RNAi Screen in Drosophila Cells Reveals the Involvement of the Tom Complex in Chlamydia Infection

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Chlamydia spp. are intracellular obligate bacterial pathogens that infect a wide range of hosts. Here, we show that C. caviae enters, replicates, and performs a complete developmental cycle in Drosophila SL2 cells. Using this model system, we have performed a genome-wide RNA interference screen and identified 54 factors that, when depleted, inhibit C. caviae infection. By testing the effect of each candidate’s knock down on L. monocytogenes infection, we have identified 31 candidates presumably specific of C. caviae infection. We found factors expected to have an effect on Chlamydia infection, such as heparansulfate glycosaminoglycans and actin and microtubule remodeling factors. We also identified factors that were not previously described as involved in Chlamydia infection. For instance, we identified members of the Tim-Tom complex, a multiprotein complex involved in the recognition and import of nuclear-encoded proteins to the mitochondria, as required for C. caviae infection of Drosophila cells. Finally, we confirmed that depletion of either Tom40 or Tom22 also reduced C. caviae infection in mammalian cells. However, C. trachomatis infection was not affected, suggesting that the mechanism involved is C. caviae specific.

Introduction

Chlamydia spp. are Gram-negative, obligate, intracellular bacterial pathogens that infect a wide range of hosts and cause various diseases. Three species infect humans. C. trachomatis is the leading cause of preventable blindness in developing countries [1] and the most common cause of bacterial sexually transmitted disease in developed countries [2]. Infection with C. pneumoniae leads to pneumonia, and in the past 10 years, C. pneumoniae has been implicated in atherosclerosis [3] and Alzheimer disease [4], although the direct links between the bacteria and these diseases is still unclear. C. psittaci infects various animals and is responsible for pneumonia in humans [5]. Many Chlamydia species are recognized as animal pathogens [6]. C. muridarum infects mice and hamsters. C. suis, C. abortus, and C. felis infect swine, ruminants, and house cats, respectively. Finally, infection with C. caviae in guinea pig resembles ocular and genital infections caused by C. trachomatis in humans.

Chlamydia are characterized by a biphasic developmental cycle that occurs exclusively in the host cell. The bacteria alternate between an infectious, metabolically inactive form called elementary body (EB) that is characterized by a condensed nucleoid, and an intracellular, metabolically active form named reticulate body (RB). Once internalized, Chlamydia resides in a membrane-bound compartment, named the inclusion. Shortly after uptake, an uncharacterized switch occurs, leading to the differentiation of EBs into RBs. The RBs then start to replicate until the inclusion occupies a large part of the cytosol of the host cells. At the end of the cycle, which lasts 2 to 3 d depending upon the species, the RBs differentiate back into EBs. The host cell is lysed, leading to the release of EBs and the infection of neighboring cells [7,8].

Both bacterial and host factors contribute to the bio-genesis of the inclusion, but little is known about the mechanisms involved. Chlamydia spp. possess a type III secretion system (TTSS) responsible for the secretion of effector proteins in the cytoplasm of the host cell. An example of such effectors is the family of highly hydrophobic Inc proteins. Some of them are present on the surface of the inclusion membrane and are thought, in combination with other bacterial effector proteins, to modify the host cell environment and allow bacterial replication [9–13]. During the cycle, Chlamydia targets various host cell functions in order to establish its replication niche and disseminate from cell to cell [14]. The bacteria acquire amino acids, nucleotides, and other precursors from the host cell. The mechanism of chlamydial entry is not well understood, but among others, heparan sulfate proteoglycans, tyrosine phosphorylation of the bacterial effector Tarp, and activation of small GTPases and signaling pathways leading to actin remodeling are involved in this process [15]. Once internal-

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Abbreviations: EB, elementary body; GAG, glycosaminoglycan; IB, intermediate body; RB, reticulate body; RNAi, RNA interference; SL2, Schneider’s Line 2; TTSS, type III secretion system

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Author Summary

*Chlamydia* spp. are intracellular bacterial pathogens that infect a wide range of hosts and cause various diseases, including preventable blindness in developing countries, sexually transmitted disease, and pneumonia. *Chlamydia* spp. are able to establish their replication niche inside the host cell, residing in a membrane-bound compartment that serves as a protector shield against immune surveillance and antimicrobial agents but also acts as a “filter” to exchange factors with the host cell. Despite the primary importance of *Chlamydia* for human health, little is known about the mechanisms underlying the infection process. The study of *Chlamydia* pathogenesis is challenging because *Chlamydia* spp. are not amenable to genetic manipulation and it is difficult to conduct extensive genetic approaches in the mammalian host. To circumvent these difficulties, we have used *Drosophila* cells to model *Chlamydia* infection. We conducted a genome-wide RNA interference screen and identified host factors that, when depleted, reduce *Chlamydia* infection. Validating our approach, we further showed that the identified factors were also required for infection in mammalian cells. This work will help us better understand the complex interaction between *Chlamydia* and its host and potentially identify novel targets for therapeutic treatment.

**Materials and Methods**

**Cell Lines and Bacterial Strains**

*Drosophila* SL2 cells [41] were cultured at 25 °C in Schneider media (Invitrogen) supplemented with 10% heat inactivated FBS (JRH).

HeLa 229 cells were cultured at 37 °C with 5% CO₂ in DMEM high glucose (Invitrogen) supplemented with 10% heat inactivated FBS (Invitrogen).

*C. caviae*, the guinea pig model of genital and ocular infection of *Chlamydomatis*, were obtained from R. Rank (University of Arkansas). *C. trachomatis Lymphogranuloma Venereum, Type II*, were obtained from ATCC (VR-902B).

SL2 cell infection with GFP-expressing *L. monocytogenes* was conducted as previously described [37].

**Chlamydia Propagation and Infection**

For propagation, HeLa 229 were incubated with *C. caviae* or *C. trachomatis* for 48 h in the presence of 2 µg/ml cycloheximide (Sigma). The infected cells were centrifuged (10 min, 1,000 rpm) and the cell pellet was resuspended in SPG buffer (218 mM sucrose, 3.76 mM KH₂PO₄, 7.1 mM KH₂PO₄, 4.9 mM glutamate [pH 7.4]). The cells were broken by passing them through a 26½ gauge needle and the unbroken cells and nuclei were pelleted by centrifugation (10 min, 1,000 rpm). The supernatant was centrifuged (30 min, 12,000 rpm), and the bacterial pellets were resuspended in SPG buffer and stored at −70 °C.

For *Drosophila* SL2 cell infection, *C. caviae* were diluted in Schneider media supplemented with 10% heat inactivated FBS and incubated with the cells at 30 °C for the indicated time. For HeLa 229 cell infection, *C. caviae* or *C. trachomatis* were diluted in DMEM high glucose supplemented with 10% heat inactivated FBS and incubated with the cells at 37 °C in the presence of 5% CO₂. One hour post infection, the bacteria were washed away and the cells were incubated with fresh media for the indicated length of time at 37 °C in the presence of 5% CO₂.

