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Chlamydiae interaction with the Endoplasmic Reticulum: contact, function and consequences

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Summary

Chlamydiae and *Chlamydiae*-related organisms are obligate intracellular bacterial pathogens. They reside in a membrane-bound compartment termed the inclusion and have evolved sophisticated mechanisms to interact with cellular organelles. This review focuses on the nature, the function(s) and the consequences of *Chlamydiae* inclusions interaction with the endoplasmic reticulum (ER). The inclusion membrane establishes very close contact with the ER at specific sites termed ER-Inclusion membrane contact sites (MCSs). These MCSs are constituted of a specific set of factors, including the *C. trachomatis* effector protein IncD and the host cell proteins CERT and VAPA/B. Because CERT and VAPA/B have a demonstrated role in the non-vesicular trafficking of lipids between the ER and the Golgi, it was proposed that *Chlamydia* establish MCSs with the ER to acquire host lipids. However, the recruitment of additional factors to ER-Inclusion MCSs, such as the ER calcium sensor STIM1, may suggest additional functions un-related to lipid acquisition. Finally, *Chlamydiae* interaction with the ER appears to induce the ER stress response, but this response is quickly dampened by *Chlamydiae* to promote host cell survival.

Introduction

The *Chlamydiae* phylum regroups gram-negative obligate intracellular bacterial pathogens of humans and animals. This phylum is sub-divided into three families. The *Chlamydiacea* family includes the major human pathogens species *Chlamydia trachomatis* and *Chlamydia pneumoniae*, which are respectively responsible for ocular and genital infections (Schachter, 1999) and pneumonia (Kuo *et al.*, 1995). The *Simkaniaceae* and the *Waddliaceae* families respectively include the *Chlamydia*-related species *Simkania negevensis* and *Waddlia chondrophila*. These emerging pathogens infect humans, animals and amoebae and are respectively linked to human pulmonary infections (Friedman *et al.*, 2003) and miscarriages in humans and abortions in ruminants (Dilbeck *et al.*, 1990, Henning *et al.*, 2002).

Chlamydiae and *Chlamydiae*-related organisms share a strictly intracellular bi-phasic developmental cycle. In the early stages, infectious bacteria (EB, Elementary Body) enter the cells and reside in a membrane bound compartment, named the inclusion. EBs then differentiate into non-infectious bacteria (RB, Reticulate Body) and undergo several rounds

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of replication. In the mid-stages, the cycle becomes asynchronous and some RBs start to differentiate back into EBs. In the late stages, EBs are released to allow a second round of infection (Moulder, 1991). Depending on the species, the cycle last 2-3 days (*C. trachomatis, C. pneumonia and W. chondrophila*) and up to 10 days (*S. negevensis*). To establish an intracellular niche permissive to bacterial replication, *Chlamydia* species rely on their ability to manipulate the host cellular environment. The bacterial type III secretion system (T3SS) and T3SS effectors proteins are key to this process (reviewed by (Mueller *et al.*, 2014)). Some effectors are released into the cytosol, while others, belonging to the family of Inc proteins, are inserted into the inclusion membrane (Dehoux *et al.*, 2011, Lutter *et al.*, 2012). The effector-dependent subversion of various cellular pathways allows the *Chlamydia* inclusion to rapidly evades the endocytic pathway, while interacting with various organelles such as mitochondria, lipid droplets, multi-vesicular bodies, the Golgi and the endoplasmic reticulum (ER) (Reviewed by (Bastidas *et al.*, 2013). Moreover, the interaction of *C. pneumoniae* with the ER was recently shown (Shima *et al.*, 2015). *W. chondrophila* also encode a T3SS (Bertelli *et al.*, 2010) and although T3SS effector proteins have yet to be identified, evasion of the endocytic pathway and interaction with mitochondria and the ER have been described (Croxatto *et al.*, 2010, Kebbi-Beghdadi *et al.*, 2011, de Barsy *et al.*, 2013). *S. negevensis* has been less studied, but the genome sequence revealed the presence of both a T3SS and a type IV secretion system (T4SS) (Collingro *et al.*, 2011) and interaction with the ER has been recently reported (Mehlitz *et al.*, 2014, Pilhofer *et al.*, 2014).

