NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF LIFE SCIENCES

Inês Pacheco Leal

BSc in Molecular and Cellular Biology

Analysis of the subcellular localization of the *Chlamydia trachomatis* effector CteG and of its homologs in other *Chlamydia* species

Master in Molecular Genetics and Biomedicine

NOVA University Lisbon

September, 2022

NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF
LIFE SCIENCES

Analysis of the subcellular localization of the *Chlamydia trachomatis* effector CteG and of its homologs in other *Chlamydia* species

INÊS PACHECO LEAL

BSc in Molecular and Cellular Biology

Adviser: Luís Jaime Gomes Ferreira da Silva Mota
Assistant Professor, NOVA School of Science and Technology

Co-adviser: Irina Luísa Saraiva Franco
Senior Researcher, NOVA School of Science and Technology

Examination Committee:

Chair: Paula Maria Theriaga Mendes Bernardo Gonçalves
Associate Professor, NOVA School of Science and Technology

Rapporteur: Pedro Matos Pereira
Assistant Researcher, ITQB NOVA University Lisbon

Adviser: Luís Jaime Gomes Ferreira da Silva Mota
Assistant Professor, NOVA School of Science and Technology

MASTER IN MOLECULAR GENETICS AND BIOMEDICINE

NOVA University Lisbon
September, 2022

Analysis of the subcellular localization of the *Chlamydia trachomatis* effector CteG and of its homologs in other *Chlamydia* species

ACKNOWLEDGMENTS

Quero agradecer a muitas pessoas que me acompanharam neste último ano, desde setembro de 2021, começando pelo professor Jaime Mota, de quem tenho um grande respeito e apreço e que, com muita paciência e assertividade, me orientou e corrigiu ao longo de todo o trabalho. Quero agradecer também à Maria da Cunha que, embora tenhamos passado pouco tempo juntas, foi quem me fez conhecer "os cantos à casa" e se mostrou sempre disponível mesmo quando já não estava no laboratório. Agradeço também às minhas colegas Irina, Inês Pereira e Maria Pequito, pessoas muito dedicadas, que me fizeram sentir em casa e com quem tive o privilégio de trabalhar lado a lado. Tornaram dias maus em dias bons às vezes apenas com uma palavra ou um sorriso.

Quero agradecer também aos meus amigos do Grupo Bíblico Universitário, ao Augusto, ao João, ao André, à Eva, ao Leo, ao Pedro e ao José. Agradeço também à minha melhor amiga, a Marília. São pessoas incríveis que me apoiaram em todos os momentos, verdadeiros amigos para a vida!

Também não podia esquecer da minha família, claro. Agradeço a todos pelo carinho e o orgulho que senti de vocês e do esforço que vi em tentarem perceber em que é que consistia o meu trabalho. Agradeço principalmente aos meus pais por ouvirem os meus desabafos e frustrações de coisas que não entendiam. Obrigada!

Para o fim, deixei o mais importante. Quero agradecer de todo o meu coração a Deus! Ele ensinou-me lições muito valiosas durante este ano. Por mais que tentasse planear as tarefas, nem sempre o dia corria como planeado. Aprendi a deixar nas Suas mãos cada dia e assim, mesmo as coisas não correndo como eu queria, eu sabia que Ele é Deus e nada foge do Seu controlo.

Não tenho palavras para descrever a gratidão que sinto por todas estas pessoas e muitas outras que não mencionei. Muito obrigada!

"Não andeis ansiosos por coisa alguma; pelo contrário, sejam os vossos pedidos plenamente conhecidos diante de Deus por meio de oração e súplica com ações de graças; e a paz de Deus, que ultrapassa todo entendimento, guardará o vosso coração e os vossos pensamentos em Cristo Jesus." (Filipenses 4:6-7).

