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The emerging complexity of *Chlamydia trachomatis* interactions with host cells as revealed by molecular genetic approaches

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Abstract*Chlamydia trachomatis* (Ct) is an intracellular bacterial pathogen that relies on the activity of secreted proteins known as effectors to promote replication and avoidance of immune clearance. Understanding the contribution of Ct effectors to pathogenesis has proven to be challenging, given that these proteins often perform multiple functions during intracellular infection. Recent advances in molecular genetic analysis of Ct have provided valuable insights into the multifaceted nature of secreted effector proteins and their impact on the interaction between Ct and host cells and tissues. This review highlights significant findings from genetic analysis of Ct effector functions, shedding light on their diverse roles. We also discuss the challenges faced in this field of study and explore potential opportunities for further research.

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Introduction

Chlamydia trachomatis (Ct) is an obligate intracellular pathogen of significant clinical and public health importance. Yet, a thorough dissection of the molecular basis for its pathogenesis was not made possible until the recent development of a system for genetic transformation. The birth of molecular genetic analysis in Ct has led to the discovery and reassessment of the function of its virulence factors. In this review, we highlight key areas where the application of molecular genetic analysis in Ct has uncovered the function of secreted effector proteins and how these discoveries have provided new insights as to how this remarkable pathogen interacts with host cells and tissues. We also discuss areas where challenges and opportunities remain.

Ever since the discovery of Ct as the causative agent of blinding trachoma in humans and later as the leading cause of sexually transmitted bacterial infections, there has been keen interest in understanding the molecular basis for its immunopathogenesis. Ct is a Gram- negative obligate intracellular pathogen that infects epithelial surfaces in the conjunctiva, urethra, and endocervix. In women, urogenital serovars of Ct (D–H) can ascend to the upper genital tract where they cause severe damage to the reproductive organs long after the pathogen has been cleared [1]. Lymphogranuloma venereum (LGV) serovars of Ct (L1, L2, and L3) are invasive strains and can be found in the rectal mucosa of both males and females where they invade and reproduce in regional lymph nodes. Recently, LGV serovars have also been associated with outbreaks of rectal infections in men who have sex with men [2]. Ct serovars A–C infect the ocular conjunctiva and are the leading cause of infectious blindness worldwide [3]. Although Ct infections can lead to disease, most individuals infected with *C. trachomatis* remain asymptomatic, and pathologies can arise from single or recurrent infections [4]. The risk of developing sequalae in response to Ct infections is compounded by the failure of the human host to mount strong, protective adaptive immune memory responses to initial infections [5,6].

Chlamydia species are obligate endosymbionts of both protist and animal hosts, which have co-evolved with eukaryotic hosts for over 600 million years [7]. Several *Chlamydia* species are pathogenic to humans (such as Ct) and animals and have adapted to specific eukaryotic hosts with the presence of genes that are restricted to each species [8]. Many of these genes encode type-III secretion (T3S) effectors, which are required for the manipulation of host cellular processes [8]. While several T3S effectors have been conserved among *Chlamydia* species that infect mammals, we predict that the emergence of host-specific effectors likely occurred to colonize tissues and evade host immune responses.

Brief timeline of genetic advances and state of the art — before the development of genetic tools in *C. trachomatis*

Chlamydia species undergo developmental transitions during infection with an environmentally stable, infectious elementary body (EB) form and a replicative reticulate body (RB) form. EBs invade epithelial cells and reside in a membrane-bound compartment that is rapidly modified by the pathogen to create an 'inclusion'. Within this vacuole, the EB form transitions to the RB form and begins to replicate. At this stage, the inclusion expands rapidly and RBs asynchronously differentiate back to the EB form to fuel the next round of infections. Under in vitro stress conditions, RBs can enter into an altered and nonreplicative form referred to as a persistence state. Upon removal of the stress, RBs revert back to their replicative state (reviewed in [9]). By the end of the infectious cycle (48–72 h in cell culture), the host cell lyses or the entire inclusion is extruded from the infected cell [10–12].

The first clues as to how Ct avoids destruction by the endolysosomal system came from the discovery by Rockey, Hackstadt, and colleagues of a protein that decorated the inclusion membrane [13]. This protein had a hydrophobic bilobed motif of 50–60 amino acids that was subsequently found to be shared among other inclusion membrane (Inc) proteins [14,15]. Inc proteins are now recognized as key players in regulating interactions between Ct and its host. The publication of the genome of *C. trachomatis* in 1998 opened up the era of functional genomics in Ct, when the complete blueprint of the organism revealed an abundant repertoire of putative Inc proteins [16•].

Early attempts at identifying Ct virulence proteins focused on potential substrates of the T3S system by expressing full-length and the N-terminal regions of Ct proteins in heterologous bacterial systems and determining if they were sufficient to drive the T3S-dependent export of reporter proteins in *Yersinia*, *Shigella*, or *Salmonella* [17–20]. Other approaches relied on the conservation between yeast and mammals to identify Ct proteins that could drive phenotypes that were consistent with the manipulation of eukaryotic cellular processes [21]. Additional functional approaches included the generation of immunoreagents to assess the localization of all Ct proteins within infected cells [22–24]. The end result of these diverse experimental strategies was the identification of a defined set of proteins, including Incs, that likely interfaced with proteins in the host cell cytoplasm.