Over the past five years, studies of *Chlamydia, Waddlia and Simkania* have brought to light the intimate association of their inclusion with the ER. This review focuses on the current knowledge of the nature of this interaction, the bacterial and host factors involved and the biological consequences for the bacteria and the host.

DIRECT CONTACT BETWEEN THE ER AND THE INCLUSION MEMBRANE

C. trachomatis and *C. pneumoniae* ER-inclusion Membrane Contact Sites (MCSs)—

Electron micrographs by Giles *et. al.* provided the first evidence that ER tubules were present in the close proximity of the *C. trachomatis* inclusion (Giles *et al.*, 2008). This observation was later on confirmed by independent studies that further characterized the connection between the *C. trachomatis* inclusion membrane and the ER (Derré *et al.*, 2011, Dumoux *et al.*, 2012). Electron micrographs of *C. trachomatis* infected cells revealed that the inclusion was covered with several patches of ER located 10-20nm away from the inclusion membrane (Figure 1A, left cartoon). In eukaryotic cells, zones of close apposition (10-50nm) between two organelles are defined as Membrane Contact Sites (MCSs). The ER is often one of the partnering organelle contacting mitochondria, endosomes, the Golgi or the plasma membrane (PM) (Levine *et al.*, 2006, Lebiedzinska *et al.*, 2009, Prinz, 2014). Based on this knowledge, the points of contact between the ER and *C. trachomatis* inclusion membrane were proposed to represent a novel type of MCSs, named ER-Inclusion MCSs (Derré *et al.*, 2011). These structures have since been recognized as *bona fid*e MCSs (Prinz, 2014) and were also detected during *C. pneumoniae* productive or persistent infection (Shima *et al.*, 2015) (Figure 1A, left cartoon).

***C. trachomatis* pathogen synapse—**Electron tomography confirmed the presence of ER patches in close apposition with *C. trachomatis* inclusion, but also revealed the presence of host ribosomes onto the cytosolic side of the ER (Dumoux *et al.*, 2012). Moreover, structures similar to ER-Inclusion MCSs, but where ordered arrays of *Chlamydia* T3SS connected luminal RBs with the inclusion membrane and the apposed rough ER (rER) were also identified and named pathogen synapses (Dumoux *et al.*, 2012).

S. negevensis* ER-inclusion and ER-mitochondria MCSs—S. negevensis* was observed in a vacuole that is tightly associated with the ER in both amoebae and human cells (Mehlitz *et al.*, 2014, Pilhofer *et al.*, 2014). Electron-cryotomography of infected amoeba revealed that *S. negevensis* inclusions were almost entirely enveloped by the rER leaving only very small areas of inclusion membrane directly in contact with the cytosol (Pilhofer *et al.*, 2014) (Figure 1A, middle cartoon). It was noted that despite the very tight association of the inclusion with the ER, fusion of the respective membranes were not observed. In human cells, an extensive network of ribosomes studded membranes was also observed in the close proximity of the *S. negevensis* inclusion (*S. negevensis* inclusion was referred to by the authors as the *Simkania* containing vacuole (SCV) because of the different morphology compare to other *Chlamydia* inclusions) (Mehlitz *et al.*, 2014). Moreover, the association with the ER correlated with a recruitment of mitochondria that were directly in contact with the ER covering the *S. negevensis* inclusion (Figure 1A, middle cartoon). These structures resemble ER-mitochondria MCSs (Kornmann, 2013) and were observed in infected human cells but not in amoeba.