**Antibodies**

The following primary antibodies were used: (FITC)-conjugated C5+C8 monoclonal antibodies directed against...
Chlamydia MOMP and LPS (1:300, Argene), rabbit polyclonal anti IncA (1:200, [42]), guinea pig polyclonal antibody directed against C. caviae EBs (Kind gift of R. Rank, University of Arkansas), rabbit polyclonal antibody anti-hTom40 (1:500, Kind gift of M. Ryan, La Trobe University, Australia [43]), mouse monoclonal anti-Tom22 (1:2000, Sigma, clone ICA9–2), and rabbit polyclonal anti-actin (1:10,000, Sigma A2066).

The following secondary antibodies were used: goat anti-rabbit AlexaFluor 594 antibody (1:1,000, Molecular Probes), fluorescein (FITC)-conjugated AffiniPure donkey anti-guinea pig IgG (1:500, Jackson ImmunoResearch), peroxidase-conjugated goat anti-rabbit IgG (1:10,000, Jackson ImmunoResearch), and peroxidase-conjugated goat anti-mouse IgG (1:10,000, Jackson ImmunoResearch).

Immunofluorescence

At the indicated time, the cells were fixed for 30 min in PBS containing 4% paraformaldehyde. Immunostainings were performed at room temperature. Antibodies were diluted in PBS containing 0.16 μg/ml Hoechst (Molecular Probes), 0.1% BSA, and 0.05% saponin. Samples were washed with PBS containing 0.05% saponin, and a final PBS wash was performed before examination under an epifluorescence microscope.

Electron Microscopy

Drosophila SL2 cells (10^6) were incubated at 30 °C with C. caviae (MOI ~ 5), fixed 45 h post infection by addition of 0.125% glutaraldehyde / 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed with osmium tetroxide, dehydrated in ethanol, embedded in epoxy resin, sectioned, stained with 1% uranyl acetate, and examined by electron microscopy [44].

HeLa 229 cells cultured on coverslips were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 1 h at room temperature, postfixed in 1% osmium tetroxide in the same buffer for 1 h at room temperature, stained in 2% uranyl acetate in 50 mM sodium maleate (pH 5.2) for 1 h at room temperature, dehydrated in ethanol, and embedded in Embed 812 epoxy resin (all reagents from Electron Microscopy Sciences). Ultra-thin sections (60 nm) were obtained on a Reichert ultra microtome, transferred onto formvar- and carbon-coated hexagonal nickel grids, stained with 1% lead citrate and 2% uranyl acetate, and examined in a Tecnai 12 Biotwin electron microscope (FEI Company). Random images of vacuoles were recorded at a magnification of 11,500 using a Morada CCD camera (Olympus Soft Imaging Solutions). For quantification of the percentage of vacuolar membrane or nuclear envelope covered by mitochondria, a grid with a distance of 560 nm between lines was superposed on top of the images, and the number of intersections of vertical and horizontal lines with membranes counted. The number of intersections of these lines with mitochondria was also counted, but mitochondria were counted as being associated with the vacuolar or nuclear membrane only if the distance between the point of intersection of the grid with the mitochondrial outer membrane and the closest vacuolar or nuclear membrane was 50 nm or less. The ratio of the number of intersection with mitochondria divided by the number of intersections with the vacuolar or nuclear membrane gives an estimate of the percentage of these membranes covered by mitochondria.

Infectious Progeny Production in Drosophila Cells

Drosophila SL2 cells (10^6) were incubated at 30 °C with C. caviae. At the indicated time, the infected cells were processed as described above for Chlamydia propagation. The bacterial pellets were resuspended in 100 μl of SPG. To test for the presence of infectious C. caviae in the preparation, 300 μl of a 1:100 dilution were incubated with 6.10^5 HeLa cells seeded onto coverslips at 37 °C in the presence of 5% CO2. After 1 h, the bacterial suspension was replaced by 500 μl of fresh medium. The cells were fixed 24 h post infection, stained, and the percentage of cells containing a large inclusion was determined by visual inspection using an epifluorescence microscope.

Infectious Progeny Production in HeLa 229 Cells

The infection was performed in 384-well format such that 75% of the cells were infected. At the indicated time, the infected cells were collected and transferred to an eppendorf tube containing 100 μl of glass beads (Sigma, G8772) and 300 μl of DMEM high glucose supplemented with 10% FBS. The cells were broken by vortexing for 1 min, and 40 μl of dilutions of the lysat were added to 4.10^3 HeLa 229 cells seeded in 384-well plate. After 1 h at 37 °C in the presence of 5% CO2, the lysat was washed away and 40 μl of fresh media was added to each well. The cells were fixed and stained 24 h post infection and the percentage of infected cells was determined.

Primary Screen

Two sets of 42 384-well plates containing 0.25 μg of dsRNA per well were provided by the Drosophila RNAi Screening Center (Harvard Medical School, Boston, Massachusetts, http://www.flyrnai.org). Drosophila SL2 cells (2.10^5), suspended in 20 μl of serum-free Schneider media, were seeded in each well and incubated 1 h at 25 °C before the addition of 20 μl of Schneider media containing serum. After 3.5 d, the cells were infected by addition of 10 μl of Schneider media containing C. caviae. The cells were centrifuged for 1 min at 1,000 rpm and incubated at 30 °C for 48 h. The cells were processed for immunofluorescence by using the DNA dye Hoescht and FITC-conjugated C5+8 monoclonal antibodies. An automated microscope was used to automatically track, focus, and capture fluorescent images of the cells within each well across an entire plate. One set of images was captured in the blue channel to detect the cells’ nuclei and one set in the green channel to detect Chlamydia. The qualitative analysis of the image data was done by visual inspection.

dsRNA Synthesis

dsRNA used for validation and secondary assays were synthesized using a MEGAscript High Yield transcription kit (Ambion) according to the recommendation of the manufacturer.

Tom40 and Tom22 siRNA in HeLa 229 Cells

The protocol used for siRNA transfection was adapted from Dharmacon’s HeLa cells transfection protocol. One volume of siRNA buffer containing 200 nM of siRNA was incubated with 1 volume of serum-free DMEM high glucose containing 5 μl/ml DharmaFECT-1 transfection reagent for 20 min at room temperature. Two volumes of DMEM high glucose supplemented with 20% FBS containing 5.10^5/ml HeLa 229 cells were added to each well and the cells were
incubated at 37 °C with 5% CO₂ for 3 d. The total volume was 40 µl in 384-well and 400 µl in 24-well. In 24-well format the transfection mix was replaced by 500 µl of fresh media 24 h post transfection.