Yet, in the absence of a method to specifically disable these proteins, it was difficult to unequivocally ascertain their function during infection. Early attempts at performing functional analysis included the microinjection of effector-specific antibodies. For instance, the role of IncA as a factor that drives the fusion of inclusions was underscored by the observation that cells that had been microinjected with anti-IncA antibodies displayed large numbers of unfused inclusions upon infection [25]. These observations have been supported by the identification of Ct clinical isolates that lack IncA and form unfused inclusions [26]. Mutagenesis of *incA* has also provided additional evidence to corroborate these findings [27•–29•]. Alternative approaches that became standard to identify host cell targets of effector proteins also included co-affinity purification schemes or yeast two-hybrid screens (i.e. [30–36]). The relevance of such interactions was then tested by disrupting the function of the host targets by expression of dominant negative forms of these proteins or by RNA interference. If blocking the function of the host protein led to an impact on bacterial replication or survival, it was inferred that the associated effector was also important for virulence. Such interpretations are fraught with caveats, as we will point out in greater detail below, but in the absence of tools to genetically manipulate *Chlamydia* species, this remained one of the few viable options to characterize putative virulence factors.

Forward and reverse genetics approaches in *C. trachomatis*

For decades after they were first cultured in the laboratory, *Chlamydia* species remained stubbornly recalcitrant to molecular genetic manipulation. Early attempts at genetic transformation yielded mixed results until Clarke and colleagues provided the first evidence of stable maintenance of a shuttle plasmid into an LGV strain of Ct [37••]. Despite the lag in the development of transformation methods for Ct, significant advances have been made in the application of genetic approaches to dissect various aspects of Ct biology. For instance, by leveraging natural lateral gene transfer among Ct, it became possible to apply genetic recombination strategies to link chemically induced mutations to specific traits [38,39]. In this fashion, banks of mutants could be assembled and used for forward and reverse genetic applications to identify phenotypes of interest and the underlying causal mutations [28••,40••–42]. As newer molecular genetic tools were developed, in particular the use of group-two introns (TargeTron) and allelic exchange for targeted gene disruption, it became increasingly feasible to generate loss-of-function mutations [27•,43•,44•] and complement them with plasmids, thus satisfying Falkow's Molecular Kochs' postulates [45] and enabling a molecular characterization of Ct virulence factors.

Invasion-associated *C. trachomatis* effectors

The best-characterized Ct effectors are those translocated during the initial stages of invasion. There are at