***W. chondrophila* mitochondria-inclusion and ER-mitochondria MCSs—**Immunofluorescence studies of *W. chondrophila* infected monocyte-derived human macrophages revealed the recruitment of mitochondria and the ER to the inclusion within the first 8 hours of infection. The mitochondria were recruited first and the association with the ER followed. Ultra-structural analysis revealed that *W. chondrophila* inclusions were surrounded by an inner layer of mitochondria that directly contacted the inclusion membrane and an outer layer composed of a dense ER network that was closely apposed to the mitochondria and occasionally contacted the inclusion membrane in small areas (Croxatto *et al.*, 2010) (Figure 1A, right cartoon).

Molecular composition of ER-Inclusion MCSs

While some ER resident proteins have been observed in the vicinity of the *Chlamydia, Simkania* and *Waddlia* inclusion, studies of *C. trachomatis* have led to the identification of proteins that are specifically enriched at ER-Inclusion MCSs.

CERT/VAPs/IncD

CERT is a functional component of ER-Golgi MCSs involved in the non-vesicular transfer of ceramide from the ER to the Golgi (Hanada *et al.*, 2003). In addition to the carboxy-terminal START domain (Ponting *et al.*, 1999) that binds ceramide, the ER-to-Golgi transfer requires a central FFAT domain (Loewen *et al.*, 2003) which binds the ER resident proteins VAPA and VAPB (Vesicle-associated membrane protein-associated protein) (Lev *et al.*,

2008) and an amino-terminal PH domain (Lemmon, 2008) which binds PtdIns(4)P and potentially Arf1 at the Golgi membrane (Balla *et al.*, 2006, Hanada *et al.*, 2009) (Figure 1B).

Immuno-fluorescence microscopy revealed that CERT and VAPA/B localized to patches associated with *C. trachomatis* inclusion membrane as early as 2h post infection (Derré *et al.*, 2011, Elwell *et al.*, 2011). Cryo-electron microscopy and immunogold-labeling confirmed their localization to ER-Inclusion MCSs, with CERT localizing at the interface between the inclusion membrane and the ER tubules and VAPB localizing to the ER (Derré *et al.*, 2011) (Figure 1B).

The CERT/VAPB localization at ER-Inclusion MCSs was therefore reminiscent of the one observed at ER-Golgi MCSs, except that the Golgi membrane was substituted with the inclusion membrane. The molecular mechanism(s) involved in CERT recruitment to *C. trachomatis* inclusion membrane were further characterized as follows.

Elwell *et. al.* investigated if CERT association with the inclusion involved PtdIns(4)P, VAP, ARF1 or the binding to ceramide. They showed that single amino acid point mutation preventing CERT ability to bind PtdIns(4)P (G67E) or VAPA/B (D324A) did not affect CERT recruitment to the inclusion. A similar result was observed upon Arf1 inhibition using Exo1. On the contrary, treatment of infected cells with HPA-12, a synthetic analogue of ceramide that inhibits CERT-mediated transfer of ceramide, prevented CERT association to the inclusion membrane (Elwell *et al.*, 2011). The authors concluded that PtdIns(4)P, VAP and Arf1 were not essential for CERT association with the inclusion, while CERT ceramide binding and/or transfer activity was.

Derré *et. al.* also showed that CERT associated with the inclusion in ARF1-depleted cells, confirming that CERT association with the inclusion was ARF1 independent. They also showed that the PH domain of CERT was necessary and sufficient for CERT association with the inclusion (Derré *et al.*, 2011). Although the PH domain of CERT is involved in PtdIns(4)P binding at the Golgi, the data obtained by Elwell *et. al.* with CERT G67E mutant and data collected by Derré *et. al.* demonstrated that in *C. trachomatis* infected cells, the PH domain of CERT is most likely not required for binding PtdIns(4)P onto the inclusion membrane. In order to identify a potential binding partner for CERT on the inclusion, Derré *et. al.* conducted pull down experiments of 3xFLAG-CERT from infected cell lysates followed by mass spectrometry analysis. This approach led to the identification of the *C. trachomatis* inclusion membrane protein IncD. The endogenous IncD protein localized to CERT-positive patches onto the inclusion membrane and co-immunoprecipitation experiments showed that 3xFLAG-IncD and GFP-CERT interacted when co-expressed in eukaryotic cells. Moreover, the PH domain of CERT was identified as the domain mediating IncD/CERT interaction. The direct interaction between IncD and the PH domain of CERT was also shown using purified proteins *in vitro* (Derré *et al.*, 2011).

