclusion membrane, as a bilayer, is fundamentally different from the phospholipid monolayer that bounds the core of LDs. Also, the inclusion membrane has a unique complement of embedded, bacterially-derived proteins. As such, we hypothesized that Lda3 contains special targeting signals for inclusion membrane interaction apart from those involved in LD localization. To test this theory, the collection of Lda3 deletion mutants was expressed in C. trachomatis-infected HeLa cells and examined for binding to the inclusion membrane. Cells were imaged by confocal microscopy for fine resolution of events at the inclusion membrane boundary.

Figure 23. Interaction of Lda3 deletion mutants with the inclusion membrane
HeLa cells were transfected with EGFP-tagged Lda3 truncation fragments and subsequently infected with *C. trachomatis* serovar L2. At 24 hr post-infection, cells were fixed and localization patterns were evaluated by confocal microscopy. Inclusions are indicated by arrows. Insets show zoomed images of the inclusion periphery. Arrowheads denote lipid droplets. Inc: inclusion; N: nuclei.

Surprisingly, in contrast to our prediction, Lda3 deletion mutants that localize to the inclusion membrane mimic those involved in binding to LDs. The 1-26 and 1-30 fragments, both which contain helix 1, are sufficient to target GFP to the inclusion periphery. Helix 2 expressed by itself (26-50) is targeted to mitochondrial tubules, and does not appear to bind the inclusion membrane. Localization to LDs is enhanced in construct 1-65 which contains all 3 helices, and inclusion membrane binding is retained. The C-terminal fragment 72-104 displays cytoplasmic localization. Interestingly, expression of helix 3 by itself occasionally exhibits localization to the inclusion membrane and small LDs (Figure 23).

From these results, we conclude that helix 1 of Lda3 is both necessary and sufficient for targeting to LDs and the inclusion membrane. Helix 3 may also play a role in these events, but more analysis is needed to verify this observation.

**5.3.4. Lda3 toxicity and self-interaction require helix 3**

In a screen aimed at assigning functions for *Chlamydia*-specific proteins, Lda2 (see Ch.3) and Lda3 were identified as cytotoxic factors to yeast growth (Sisko et al., 2006). Cytotoxicity indicates that the heterologously expressed protein disrupts essential cellular processes (Valdivia, 2004). Additionally, Lda3 interacts with itself by yeast 2-hybrid suggesting that it could dimerize or form higher-order oligomeric
complexes at the surface of the inclusion. Theoretically, this might aid in the clustering of Lda3 or other interacting factors which are important for recruitment of LDs to the inclusion. We expected that the domains controlling Lda3 functions would be distinct from the cellular localization signals.

![Diagram of Lda3 domains](image)

**Figure 24. Identification of Lda3 domains involved in cytotoxicity and self-interaction**

**Phenotypic characterization of Lda3 truncation expression on yeast growth.** Overnight cultures of yeast containing Lda3 truncation fragments were serially diluted and spotted onto agar plates containing 2% dex or 2% gal and toxicity was scored after 3 days of growth at 30°C. For toxicity, +++ denotes toxicity equal to the full-length (FL) Lda3 protein, and – indicates growth comparable to the vector alone control (not toxic). **Yeast two-hybrid interactions.** PJ69-4A yeast containing activation domain vectors with truncated Lda3 fragments were crossed with AH109 containing the full-length Lda3 protein in the binding domain vector. Protein-protein interactions were detected by growth on media lacking histidine. For 2-hybrid, + and – signify the level of growth on reporter media.

To identify toxic fragments, Lda3 deletion mutants were evaluated for decreased yeast growth in response to protein induction. Toxicity is abrogated in Lda3 fragments containing amino acids 1-50 or less (Figure 24). Yeast growth is inhibited at levels similar to the full-length Lda3 protein when helix 3 is included (fragment 1-65).
Addition of helix 3 to just the C-terminal portion of the protein also restores cytotoxicity (fragment 50-104) (Figure 24). Taken together, these results suggest that cytotoxic functions of Lda3 are mediated by helix 3. The cause for loss of toxicity in constructs from the N-terminal 1-50 amino acids is unknown. However, in contrast to Lda2, we do know that Lda3 protein function and stability is not dependent on LD formation (Ch.3, Fig. 10A), and protein expression levels of these constructs are normal (not shown). This suggests that cytotoxic and LD binding functions are directed by discrete domains within Lda3.