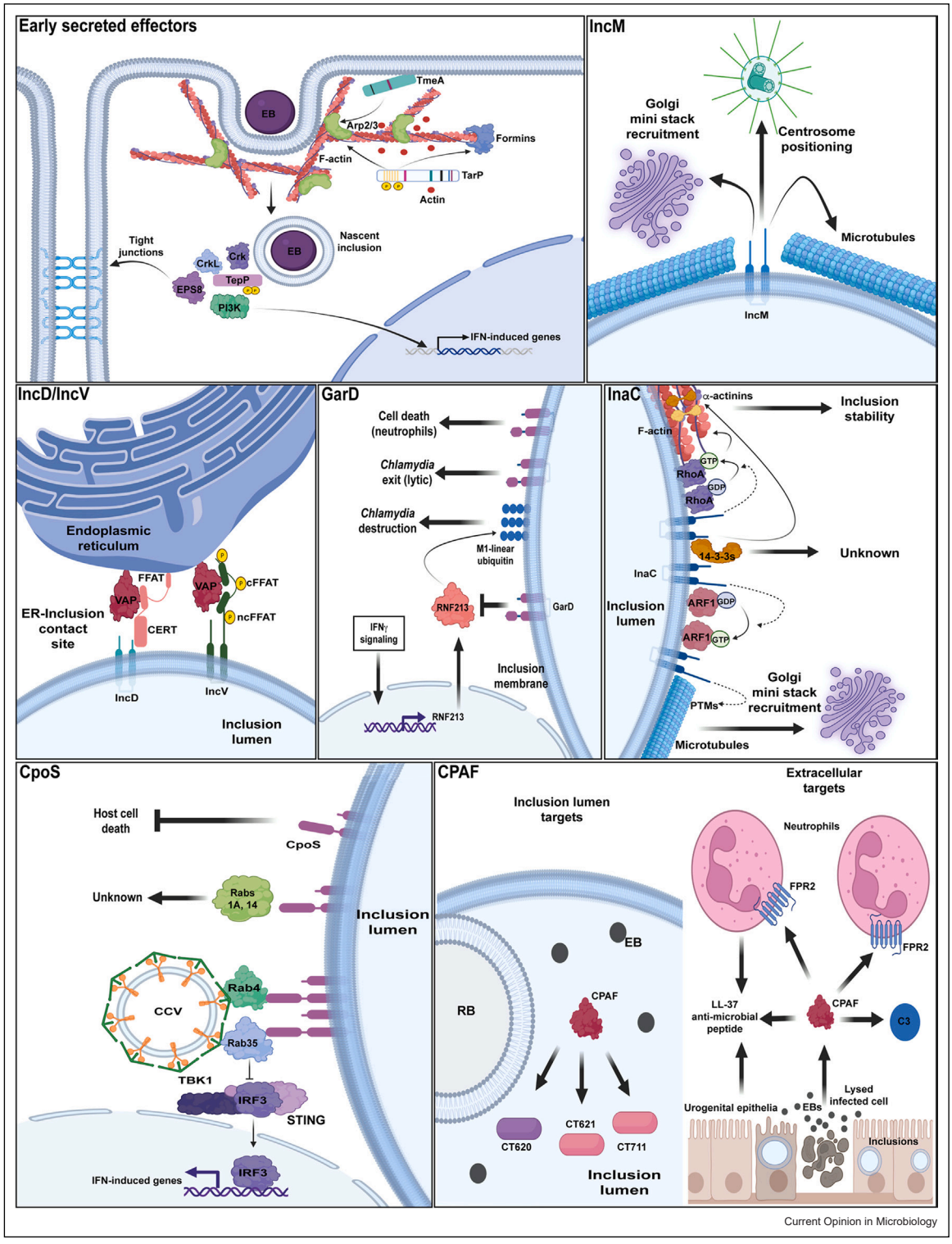
least five unique factors that are translocated during EB attachment and invasion of the target host cell, including the effectors *Translocated actin recruiting phosphoprotein* (Tarp), *Translocated membrane effector A* (TmeA), *Translocated membrane effector B* (TmeB), *Translocated early phosphoprotein* (TepP), and *Translocated ATG16L1 interacting protein* (TaiP). Tarp is a multidomain protein that can nucleate actin filaments and recruit and activate the host cell Arp2/3 complex (an actin nucleator complex that promotes formation of branched actin filaments) through tyrosine-phosphorylated repeats to promote actin polymerization at bacterial entry sites [46•–48]. Through the use of mutant strains expressing *tarP* alleles carrying in-frame deletions of the Tyr phosphorylation and F-actin-binding domains, these regions were shown to be required for TarP-dependent invasion [49]. However, while TarP clearly plays a role in invasion, *tarP* mutants only exhibit a partial reduction in invasion efficiency, suggesting that additional Ct factors enable entry into epithelial cells [49].

Another effector implicated with the process of invasion is TmeA. Loss of *tmeA* or *tarP* results in a moderate decrease in invasion efficiencies of host cells [49–51]. A double *tmeA tarP* mutant strain exhibits an additive effect on Ct invasion efficiency, indicating that both secreted effectors act synergistically in orchestrating Ct entry into host cells [51]. Proximity- labeling experiment using TmeA–BirA in transfected cells and TmeA–APEX (Enhanced ascorbate peroxidase) chimeras expressed in Ct, identified *Neural Wiskott-Aldrich syndrome protein* (N-WASP) as a host factor that interacts with TmeA [51]. The interaction of TmeA with N-WASP is sufficient to activate Arp2/3-dependent actin polymerization [51]. Interestingly, TarP can also induce Arp2/3-dependent actin polymerization via recruitment of factors that activate the small regulatory GTP binding protein (GTPase) Rac1 [52]. In this fashion, both TmeA and TarP promote actin polymerization independently to promote Ct entry into host cells [51]. In addition, Formin 1, another actin-nucleating factor that is required for rapid actin polymerization at sites of entry, is recruited to bacterial entry sites in a TarP-dependent but TmeA-independent manner, suggesting that TarP also promotes actin polymerization by multiple mechanisms [53].

TepP, such as TarP, is a Ct effector secreted early during infection that is phosphorylated at tyrosine residues by Src kinases [54•,55]. However, *tepP*-deficient mutants are not defective for the invasion of mammalian cells but they display decreased replication during infection of endocervical epithelial cells [55]. TepP associates with CrkI/II, CrkL, GSK-3, and *Phosphoinositide 3-kinase* (PI3K) at nascent inclusions and loss of *tepP* results in increased expression of several cytokine genes such as *IL6* and Chemokine (C-X-C motif) ligand 3 (*CXCL3*) [54•,55]. Paradoxically, overexpression of *tepP* leads to an increase in the expression of type-I Interferon (IFN) genes *IFIT1* and *IFT2* [54•,55]. TepP activates PI3K and PI3K activity is required for TepP-dependent induction of *IFIT1* and *IFT2* gene expression [55]. How TepP promotes activation of PI3K and Crk and GSK binding remains to be determined, however, TepP association with these factors is probably necessary for optimal Ct fitness in cervical epithelial cells and to modulate the transcription of genes associated with type-I IFN responses [55]. Like TarpP, TepP is likely to perform multiple functions that can be genetically separable. Indeed, TepP co-opts the function of the F-actin regulator EPS8 to disassemble tight junctions of polarized epithelial cells to further enhance the invasion by EBs, presumably by providing access to additional receptors [56]. In addition, endometrial organoids (EMOs) infected with a *tepP* mutant significantly increased neutrophil recruitment to EMOs relative to EMOs infected with wild-type Ct [57]. These results suggest that TepP also functions in limiting the influx of neutrophils at sites of infection, possibly through its role in dampening expression of IL-6 and CXCL3. The role of TepP in Ct infections is further underscored by the observation that *C. muridarum (Cm) tepP* mutants are severely attenuated in their ability to ascend to the upper genital tract after vaginal infections [56].