Assay for Tom40 and Tom22 Protein Depletion
The knock down of Tom40 or Tom22 was performed as described above in 24-well plate. Three days post transfection, the cells were harvested in 100 µl of protein sample buffer and 20 µl of cell lysates were run on SDS-PAGE gels and analyzed by western blot using HPR-conjugated secondary antibodies and Amersham ECL western blotting detection reagents.

Computer Assisted Image Analysis
Images were acquired using the Metamorph software (Molecular Devices). The integrated morphometry analysis module was used to quantify the size of C. caviae inclusions.

Results
C. caviae Infect and Replicate in Drosophila SL2 Cells
In an attempt to use Drosophila as a model system to study Chlamydia pathogenesis, we investigated C. caviae replication in Drosophila SL2 cells. For this purpose, 80% confluent Drosophila SL2 cells cultured in 96-well dish were incubated with C. caviae. At various times post infection, the cells were transferred to Concavalin A–coated coverslips (Sigma, 2 mg/ml) in Schneider media for 2 h. The samples were then fixed and stained with the DNA dye Hoescht and a FITC-conjugated antibody directed against Chlamydia to stain the inclusion.

As shown in Figure 1, C. caviae is able to infect and replicate in Drosophila SL2 cells. Although most of the cells contained at least one bacterium 1 h post infection, only 20% to 30% of the cells had an inclusion 48 h post infection (not shown), suggesting that some bacteria were actually cleared in the phagocytic SL2 cells. However, when the bacteria were successful in establishing their niche, the infected cells displayed a perinuclear inclusion whose size increased between 24 and 72 h post infection. At 96 h post infection, the size of the inclusions was more heterogeneous (not shown) and some cells displayed disrupted inclusions, suggesting that the developmental cycle was completed and that reinfection was occurring between 72 and 96 h post infection.

C. caviae Complete a Full Developmental Cycle in Drosophila SL2 Cells
We next determined whether C. caviae were undergoing a full developmental cycle in Drosophila SL2 cells. To this end, we determined whether the different developmental forms of C. caviae were present in the inclusion by electron microscopy. As shown in Figure 2A, 45 h post infection the bacteria were found in a membrane-bound compartment that occupies most of the cytosolic space. The inclusions mainly contained RBs and intermediate bodies (IBs) in the process of differentiating to EBs and are characterized by their DNA condensation stage, but they also contained some bacteria with an EB morphology (Figure 2B), suggesting that in Drosophila SL2 cells, RBs start to differentiate back to EBs 45 h post infection.

In order to demonstrate that infectious progeny was produced, C. caviae harvested from Drosophila SL2 cells at different times post infection were used to infect HeLa cells (Materials and Methods; Figure 2C). When C. caviae were harvested 3 h post SL2 infection, 10% of the HeLa cells displayed an inclusion. This number decreased to less than 5% when the bacteria were isolated 24 or 48 h post infection, suggesting that a substantial amount of bacteria were either cleared or had differentiated into non-infectious RBs. In contrast, 12.5% and 19% of the HeLa cells contained a large inclusion when the bacteria were harvested 72 and 96 h post SL2 infection, respectively. After 96 h, the number of infected HeLa cells remained constant. These results indicate that infectious forms of C. caviae are produced in Drosophila SL2 cells. Moreover, they are in agreement with the immunofluorescence (Figure 1) and electron microscopy (Figure 2A and 2B) data and confirm that 48 h post infection of Drosophila SL2 cells, the inclusion mainly contains RBs and IBs, whereas EBs are produced in the next 24 h. Taken together, these data show that C. caviae undergo a full developmental cycle in Drosophila SL2 cells and suggest that the cycle lasts 72 to 96 h.

C. caviae TTSS Is Functional in Drosophila SL2 Cells
The TTSS of C. caviae was functional in Drosophila SL2 cells as shown by determining the presence of the Inc family protein, IncA, on the C. caviae inclusion membrane (Figure 3). Drosophila SL2 cells were fixed 48 h post infection with C. caviae, stained with the DNA dye Hoescht to visualize the nuclei (N) and the inclusions (Inc), and antibodies directed against IncA. A ring-like signal (IncA, red) that surrounded the inclusion (Inc, blue) was observed, indicating that, in Drosophila SL2 cells, the TTSS of C. caviae is functional and that TTSS substrates such as IncA, are delivered to the inclusion membrane.

Host Factors That, When Depleted, Inhibit C. caviae Infection
Sixteen thousand Drosophila genes were individually knocked down by RNAi and screened for their ability to reduce C. caviae infection of Drosophila SL2 cells. The assay was performed as follows (Materials and Methods; Figure 4A). After 3.5 d of RNAi treatment, the Drosophila SL2 cells were incubated with C. caviae for 48 h. The infected cells were fixed and stained with a DNA dye and a Chlamydia-specific FITC-conjugated antibody. An automated microscope was used to capture fluorescence images that were subsequently analyzed by visual inspection. The primary screen was performed in duplicate. We identified 162 candidates that, when depleted, reduced C. caviae infection (Table S1). Figure 4B is representative of the phenotype observed: few cells displayed wild-type size inclusion (middle top panel) and the number of infected cells, as well as the size of the inclusion, was largely reduced (middle bottom panel). The candidates were grouped into 14 functional categories (Figure 5): miscellaneous (32), unknown (32), metabolism (18), transcription (14), vesicular trafficking (12), cytoskeleton (9), mitochondria (8), transporter (8), kinase and phosphatase (7), chromatin organization (5), endosome and lysosome (5), protein biosynthesis (5), RNA processing (4), and cell cycle (3).

The dsRNA targeting most of the candidates of the miscellaneous, metabolism, vesicular trafficking, cytoskeleton, mitochondria, transporter, kinase and phosphatase, and
endosome and lysosome categories were resynthesized to confirm the phenotype observed in the primary screen (Table S1). Out of the 100 candidates retested, the phenotype was confirmed for 54 candidates in at least two out of three replicates (Table 1). The validation rate varied among the categories: miscellaneous (40%), metabolism (47%), vesicular trafficking (75%), cytoskeleton (75%), mitochondria (67%), transporter (37%), kinase and phosphatase (57%), and endosome and lysosome (100%).

In an attempt to assay for Chlamydia specificity, the knock

Figure 1. C. caviae Replicate in Drosophila SL2 Cells
Immunofluorescence images of Drosophila SL2 cells incubated for 1, 24, 48, 72, and 96 h with C. caviae. After fixation, the samples were stained with the DNA dye Hoechst (DNA, blue) and a FITC-conjugated monoclonal C5+c8 antibody directed against MOMP and LPS (C. caviae, green). Merge: overlay of the two images.
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down of the candidates was tested for the inhibition of *L. monocytogenes* infection (Table 1). The knock down of most vesicular trafficking (9/9), cytoskeleton (4/6), and endosome and lysosome (5/5) candidates inhibited both *C. caviae* and *L. monocytogenes* infection, and an equal number of kinase and phosphatase candidates inhibited *C. caviae* or *L. monocytogenes* infection. The knock down of most miscellaneous (11/13) and metabolism (6/8) candidates and of all mitochondria (6/6) and transporter (3/3) candidates inhibited *C. caviae* infection only. These results suggest that the latter categories are likely to represent candidates specifically involved in *C. caviae* infection.