The advances in *Chlamydia* genetics (Wang *et al.*, 2011) allowed for probing the IncD/CERT/VAPB interaction during infection, when IncD-3xFLAG was expressed from *C. trachomatis* under the control of an anhydrotetracyclin inducible promoter and inserted into the inclusion membrane (Agaisse *et al.*, 2014). In the absence of IncD-3xFLAG expression,

CERT localized to discrete patches onto the inclusion membrane, however CERT was massively associated with the inclusion membrane upon (over)-expression of IncD-3xFLAG. This association was dependent on the PH domain of CERT. In addition, the IncD-dependent massive recruitment of CERT to the inclusion correlated with a massive association of VAPB that was dependent on the FFAT domain of CERT (Agaisse *et al.*, 2014). Altogether these results experimentally validated a model in which, at ER-Inclusion MCSs, the *C. trachomatis* effector protein IncD recruits CERT to the inclusion membrane by direct interaction with CERT PH domain, which mediates the FFAT motif-dependent recruitment of the ER-resident protein VAPB to the inclusion (Figure 1B). It is unclear at this point whether the IncD-CERT-VAP interaction is sufficient to bring the ER in close apposition to the inclusion membrane or if additional factors are required to establish the ER-Inclusion MCSs.

STIM1

The ER calcium sensor, STIM1, and the plasma membrane localized calcium channel, Orai1, play a central role at ER-PM MCSs during store-operated calcium entry (SOCE) (Soboloff *et al.*, 2012, Srikanth *et al.*, 2012, Prakriya, 2013). In resting cells, STIM1 is bound to Ca^{2+} and localized to the bulk of the ER. Upon ER Ca^{2+} store depletion, the unbinding of Ca^{2+} triggers STIM1 oligomerization and redistribution to ER-PM MCSs, where the CAD domain of STIM1 interacts with and activates the Orai1 Ca^{2+} channel leading to Ca^{2+} influx and replenishment of the ER store.

Agaisse *et. al.* showed that, compared to general ER markers, such as Rtn3C and Sec61ß, that appeared evenly distributed throughout the bulk of the ER and in the vicinity of the inclusion, STIM1 was highly enriched in patches at the inclusion membrane (Agaisse *et al.*, 2015). Ultra-structural studies indicated that STIM1 localized to ER structures closely apposed to the inclusion membrane confirming its localization to ER-Inclusion MCSs. A time course of infection revealed that STIM1 associated with the inclusion throughout the developmental cycle. The Ca^{2+} channel Orai1 was never found in contact with the inclusion, however CERT co-localized with STIM1 at all stages of the developmental cycle (Agaisse *et al.*, 2015). It was therefore suggested that the ER-inclusion MCSs formed during *C. trachomatis* infection are hybrid MCSs composed of proteins usually found at ER-Golgi or ER-PM MCSs (e.g. CERT/VAP and STIM1 respectively) (Figure 1B).

The CAD domain of STIM1, which mediates the interaction with Orai1, was identified as the minimal domain required for STIM1 association with the inclusion (Agaisse *et al.*, 2015). It was proposed that, as shown for CERT, a *Chlamydia* effector protein might be involved in the CAD-domain-dependent recruitment of STIM1 to the inclusion.

It was shown that, similar to the situation observed in non-infected cells, STIM1 molecules localized to ER-PM MCSs upon Ca^{2+} store depletion of *C. trachomatis* infected cells. Moreover, a pool of STIM1 molecules remained associated with the inclusion after Ca^{2+} store depletion, suggesting that *de novo* formation of ER-PM MCSs did not disengage STIM1 from ER-Inclusion MCSs (Agaisse *et al.*, 2015).