TaiP was originally identified as a Ct protein with a functional T3S signal that is highly enriched in EBs and localizes to the cytoplasm of host cells [19,58–60]. Based on these observations, TaiP has been proposed to be secreted early in infection [60]. TaiP unlike TarP and TepP is not phosphorylated upon translocation [60], and *taiP* mutants are internalized less effectively and are attenuated for growth in Hela cells [60]. TaiP binds the autophagy-related protein 16–1 (ATG16L1) through an ATG16L1-binding motif in its carboxy tail domain [61]. ATG16L1 restricts inclusion growth through its association with the transmembrane protein Transmembrane protein 59 (TMEM59) rather than by its well-known role in promoting lipidation of the microtubule-associated and autophagy-promoting protein Microtubule-associated protein 1A/1B-light chain 3 (LC3) [61]. TaiP binds to the WD or beta-transducin repeat (WD40) domain of ATG16L1 and disrupts ATG16L1 association with TMEM59, leading to the rerouting of Rab6 (Golgi-associated GTPase)-positive vesicles to the inclusion [61]. These results suggest that Rab6-mediated membrane trafficking is important for inclusion growth and that Ct utilizes a unique effector for the acquisition of nutrients. The role for TaiP in promoting invasion remains to be further characterized.

Figure 1



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The multiple roles of *C. trachomatis* effectors as determined by molecular genetic approaches. Early secreted effectors. TarP and TmeA are translocated into host epithelial cells during Ct invasion of host cells. TarP directly binds to actin and promotes the polymerization and bundling of actin filaments [46–48,51,53]. TarP is phosphorylated by host cell kinases at tyrosine residues residing in a tyrosine-rich domain at its N-terminal region [46]. Phosphorylated TarP activates Rac1 signaling, leading to Arp2/3-mediated polymerization of actin at sites of Ct invasion [48,52]. Phosphorylation of TarP also leads to recruitment of Formins, which are actin nucleators, to sites of invasion [53]. TmeA acts synergistically with TarP to promote actin polymerization by activating Arp2/3 via N-WASP signaling [51,53]. After delivery into host cell cytoplasm, TepP localizes near the nascent Ct inclusion [54•]. TepP is rapidly phosphorylated at tyrosine residues by host cell Src family kinases [54•]. TepP mediates recruitment of Adapter molecule crk (CRK), CRK like protein (CRKL), *Glycogen synthase kinase-3 beta* (GSK3B), and subunits of PI3K to the Ct nascent inclusion [54•, 55]. TepP- dependent induction of host *IFIT* genes requires its interaction with PI3K [55]. The interaction between TepP and Eps8 is necessary for Ct to remodel tight junctions between host epithelial cells [56]. This leads to disruption of epithelial barriers to promote secondary invasion events [56]. **IncM** is an inclusion membrane protein with multiple functions. It promotes the stabilization of microtubules and repositioning of Golgi ministacks around the inclusion. IncM also is involved in repositioning of host cell centrosomes [67]. **IncD** and **IncV**. IncD interacts with the human ceramide transfer protein Ceramide transport protein (CERT), which binds to the ER-resident protein VAMP-Associated protein (VAP) through a Phenylalanines (FF) in an acidic tract (FFAT) motif. Formation of IncD and CERT might facilitate transport of ceramides to the inclusion membrane [68•,70]. IncV independently binds to VAPs through noncanonical (ncFFAT) and canonical (cFFAT) FFAT motifs present in its C-terminus [69]. Phosphorylation of IncV by host protein kinases is necessary for IncV interactions with VAPs [71]. Both IncD and IncV play roles in promoting tethers (contact sites) at the inclusion and ER interphase [68•,69,71]. **GarD** shields inclusions from targeted ubiquitination by the IFNγ-inducible E3 ligase RNF213. When infected with a *garD*-null strain, IFNγ-primed host cells trigger M1-linked polyubiquitination of the inclusion, leading to its subsequent destruction [83•]. GarD is also required for the lytic exit of Ct from host cells [85]. In murine primary neutrophils infected with Ct serotype D, GarD (CT135) is necessary for the Ct-dependent activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome and host cell death [86•]. **InaC** interacts with multiple 14–3–3 proteins during infection of human host cells [28••]. The function of these interactions remains unknown. InaC also promotes activation of the GTPase ARF1 (ARF1–GTP) in early stages of *Chlamydia* infection of host cells. Both InaC and activated ARF1 promote PTMs (acetylation and detyrosination) of microtubules present at the periphery of the inclusion [74]. Modified microtubules are necessary for repositioning of Golgi ministacks around the inclusion periphery [74]. At later stages of infection, InaC promotes activation of Ras homolog family member A (RhoA) that is recruited to the inclusion in an InaC-independent manner. Activated RhoA (RhoA–GTP) inhibits ARF1 activation and subsequent PTMs of microtubules, while ARF1–GTP promotes further activation of RhoA. As levels of activated RhoA increase, activated RhoA promotes formation of F-actin scaffolds around the inclusion, which provides stability to the inclusion [76,77]. InaC is also required for recruitment of alpha-actinin 1 and alpha-actinin 2 to the inclusion periphery where they promote stabilization of actin scaffolds and inclusion stability [109]. **CpoS** interacts with multiple human Rab GTPases during infection of human cells [29•,79]. The interaction of CpoS with both Rab4 and Rab35 mediates recruitment of clathrin-coated vesicles to the inclusion [80], while the interaction of CpoS with Rab35 is required for *Chlamydia* to block activation of (Stimulator of interferon genes/Interferon regulatory factor 3) STING/IRF3-mediated transcription of ISGs [81]. CpoS interactions with Rabs 1A and 14 remain to be further characterized. CpoS also blocks activation of host cell death programs by an unknown mechanism and this activity is uncoupled from its interactions with Rab GTPases [29•]. **CPAF**. CT620, CT621, and CT711 are secreted into the inclusion lumen and may be targeted by CPAF, which also localizes to the inclusion lumen [93–95]. CPAF is likely released from infected cells during the lytic exit of Ct from host cells. Once released, CPAF can cleave the FPR2, which is present on the surface of infiltrating neutrophils, to block their activation and NETosis [96•]. In addition, CPAF can cleave LL-37, an antimicrobial peptide produced by urogenital epithelia and neutrophils, as well.

