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Recent Advances in *Chlamydia* Subversion of Host Cytoskeletal and Membrane Trafficking Pathways

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Abstract

Chlamydia species are obligate intracellular bacteria that cause sexually transmitted disease, ocular infections and atypical pneumonia. This review highlights recent advances describing the mechanisms by which *Chlamydia* subvert host cytoskeleton and membrane trafficking pathways to create a replication competent niche.

Keywords

Chlamydia; inclusion; actin; Rab GTPases; sphingomyelin; phosphoinositides; recycling endosomes; mitochondria

1. Introduction – *Chlamydia* developmental cycle

Chlamydia species are medically important obligate intracellular bacteria that cause sexually transmitted disease, ocular infections and atypical pneumonia [1]. Of the nine species within the family Chlamydiaceae, *Chlamydia trachomatis* and *Chlamydia pneumoniae* are responsible for the majority of human *Chlamydia* infections. Despite differences in clinical manifestations and tissue tropism, all *Chlamydia* species undergo a unique biphasic developmental cycle that takes place within a non-acidified membrane bound vacuole, termed the inclusion [2]. Infection is initiated by the attachment and subsequent parasite mediated endocytosis of the infectious but metabolically inactive elementary body (EB) into epithelial cells. Once internalized, EBs differentiate into the metabolically active but non-infectious reticulate bodies (RB), which replicate by binary fission. In an asynchronous manner, while some RBs continue to divide, others are triggered by unknown signals to differentiate back into infectious EBs. The developmental cycle ends with release and dissemination of infectious EBs within the host.

Immediately after formation, the properties of the nascent inclusion are modified by processes that are dependent on early *Chlamydia* gene expression resulting in avoidance of lysosomal fusion and microtubule-dependent trafficking of the inclusion to the microtubule organizing center (MTOC) [2]. Subsequently, the inclusion intercepts vesicular and non-vesicular mediated pathways to obtain host derived lipids such as sphingomyelin [3], cholesterol [4], glycerophospholipids [5] and neutral lipids [6]. Exploitation of host cellular pathways creates an environment not only conducive to differentiation and replication, but

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also one protected from innate and adaptive host immune responses in part through the inhibition of apoptosis [7] and decreased expression of MHC class I and II molecules [8]. Failure to modify the inclusion results in the eventual targeting of *Chlamydiae* to and destruction within lysosomes [2]. These interactions between the inclusion and the host are likely mediated by both type three secretion (T3S)-dependent and -independent cytosolic and inclusion membrane localized (Incs) [9] *Chlamydia* effectors, which are thought to directly target and exploit host cellular pathways [10,11].

Here we review recent advances in two rapidly developing areas of *Chlamydia* cell biology, exploitation of the host cytoskeleton and membrane trafficking pathways. Coopting these two basic cellular processes enable *Chlamydiae* to gain entry into non-phagocytic cells, obtain essential nutrients and precursors, modulate the fusogenicity and direct the intracellular trafficking of the inclusion, and exit the host cell.

2. Exploitation of the host cytoskeleton

Subversion of the host cytoskeleton via translocation of bacterial effectors or activation of signal transduction pathways via receptor binding is a pathogenic trait shared by many microorganisms including *Chlamydia* species [12]. *Chlamydiae* target actin, microtubules and intermediate filaments to regulate diverse aspects of their intracellular survival from entry into and exit from the host cell (Fig. 1).