To determine the domains involved in Lda3 self-interaction, yeast 2-hybrid analysis was performed between the full-length (FL) Lda3 protein and the collection of deletion constructs. The minimal region of Lda3 required for a robust positive interaction with FL Lda3 is fragment 1-65 (Figure 24). A small amount of growth is observed with fragment 1-50. No protein-protein interaction is found between Lda3 FL and C-terminal fragments lacking helix 1 or helix 2. These data indicate that helix 3 is essential for the ability of Lda3 to interact with itself (Figure 24).

5.3.5. Mobility of Lda3 in the inclusion membrane and LDs

The fact that the molecular determinants of Lda3 binding appear to be the same for LDs and the inclusion membrane implies that these two membranes are more similar in nature than originally predicted. Despite this, biophysical differences between these membranes may exist and would not be seen by static imaging. Due to its dual targeting properties, the chlamydial Lda3 protein provides the opportunity to directly compare the characteristics of protein movement between LD and inclusion membranes.
Fluorescence recovery after photobleaching (FRAP) and tracking of photoactivated fluorescence molecules provide information about a variety of parameters including the viscosity of membrane environments, whether proteins are part of complexes, and if transport occurs through diffusion or is motor driven (Snapp et al., 2003). We applied these techniques to follow the mobility of ectopically expressed Lda3 in Chlamydia-infected cells.

5.3.5.1. Lda3 mobility into LD membranes

We postulated that LDs serve to directly transport lipids to the inclusion membrane by flow through membrane contact, and that Lda3 may provide a structural link for this process. If this were true, we would expect to see rapid lateral transfer of Lda3 between LD and inclusion membranes. To follow Lda3 protein mobility, we performed live cell imaging of HeLa cells that were transfected with Lda3-EGFP and infected with C. trachomatis L2. Regions of contact between LDs and the inclusion membrane were selectively photobleached (Figure 25A).

When Lda3-EGFP is photobleached in a region of interest (ROI) on the inclusion membrane, fluorescence in the bleached area rapidly recovers (Figure 24A). The ROI was pulsed with high intensity light at 488nm for ~7sec. However, the decrease in fluorescence only reached 40% of the original value. Lateral diffusion within membranes typically occurs on the order of ~0.01μm²/sec (Chen et al., 2006), and our experimental set-up took images every 1.314 sec. This suggests that Lda3 is moving very rapidly within the inclusion membrane, and recovery starts to occur before the first post-bleach images are taken (Figure 25A, top panel).
Figure 25. Comparison of Lda3 protein mobility in the inclusion membrane and LDs
A. Fluorescence recovery after photobleaching (FRAP) of Lda3-EGFP. Pre-bleach, post-bleach, and post-recovery images of cells in which a region of the inclusion membrane (top panel) or a lipid droplet at the periphery (bottom panel) were selectively photobleached using high intensity light at 488nm for 4-5 sec. B. Fluorescence intensity recovery plot. Fluorescence intensity in the photobleached regions of interest (ROI) was measured in pre-bleach and post-bleach images over the 60 sec recovery. The integrated intensity values were corrected for background fluorescence and normalized to pre-bleach intensity. Mobile fractions indicate the level of recovery to pre-bleach values from the value recorded directly after photobleaching. Plots are color coded with their respective ROI. Dotted lines show nuclei boundaries. Large arrows indicate inclusions and small arrows point to LDs. N: nuclei. Scale bars = 12.5 μm.

In contrast, when an Lda3-positive LD at the surface of the inclusion was photobleached, fluorescence was not recovered in the same manner. Examination of the bleached ROI at the end of the series reveals that while fluorescence recovers in the inclusion membrane adjacent to the LD, Lda3 does not rapidly re-populate the surface of the bleached LD (Figure 25A, bottom panel).