ABSTRACT

Chlamydiae are a large group of phylogenetically related obligate intracellular Gram-negative bacteria, that only grow within eukaryotic host cells. Among *Chlamydiae*, *Chlamydia trachomatis* is an exclusive human pathogen, causing ocular and genital infections. As all *Chlamydiae*, *C. trachomatis* manipulates human cells through a type III secretion system that enables the delivery into host cells of effector proteins that, in general, promote chlamydial growth and survival. In this work, the *C. trachomatis* effector associated with the Golgi (CteG) and its homologs in other *Chlamydia* species were studied. *C. trachomatis* CteG initially localizes at the Golgi complex, from ~20 h post-infection, and then starts localizing at the host plasma membrane (PM), from ~30 h post-infection. First, we aimed to test if twelve CteG homologs that are type III secreted by *Yersinia* can be transported by *C. trachomatis* into the cytoplasm of infected cells (i.e., translocated). For this, several *C. trachomatis* strains were generated. Immunofluorescence microscopy revealed that seven (out of twelve) CteG homologs were translocated into host cells. Furthermore, analysis of their subcellular localization indicated that some localized at the Golgi and PM, while others only at the Golgi or PM. Based on this, and considering the predicted secondary structure of CteG, we deduced regions in CteG that may determine its subcellular localization. To analyze this, several transfection plasmids and *C. trachomatis* strains were generated, enabling the expression of defined CteG truncated proteins. In transfected cells, immunofluorescence microscopy revealed that the C-terminal region of CteG was implicated in its localization at the plasma membrane. In infected cells, the immunofluorescence microscopy analysis was hampered by the low expression of CteG truncated proteins. Overall, this work enabled to define a group of *Chlamydia* CteG homolog effectors and to set the basis for additional analysis of the determinants of the subcellular localization of CteG.

Keywords: Host-pathogen interactions, *Chlamydia trachomatis*, type III secretion system, effector proteins, CteG

RESUMO

Chlamydiae é um grupo de bactérias Gram-negativas intracelulares obrigatórias, que se desenvolvem unicamente dentro de células hospedeiras. Dentre *Chlamydiae*, *Chlamydia trachomatis* é um organismo patogénico exclusivo de humanos, que causa infeções oculares e genitais. *C. trachomatis* promove o seu crescimento e sobrevivência em células humanas através de um sistema de secreção do tipo III, que transporta proteínas efetoras para células hospedeiras. Neste trabalho, o efetor de *C. trachomatis* associado com o Golgi (CteG) e os seus homólogos noutras espécies de *Chlamydia* foram estudados. CteG localiza-se inicialmente no complexo de Golgi a partir das 20 h pós-infeção, e depois começa a localizar-se na membrana plasmática do hospedeiro (PM), a partir das 30 h pós-infeção. Primeiro, quisemos testar se doze homólogos de CteG, excretados pelo sistema de secreção de *Yersinia* podiam ser transportados por *C. trachomatis* para o citoplasma de células infetadas. Para isto foram geradas várias estirpes de *C. trachomatis*. Microscopia de imunofluorescência revelou que sete (dos doze) homólogos de CteG foram transportados para células hospedeiras. Para além disso, a análise da sua localização celular indicou que alguns se localizavam no Golgi e na PM, outros só no Golgi ou na PM. Baseando-nos nisto, e considerando a estrutura secundária prevista de CteG, deduzimos regiões que podem determinar a sua localização celular. Para analisar isto foram gerados vários plasmídeos de transfeção e estirpes de *C. trachomatis*. Em células transfetadas, microscopia de imunofluorescência revelou que a região C-terminal de CteG estava envolvida na sua localização na membrana plasmática. Em células infetadas, a análise de microscopia de imunofluorescência foi dificultada pela baixa expressão das proteínas truncadas de CteG. Concluiu-se que este trabalho permitiu definir um grupo de efetores homólogos de CteG e criar a base para análises adicionais dos determinantes da localização celular de CteG.