Finally, while CERT or VAPA/B depletion have an adverse effect on *C. trachomatis* intracellular replication (Derré *et al.*, 2011, Elwell *et al.*, 2011), STIM1 depletion was not associated with a growth defect which could suggest redundancy and leaves the role of STIM1 during *C. trachomatis* infection unanswered (Agaisse *et al.*, 2015).

Chlamydia interaction with the ER and potential connection with lipid transfer and acquisition

Host lipids, such as sphingomyelin (SM) and cholesterol, are essential for *Chlamydia* development (original observation by (Hackstadt *et al.*, 1996) and reviewed by (Elwell *et al.*, 2012)). The bulk of SM is synthetized at the Golgi by the sphingomyelin synthase SMS1, while some SM synthesis also occurs at the PM *via* SMS2 (Huitema *et al.*, 2004, Tafesse *et al.*, 2007). At the Golgi, SM synthesis results from the CERT/VAP-dependent non-vesicular transfer of the SM precursor, ceramide, from the ER to the Golgi at ER-Golgi MCSs (Figure 1B). In the context of *C. trachomatis* infection, it was shown by labeling infected cells with fluorescent ceramide, which was subsequently metabolized into fluorescent SM in the Golgi, that SM-containing vesicles trafficked from the Golgi to the inclusion, where SM was transiently incorporated into the inclusion membrane before accumulating in the cell wall of the *Chlamydia*. These results highlighted the existence of a vesicular-dependent pathway of SM acquisition by *C. trachomatis* (Hackstadt *et al.*, 1996, Heuer *et al.*, 2009) (Figure 1B).

Three complementary approaches (the pharmacological inhibitor HAP-12, down-regulation of CERT activity through phosphorylation and protein depletion) were used by Elwell *et. al.* and Derré *et. al.* to show that CERT inhibition resulted in a decrease in the size of *C. trachomatis* inclusion and in the numbers of infectious bacteria recovered at the end of the developmental cycle (Derré *et al.*, 2011, Elwell *et al.*, 2011). A similar phenotype was observed upon VAPA/B depletion (Derré *et al.*, 2011) and SMS1 or SMS2 depletion (Elwell *et al.*, 2011). Moreover, while CERT depletion did not seem to affect the trafficking of fluorescently labeled SM to the inclusion (Derré *et al.*, 2011), HAP-12 treatment had a strong inhibitory effect (Elwell *et al.*, 2011). The decrease of *C. trachomatis* replication and lipid trafficking to the inclusion described above could originated from interference with the CERT/VAP-dependent transfer of ceramide at ER-Golgi MCSs or SM synthesis at the Golgi, which would in turn affect the vesicular pathway of SM acquisition by *C. trachomatis* (Figure 1B). However, the fact that anti-Ceramide antibodies partially labeled the inclusion membrane (Elwell *et al.*, 2011) and the fact that SMS2, but not SMS1, was recruited to the inclusion membrane ((Elwell *et al.*, 2011), Agaisse and Derré Unpublished), led to propose that, in addition to the vesicular-dependent pathway of SM acquisition, ER-Inclusion MCSs might play a role in the non-vesicular transfer of lipids to *C. trachomatis* inclusion (Derré *et al.*, 2011, Elwell *et al.*, 2011). In this model (Figure 1B), CERT and VAPA/B would participate to the non-vesicular trafficking of ceramide from the ER to the inclusion membrane, where ceramide would be further synthetized into SM by SMS2 and then transferred to the *Chlamydia*.

Altogether, these data suggest that *C. trachomatis* interaction with the ER could facilitate the transfer of lipids from the ER to the inclusion membrane and/or to the bacteria. These lipids could serve as a source of nutrient during bacterial replication. In addition, these lipids could

be used to change the lipid composition of the inclusion membrane and avoid immune recognition and/or facilitate/prevent interaction with certain organelles or proteins. It would be interesting to investigate whether additional lipid transfer proteins are recruited to ER-Inclusion MCSs to increase the panel of host lipids that are scavenged during *C. trachomatis* infection. Finally, while the IncD-dependent recruitment of CERT to the inclusion membrane has been established, how SMS2 associates with the inclusion membrane and how the SM synthetized at the inclusion membrane would be transferred to the bacteria remain to be explored.