When we compare the rate of percent fluorescence recovery between the two ROIs, fluorescence intensity reaches the half-maximal value at approximately the same time, but the percent mobile fractions are drastically different (Figure 25B). The interpretation of these data is that the fluorescence recovery seen in the LD-bleached ROI likely corresponds to the repopulation of the inclusion membrane signal that was included within the region. This accounts for the similarity in rates of recovery. The small mobile fraction that recovers in the LD sample is due to lack of Lda3 movement into the LD membrane within the imaging time frame.

Overall, these results suggest that the inclusion membrane and LD membranes have different properties, at least with respect to the characteristics which control Lda3
protein targeting to these membranes. Additionally, we do not observe the transport of Lda3 protein from the inclusion membrane into LDs. If we assume that the LD has not moved out of the ROI, this indicates that LDs and the inclusion membrane are not directly connected, or that transport between the two organelles may take longer than the time frame that was documented.

5.3.5.2. Lda3 mobility from LD membranes

The results of the previous experiment implied that Lda3 does not move between LD and inclusion membranes or that the mechanisms involved are much slower than predicted. In FRAP experiments, the fact that LDs are mobile complicates analysis since LDs can move in and out of the ROI during imaging. Therefore, we also examined mobility of Lda3 tagged with photoactivatable GFP (PAGFP).

HeLa cells expressing Lda3-PAGFP and treated with oleic acid were imaged live. Lda3-PAGFP was activated at an area adjacent the Chlamydia inclusion that was packed with large LDs. Exposure to light at 405nm for 1.776 sec resulted in an approximately 40% increase in fluorescence intensity (Figure 26A and 26C). The movement of fluorescent Lda3 was tracked, but during the image acquisition series, little transport of Lda3 out of the photoactivated region was observed. The fluorescent signal eventually dispersed and was noted at the start of a new imaging series ~17 min later (Figure 26A).
Figure 26. Diffusion of Lda3 protein from lipid droplet membranes

A. Lda3-PAGFP photoactivation series. Infected cells expressed Lda3-PAGFP and soluble mCherry. Photoactivation was achieved with high intensity light pulsed at 405nm for 1.776 sec. Insets show the ROI fluorescence before and after activation. B. Regions of interest for fluorescence intensity measurements. Positions of the ROIs used to calculate values in the fluorescence recovery graph. C. Measurements of fluorescence diffusion. Fluorescence intensity values in the photoactivated region and adjacent regions were calculated and plotted over time. Integrated intensity values were
corrected for background and normalized to pre-activation. Arrows denote inclusions, N: nucleus.

Measurements of fluorescence intensity in several ROIs were analyzed for 8.3 min following activation to assess transport of Lda3 to other regions of the cell including the inclusion membrane (Figure 26B and 26C). While Lda3 signal in the activated region did eventually decay, the fluorescence intensity values never reached an asymptote indicating that the signal was still in the process of dispersing at the end of imaging. If, as we originally hypothesized, LDs and the inclusion membrane are in direct contact and Lda3 is able to transfer between the two compartments, we would have expected to see an increase in the level of Lda3-EGFP in the inclusion membrane adjacent to the photoactivated LDs. However, gains in fluorescence intensity for regions adjacent to the LDs in the activated ROI were minimal, and no increase GFP signal was detected in the ROI at the inclusion membrane (Figure 26C).

These results can be interpreted several ways. It suggests that protein is not transferred from LDs to the inclusion membrane, or that the LD surface may act as a terminal sink once a protein is bound. However, the enhancement of LD production using oleic acid was particularly effective in this experiment. If Lda3 has a higher affinity for LD membranes compared to the inclusion membrane, then Lda3 would be less likely to be lost from the LD surface particularly with the large level of LD membranes that were present in these cells to act as acceptors.
5.4. Conclusions

In this chapter, we aimed to identify the determinants within Lda3 that regulate its various functions. Through studies using deletion mutants, we examined the domains involved in mediating Lda3 cytotoxicity, yeast 2-hybrid interaction, LD targeting and inclusion membrane binding.