2.1 Subversion of the host actin cytoskeleton

In vitro, *Chlamydiae* enter non-professional phagocytic cells by multiple mechanisms with the choice of strain, method of inoculation, host cell type and culture methods influencing which specific mechanism predominates. Mechanisms dependent and independent of clathrin, microfilaments and lipid rafts have all been documented [13]. Given the essential nature of entry into host cells and the multiple mechanisms that *Chlamydiae* utilize, it is no surprise that numerous *Chlamydia* ligands and host cell receptors have been reported [13] [14,15]. Despite the various entry mechanisms, ligands and receptors that have been proposed, there is a growing consensus that a Rac1-dependent actin remodeling entry mechanism is shared by many *Chlamydia* species [16–19]. Binding of EBs to the cell surface activates and recruits Rac1 to sites of EB entry of at least two *Chlamydia* species [18,19]. During *Chlamydia caviae* infection, Cdc42 [19] and Arf6 [20] are also activated and are required for entry. Cdc42 is not required for the entry of *C. trachomatis* [18] and the role of Arf6 during *C. trachomatis* infection has not been examined. It is not clear why the requirements for small GTPases differ between the *Chlamydia* species, but highlights the fact that multiple entry mechanisms can be utilized by *Chlamydia*. During *Chlamydia trachomatis* invasion, Rac1 is activated by Sos2 and Vav2 [14,21], Rac1 guanine nucleotide exchange factors (GEFs), and results in the recruitment of the actin regulators WAVE2 and Abi-1 and Arp2/3-dependent actin remodeling and transient pedestal formation [22]. Inhibition of actin polymerization or Rac-dependent signaling pathways by pharmacological inhibition, expression of dominant negative mutants or by siRNA knock down, results in inhibition of *Chlamydia* invasion demonstrating the importance of these pathways in multiple *Chlamydia* species [14,16–19,21]. For *C. trachomatis*, Rac1 activation may be mediated by tyrosine phosphorylation of the *Chlamydia* translocated actin-recruiting phosphoprotein (TARP) protein [21] (see below).

Bacterial pathogens subvert actin remodeling through translocation of effector proteins that modulate the activity of Rho family GTPases or directly nucleate actin polymerization. The *Chlamydia*-specific T3S-dependent effector TARP [23], which is translocated across the host plasma membrane during entry, regulates actin reorganization by both mechanisms. *In vitro*, TARP nucleates actin polymerization through a direct interaction with actin [24].

Actin polymerization is mediated by a carboxy-terminal WH2-like actin-binding domain common to all *Chlamydia* TARP orthologues and is essential for invasion [25]. TARP proteins that contain only a single actin-binding domain require oligomerization of TARP via the presence of proline rich repeat domains to nucleate actin [25]. In contrast, some TARP proteins contain multiple actin-binding domains that bypass the need to oligomerize [25]. A possible model that incorporates actin polymerization by both TARP and Arp2/3 proposes that Arp2/3 acts on TARP-nucleated filaments to produce branched chain actin filaments. In addition, at least for *C. trachomatis*, TARP may also mediate actin polymerization through an alternate Rac-dependent mechanism [14,21]. TARP orthologues from *C. trachomatis*, but not other *Chlamydia* species, are tyrosine phosphorylated by multiple host kinases including Src family [26], Abl [14] and Syc [27] kinases upon translocation into host cells via amino terminal tyrosine rich domain repeats. EB binding and activation of Platelet derived growth factor receptor (PDGFR) or PDGFR-independent activation of Abl kinase activates Rac-dependent signaling pathways via phosphorylation of Tarp, Vav2, and other actin binding proteins [14]. In addition, TARP interacts with Vav2 and Sos in a phosphorylation-dependent manner [21,28] suggesting that phosphorylation of TARP regulates actin polymerization by Rac-dependent signaling mechanisms via recruitment and activation of the Rac GEFs, Sos1 and Vav2. However, since tyrosine phosphorylation of TARP is not required for entry [29], it may be important for post-entry signaling events [28].