CONSEQUENCES OF THE INTERACTION WITH THE ER: MODULATION OF THE ER STRESS RESPONSE

The ER plays a major role in controlling cellular homeostasis and perturbations of ER functions lead the activation of a process called the unfolded protein response (UPR) (reviewed by (Celli *et al.*, 2015) in the context of host-pathogens interaction). UPR relies on the ER membrane localized sensors activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE1) and double-stranded RNA-dependent protein kinase R (PKR)-like ER kinase (PERK) which at steady state are bound to the ER chaperone immunoglobulin protein (BiP), also known as 78kDa glucose related protein (GRP78). If unfolded proteins accumulate within the ER, BiP/GRP78 associates with these unfolded proteins and dissociates from ATF6, IRE1 and PERK leading to their activation and the initiation of the UPR signaling cascades. These signaling events are complex and lead to the increased production of chaperones such as BiP/GRP78, to the phosphorylation of the eukaryotic translation initiation factor-a (elF2a) and to the C/EBP-homologous protein (CHOP) translocation to the nucleus and activation of apoptotic cell death, to the proteolytic cleavage of ATF6 and to the splicing of X-box binding protein 1 (XBP1) mRNA.

The association of pathogens with the ER could have adverse effects on ER functions, especially when, as observed for *S. negevensis*, the inclusion is massively and tightly associated with the ER. This idea prompt Mehlitz *et. al.* to investigate the effect of *S. negevensis* infection on the UPR (Mehlitz *et al.*, 2014). During the early stages of *S. negevensis* infection, the increase of in BiP/GRP78 mRNA levels was indicative of an initial pulse of UPR, which was however not sustained during the rest of the developmental cycle. Accordingly, in comparison to un-infected cells, *S. negevensis* infected cells treated with the ER-stress inducers Thapsigargin or Tunicamycin displayed reduced levels of BiP/GRP78 and ERp72 proteins, reduced elF2a phosphorylation and decreased nuclear translocation of CHOP. Finally, *S. negevensis* replication was affected at high dose of ER-stress inducer. It was therefore proposed that although ER-stress is initially induced upon *S. negevensis* interaction with the ER, the bacteria is able to down-regulate this response by inhibiting the UPR signaling cascades.

Shima *et. al.* also investigated the effect of *C. pneumoniae* infection on the UPR in the context of persistent infection (Shima *et al.*, 2015). Persistent *Chlamydia* infections are characterized by the formation of small inclusions containing non-infectious but viable enlarged RBs and can be induced by stimuli such as INFγ, antimicrobial treatment or nutrient starvation (Hogan *et al.*, 2004, Wyrick, 2010, Lewis *et al.*, 2014). As observed

during *S. negevensis* infection, BiP/GRP78 expression was also induced during the early phase, but not in the late phase of *C. pneumoniae* INFγ-induced persistent infection. Interestingly, this effect was not observed in penicillin-induced persistent infection, suggesting that in the case of *C. pneumonia*, interaction with the ER alone was not sufficient to induce BiP/GRP78 expression. The analysis of UPR-mediating signaling pathways revealed that elF2a was phosphorylated during *C. pneumoniae* INFγ-induced persistent infection, however ATF6 proteolytic cleavage and XBP1 splicing did not occur. Moreover, the analysis of the UPR induced by the exogenous ER-stress inducer dithiothreitol (DTT) revealed that DTT-mediated phosphorylation of elF2a was attenuated in *C. pneumoniae* INFγ-induced persistent infection. However in BiP/GRP78 depleted cells, elF2a phosphorylation was increased and an increase in the percentage of apoptotic cells was observed. It was therefore proposed that during the early stages of *C. pneumoniae* INFγ-induced persistent infection the increase in BiP/GFP78 expression level activates the UPR signaling cascade and that failure to do so result in host cell death.