For both toxicity and yeast 2-hybrid interaction, we found that the region of Lda3 containing the third alpha-helix was required for these functions. We expected that these regions would stand apart from the LD and inclusion membrane binding properties of Lda3.

We were particularly interested in identifying the Lda3 domains which control binding to the inclusion membranes or LDs. Lda3 is unique in the ability to target to both of these compartments, and this ability is hypothesized to have a potential role in the process of LD translocation. Since other LD-associated proteins do not target to the inclusion membrane, we hypothesized that targeting to LDs would occur similarly among proteins and that different domains would be important in inclusion binding.

Surprisingly, experiments revealed that the amphipathic helix at the N-terminus of Lda3 appeared to be involved in the binding to both LDs and the inclusion membrane. At face value, this result implies that the inclusion membrane and LDs may share similarities in composition.
Some studies have implicated LDs in pathways of lipid and membrane traffic in the cell, and we have theorized that LDs may serve as a source of phospholipids to the inclusion. The direct transfer of lipids at membrane contact sites is the proposed mechanism for these events. Therefore, we postulated that the chlamydial inclusion may obtain membrane lipids and precursors by direct contact with LD membranes.

If this hypothesis was true, we would expect to see the transfer of lipid between LDs and the inclusion membrane. Since Lda3 targeted to both of LDs and inclusion membranes, it was a perfect tool to observe these events and to be able to compare the biophysical characteristics of LDs and inclusion membranes.

First we looked at the mobility within the inclusion membranes compared to individual lipid droplet or clusters of droplets. Our results showed that recovery of fluorescence within the inclusion membrane happens very quickly, whereas, the recovery of fluorescent proteins back into LDs is not observed. These results suggest that LD and inclusion membranes are indeed different from each other as expected, but it also implies that membrane transport between the two compartments may not occur as readily as our model predicts.

Similar results were seen when we used photoactivated GFP to track the flow out of LD membranes into the cell. Dispersion of the fluorescent signal out of the photoactivated area did not reach a minimum over the 8 min acquisition window. It was not until almost 25 min later that protein seemed to have travel out of the LDs.
These results suggest that once proteins are targeted to LDs they do not readily diffuse from this compartment, and this would prohibit the ability of LDs to transport proteins to the inclusion membrane through transient contact.

Going forward from this point, we would like to compare the transport of the individual Lda proteins and determine whether signals similar to those seen for Lda3 are involved the targeting of these proteins to LD and other host organelles. Additionally, we plan to explore the N-terminal helix of Lda3 to determine if specific residues are involved in LD versus inclusion membrane binding.
Chapter 6. Future Directions

The work presented here reveals lipid droplets as novel target organelles of *Chlamydia* modulation, and identifies neutral lipid as a new class of host lipid that is essential for *Chlamydia* development. We have shown that *Chlamydia* targets and modulates the normal cellular transport and functions of host LDs. Neutral lipid biosynthesis led to decreased chlamydial growth and replication, suggesting that LD formation or LD-associated host factors are crucial for proper bacterial development. Most surprisingly, we find that whole, intact LDs are translocated into the lumen of the inclusion. Some of our findings provide new answers to many of the open questions in the subject of lipid transport to the inclusion. However, we are left with many more exciting questions and challenges to answer.

**New source for non-vesicle mediated uptake of phospholipids**

A unique feature of *Chlamydia* that has been studied for several years is that the phospholipid (PL) content of bacterial membranes is akin to that of the host cell, containing several PL species not generally found in prokaryotes (Hatch and McClarty, 1998). Phosphatidylcholine (PC) in particular makes up a large proportion of bacterial membranes, and is acquired from the host cell by *Chlamydia*. Host PLs are cleaved at the sn-2 position, presumably by cytosolic phospholipase A2 (cPLA2), to generate lysophospholipids (lyso-PLs) and arachidonic acid (Su et al., 2004). Lyso-PLs are then taken up by the bacteria through an unknown mechanism and modified with bacterially-derived branched chain fatty acids.
(Wylie et al., 1997). Golgi-mediated transport events were ruled out for PL trafficking, but direct membrane contact and other non vesicle-mediated pathways were proposed to be involved. Recent lipidomic analysis reveals that the LD phospholipid monolayer is specifically enriched for lyso-PC and PC, suggesting that LDs may represent a novel source for the direct acquisition of pre-processed building blocks for chlamydial membrane lipid synthesis (Bartz et al., 2007).