Actin reorganization induced by *Chlamydia* is transient, and therefore *Chlamydiae* may also secrete effectors that regulate actin depolymerization. CT166, a *C. trachomatis* protein that contains regions homologous to clostridial glucosylating toxins (GCTs) is a likely candidate for this process [30,31]. *Chlamydia* strains expressing CT166 that contain domains homologous to GCTs induce actin disassembly and are cytotoxic when infected at high multiplicities of infection [30]. Ectopic expression of *C. trachomatis* CT166 reproduces this cytotoxicity and actin reorganization phenotype via glucosylation and inhibition of Rac1. Ectopic expression also reduces internalization of *Chlamydia* suggesting that under conditions of a normal infection, CT166 may act to turn off Rac-dependent actin reorganization after entry. Another candidate may be *C. trachomatis* CT694. Similar to TARP, *C. trachomatis* CT694 is translocated into the host cell and localizes adjacent to EBs during entry [32]. CT694 interacts and co-localizes with eukaryotic AHNAK, an actin-binding protein, and ectopic expression of CT694 results in loss of actin stress fibers in an AHNAK-dependent manner [32]. The exact function of CT694 during *C. trachomatis* is unclear, but may play a role in regulating membrane fluidity or actin depolymerization during invasion or early stages of *Chlamydia* infection.

The inclusion is stabilized by the formation of an actin and intermediate filament scaffold that surrounds the mature inclusion [33]. Scaffold formation is dependent on RhoA, a regulator of actin stress fiber formation, but independent of known RhoA effector mechanisms [33]. Chlamydial proteasome/protease-like activity factor (CPAF)-mediated structural alterations in vimentin are thought to create a dynamic structure that allows for expansion of the inclusion at late stages of *Chlamydia* development [33]. CPAF is a protease secreted into the host cytosol that targets multiple host proteins with important roles in immune evasion [8]. As mentioned below, sphingomyelin biosynthesis is also required to maintain the integrity of inclusion, but the relationship between sphingomyelin and the actin scaffold is presently unknown [34]. By providing structural integrity to the inclusion, the presence of the cytoskeletal scaffold has been proposed to limit activation of host cytosolic immune surveillance pathways by preventing leakage of luminal contents into the cytosol [33].

Finally, *Chlamydiae* subvert actin remodeling during exit from the host cell. Live cell imaging studies revealed that *Chlamydiae* exit by two mutually exclusive mechanisms, cell lysis and inclusion “extrusion” [35]. Cell lysis is dependent on proteases and intracellular calcium signaling and involves sequential lysis of the inclusion, the nucleus and then the plasma membrane. Host cell survival following cell lysis is dependent on lysosomal repair of the plasma membrane, a process requiring actin depolymerization [36]. Inclusion extrusion involves release of an intact inclusion that has been packaged and extruded through the plasma membrane and requires actin polymerization, neuronal Wiskott-Aldrich syndrome protein (WASP), myosin II and Rho GTPase [35].

2.2 Subversion of host microtubules

During the initial stages of infection, the nascent inclusion is trafficked by a dynein and microtubule-dependent mechanism to the peri-Golgi region of the cell where it resides in close contact with the MTOC [37]. Although p150^{Glued}, a component of dynactin, a multiprotein complex normally required for cargo binding to the dynein motor, localizes to the inclusion, trafficking is independent of dynactin [37]. Interaction with the dynein motor complex is likely mediated through interactions with inclusion membrane proteins, which may mimic the cargo binding activity of dynactin. Consistent with this model, microdomains within the inclusion membrane that contain several Incs, active Src family kinases and cholesterol have been identified that co-localize with centrosomes and dynein [38]. One of these proteins, CT850 co-localizes with centrosomes when ectopically expressed suggesting that these inclusion protein-containing microdomains may play a role in inclusion-centrosome interactions [38]. The importance of Src family kinases in inclusion-centrosome interactions is unclear since their depletion does not affect inclusion development [38]. The inclusion maintains a dynein-dependent association with the centrosome during transit through the host cell cycle resulting in an increase in supranumerary centrosomes, abnormal spindle poles and segregation defects [39]. Chromosomal segregation defects are even observed in cells cured of *Chlamydia* [39]. Although cytokinesis is inhibited at late stages in *Chlamydia* infected cells [40], the increase in centrosome numbers does not arise as a result of this defect but instead results from dysregulation of centrosome duplication by a mechanism that requires known regulators of centrosomal duplication and progression through S phase [41]. The relevance for these interactions are not clear since in vivo *Chlamydia* replicate in terminally differentiated columnar epithelial cells. However, *Chlamydia*-induced chromosomal segregation defects may explain the observed link between *Chlamydia*-infections and cervical cancer [42].