Inclusion membrane dynamics

The membrane of the inclusion enables the bidirectional flow of nutrients and metabolic waste while limiting the engagement of cytoplasmic innate immune sensors. Inc proteins are predicted to perform many of these functions by engaging the membranes of host organelles adjacent to inclusions, regulating protein and membrane trafficking, or by providing structural integrity to the inclusion (reviewed in [62••]). Inc proteins may also act as hubs for protein–protein interactions that control signaling cascades important for bacterial fitness within infected cells and tissues. One of the challenges in studying Inc proteins is that any one Inc likely performs multiple functions (Figure 1). This was first predicted by findings from proteomic screens for host-binding partners of Inc proteins, which often yielded multiple high- confidence binding partners for any one Inc [35••]. The modular and multifunctional nature of Incs has further been evidenced through molecular genetic approaches as detailed below.

The N- and C-terminal domains of Inc proteins are exposed to the host cell cytosol, where they have the potential to interact with multiple host proteins essential for bacterial survival. One such example is the inclusion membrane protein IncS, which is required early in the Ct developmental cycle of both Ct and Cm [63••]. IncS is likely essential since *incS*-null strains cannot be recovered, unless they are generated in the presence of conditionally expressed *incS* [63••]. The number of RBs detected within Ct and Cm inclusions drastically decreases in the absence of IncS, suggesting that IncS is necessary for EB to RB developmental transitioning [63••]. This work is a significant advance in the field as it describes how to generate targeted conditional mutant strains for characterizing the function of essential effectors. The next step will be to elucidate the mechanism by which IncS facilitates this early developmental transition.

During Ct infection, Ct traffics to the microtubule-organizing center where Inc proteins mediate interactions with organelles, centrosomes, and cytoskeletal proteins (reviewed in [62••]). One consequence of these interactions is disruption of the cell cycle as evidenced by the presence of multinucleated cells in Ct-infected cells [64–66]. This phenomenon is mediated in part by IncM (Inc-mediating multinucleation) [67]. Epithelial cells display fewer multinucleated cells when infected with *incM*-null strains [67]. IncM also contributes to the

positioning of centrosomes relative to the host cell nucleus and the repositioning of Golgi ministacks around the inclusion periphery [67]. Additionally, IncM stabilizes a network of microtubules that surround the inclusion, but it remains unclear whether IncM directly interacts with microtubules [67]. The direct interactions between IncM and microtubules could be a mechanism by which IncM facilitates centrosome positioning and Golgi dispersion around the inclusion.