**A source of lipids for nutrients, energy, or membranes?**

The neutral lipid cores of LDs serve as a readily mobilizable source of precursors for energy generation and membrane synthesis in eukaryotic cells. Both of these LD functions may be important for chlamydiae since they do not encode for the full complement of enzymes necessary to generate their own ATP, and they require host phospholipids to supplement to their membrane synthesis needs (Moulder, 1991; Su et al., 2004).

**What is the source of lipase?**

In order to harness LDs for nutrients and metabolites, neutral lipids must first be broken down into their component parts by the action of lipases. It is unknown whether *Chlamydia* can generate the lipolytic enzymes necessary for this process. *Chlamydia* does not appear to have proteins homologous to those found in *M. tuberculosis*, which can generate its own triacylglycerol (TAG) storage inclusions and produces lipases that can degrade lipids (Cotes et al., 2007; Garton et al., 2002). From the EM images, we observe few whole LDs and a variety of membrane remnants litter the inclusion lumen hinting
that LDs are likely degraded after they are internalized. Additionally, we found that treatment of infected cells with a neutral lipase inhibitor (Gilham et al., 2003) severely restricted inclusion expansion indicating that the ability to catabolize neutral lipids is required for chlamydial growth.

How does translocation work? Are LDs the only internalized organelle?

The unique translocation and sequestration of LDs by the *Chlamydia* inclusion is one of the most intriguing findings from these studies. Determining the mechanisms involved in translocation will be necessary for examining if this phenomenon is an essential feature of *Chlamydia* pathogenesis. Whether other organelles are acquired in a similar fashion is something that we will actively pursue. Interestingly, when we look back at infected cells, Mitotracker staining of the infected cells indicate that activated mitochondria or mitochondrial products may be taken into the inclusion.

Is the translocation of LDs unique to *Chlamydia*?

The finding that whole, intact organelles can be transported into the inclusion lumen redefines our traditional view of the inclusion membrane as an impermeable barrier, and opens up new avenues in the field of *Chlamydia* basic research. Since several bacteria and parasites live within parasitophorous vacuoles similar to *Chlamydia*, it will be interesting to see if the targeting and translocation of lipid droplets is a universal feature of the intracellular lifestyle.
References


Murphy, D.J. (2001). The biogenesis and functions of lipid bodies in animals, plants and microorganisms. Prog Lipid Res 40, 325-438.


Chlamydophila abortus genome sequence reveals an array of variable proteins that contribute to interspecies variation. Genome Res 15, 629 - 640.


Biography

Jordan Lindsey Cocchiaro was born on September 11, 1978 in Weisbaden, Germany. She subsequently lived in California, England, and the small town of Altus, Oklahoma. Her father’s retirement from the Air Force sent her family home to Portsmouth, New Hampshire at the beginning of her Junior year in high school where she graduated as the Valedictorian of her class from Portsmouth High School in 1996. She attended Bates College in Lewiston, Maine graduating *cum laude* with a B.S. Degree in Biology in May of 2000. Following graduation, she worked for 3 years as a Research Assistant in the lab of Dr. Jean C. Lee at the Channing Laboratory in Boston, Massachusetts studying the role of *S. aureus* capsular polysaccharide in nasal colonization. She entered the Cell and Molecular Biology Program at Duke University in Fall 2003, and began her graduate work in the lab of Dr. Raphael Valdivia the following summer. While at Duke, she was awarded a Predoctoral Fellowship from the American Heart Association in 2007. In 2008, she received a Bacteriology Meritorious Research Travel Award from the Center for Microbial Pathogenesis at Duke and a Richard and Mary Finkelstein Student Travel Grant Award from the American Society for Microbiology to present her work at the 108th ASM General Meeting.

Publications