3. Subversion of membrane trafficking pathways

3.1 Rab GTPases and phosphoinositides

The biogenesis and maintenance of vacuoles harboring numerous pathogenic organisms involve the selective recruitment or exclusion of Rab GTPases [43]. Rab GTPases are small Ras-like GTPases that function in vesicular trafficking and organelle identity (reviewed in [44]). Localized to distinct organelles or organellar subdomains, Rab GTPases cycle between an inactive cytosolic GDP-bound form and an active membrane associated GTP-bound form. Interaction between the GTP-bound Rab and downstream effectors regulate formation of vesicles at donor membranes, trafficking and fusion of transport vesicles with acceptor membranes.

Chlamydiae target multiple Rab-dependent pathways. Both endocytic (Rab4, 11, 14) [45,46] and ER-Golgi related (Rab1, 6 and 10) [45] Rab GTPases and their effectors [47,48] associate with inclusions at different times during the biogenesis of the inclusion by species-dependent and –independent processes. Recruitment of at least several Rab GTPases is

mediated through direct interactions with Incs. For example, *C. trachomatis* CT229 interacts with Rab4 [49], while Cpn0585, interacts with multiple Rab GTPases including Rab1, 10 and 11 [50]. Roles for specific Rab GTPases are only now being elucidated but current data suggests they play multiple essential roles in various aspects of *Chlamydia* development. Depletion of Rab6, Rab11 [51] or Rab14 [46] and co-expression of dominant negative Rab4 and Rab11 mutants [52], result in varying degrees of impaired *Chlamydia* growth demonstrating that Rab GTPases play crucial and at times functionally redundant roles during *Chlamydia* development. As described below, Rab6, Rab11 and Rab14 function in sphingomyelin delivery to the inclusion [46,51] while Rab4 and Rab11 regulate a slow transferrin recycling pathway intercepted by the inclusion [52]. Genome-wide RNA interference screens confirmed requirements for Rab1 and Rab14 in *C. trachomatis* development [14] and Rab11 in *C. caviae* development in *Drosophila melanogaster* S2 cells [53] as well as identifying additional Rab GTPases (Rab8, 32, 39 and 35) that may play both positive and negative roles during *Chlamydia* development [14,53]. The localization of Rab35 to the *Chlamydia* inclusion has recently been confirmed (M. Scidmore, unpublished data). Further work is needed to clarify roles of specific Rab GTPases, as loss of Rab1 has also been shown to both increase [51] and decrease *Chlamydia* development [14].

In addition to Rab GTPases, organelle identity is also determined in part by the presence or absence of specific phosphoinositides (PI) and thus many pathogenic bacteria often target PI metabolism [54]. Phosphoinositides are short lived phosphorylated derivatives of inositol that play key roles in intracellular signaling and vesicle transport by controlling the subcellular localization and activity of PI-binding proteins. The intracellular localization of PI is tightly regulated by PI-specific kinases and phosphatases that add or remove specific phosphate groups, respectively, generating seven distinct PI species. Since Rab GTPases function through their interaction with downstream effectors, efforts are currently focused on identifying inclusion localized Rab GTPase effectors [47,48]. Oculocerebral syndrome of Lowe protein 1 (OCRL1), a Golgi-localized PI-5' phosphatase that primarily hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 4-phosphate (PI4P) and binds to multiple Rab GTPases localizes to all *Chlamydia* inclusions [48]. PI4P, the product of OCRL1, is predicted to localize to the inclusion membrane based upon inclusion localization of GFP-tagged PI4P-specific pleckstrin homology (PH) domains of oxysterol binding protein (OSBP) and Good Pasture Binding Protein (GPBP) [48]. A second host enzyme, phosphatidylinositol 4-kinase (PI4KII α), which produces PI4P from PI, is also present at the inclusion [48]. Although studies with BFA, an inhibitor of anterograde Golgi-to-ER trafficking suggest that Golgi-trafficking is not required for the presence of PI4P at the inclusion, it is currently unknown whether PI4P is produced by OCRL1 or PI4KII α directly at the inclusion and what specific role it plays. However, the absence of OCRL1 or PI4KII α reduces the ability of *Chlamydia* to infect cells suggesting roles in entry or early inclusion modification possibly through recruitment of PI4P-binding proteins or other mechanisms [48].