Palavras chave: Interações hospedeiro-organismo patogénico, *Chlamydia trachomatis*, sistema de secreção do tipo III, proteínas efetoras, CteG

CONTENTS

LIST OF FIGURES

List of Tables

Acronyms

2HA	Double human influenza hemagglutinin epitope
Cap1	Class 1 accessible protein
CteG	*Chlamydia trachomatis* effector associated with the Golgi
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
EB	Elementary body
ECACC	European Collection of Authenticated Cell Cultures
EGFP	Enhanced green fluorescent protein
FITC	Fluorescein isothiocyanate
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
Hsp60	Heat shock protein 60
IFUs	Inclusion forming units
Incs	Inclusion membrane proteins
LB	Lysogeny broth
Lda Proteins	Lipid droplets-associated proteins
LGV	Lymphogranuloma venereum
mEGFP	Monomeric enhanced green fluorescent protein
MOI	Multiplicity of Infection
MOMP	Major outer membrane protein
NUE	Nuclear effector
p.i.	Post-infection
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PM	Plasma membrane
Ptet	Tetracycline promoter
PcteG	CteG promoter
RB	Reticulate body
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SPG	Sucrose-phosphate-glutamate buffer
T3SS	Type III secretion system
TarP	Translocated actin recruiting phosphoprotein
TmeA	Translocated membrane-associated effector A

1

INTRODUCTION

1.1 *Chlamydiae*

Chlamydiae are obligate intracellular Gram-negative bacteria that parasitize eukaryotic host cells. The *Chlamydiae* Phylum comprises one Class (*Chlamydiia*) and one Order (*Chlamydiales*). The *Chlamydiales* Order includes nine Families: *Chlamydiaceae*, which is the most studied and comprises the *Chlamydia* genus gathering pathogens of different mammals[1], and eight Families (*Parachlamydia*, *Waddliaceae*, *Simkaminiaceae*, *Rhabdochlamydiaceae*, *Criblamydiaceae*, *Piscichlamydiaceae*, *Clavichlamydiaceae* and *Parilichlamydiaceae*) of so called "*Chlamydia*-like organisms" that are symbionts of amoebae and other eukaryotic hosts, but which could also infect humans and other animals[2].

1.2 *Chlamydia* species

Within *Chlamydiaceae*, there are eleven recognized *Chlamydia* species: *C. trachomatis*, *C. suis*, *C. pneumoniae*, *C. muridarum*, *C. abortus*, *C. caviae*, *C. pecorum*, *C. psittaci*, *C. felis*, *C. avium* and *C. gallinaceae*. Each one infects different animals and causes different diseases. *C. suis* infects pigs and causes conjunctivitis, abortion, and enteritis. It also has the particularity of having genes that confer tetracycline resistance[3]. *C. pneumoniae* infects humans, amphibians, reptiles and other animals, and is a major cause of community acquired pneumonia[4]. *C. muridarum* infects mice and causes pneumonitis. Mice infected with *C. muridarum* are common animal models to study chlamydial infections[5]. *C. abortus* infects sheep and goats, and causes abortion[6]. *C. caviae* infects guinea pigs and causes conjunctivitis. Guinea pigs infected with *C. caviae* have been used as animal models to study upper genital tract infections[7]. *C. pecorum*

infects cattle, sheep, goats, pigs and koalas, and causes arthritis, conjunctivitis and encephalomyelitis[8].

1.3 *C. trachomatis* pathology

C. trachomatis is the leading cause of bacterial sexually transmitted infections worldwide. It infects exclusively humans, causes ocular or genital infections, depending on the serovars, and it is the most studied *Chlamydia* species. Serovars A to C infect the conjunctive epithelium, leading to ocular infections and trachoma (main cause of preventable blindness)[9]. It is responsible for blindness of about 2.2 million people[10]. Serovars D to K infect the genital epithelium, causing urogenital tract infections, as cervicitis in women and urethritis in men. These infections are normally asymptomatic, allowing the silent progression of bacteria through the upper genital tract and culminating in serious conditions as pelvic inflammatory disease, tubal infertility, ectopic pregnancy and chronic pelvic pain[11]. Serovars L1 to L3 infect the genital epithelium and monocytes, promoting lymphogranuloma venereum (LGV)[9]. LGV causes genital ulceration and painful inguinal lymphadenopathy[12].