**Tom40 and Tom22 Depletion Reduce *C. caviae* Infection in Mammalian Cells**

The RNAi screen in *Drosophila* cells revealed that the silencing of six mitochondrial genes inhibited *C. caviae*, but not *L. monocytogenes* infection. Moreover, four out of the six candidates were members of the mitochondrial membrane translocase, a multiprotein complex involved in the recognition and import of nuclear-encoded mitochondrial proteins to the mitochondria [43,45]. Taken together, these results suggested a specific role of this machinery for optimal *C. caviae* infection in *Drosophila* cells. To address the relevance of these findings in *Chlamydia* pathogenesis, this observation was further investigated in mammalian cells.

Tom40 or Tom22 expression was knocked down in HeLa 229 cells using a mix of four siRNA duplexes directed against their respective mRNA (ThermoFisher). In addition, each siRNA was tested individually to rule out any potential off-target effects. The depletion of either Tom40 or Tom22 was assayed 3 d post transfection of the siRNAs by western blot analysis. As shown in Figure 6A, both Tom40 and Tom22 were efficiently depleted after incubation with the mix of four siRNAs or with individual siRNA duplexes.

The effect of Tom40 or Tom22 depletion on *C. caviae* infection was analyzed. HeLa 229 cells were incubated for 3 d with either CDH1 siRNA control directed against E-Cadherin, or Tom40 or Tom22 siRNAs pooled (mix), or individually (1, 2, 3, 4), infected with *C. caviae* for 24 h, and processed for immunofluorescence. The corresponding low and high magnification images are depicted in Figure 6B and 6C, respectively. The nuclei were labeled with the DNA dye Hoeschst (Figure 6B and 6C: left panel, DNA, blue) and the inclusions were stained with a guinea pig polyclonal antibody against *C. caviae* (Figure 6B and 6C: middle panels, *C. caviae*, green). Although the number of infected cells was similar, the inclusions appeared smaller upon Tom40 or Tom22 depletion (compare CDH1 middle panels to Tom40 or Tom22 middle panels).

A computer-assisted analysis of the images was used to quantify the size of the inclusions (Materials and Methods). In
In the control situation, we determined that each inclusion could be defined as a 10- to 150-μm² object and 40% of the inclusions were larger than 30 μm². We defined 10- to 30-μm² and 30- to 150-μm² objects as small and large inclusions, respectively. The impact of Tom40 or Tom22 knock down on C. caviae ability to form large inclusions was analyzed (Figure 6D). A 5- to 3-fold reduction in the percentage of large inclusions was observed upon depletion of either Tom40 or Tom22, confirming the overall reduction in the size of the inclusions and suggesting that upon Tom40 or Tom22 depletion, C. caviae intracellular growth is impaired.

Electron microscopy analysis of C. caviae inclusions in control or Tom40 depleted cells confirmed the immunofluorescence results. Although a mixed population of small and large inclusions was observed 24 h post infection, the overall size of Tom40 depleted cell inclusion was smaller (Figure 7). In addition, RBs had already started to differentiate back into EBs in control cells, and 85% of the inclusions contained more than 25% EBs. In contrast, although some IBs were present, very few EBs were visible in Tom40-depleted cells, and only 25% of the inclusions contained more than 25% EBs. This result suggested that, in addition to a reduction in intracellular growth, differentiation back into EBs is also lessened in Tom40-depleted cells.

C. caviae, but Not C. trachomatis, Infectious Progeny Production Is Impaired upon Tom40 and Tom22 Depletion in Mammalian Cells

The electron microscopy results suggested that RB differentiation into EBs was reduced upon Tom40 or Tom22 depletion. We therefore investigated the production of infectious progeny by Tom40- or Tom22-depleted cells. The cells were incubated with the siRNA in pool or individually for 3 d before incubation with C. caviae for 48 h to allow completion of the developmental cycle. The infected cells were collected, lysed with glass beads, and dilutions of the lysate were used to infect fresh HeLa 229 cells (see Materials for details).
and Methods). The cells were fixed 24 h post infection and the number of inclusion forming units (IFUs) was determined after assessment of the number of infected cells by immunolabeling (Figure 8A). We observed a 2- to 3-fold reduction in the production of infectious progeny upon Tom40 or Tom22 depletion. On the contrary, a similar number of infectious C. trachomatis were recovered from control or Tom40- or Tom22-depleted cells (Figure 8B).

These results demonstrate that, as suggested by the electron microscopy analysis, the reduction in the size of C. caviae inclusions is accompanied with a decrease in the number of infectious progeny produced. Altogether, our