Requirements for additional PI species during *Chlamydia* infection have also been documented. Inhibition or depletion of ADP-ribosylating factor 6 (Arf6), the Arf6 effector phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase), or PIP2, the product of PIP 5-kinase, at the plasma membrane alters actin remodeling and reduces the internalization of *C. caviae* [20]. A role for Arf6 and PIP2 in entry of other *Chlamydia* species has not been examined. Finally, PI3K-dependent pathways have multiple roles during *Chlamydia* development including *C. pneumoniae* [17] and *C. caviae* entry [19], interaction of the Rac GEF, Vav2 with *C. trachomatis* TARP [21] and resistance to apoptotic stimuli [7].

3.2 Lipid transport pathways

As obligate intracellular bacteria, *Chlamydiae* rely on essential nutrients and biosynthetic precursors from its eukaryotic host. It has become increasingly evident, that *Chlamydiae* are extremely proficient at acquiring host-derived lipids. Using the long chain fluorescent derivative of ceramide, N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)] aminocaproylsphingosine (C6-NBD-Cer), Hackstadt and colleagues demonstrated for the first time, the delivery of a host-derived lipid to the inclusion and uptake of this lipid by EBs [3]. Sphingomyelin is a unique and defining property of all *Chlamydia* species and is required for *Chlamydia* development [34,55]. Furthermore, sphingomyelin trafficking is dependent on a Golgi-dependent vesicular-mediated trafficking pathway [56] that may transport sphingomyelin-containing vesicles to the basolateral surface of polarized cells [57]. These data together, with the close physical proximity to the Golgi apparatus, prompted researchers to propose that the inclusion is trafficked to the peri-Golgi region of the cell where it intercepts a subset of Golgi-derived vesicles normally destined for the plasma membrane as a mechanism to obtain host cell nutrients. This discovery revealed for the first time that inclusion selectively fused with host-derived vesicles and was, in fact, not completely disconnected from host membrane trafficking pathways.

Ten years after the initial discovery, the first clues of how *Chlamydiae* co-opt sphingomyelin transport pathways were revealed and indicate that *Chlamydiae* target multiple sources of sphingomyelin. Beatty and colleagues demonstrated that MVBs, components of late endosomes, were trafficked and their contents delivered to the lumen of the inclusion [58]. Disruption of MVB trafficking prevents transport of both cholesterol and sphingomyelin to the inclusion and results in impaired growth of *Chlamydiae* [58,59]. Furthermore, inhibition of sphingomyelin synthesis results in loss of inclusion membrane integrity, inhibition of homotypic inclusion fusion and early re-differentiation of RBs into EBs [34]. Inhibition of MVB trafficking, as compared to inhibition of sphingomyelin synthesis, results in a greater reduction of *Chlamydia* growth suggesting that additional MVB components are essential for *Chlamydia* development [34].