The current treatment is based on antibiotic administration such as azithromycin and doxycycline, but the development of a vaccine should be a better solution, since 10-15% of treated women suffers a recurrent infection[13,14].

1.4 *Chlamydia* genetic manipulation

Due to *Chlamydia* obligate intracellular nature, it has been a challenge to study it. An established method for transformation of *C. trachomatis* was only described in 2011[15].

The first successful transformation of *Chlamydiae* was in 1994 by electroporation of elementary bodies (EBs; see section 1.5 below). Although the plasmid could not be maintained through some passages, it was a key advancement in genetic manipulation of *Chlamydia*[16]. In 2011, a major turning point occurred with the establishment of the *C. trachomatis* transformation protocol by $CaCl_2$ treatment, using a shuttle vector encoding penicillin-resistance. This protocol not only allows to distinguish between aberrant and transformed bacteria, but it also increases the transformation rate using penicillin as selection, since it grants time for phenotypic conversion from aberrant to normal transformed *Chlamydia*[15].

1.5 *C. trachomatis* developmental cycle and pathogenesis

As all *Chlamydiae*, *C. trachomatis* has a biphasic developmental cycle with two different forms: an infectious non-replicative form called elementary body (EB) and a non-infectious replicative form called reticulate body (RB). Elementary bodies have a small size (~0.3 μm) and a crosslinked cell wall. This allows bacteria to survive outside the host cell between infections and resist to osmotic and physical stress. Reticulate bodies are larger (~1 μm), do not have the crosslinked cell wall, but are metabolically very active, with the purpose of nutrient acquisition and replication[17].

The developmental (or infectious) cycle of *C. trachomatis* lasts between 48-72 h, depending on the serovar and strain. It begins with the attachment of an EB to the epithelial cell surface. This process has two stages. The first one is reversible and occurs through electrostatic interactions of the bacteria with heparan sulfate containing glycosaminoglycans, while the second is irreversible. Then, the type III secretion system (T3SS; see section 1.6 below) is activated and starts secreting proteins that will allow the internalization of the EB into a membrane-bound vacuole, named inclusion. Afterwards, some molecular changes happen to convert the EB into RB, one of these is the reduction of cross-linking in the outer membrane. The expression of RB genes is promoted for acquiring nutrients and avoiding the endocytic pathway, and RBs replicate by binary fission, enlarging and filling the inclusion with progeny. Later in the cycle, RBs re-differentiate into EBs and exit the host cell through lysis of the inclusion and host cell membranes, or by extrusion of the inclusion, leading to the infection of the neighboring cells[17] (Figure 1.1).