Conclusion and Perspectives

Over the past five years, it has become clear that *Chlamydiae* and *Chlamydiae*-related organisms interact with the ER. The *Chlamydiae*-containing inclusions do not appear to fuse with the ER, but instead very close contacts, the ER-Inclusion MCSs, are established between the membrane of the inclusion and the ER. How MCSs are established and maintained are open questions. It is possible that as shown for ER-mitochondria MCSs (Kornmann, 2013), special structural components of host and/or bacterial origin play a role in this process. In addition, the bacteria recruit specific host factors. The role of some of these factors, such as CERT and VAPA/B, in the non-vesicular trafficking of lipids suggests that *Chlamydia* could target the ER to directly harvest host lipids. Several lipid transfer proteins localize to MCSs between the ER and various organelles (Prinz, 2014) and it would be interesting to investigate whether any of them are also recruited to ER-Inclusion MCSs. In addition, the identification of additional factors, such as STIM1, that are recruited at point of contact between the ER and the inclusion, may uncover functions un-related to lipid acquisition. Finally, it appears that *Chlamydiae* interaction with the ER may have an adverse effect on the host cell by inducing the ER stress response. However, this response is quickly dampened, suggesting that *Chlamydiae* have evolved specific mechanisms to inhibit the ER stress response. It would be interesting to investigate whether any of the type III secretion effectors are involved in this process.

Altogether the discovery that *Chlamydiae* interacts with the ER has opened a novel and exciting area of research which will lead to a better understanding of the mechanisms involved in the subversion of cellular organelles by these obligate intracellular bacteria.

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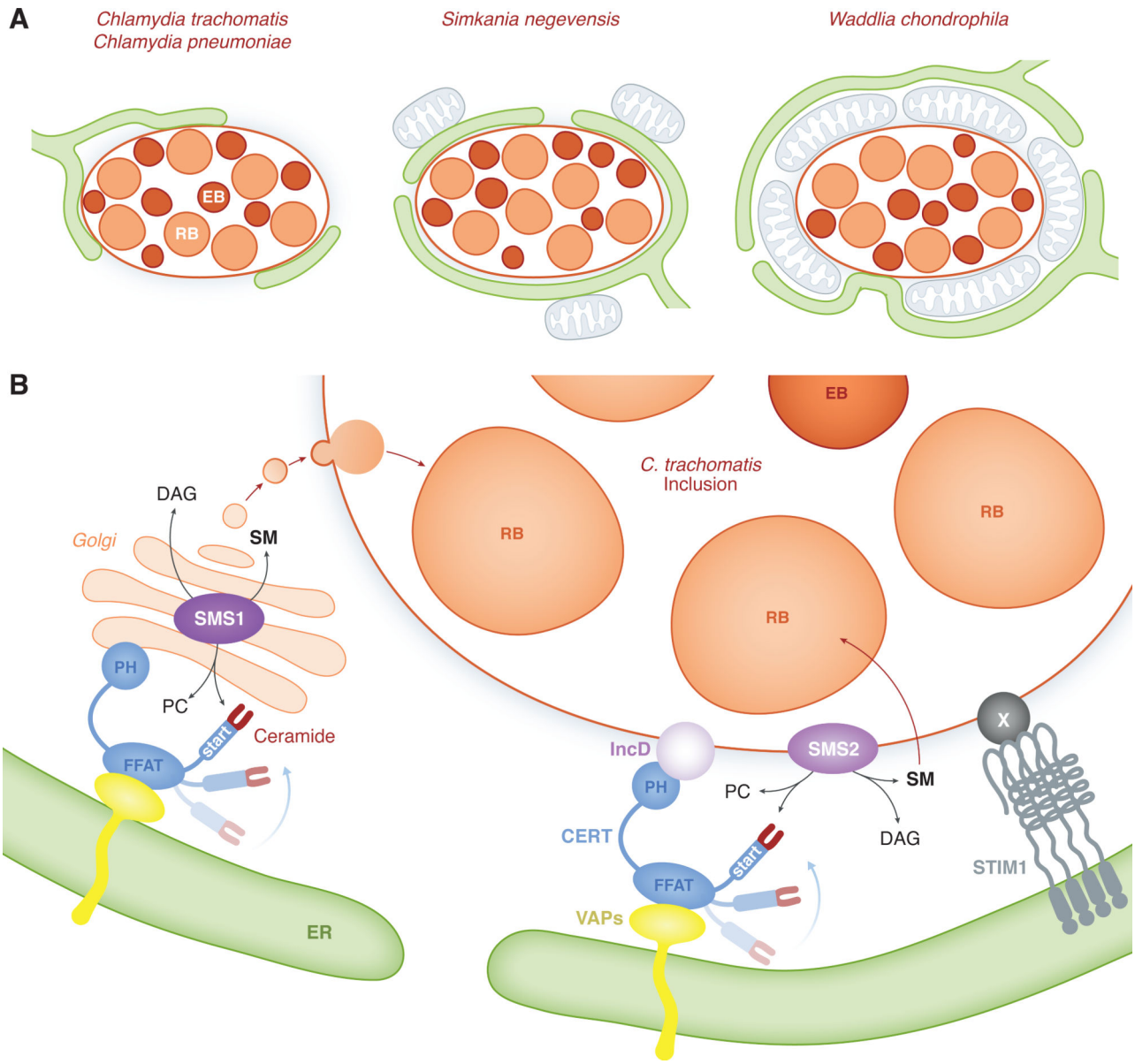