Subsequently, Myers and co-workers discovered that *Chlamydiae* induce fragmentation of the Golgi apparatus resulting in the localization of Golgi mini stacks to the inclusion [60]. Golgi fragmentation is mediated by cleavage of Golgin-84, a component of the Golgi matrix, by both inflammatory caspase- and calpain-dependent mechanisms [60]. Inhibition of Golgi-fragmentation reduces transport of sphingomyelin to the inclusion and *Chlamydia* growth, while the induction of Golgi fragmentation via depletion of the Golgin p115 increases *Chlamydia* growth [60]. Collectively these data suggest that *Chlamydia* co-opt an entire organelle to facilitate the essential delivery of Golgi-derived lipids to the inclusion. Rab GTPases also function to maintain Golgi structure [61]. Through siRNA gene induced silencing experiments, it was shown that Rab6 and Rab11, both of which localize to inclusions [45], regulate the ability of *Chlamydiae* to induce Golgi fragmentation and that Rab6 acts downstream of Golgin 84 cleavage since in the absence of Rab6, Golgin 84 is still cleaved [51]. Rab14, a regulator *trans*-Golgi to early endosome trafficking, is also implicated in the delivery of sphingomyelin to the inclusion [46]. In contrast to Rab6 and Rab11, there is no evidence that Rab14 destabilizes the Golgi apparatus in infected cells. Further experiments are needed to clarify the roles of Rab GTPases in the destabilization of the Golgi apparatus and in the transport of sphingomyelin and how the formation of mini stacks facilitates sphingomyelin transport to the inclusion. Additionally, the *Chlamydia* proteins that target Golgin-84 cleavage or regulate sphingomyelin trafficking have not been identified.

The ectopic expression of putative *Chlamydia* effectors as GFP-tagged proteins in *Saccharomyces cerevisiae* revealed a tropism for lipid droplets (LD) by four *Chlamydia*

proteins [6,62] suggesting that in addition to sphingomyelin, *Chlamydiae* target LDs as a source of lipids. Lipid droplets, ER-derived storage organelles for neutral lipids or long chain fatty acids, were subsequently shown to be recruited to and translocate into the lumen of the inclusion [6,63]. Lipid homeostasis is altered during *Chlamydia* infection and pharmacological inhibition of LD formation results in a decrease in inclusion size and a reduction in EB production demonstrating the importance of LDs for *Chlamydia* development. The presence of neutral lipids in RBs and the absence of a net accumulation of LDs in the lumen suggest that LDs are metabolized possibly by direct scavenging or lipolysis of the associated lipids. Two of the three proteins that display tropism for LDs, Lda1 and Lda3, are translocated into the host cytosol and localize to LDs that are adjacent to the inclusion membrane [6]. Furthermore, ectopically expressed GFP-Lda3 accumulates with LDs that have reduced staining for adipocyte differentiation related protein (ADRP), a surface exposed inhibitor of lipases [63]. Finally, IncA, a protein localized to the inclusion membrane, co-fractionates and partially co-localizes with intralumenal LDs, suggesting that IncA may mark sites of the inclusion that are entry sites for LDs [63]. Based upon the current data, it has been proposed that *Chlamydiae* translocate Lda3 into the host cytosol, where it acts as bridge to anchor LDs to the inclusion membrane and also may function to remove the protective coat protein ADRP from LDs. Invagination, by as yet some undetermined mechanism, of the inclusion membrane at sites of LD association, traps LDs within the lumen allowing RBs to directly scavenge or metabolize associated lipids, possibly by LD-associated lipases [63]. Scavenging lipids from an organelle from within the lumen of the inclusion may be advantageous as cytosolic surveillance mechanisms and the generation of inflammatory byproducts of fatty acid lipolysis would be avoided.