Additional Inc proteins also facilitate interactions between Ct and host organelles. For instance, IncV and IncD facilitate the tethering of the endoplasmic reticulum (ER) to the inclusion membrane to form ER-inclusion membrane contact sites (MCSs) [35••,68•,69]. Formation of these MCSs at the ER-inclusion interface mediates the transfer of lipids to the inclusion [68•,70]. IncV interacts with the proteins VAMP-Associated protein A (VAPA) and VAMP-Associated protein B (VAPB) that are normally enriched at ER MCSs [69]. These interactions are dependent on two FFAT motifs present in IncV as demonstrated by molecular genetic approaches. GFP-tagged VAPA and VAPB co-IP with Flag-tagged IncV were expressed in Ct during infection and this interaction was dependent on both its canonical FFAT motifs [69]. IncV is also phosphorylated by the protein kinase *Casein kinase 2* (CK2) and this phosphorylation is necessary for its interactions with VAPs and for assembly of the ER-inclusion MCS [71]. Furthermore, the expression of IncV variants with amino acid substitutions in S345A, S346A, and S350A in Ct *incV* mutant strains showed that all three serine residues, which are part of the CK2 recognition site in the C-terminal domain of IncV, are necessary for the recruitment of CK2 to the inclusion membrane [71]. While many FFAT domain proteins contain acidic tracts that are required for interactions with VAPS, IncV's phosphoserine-rich tracts may mimic these acidic patches [71]. Interestingly, replacing the IncV serine patches with acidic amino acids and expression of these variants in *incV* mutant strains prevents its translocation to the inclusion membrane [71]. These results suggest that the serine tracts in IncV ensure proper secretion of IncV and subsequent phosphorylation by host cell kinases promotes interactions with eukaryotic VAPs [71]. While these observations suggest that IncV plays a role in ER-inclusion MCS formation, a Ct mutant strain lacking *incV* only exhibits a moderate decrease in recruitment of VAPS to ER-inclusion MCSs suggesting that additional factors contribute to formation of MCS at the inclusion [69].

Identification of new *Chlamydia* effectors by forward genetic approaches

The Ct protein CT813 was originally identified as an inclusion membrane protein based on its localization by indirect immunofluorescence [72]. It was subsequently renamed *In*clusion-*a*ssociated A*c*tin (InaC) when a mutant in this gene was identified from a screen of mutagenized Ct strains that failed to recruit F-actin scaffolds to the inclusion membrane [28••]. InaC was also observed to interact with (Adenosine diphosphate) ADP-ribosylation factor (ARF) GTPases, which are major regulators of intracellular trafficking [73], and to be necessary for repositioning of Golgi ministacks around the inclusion [28••,74] with Arf1 and Arf4 recruitment to inclusion membranes being lost in *inaC* mutants [28••,74]. InaC promotes activation of Arf GTPases, which in turn leads to the acetylation and detyrosination of microtubules adjacent to the inclusion membrane [74]. These microtubule modifications promote Golgi ministack repositioning around the inclusion [74]. InaC also promotes activation but not recruitment of the small GTPase RhoA (a regulator of the actin cytoskeleton (reviewed in [75])), which has previously been shown to be required for the formation of scaffolds of actin around the inclusion [66,67•]. The scaffolding of actin and of modified microtubules around the inclusion occurs at different stages of the Ct lifecycle. Modified microtubules are primarily assembled around the Ct inclusion around 24 h post infection (hpi) ,whereas recruitment of actin scaffolds begins at 32 hpi [76,77•]. The temporal regulation of actin and microtubule assembly around the inclusion is also InaC dependent. Small GTPases cycle between an active (Guanosine-5'-triphosphate) GTP-bound state and an inactive GDP-bound state. InaC-dependent activation of Arf1 (Arf1–GTP) occurs early in infection (~16 hpi). Midway through infection (~24 hpi), RhoA is recruited to the inclusion independently of InaC where it proceeds to inhibit Arf1 activation. Inactivation of Arf1 concomitantly leads to an InaC- dependent activation of RhoA [77•]. This complex coordination of the activity of host cell small GTPases (Arf1 and RhoA) by a single Ct effector highlights the modular and multifaceted activities inherent to Ct effectors for fine-tuning rearrangements of the host cytoskeleton during infection. Importantly, the use of engineered Ct strains was instrumental for dissecting these functional intricacies since relying on pharmacological inhibitors of cytoskeleton assembly can easily obfuscate results due to the pleiotropic effects they elicit. Unresolved questions as to the function of InaC include its role in the recruitment of 14–3–3 proteins to the inclusion membrane [28••], a function that has also been ascribed to IncG [30].

Chlamydia p romoter *o*f *S*urvival (CpoS) is another Inc with multiple roles during Ct infection of host cells. CpoS was identified in a screen for Ct mutants that induced enhanced cytotoxicity in Hela and Human leukemia monocytic cell line (THP-1) cells [29•]. CpoS binds to Rab small GTPases, which are regulators of membrane trafficking between intracellular