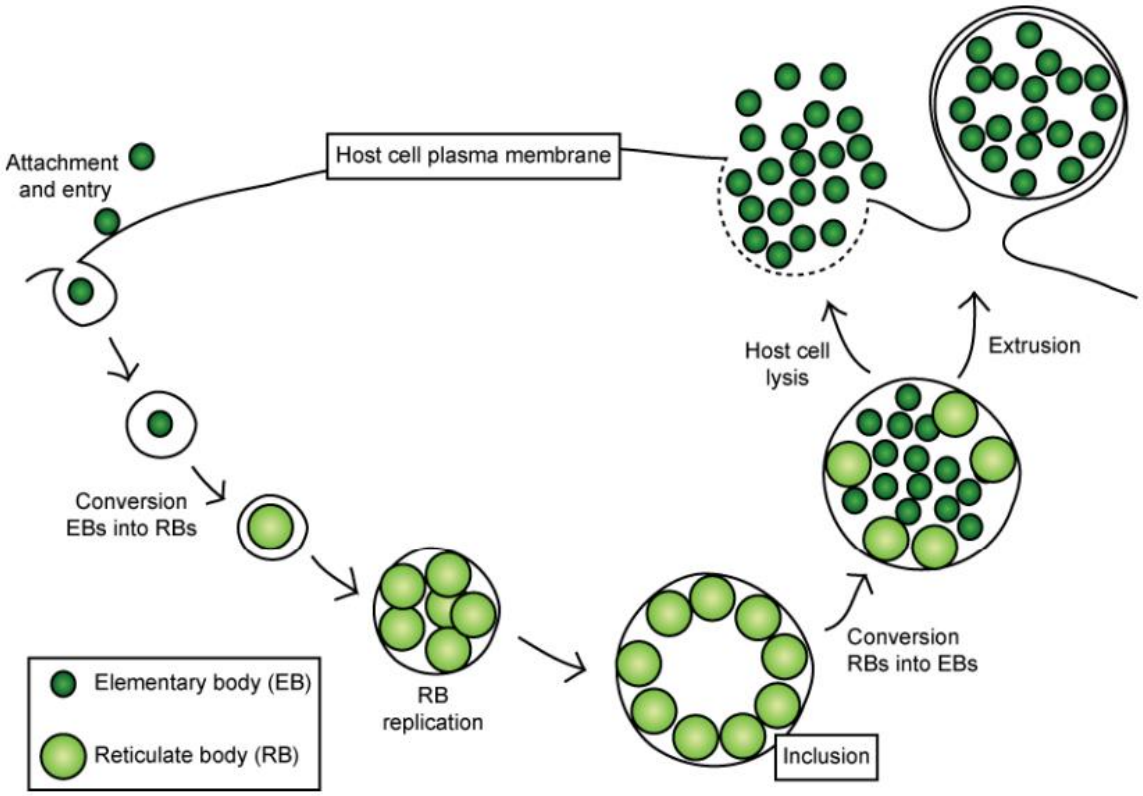

Figure 1.1 - *Chlamydia* developmental cycle. The life cycle of *C. trachomatis* begins with the attachment of an elementary body (EB) to the epithelial cell surface, followed by the internalization of the EB into a membrane-bound vacuole (inclusion). In this compartment, EB converts into reticulate body (RB) and starts replicating. Later, RBs re-differentiate into EBs and exit the host cell through host cell lysis or extrusion. Figure from Pais, S. (2018). PhD Thesis, NOVA School of Science and Technology, NOVA University Lisbon.

1.6 Type III secretion system

Protein secretion systems are important multiprotein complexes that allow the delivery of proteins to the extracellular space, which could be the environment, another bacterium cytoplasm, or a eukaryotic cell. Type III secretion system (T3SS) is exclusive of Gram-negative bacteria (as *C. trachomatis*) and allows the direct delivery of bacterial virulence effector proteins into the cytoplasm of the eukaryotic host cell. In general, these effectors promote bacterial growth and survival by manipulating host cell processes[18].

T3SS consists in a needle-like molecular syringe, named injectisome, composed by a basal apparatus, composed by two rings inserted in bacterial inner membrane connected to two rings in outer membrane; an extracellular needle complex, linked to the base and to the tip; and a translocation pore inserted in a host cell membrane (or in the inclusion membrane, in the case of intravacuolar *Chlamydia*)[18] (Figure 1.2).