### Table 1. Host Factors Required for Optimal C. caviae Infection and Their Effect on L. monocytogenes Infection

| Gene Symbol | CG Number | Predicted Biological Process | Function | Confirmed | Inhibit Lm Infection |
|-------------|-----------|------------------------------|----------|-----------|----------------------|
| Pav         | CG1258    | Cytoskeleton                 | Microtubule-based movement | 3         | No                   |
| Act57B      | CG10067   | Cytoskeleton                 | Actin filament | 3         | Yes                  |
| Act15C      | CG4027    | Cytoskeleton                 | Actin filament | 3         | Yes                  |
| Act79B      | CG7478    | Cytoskeleton                 | Actin filament | 3         | Yes                  |
| Katanin-60  | CG10229   | Cytoskeleton                 | Microtubule severing | 2         | No                   |
| Arc-p20     | CG5972    | Cytoskeleton                 | Actin binding | 2         | Yes                  |
| CG11814     | CG11814   | Endodome and lysosome        | Lysosomal transport | 3         | Yes                  |
| CG5691      | CG5691    | Endodome and lysosome        | Lysosomal organization | 3         | Yes                  |
| Rab5        | CG3664    | Endodome and lysosome        | Endosome to lysosome transport | 3         | Yes                  |
| VhaAC39     | CG2934    | Endodome and lysosome        | v-ATPase V(0) | 3         | Yes                  |
| VhaPPA1-1   | CG7007    | Endodeme and lysosome        | v-ATPase V(0) | 3         | Yes                  |
| Ckibeta     | CG15224   | Kinasases and phosphatases   | Casein serine-threonine kinase | 3         | Yes                  |
| Tor         | CG5092    | Kinasases and phosphatases   | Kinase | 3         | Yes                  |
| ssh         | CG6238    | Kinasases and phosphatases   | Phosphatase/actin (de)polymerization | 2         | No                   |
| tivs        | CG6235    | Kinasases and phosphatases   | Phosphatase type A2 | 2         | No                   |
| CG16905     | CG16905   | Metabolism                   | Fatty acid elongation | 3         | No                   |
| CG5844      | CG5844    | Metabolism                   | Fatty acid beta oxidation | 3         | No                   |
| Gapdh2      | CG8893    | Metabolism                   | Glycolysis | 3         | No                   |
| desat1      | CG5887    | Metabolism                   | Fatty acid desaturation | 3         | Yes                  |
| Pde9        | CG32648   | Metabolism                   | Cyclic nucleotide metabolism | 2         | No                   |
| Pect        | CG5547    | Metabolism                   | Ethanolamine and derivative metabolism | 2         | No                   |
| PR          | CG4001    | Metabolism                   | Glycolysis | 2         | No                   |
| CG3523      | CG3523    | Metabolism                   | Fatty acid synthase | 2         | Yes                  |
| dpr8        | CG32600   | Miscellaneous                | Adhesion molecule | 3         | No                   |
| ERR         | CG7404    | Miscellaneous                | Estrogen-related receptor | 3         | No                   |
| gig         | CG6975    | Miscellaneous                | GTPase regulator | 3         | No                   |
| li(1)G0060  | CG3125    | Miscellaneous                | Cell adhesion | 3         | No                   |
| Dip         | CG32146   | Miscellaneous                | Glycan | 3         | No                   |
| Mask        | CG33106   | Miscellaneous                | Multiple ankyrin repeats | 3         | No                   |
| Obp99a      | CG18111   | Miscellaneous                | Oorant binding | 3         | No                   |
| Tsc1        | CG6147    | Miscellaneous                | Signal transduction | 3         | No                   |
| smt3        | CG4494    | Miscellaneous                | Protein modification | 3         | Yes                  |
| th          | CG12284   | Miscellaneous                | Negative regulator of apoptosis | 3         | Yes                  |
| CG7974      | CG7974    | Miscellaneous                | Hepatocellular carcinoma-associated Ag59 | 2         | No                   |
| Chit        | CG52150   | Miscellaneous                | Glycosyl hydrolase family | 2         | No                   |
| Cyp48d      | CG4321    | Miscellaneous                | Cholesterol P450 | 2         | No                   |
| Hsc70-4     | CG4264    | Mitochondria                 | Chaperone | 3         | No                   |
| Hsc70-5     | CG8542    | Mitochondria                 | Protein targeting to mitochondria | 3         | No                   |
| mge         | CG14981   | Mitochondria                 | Protein targeting to mitochondria | 3         | No                   |
| Tom40       | CG12157   | Mitochondria                 | Protein targeting to mitochondria | 3         | No                   |
| CG3731      | CG3731    | Mitochondria                 | Mitochondrial-processing peptidase | 3         | No                   |
| Tim9a       | CG1660    | Mitochondria                 | Protein targeting to mitochondria | 2         | No                   |
| CG17922     | CG17922   | Transporter                  | Potassium transport | 2         | No                   |
| CG30035     | CG30035   | Transporter                  | Glucose transporter | 2         | No                   |
| CG31729     | CG31729   | Transporter                  | Lipid/cation transport | 2         | No                   |
| alphacop    | CG7961    | Vesicular trafficking        | COP vesicle coat | 3         | Yes                  |
| Arf102F     | CG11027   | Vesicular trafficking        | GTPase activity | 3         | Yes                  |
| betacop     | CG6223    | Vesicular trafficking        | COP vesicle coat | 3         | Yes                  |
| betaCOP     | CG6223    | Vesicular trafficking        | COP vesicle coat | 3         | Yes                  |
| cdc42       | CG12530   | Vesicular trafficking        | GTPase activity | 3         | Yes                  |
| gamma cop   | CG1528    | Vesicular trafficking        | COP vesicle coat | 3         | Yes                  |
| Rab11       | CG5771    | Vesicular trafficking        | Rab GTPase activity | 3         | Yes                  |
| Rab35       | CG9575    | Vesicular trafficking        | Rab GTPase activity | 3         | Yes                  |
| zetacop     | CG3948    | Vesicular trafficking        | COPi vesicle coat | 3         | Yes                  |

100 newly synthesized dsRNA from eight functional categories were used to confirm the phenotype observed in the primary screen (see Table S1). The table lists the 54 candidates for whom the phenotype was confirmed in three (3) or two (2) out of the three independent replicates. The last column indicates whether the knock down of candidate expression had an effect (Yes) or not (No) on Listeria infection indicate when tested in secondary screens.

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Host Factors Involved in Chlamydia Infection

results indicate that depletion of members of the Tom complex in mammalian cells have a detrimental effect on C. caviae intracellular replication, which impairs bacterial replication and differentiation. Since Tom40 or Tom22 depletion had no effect on C. trachomatis infection, our results also indicate that the mechanism involved is C. caviae–specific.

Discussion

Chlamydia infections represent an enormous burden to human health, and although some of the host cellular processes targeted by Chlamydia have been identified, most of the factors involved in the infection process remained to be identified. This paucity of knowledge is mainly due to the fact that Chlamydia is not genetically tractable and to the difficulty of conducting genetic approaches in the mammalian host. Drosophila has recently emerged as a powerful alternative model to dissect microbial pathogenesis, and we show here that Drosophila SL2 cells constitute a viable model to study Chlamydia infection and identify host factors involved in the infection process.

C. caviae Replication in Drosophila Cells

We demonstrated that, similar to the situation in mammalian cells [8], infectious forms (EB) of C. caviae enter Drosophila SL2 cells, differentiate into the replicative form (RB), replicate within a membrane-bound compartment, and differentiate back from RBs to EBs.

A previous report showed that different serovars of C. trachomatis, including C. trachomatis LGV serovar L2, could initiate their developmental cycle in Drosophila S2 cells [30]. However, the later stages of the developmental cycle were not achieved. Similarly, we found that when Drosophila SL2 cells were incubated with C. trachomatis LGV serovar L2 most cells

Figure 6. Tom40 and Tom22 Depletion Reduce C. caviae Infection of Mammalian Cells
(A) HeLa 229 cells were transfected for 3 d with a CDH1 control siRNA (Ctrl), or four siRNA duplexes against Tom40 or Tom22 either pooled (Mix) or individually (1, 2, 3, 4) and analyzed by immunoblotting using antibodies directed against Tom40, Tom22, or actin.
(B and C) Immunofluorescence images of HeLa 229 cells transfected for 3 d with CDH1, Tom40, or Tom22 siRNA and subsequently incubated for 24 h with C. caviae. After fixation the cells were stained with the DNA dye Hoechst (DNA, blue) and polyclonal antibodies against C. caviae (C. caviae, green). Merge: overlay of the two images. (B) Low magnification images (10×). (C) High magnification images (100×).
(D) Quantification of the percentage of large C. caviae inclusions upon CDH1, Tom40, or Tom22 siRNA. Inclusions were defined as objects whose size ranged from 10 to 150 μm². Objects whose size ranged from 30 to 150 μm² were defined as large inclusions. Mix: pool of four siRNAs; 1, 2, 3, 4: individual siRNA.