compartments, suggesting that CpoS mediates interactions with vesicular trafficking pathways [29•,78,79]. These GTPases are not recruited to the inclusion membrane of *cpoS* mutants [29•,79] and the recruitment of transferrin and the mannose-6-phosphate receptor to the inclusion is diminished in host cells infected with a *cpoS* mutant indicating that CpoS mediates interactions with clathrin-dependent transport pathways from the plasma membrane and trans-Golgi network [79]. CpoS possesses a coiled-coil domain at its C-terminus domain [69]. Ectopic expression of a CpoS variant carrying an amino acid substitution in its coiled-coil domain (L120D) in a *cpoS*- null background suggests that the C-terminal region of CpoS is necessary for binding to Rab GTPases [79]. While the repurposing of vesicle trafficking by CpoS might be necessary for nutrient acquisition by Ct, these findings should be interpreted in the context of the severe growth phenotype of *cpoS* mutants and the rapid onset of death in cells infected with these mutants [29•,80], and that mutant alleles of *cpoS* that cannot recruit Rab GTPases still protect the host cell from death [81]. Loss of *cpoS* also results in a significant increase in the transport of the innate immune receptor STING from the ER compartment and the hyperactivation of interferon-stimulated genes (ISGs) [29•]. The same *cpoS* alleles that fail to recruit Rab GTPases are also unable to block the enhanced translocation of STING, suggesting that one prominent role of co-opting Rab GTPases is to dampen innate immune signaling. Interestingly, while the expression of ISGs is not sufficient to induce death in cells infected with *cpoS* mutants, STING is still partially required to induce cell death [29•]. This is consistent with a role of STING in controlling the activation of type-I IFNs and cell death through distinct mechanisms (reviewed in [82]). How these activities impact the expression of IFN-regulated genes or protection from cell death remains to be better characterized but will be possible through the use of separation-of-function *cpoS* alleles.

Forward genetic screens of Ct mutant strains also resulted in the identification of GarD (gamma-resistance determinant) as an Inc required for Ct to protect itself from (interferon gamma) INFγ-induced antimicrobial host defenses [83•]. In infected human cells, Cm is susceptible to INFγ- mediated clearance while Ct is not, indicating that Ct actively attenuates human INFγ-based defenses [83•,84]. In a forward genetic screen for INFγ-sensitive Ct mutants, strains with nonsense and loss-of-function mutations in *garD* (CTL0390/CT135) were identified that displayed attenuated growth in INFγ-primed A549 cells [83•]. Inclusions of *garD*-deficient strains were targeted for INFγ-dependent M1-linked ubiquitination by the E3 ligase RNF213 [83•]. Cells infected with *garD* mutants also recruited the M1-linked ubiquitin-binding adaptor proteins Optineurin (OPTN), *Nuclear dot protein 52* (NPDP52), and Tax1-binding protein 1 (TAX1BP1) and to a lesser extent the ubiquitin-like proteins LC3 and Gamma-aminobutyric acid receptor-associated protein (GABARAPs) to the inclusion membrane [83•]. The recruitment of xenophagy receptors to *garD* mutant inclusions suggests that *garD*-deficient strains might be routed for destruction through lysosomal degradation since lysosomal *Lysosomal-associated membrane protein 1* (LAMP1) also colocalizes with *garD* mutant inclusions [83•]. Some questions remain to be addressed such as identifying the targets recognized by RNF213, the mechanism by which RNF213 is directed to the *Chlamydia* inclusion, and weather RNF213-mediated immune defenses are deployed against other intracellular pathogens. GarD also plays additional roles such as promoting exit of Ct from host cells via lysis [85]. Interestingly, GarD-dependent host cell lysis requires STING, which is also partly required for host cells to induce cell death during infection of epithelial cells with *cpoS*-deficient strains [29•,85]. In addition, GarD in Ct serovar D (CT135) has also been linked to activation of NLRP3 inflammasomes and cytotoxicity in infected murine primary neutrophil cells, suggesting that GarD and its homologs can also promote Ct immune evasion by neutralizing neutrophil host defenses [86•].

Additional genetic screens have helped identify factors required for *Chlamydia* to evade INFγ-mediated immunity. Screening of Cm mutagenized with EMS, for example, identified 31 IFNγ-sensitive mutants, including a strain with a missense mutation in a gene encoding the putative Cm inclusion membrane protein TC0574, which was linked to sensitivity to IFNγ treatment [87]. Inclusions of strains carrying mutations in TC0574 lyse in the presence of IFNγ and elicit host cell death that is dependent on host cell caspases [87]. The mechanism by which TC0574 protects Cm from IFNγ-mediated clearance remains to be elucidated. IFNγ also triggers *Chlamydia* species to enter a viable but non-replicating state in vitro called persistence [reviewed in [9]). Screens of chemical mutants have also been employed to identify Ct factors necessary for Ct to reactivate from IFNγ- induced persistence. From these screens, *trpB*, and genes previously unlinked to persistence such as CTL0225, CTL0694, and *ptr* were linked to Ct ability to exit from a persistence state [88,89]. *trpB* encodes the beta-subunit of tryptophan synthase and has been linked to Ct persistence [41••], while CTL0225 and CTL0694 encode a putative membrane protein and an oxidoreductase, respectively. The mechanisms by which the latter promote reactivation from persistence remain unknown. Ptr-deficient mutants showed a reduction in the generation of infectious progeny after exiting from IFNγ-induced persistence [89]. Interestingly, Ptr is a putative protease that is secreted into the inclusion lumen [89]. Considering that persistence is thought to be a stress response triggered by amino acid

starvation (reviewed in [9]), Ptr might be playing a role in scavenging amino acids from the lumen of the Ct inclusion, thereby aiding in the recovery of Ct from a persistent state.