Eukaryotic glycerophospholipids such as phosphatidyl choline and phosphatidyl inositol are present in purified EBs and delivered to the inclusion by non-vesicular mediated pathways [5]. In contrast to cholesterol and sphingomyelin, glycerophospholipids are selectively modified by *Chlamydiae*. *Chlamydia*-induced ERK1/2 dependent activation of cytosolic cPLA₂ results in the replacement of eukaryotic-derived straight chain fatty acid at the sn-2 position with a *Chlamydia*-derived branch chain fatty acid [64]. Inhibition of ERK signaling during *Chlamydia* infection, results in reduction of glycerophospholipid uptake by *Chlamydiae* and a decrease in infectivity [64]. Interestingly, although Ras is also activated during *Chlamydia* infection and usually functions to activate ERK1/2 through Raf, ERK1/2 is activated independently of Ras and Raf1 [65,66]. In fact, Ras and its down stream target, Raf1, negatively regulate *Chlamydia* infections [66]. These data highlight the ability of *Chlamydiae* to activate eukaryotic signaling pathways by non-canonical mechanisms. As we begin to unravel the interactions between *Chlamydiae* and its eukaryotic host, it is becoming clear that these interactions are quite complex. This is best exemplified by the role of cPLA₂, which in addition to being essential in *Chlamydia*-mediated modification of host derived glycerophospholipids, cPLA₂ also regulates the expression of type I interferons and intracellular immunity to *C. trachomatis* in mouse cells [65].

3.3 SNARE mimicry

The selective targeting of host vesicles suggests that *Chlamydiae* have evolved mechanisms to regulate the fusogenicity of host transport vesicles and organelles. A clue to unraveling these phenomena was revealed by the discovery that several Incs including IncA, CT223 and CT813, contain eukaryotic soluble NSF (N-ethylmaleimide-sensitive) attachment protein receptors (SNARE) protein domains [67]. SNARE proteins present on opposing membranes drive membrane fusion by bringing vesicle and target membranes close together through the assembly of a SNARE-containing four-helix bundle. Vesicles containing endocytic SNAREs, Vamp3, Vamp7 and Vamp8, accumulate around the inclusion via interactions with IncA that are dependent on IncA's SNARE motifs [67]. Functional

significance for these interactions was revealed during *in vitro* fusion assays where the presence of IncA inhibited the fusion activity of several endocytic SNAREs [68]. In contrast, IncA has also been shown to be involved in homotypic fusion of *Chlamydia*-containing vacuoles [67,69]. Based upon the inhibitory effect of IncA on SNARE-mediated membrane fusion and its role in promoting homotypic vesicle fusion, Subtil and colleagues propose that IncA may act as a switch regulating the maturation of the inclusion. In their model, newly synthesized IncA would first inhibit SNARE-mediated fusion events with the inclusion by binding host SNAREs, and then once IncA-SNARE interactions have been saturated, continued synthesis of IncA would promote IncA-IncA interactions and homotypic vesicle fusion [68]. Since IncA is not expressed until 10 hours post infection and early inclusions resist fusion with lysosomes, additional SNARE-inhibitory Inc activity would be required prior to the expression of IncA.

In addition to SNARE proteins, the Arf family of small Ras-like GTPases, also regulate membrane trafficking. The Golgi-localized Arf1 regulates production of COPI transport vesicles via recruitment of coat proteins and PI homeostasis and membrane trafficking via recruitment of PI4K enzymes and PI4P-binding proteins. Although Arf1 localizes to *Chlamydia* inclusions in a species-specific manner [48], its role during *Chlamydia* development is uncertain since loss of Arf1 function has been shown to both inhibit [48] and increase *Chlamydia* development [66].