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were also infected 1 h post infection. However, the pattern of staining did not change over a 72-h period post infection and the cells never displayed large perinuclear inclusions (not shown), confirming that *C. trachomatis* developmental cycle is not complete in *Drosophila* SL2 cells.

We noticed a difference in the morphology of *C. caviae* inclusion in *Drosophila* cells compared to mammalian cells. The inclusions appear multilobed in mammalian cells [46], whereas they appeared as a single membrane-bound compartment in *Drosophila* SL2 cells. This morphology resembles that of *C. trachomatis* inclusions that are known to undergo homotypic fusion and are therefore monovacuolar. This observation suggests that *C. caviae* inclusions may also undergo homotypic fusion in *Drosophila* SL2 cells.

IncA, a type III secretion (TSS) substrate known to be involved in the homotypic fusion of the *C. trachomatis* inclusions [47], was present on the surface of *C. caviae* inclusion in *Drosophila* SL2 cells. Since IncA from *C. caviae* can interact with itself [48], it is possible that in *Drosophila* SL2 cells it participates to the biogenesis of a single large inclusion. If it is the case, some *Drosophila* factors probably interact with IncA and help promote the fusion. However, one cannot exclude that in *Drosophila* cells the homotypic fusion of *C. caviae* inclusions is IncA independent.

**Host Factors Identified in RNAi Screen**

Using the *Drosophila* cell / *C. caviae* model system, we have performed an RNAi screen and identified 54 host factors that, when depleted, reduced *C. caviae* infection. By testing the effect of the candidates' knock down on *L. monocytogenes* infection, we have identified candidates presumably specific of *C. caviae* infection. In the following section, we discuss their potential relevance in *Chlamydia* pathogenesis.

The attachment of most *Chlamydia* species to the host cell is dependent on host cell heparan sulfate glycosaminoglycans (GAGs) [15]. *C. caviae* is no exception, because its adhesion is GAG dependent and can be blocked by heparin [49]. *Drosophila* contains two main glypicans: Dally (Division abnormally delayed) [50] and Dlp (Dally-like protein)

![Figure 7](image7.png)

**Figure 7.** Tom40 Depletion Affects *C. caviae* Inclusion Size and Differentiation into EBs

Electron micrographs of HeLa229 cells depleted for CDH1 (top panels, CDH1 siRNA) or Tom40 (bottom panels, Tom40 siRNA) 24 h post *C. caviae* infection. Representative images of small (left panels) and large (right panels) inclusions are shown. Bar: 2 μM.
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![Figure 8](image8.png)

**Figure 8.** Tom40 and Tom22 Depletion Reduces the Production of *C. caviae*, but Not *C. trachomatis*, Infectious Progeny

Quantification of the infectivity of *C. caviae* (A) or *C. trachomatis* (B) progeny isolated from CDH1 or Tom40- or Tom22-depleted cells. The progeny was isolated 48 h post infection and dilutions were incubated with fresh HeLa 229 cells for 24 h. After fixation and staining, the IFUs/ml was determined by assessment of the number of infected cells. Cc: *C. caviae*; Ct2: *C. trachomatis*.
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and further investigation may shed light on the host such enzymes reinforces the idea that the acquisition of lipids desaturation, elongation, and oxidation. The identification of several enzymes involved in fatty acid synthesis, from the Golgi [18] and targets lipid droplets [20]. We have identified two candidates involved in fatty acid synthesis, as well as two subunits of the v-ATPase (VhaAC39 and Metabolism. Mitochondria are involved in carbohydrate and lipid metabolism. Depletion of Tom complex components in mitochondria may lead to the reduction of the intracellular ATP pool and interfere with C. caviae infection. Although Chlamydia species have the capacity to produce their own ATP [61], they also encode ATP transporters [62–64], suggesting that Chlamydia may utilize host cell ATP. In agreement with this assumption, it has been reported that Chlamydia infection leads to an increase in ATP production [65]. Using a Luminescent Cell Viability Assay (Promega), we have shown that the intracellular ATP levels were comparable in control, Tom40-, or Tom22-depleted cells (between 1,500 and 2,000 luminescence arbitrary units / 384 well) (not shown). This result suggests that production of energy is not dramatically affected upon depletion of Tom40 or Tom22. Moreover, addition of extra glucose up to 20 g/l, to increase the source of energy available, did not rescue the phenotype (not shown). Finally, Listeria infection, which requires ATP for the bacteria to polymerize actin and spread from cell to cell, was not affected (not shown), but most importantly, a similar amount of C. trachomatis infectious progeny was recovered from control or Tom40- or Tom22-depleted cells, suggesting that the C. trachomatis developmental cycle was not affected (Figure 8B).Taken together, these results suggest that energy depletion does not account for the phenotype observed.

Apoptosis. Mitochondria play an important role in the control of apoptotic events, and Chlamydia inhibits apoptosis of the host cell by a mechanism that prevents mitochondrial release of cytochrome c [21,22]. The depletion of members of the Tom complex could interfere with this process and therefore affect C. caviae replication. However, Tom40- or Tom22-depleted cells infected with C. caviae did not display the characteristic condensed nuclear morphology of apoptotic cells (Figures 6B, 6C, and 7). This result suggests that premature cell death of the infected cells is not responsible for the inhibition of C. caviae infection.

Mitochondria recruitment to the inclusion. It was previously shown that mitochondria are associated with C. caviae inclusions but not C. trachomatis inclusions [66,67]. We therefore investigated whether mitochondria were still recruited to the C. caviae inclusions in Tom40-depleted cells (Figure S1). Our results indicate that mitochondria were found in vicinity (<50 nM) of 8.5% of C. caviae inclusion membrane in control cells. Consistent with the specific recruitment of mitochondria to the inclusion membrane, only 1% of the nuclear membrane was covered with mitochondria. In Tom40-depleted cells, mitochondria were found in the vicinity of 5.8% of C. caviae inclusion membrane. Further experiments will be required to determine whether
this slight reduction in mitochondria recruitment may account for the inhibition of C. caviae infection in Tom40-depleted cells.

Import of bacterial effectors. Finally, it was previously shown that two enteropathogenic E. coli type III effectors, EspF and Map, are recognized and imported to the mitochondria through the Tim-Tom complex [68,69]. It is therefore possible that similar C. caviae type III effectors are targeted to the mitochondria via the Tom complex. Depletion of Tom complex components may therefore interfere with C. caviae ability to specifically modulate mitochondrial functions and impair the intracellular replication of the bacteria. Identification of such effectors, possibly through their mitochondria targeting sequence, and their further characterization might shed light on the significance of the association C. caviae inclusion with the mitochondria.