Reassessing the function of proteases and the cryptic plasmid

Before the development of molecular genetic tools, multiple approaches had been implemented to assign a function to *Chlamydia* effectors and other potential virulence factors. These included implying functions from predicted and confirmed biochemical activities, or their impact on mammalian cells following ectopic expression. While in many instances these approaches have identified bonafide substrates and functions of *Chlamydia* effectors, there are caveats given that overexpressed effectors, for instance, during transient transfections, may not reflect the true localization or function of the native proteins during infection given the differences in stoichiometry, spatial constraints in relation to inclusions, post-translational modifications (PTMs), or by activities provided by other *Chlamydia* factors present during infection. An example of the challenges associated with elucidating the function of effectors solely based on enzymatic activities is illustrated by the secreted protease CPAF. Numerous studies suggested that CPAF modulated multiple processes given the many substrates identified in vitro (reviewed in [90]). However, in retrospect, caution in the interpretations of these results was warranted given that CPAF's activity is difficult to inhibit and can cleave substrates during sample preparation [91]. The identification of a *C. trachomatis cpa* (CPAF)-null strain provided genetic evidence that many potential CPAF substrates may not be targeted for proteolysis during Ct's intracellular stage [92] although this observation does not preclude the possibility that some of the identified targets are cleaved at a different stage during infection.

Renewed efforts at identifying CPAF substrates using *cpa*-null strains included a comparative analysis of proteomes from Hela cells infected with wild-type Ct and a *cpa*-null strain that revealed 10 Ct proteins whose abundance increased during infection with a *cpa*- null strain [93]. Of these, the T3S substrates CT620 and CT711 appear to be proteolytically processed in wild-type Ct cells [93]. CT620 and CT621 (another T3S substrate) also localize to the inclusion lumen where CPAF has also been shown to localize [94,95]. These results suggest that CT620, CT621, and CT711 are potential bacterial in vivo targets of CPAF. Mutant *cpa* strains also support a role for CPAF in degrading targets that are not in the host cell cytoplasm, presumably after being released during lytic exit of Ct from host cells. For instance, CPAF cleaves formyl peptide receptor 2 (FPR2) present on the surface of neutrophils to block the activation of neutrophils and NETosis [96••]. CPAF can also cleave the antimicrobial peptide LL-37 produced by urogenital epithelia and recruited neutrophils as well as complement factors C3 and factor B [97,98]. Targeting of these extracellular factors may be linked to CPAF's role in promoting Ct survival in the upper genital tract [99].

Native plasmids of both Ct and Cm contribute to infectivity and virulence in several animal models of *Chlamydia* infection [100–103]. *Chlamydia* strains harboring plasmids with *pgp3* deletions are as attenuated in virulence as plasmidless *Chlamydia* strains [104,105]. Notably, *pgp3*-deficient Cm is less invasive in the lower genital tract and fails to ascend to murine upper genital tracts [105]. Cm *pgp3*-deficient strains are also defective in colonization of the gastrointestinal tract in infected mice [106]. The mechanism by which pGP3 promotes Cm colonization of genital and intestinal tracts requires further characterization, especially with the aid of *pgp3*-deficient strains. Recombinant pGP3 binds and neutralizes the antimicrobial peptide cathelicidin LL-37, which is also targeted by CPAF hinting to synergistic functions between the two virulence factors [97,107,108].

Challenges for *Chlamydia* effector biology and analysis

The application of a full spectrum of genetic tools in *Chlamydia*, including tools for generating conditional null strains for identifying the function of essential effectors (i.e. [42,63]), will invariably increase the rigor of future molecular analysis of effector proteins and their role in infection. Given the emerging theme that *Chlamydia* effectors perform multiple functions (Figure 1), the generation of separation-of-function mutants will be essential to understand whether these functions are acting independently from each other or have evolved to function synergistically. Separation-of-function mutants will also aid in defining which functions and pathways are most critical for pathogenesis. Similarly, it will be important to use *Chlamydia* effector mutants in the context of infection of cells defective in the proposed pathways targeted by the effector to distinguish between indirect effects resulting from the disruption of major host cellular pathways and those perturbed by the effector. It would not be surprising if virulence phenotypes are not as penetrant as first envisioned and that multiple effectors act in a coordinated manner. In this case, mutant strains bearing multiple null alleles of effectors in various combinations and genetic screens such as synthetic lethal screens will be useful in uncovering synergistic interactions among effectors. The analysis of *Chlamydia* effector proteins will also benefit from new cellular models of infection that better mimic the cervical and uterine epithelium and animal models that are

closer to both the acute and chronic stages of infection, as many relevant phenotypes will not become apparent until infections with mutants are performed in the most relevant tissues.

CRediT authorship contribution statement

Bastidas Robert: Writing – review & editing. **Valdivia Raphael**: Writing – review & editing.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

R.H. Valdivia is co-founder at Bloom Sciences. The company did not sponsor any of the work mentioned in this article.

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