3.4 Recycling endosomes and mitochondria

Although non-fusogenic with lysosomes, the inclusion associates with but does not fuse with recycling endosomes containing transferrin [2]. Consistent with these data, the endocytic Rab GTPases, Rab4 and Rab11, also co-localize with the inclusion [45]. Clues to the biological significance of these interactions were uncovered in a screen for low-molecular weight inhibitors of *Chlamydia* growth [52]. One inhibitor identified as a result of this screen, FR179254, reduces the formation of transferrin-containing Rab4/11 hybrid vesicles and causes a delay in the slow transferrin recycling pathway. Similar phenotypes, reduction in *Chlamydia* growth and a defect in transferrin recycling, are observed upon co-expression of dominant negative mutants of Rab4 and Rab11 [52]. Surprisingly, growth inhibition caused by FR179254 is reversed by the omission of transferrin-containing serum from the media [52]. Collectively, these data suggest that alteration of the slow recycling pathway and accumulation of iron-laden transferrin-containing endosomes at the inclusion is toxic to the development of *Chlamydiae* and that *Chlamydiae* may target Rab4 and Rab11 to ensure proper transferrin recycling as a potential mechanism for iron acquisition.

A genome wide RNA interference screen in *D. melanogaster* S2 cells revealed a *C. caviae*-specific requirement for the Tom complex, a multiprotein complex involved in mitochondrial protein export, supporting previous studies that *C. caviae*, but not *C. trachomatis*, targets mitochondria [53]. Depletion of two components of the complex, Tom40 and Tom22, reduces *C. caviae* EB production, but no defects in energy metabolism or susceptibility to apoptotic stimuli are detected and only a small defect in the association of mitochondria with the *C. caviae* inclusion is observed. Although important for *C. caviae* development, it is still unclear how mitochondria might specifically benefit *C. caviae*.

4. Concluding remarks

Once thought to be disconnected from membrane trafficking pathways, we are now discovering that the inclusion intercepts a complex array of host cellular pathways (summarized in Fig. 2) that promote the intracellular survival of *Chlamydia*. And despite the genetic intractability of *Chlamydia*, the mechanisms by which these pathways are exploited are rapidly being unraveled. The recent development of potentially powerful genetic tools

should hopefully further our understanding of how specific *Chlamydia* effector proteins target these cellular pathways.

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Figure 1. *Chlamydia* subvert the host cytoskeleton

A. *Chlamydiae* enter non-professional phagocytes in a Rac1 and actin-dependent mechanism. In *C. trachomatis*, the T3SS effector, TARP, is tyrosine phosphorylated by Src family, Abl and Syc kinases resulting in the activation and recruitment of Rac1 GEFs, Sos and Vav2. Rac1 activation leads to Arp2/3-dependent actin polymerization. EB binding and activation of PDGFR is one mechanism that promotes Abl kinase and Rac1 activation. In addition, TARP orthologues from all *Chlamydia* species nucleate actin via their actin-binding WH2-like domain. TARP mediated actin nucleation is essential for chlamydial invasion. * In *C. caviae*, Cdc42 and Arf6 also promote actin polymerization. CT166 and CT694, secreted effectors, may also function during entry via inhibition of Rac1 and interaction with AHNK, respectively. B. EBs are transported via a dynein-dependent, but dynactin-independent mechanism to the MTOC. C. The inclusion is stabilized by the formation of a scaffold consisting of actin and vimentin by a RhoA-dependent mechanism. CPAF-dependent cleavage of vimentin may allow flexibility in the scaffold to accommodate the growth of the inclusion at late stages of development. D. *Chlamydiae* can exit the host cell via a mechanism that is dependent on actin, myosin II, WASP and Rho GTPases.

Figure 2. *Chlamydia* subvert host membrane trafficking pathways

Chlamydiae obtain host derived lipids by intercepting MVB and Golgi-derived vesicles containing sphingomyelin and cholesterol, and lipid droplets (LD) containing neutral lipids via interactions with secreted Lda. Multiple Rab GTPases are associated with *Chlamydia* inclusions via interaction with Incs and function to mediate Golgi fragmentation, sphingomyelin trafficking and interaction with a slow transferrin recycling pathway. Inca and CT813 interact with and can inhibit host-mediated SNARE trafficking pathways. PI4P is localized to inclusions possibly via recruitment of OCRL1 and PI4KIIa. For *C. caviae*, the mitochondrial Tom complex is required for infection.