In conclusion, we have used Drosophila as a model system to identify host factors important in C. caviae replication. By comparative analysis, we have identified factors that are probably specific to Chlamydia pathogenesis and we validated the effect of two candidates (Tom40 and Tom22) in mammalian cells. Our results indicate that upon Tom 40 or Tom22 depletion, C. caviae replication and differentiation is lessened, whereas C. trachomatis infection is not affected. Further investigations are under way to elucidate the specific role of the Tom complex in C. caviae infection.

Supporting Information

Figure S1. Mitochondria Recruitment to C. caviae Inclusion
(A) Electron micrographs of HeLa229 cells depleted for CDH1 (left panels, CDH1 siRNA) or Tom40 (right panels, Tom40 siRNA) 24 h post C. caviae infection. Representative images of mitochondria apposed to the inclusions membrane are shown. M: mitochondria. (B) Quantification of the fraction of inclusion or nuclear membrane covered with mitochondria (See Materials and Methods).

References

1. Thylefors B, Negrel AD, Pararajasegaram R, Dadzie KY (1995) Global data on blindness. Bull World Health Organ 73: 115–121.
2. World Health Organization (2001) Global prevalence and incidence of selected curable sexually transmitted infections: overview and estimates. WHORDH_ABDS/001.02. Geneva: World Health Organization.
3. Grayston JT (2000) Background and current knowledge of Chlamydia trachomatis. Infection 28: 143–150.
4. Itzhaki RF, Wozniak MA, Appelt DM, Balin BJ (2004) Infiltration of the parasite and host responses to its development. J Bacteriol 110: 706–721.
5. Gregory DW, Schaffner W (1997) Psittacosis. Semin Respir Infect 12: 7–11.
6. Everett KD, Bush RM, Andersen AA (1999) Emended description of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms. Int J Syst Bacteriol 49 (Pt 2): 415–440.
7. Friis RR (1972) Interaction of L cells and Chlamydia psittaci: entry of the parasite and host responses to its development. J Bacteriol 110: 706–721.
8. Moulder JW (1991) Interaction of Chlamydia trachomatis and host factors. Annu Rev Immunol 25: 419–448.
9. Bannantine J, Stamm W, Suchland R, Rockey D (1998) Chlamydia trachomatis IncA is localized to the inclusion membrane and is recognized by antibodies from infected humans and primates. Infect Immun 66: 6017–6021.
10. Kalman S, Mitchell W, Marathe R, Lammel C, Fan J, et al. (1999) Comparative genomes of Chlamydia pneumoniae and C. trachomatis. Nature genetics 21: 385–389.
11. Rockey DD, Heinen RA, Hackstadt T (1995) Cloning and characterization of a Chlamydia psittaci gene coding for a protein localized in the inclusion membrane of infected cells. Mol Microbiol 15: 617–626.
12. Stephens RS, Kalman S, Lammel C, Fan J, Marathe R, et al. (1998) Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis. Science 282: 754–755.
13. Subtil A, Parsot C, Dautry-Varsat A (2001) Secretion of predicted Inc proteins of Chlamydia pneumoniae by a heterologous type III machinery. Mol Microbiol 39: 792–800.
14. Field KA, Hackstadt T (2002) The chlamydial inclusion: escape from the endocytic pathway. Annu Rev Cell Dev Biol 18: 221–245.
15. Dutry-Varsat A, Subtil A, Hackstadt T (2005) Recent insights into the mechanisms of Chlamydia entry. Cell Microbiol 7: 1714–1722.
16. Grieshaber SS, Grieshaber NA, Hackstadt T (2005) Chlamydia trachomatis uses host cell dynein to traffic to the microtubule-organizing center in a p50 dynamin-independent manner. J Cell Sci 118: 5793–5802.
17. Scidmore MA, Fischer ER, Hackstadt T (2003) Restricted fusion of Chlamydia trachomatis vesicles with endocytic compartments during the initial stages of infection. Infect Immun 71: 973–984.
18. Hackstadt T, Rockey DD, Heinzen RA, Scidmore MA (1996) Chlamydia trachomatis interrupts an exocytic pathway to acquire endogenously synthesized sphingomyelin in transit from the Golgi apparatus to the plasma membrane. EMBO J 15: 964–977.
19. Rozmp KA, Scholtes LD, Briggs BJ, Whittaker GR, Scidmore MA (2005) Rab GTases are recruited to chlamydial inclusions in both a species-dependent and species-independent manner. Infect Immun 73: 5853–5870.
20. Sisko JL, Spaeht K, Kumar Y, Valdivia RH (2006) Multifunctional analysis of Chlamydia-specific genes in a yeast expression system. Mol Microbiol 60: 51–66.
21. Byrne GI, Ojcius DM (2004) Chlamydia and apoptosis: life and death decisions of an intracellular pathogen. Nat Rev Microbiol 2: 802–808.
22. Miyairi I, Byrne GI (2006) Chlamydia and programmed cell death. Curr Opin Microbiol 9: 102–108.
23. Lemaire B, Hofmann J (2007) The host defense of Drosophila melanogaster. Annu Rev Immunol 25: 697–743.
24. D’Argenio DA, Gallagher LA, Berg CA, Manoil C (2001) Drosophila as a model host for Pseudomonas aeruginosa infection. J Bacteriol 183: 1466–1471.
25. Dionne MS, Ghori N, Schneider DS (2003) Drosophila melanogaster is a genetically tractable model host for Mycobacterium marinum. Infect Immun 71: 3540–3550.
26. Brandt SM, Dionne MS, Khush RS, Pham LN, Vigdal TJ, et al. (2004) Secreted bacterial effectors and host-produced eiger/TNF drive death in a Salmonella-infected fruit fly. PLoS Biol 2: e418. doi:10.1371/journal.pbio.0020418
27. Mansfield BE, Dionne MS, Schneider DS, Freitag NE (2003) Exploration of host-pathogen interactions using Listeria monocytogenes and Drosophila melanogaster. Cell Microbiol 5: 901–911.
28. Cheng LW, Portnoy DA (2003) Drosophila S2 cells: an alternative infection model for Listeria monocytogenes. Cell Microbiol 5: 875–885.
29. Dorer MS, Kirton D, Bader JS, Iserberg RR (2006) RNA interference analysis