Figure 1. Nature, composition and function of membrane contact sites between *Chlamydiae* inclusion and the ER

A. Nature of the interaction between *Chlamydiae* inclusion and the ER. Schematic representation of *C. trachomatis, C. pneumoniae, S. negevensis* and *W. chondrophila* inclusions filled with RBs (light orange) and EBs (dark orange). The ER (green) is found in the vicinity of the inclusion membrane for each species. *C. trachomatis* and *C. pneumoniae* inclusion membrane is in close contact with and partially covered by the ER (left cartoon). *S. negevensis* inclusion is almost entirely covered by and tightly associated with the ER and ER-associated mitochondria (blue) (middle cartoon). *W. chondrophila* inclusion is surrounded by an inner layer of tightly associated mitochondria, which interact with a dense ER network (right cartoon).

B. Molecular composition of ER-Golgi and ER-Inclusion MCSs and their role in the vesicular and non-vesicular trafficking of lipids to *C. trachomatis* inclusion. CERT (blue)

localize to ER-Golgi MCSs where it participates to the transfer of ceramide from the ER to the Golgi, via the START domain. The FFAT motif of CERT binds to the ER-resident proteins VAPA/B (yellow) and the PH domain of CERT binds to the Golgi. CERT and VAPA/B also localize to ER-Inclusion MCSs. The PH domain of CERT binds to the *C. trachomatis* inclusion membrane protein IncD (light purple) and the FFAT motif of CERT allows CERT association with VAPA/B on the ER. In addition to CERT, VAPA/B and IncD, the ER Calcium sensor STIM1 (grey) also localize to ER-Inclusion MCSs. A *Chlamydia* factor X (dark grey) may be involved in this process. At ER-Golgi MCSs, the CERT-dependent transfer of ceramide from the ER to the Golgi-localized Sphingomyelin synthase SMS1 (dark purple) supports Sphingomyelin (SM) synthesis at the Golgi. In the context of *Chlamydia* infection, Golgi-derived vesicles traffic to the inclusion and SM is incorporated into the inclusion membrane and the cell wall of the bacteria. It is proposed that at ER-Inclusion MCSs, the CERT-dependent transfer of ceramide from the ER to the inclusion membrane-localized Sphingomyelin synthase SMS2 (purple) supports SM synthesis directly at the inclusion membrane. SM would then be incorporated into the bacteria.