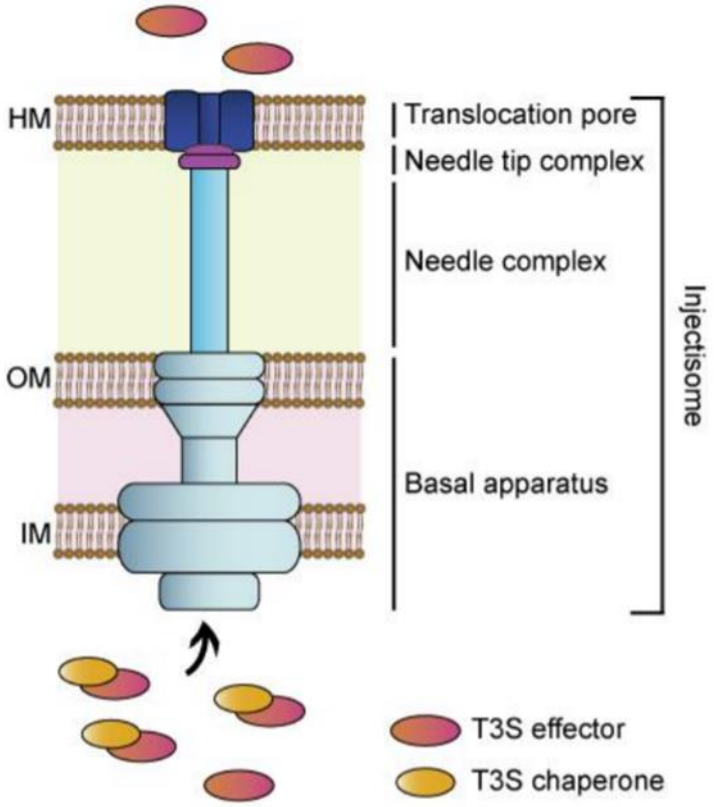

Figure 1.2 - Schematic representation of chlamydial T3SS. The needle-like molecular syringe (injectisome) is composed by a basal apparatus inserted in bacterial inner membrane (IM) and outer membrane (OM), an extracellular needle complex with a tip and a translocation pore inserted in a host membrane (HM). Figure from Pais, S. (2018). PhD Thesis, NOVA School of Science and Technology, NOVA University Lisbon.

1.7 *Chlamydia* effector proteins

Approximately 10% of the *Chlamydia* genome encodes for virulence effector proteins translocated by the chlamydial T3SS. Among these effectors, there are inclusion membrane proteins (Incs), which insert in the inclusion membrane through characteristic bilobed hydrophobic domain; many Incs have also coiled-coil domains in host cytosolic domains enabling protein-protein interactions[19,20]. There are about 60 known Inc proteins, most of them with the function still to be discovered, that interact with different host cell compartments. Some examples of Incs and their function are IncV that tethers the endoplasmic reticulum to the inclusion[21], MrcA which regulates chlamydial release by extrusion[22], IncD that recruits endoplasmic reticulum proteins to the inclusion membrane[23], IncA which promotes fusion of membranes[24], and IncC that stabilizes the inclusion membrane, avoiding its destruction and cytosolic exposure of the bacteria[25].

Other *C. trachomatis* effectors (non-Inc proteins) are directly translocated to the host cell cytoplasm, like the translocated actin recruiting phosphoprotein (TarP), the most studied *C. trachomatis* effector protein, which not only interacts with actin to promote chlamydial invasion, but also appears to induce host cell survival[26]; the translocated membrane-associated effector A (TmeA), which is important for infectivity and invasion of *C. trachomatis*[27], and the lipid droplets-associated (Lda) proteins[28]. However, not every non-Inc protein localizes in the host cell cytoplasm, the nuclear effector (NUE), for example, is eventually transported into the host cell nucleus and interacts with chromatin[29], and the class 1 accessible protein (Cap1), despite not having a bilobed hydrophobic domain, localizes at the inclusion membrane[30].

1.8 *C. trachomatis* effector associated with the Golgi (CteG)

C. trachomatis effector associated with the Golgi (CteG) is a non-Inc protein, with 656 amino acid residues, mostly produced by LGV strains (serovars L1-L3)[31]. CteG is translocated by the chlamydial T3SS into the cytoplasm of infected host cells, and as its name acknowledges, it was discovered that it localizes at the Golgi complex of the host cell at ~20 h post-infection. However, as the infection progresses CteG starts localizing also at the host cell plasma membrane and later in the cycle, at ~40h post-infection, that is its predominant localization. This change of localization is independent of intact host cell microfilaments and microtubules, and so it remains unknown how it processes[31]. Through an experiment where mammalian cells were transfected with plasmids encoding different truncated CteG proteins (as EGFP fusion proteins) followed by immunofluorescence microscopy, it was shown that mEGFP-CteG$_{1\text{-}100}$ localizes at the Golgi complex, indicating that the first 100 amino acids of CteG contain a Golgi-targeting region[31].