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of Legionella in Drosophila cells: exploitation of early secretory apparatus dynamics. PLoS Pathog 2: e34. doi:10.1371/journal.ppat.0020034
30. Elwell C, Engel JN (2005) Drosophila melanogaster S2 cells: a model system to study Chlamydia interaction with host cells. Cell Microbiol 7: 725–739.
31. Clemens JC, Worby CA, Simonson-Leff N, Muda M, Machama T, et al. (2000) Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc Natl Acad Sci U S A 97: 6499–6503.
32. Armknecht S, Bourtos M, Kiger A, Nybakken K, Mathey-Prevot B, et al. (2005) High-throughput RNA interference screens in Drosophila tissue culture cells. Methods Enzymol 392: 55–75.
33. Bourtos M, Kiger AA, Armknecht S, Kerr K, Hild M, et al. (2004) Genome-wide RNAi analysis of growth and viability in Drosophila cells. Science 303: 832–835.
34. Eggert US, Kiger AA, Richter C, Perlmann ZE, Perrimon N, et al. (2004) Parallel chemical genetic and genome-wide RNAi screens identify cytokinesis inhibitors and targets. PLoS Biol 2: e379. doi:10.1371/journal.pbio.0020379
35. DasGupta R, Kaykas A, Moon RT, Perrimon N (2005) Functional genomic analysis of the Wnt-wingless signaling pathway. Science 308: 826–833.
36. Baeg GH, Zhou R, Perrimon N (2005) Genome-wide RNAi analysis of Jak/Stat signaling components in Drosophila. Genes Dev 19: 1861–1870.
37. Agaisse H, Burrack LS, Philips JA, Rubin EJ, Perrimon N, et al. (2005) Genome-wide RNAi screen for host factors required for intracellular bacterial infection. Science 309: 1248–1251.
38. Cheng IW, Viau JP, Sturman N, Wiedermann U, Vale RD, et al. (2005) Use of RNA interference in Drosophila S2 cells to identify host pathways controlling compartmentalization of an intracellular pathogen. Proc Natl Acad Sci U S A 102: 13646–13651.
39. Philips JA, Rubin EJ, Perrimon N (2005) Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection. Science 309: 1251–1253.
40. Stroschein-Stevenson SL, Foley E, O’Farrell PH, Johnson AD (2005) Identification of Drosophila gene products required for phagocytosis of Candida albicans. PLoS Biol 4: e14. doi:10.1371/journal.pbio.0040004
41. Bruckner K, Perez L, Clausen H, Cohen S (2000) Glycophosphotransferase activity of Fringe modulates Notch-Delta interactions. Nature 406: 411–415.
42. Boleti H, Benmerah A, Ojcius DM, Cerf-Bensussan N, Dautry-Varsat A (1999) Chlamydia infection of epithelial cells expressing dynamin and Eps15 mutants: clathrin-independent entry into cells and dynamin-dependent productive growth. J Cell Sci 112 (Pt 10): 1487–1496.
43. Hsu SW, Zhang Y, Streimann KC, Stoianovski D, Johnston AJ, Yano M, et al. (2005) Dissection of the mitochondrial import and assembly pathway for human Tom40. J Biol Chem 280: 11535–11543.
44. Webster P (2002) Early intracellular events during internalization of Listeria monocytogenes by J774 cells. J Histochem Cytochem 50: 503–518.
45. Bauer MF, Hofmann S, Neupert W, Brunner M (2000) Protein translocation into mitochondria: the role of TIM complexes. Trends Cell Biol 10: 25–31.
46. de Vries EF, Geuze HJ, van Deurs B, Simons K (1990) Clathrin-dependent and -independent endocytosis in animal cells. Cell 60: 819–824.
47. Hackstadt T, Scidmore-Carlson MA, Shaw EI, Fischer ER (1999) The division abnormally delayed of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell 108: 233–246.
48. Adams RR, Tavares AA, Salzberg A, Bellen HJ, Glover DM (1998) pavarotti encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. Genes Dev 12: 1483–1494.
49. Escalante-Ochoa C, Ducatelle R, Charlier G, De Vos K, Haesebrouck F (1999) Significance of host cell kinesis in the development of Chlamydia psittaci. Infect Immun 67: 5441–5446.
50. Baas PW, Karabay A, Qiang L (2005) Microtubules cut and run. Trends Cell Biol 15: 518–524.
51. Grieshaber SS, Grieshaber NA, Miller N, Hackstadt T (2006) Chlamydia trachomatis causes centrosomal defects resulting in chromosomal segregation abnormalities. Traffic 7: 940–949.
52. Beatty WL (2006) Trafficking from CD63-positive late endocytic multivesicular bodies is essential for intracellular development of Chlamydia trachomatis. J Cell Sci 119: 350–359.
53. Rapaport D (2005) How does the TOM complex mediate insertion of precursor proteins into the mitochondrial outer membrane? J Cell Biol 171: 419–423.
54. Niwa R, Nagata-Ohashi K, Takeichi M, Mizuno K, Uemura T (2002) Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell 108: 233–246.
55. Adams RR, Tavares AA, Salzberg A, Bellen HJ, Glover DM (1998) pavarotti encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. Genes Dev 12: 1483–1494.
56. Escalante-Ochoa C, Ducatelle R, Charlier G, De Vos K, Haesebrouck F (1999) Significance of host cell kinesis in the development of Chlamydia psittaci. Infect Immun 67: 5441–5446.
57. Haferkamp I, Schmitz-Esser S, Linka N, Urbany C, Collingo A, et al. (2004) A candidate NAD+ transporter in an intracellular bacterial symbiont related to Chlamydiae. Nature 432: 622–625.
58. Haferkamp I, Schmitz-Esser S, Wagner M, Neigel N, Horn M, et al. (2006) Tapping the nucleotide pool of the host: novel nucleotide carrier proteins of Protochlamydia ambaphila. Mol Microbiol 53: 177–187.
59. Tjaden J, Winkler HH, Schwoppe C, Van Der Laan M,Mohllmann T, et al. (1999) Two nucleotide transport proteins in Chlamydia trachomatis, one for net nucleoside triphosphate uptake and the other for transport of energy. J Bacteriol 181: 1196–1202.
60. Ojcius DM, Degani H, Wispeljer J, Dautry-Varsat A (1998) Enhancement of ATP levels and glucose metabolism during an infection by Chlamydia. NMR studies of living cells. J Biol Chem 273: 7052–7058.
61. Matsumoto A, Besoho H, Uehira K, Suda T (1991) Morphological studies of the association of mitochondria with chlamydial inclusions and the fusion of chlamydial inclusions. J Electron Microsc (Tokyo) 49: 356–363.
62. Peterson EM, de la Maza LM (1988) Chlamydia parasitism: ultrastructural characterization of the interaction between the chlamydiyal cell envelope and the host cell. J Bacteriol 170: 1389–1392.
63. Nagai T, Abe A, Sasakawa C (2005) Targeting of enteropathogenic Escherichia coli EspF to host mitochondria is essential for bacterial pathogenesis: critical role of the 16th leucine residue in EspF. J Biol Chem 280: 2908–3011.
64. Papatheodorou P, Domanska G, Oxle M, Mathieu J, Selchow O, et al. (2006) The enteropathogenic Escherichia coli (EPEC) Map effector is imported into the mitochondrial matrix by the TOM/Hsp70 system and alters organelle morphology. Cell Microbiol 8: 677–689.