A *C. trachomatis* CteG-deficient strain (*C. trachomatis cteG::aadA*) was previously generated[31]. It contains a modified group II intron with a spectinomycin-resistance gene (*aadA*) that inactivates the *cteG* gene. This mutant strain shows a production of smaller inclusions and a defect in progeny generation, in comparison to the wild type (L2/434). Its phenotype is not complemented by *C. trachomatis cteG::aadA* harboring a plasmid encoding CteG[31,32]. The reason for this lack of complementation is unknown, but could be due to the presence of nucleotide changes in the mutant strain that lead to missense mutations[32]. However, it was discovered that in comparison to the wild type or the complemented strain, the *cteG::aadA* strain had significant less *C. trachomatis* infectious particles in the cell culture supernatant[32]. This revealed that CteG is involved in *Chlamydia* exit from the host cell. Beyond that, this

significant difference was accompanied by the destruction of the monolayer of infected cells, much more visible in wild type or complemented strain, showing that CteG promotes the exit specifically by host cell lysis[32].

Another previous observation is the detection by immunoblotting of several migrating bands corresponding to CteG, but at a different molecular mass from the predicted one, in whole extracts of cells infected with *C. trachomatis*[31,32]. A possible explanation for this can be the CteG degradation or processing during the *Chlamydia* developmental cycle; however, it is unknown if it has functional relevance, or it is a consequence of plasmid-mediated overexpression of CteG-2HA[31,32].

Researching potential homologs of CteG in other *Chlamydia* species, several proteins were discovered that show significant similarity to CteG[31] (Table 1.1). Beyond that, through a heterologous type III secretion assay in *Yersinia*[33], it was possible to find which ones were secreted by T3SS (da Cunha *et al*, unpublished data), indicating that they have a determinant region that recognizes T3SS (Table 1.2).

Table 1.1 - Potential homologs of CteG in other *Chlamydia* spp.

Chlamydia spp.	Identity (at amino acid level)	Protein	Protein length (aa)
C. abortus	28%	CAB376	732
C. avium	30%	M832_01180	304
	22%	M832_01160	479
C. caviae	27%	CCA_00389	726
	26%	CCA_00390	898
	27%	CCA_00297	445
	25%	CCA_00298	533
C. felis	26%	CF0619	737
	24%	CF0618	816
	25%	CF0705	536
	23%	CF0706	447
C. gallinaceae	22%	M787_003335	439
	24%	M787_003340	451
C. muridarum	53%	TC_0381	650
C. pecorum	28%	CpecS_0642	597
	26%	CpecS_0639	566
	23%	CpecS_0640	463
C. pneumoniae	30%	Cpn_0405	258
	22%	Cpn_0404	339
C. psittaci	25%	CPSIT_0422	610
	27%	CPSIT_0421	733
C. suis	47%	Q499_0113	650
	28%	Q499_0114	607

Table 1.2 - CteG homologs secreted by *Yersinia* in a type III secretion assay.

Chlamydia spp.	Identity (at amino acid level)	Protein	Protein length (aa)
C. abortus	28%	CAB376	732
C. caviae	27%	CCA_00389	726
	26%	CCA_00390	898
	27%	CCA_00297	445
	25%	CCA_00298	533
C. muridarum	53%	TC_0381	650
C. pecorum	27%	G5S_0729	187
	27%	G5S_0733	594
C. pneumoniae	30%	Cpn_0405	258
	22%	Cpn_0404	339
C. suis	46%	Q499_0113	650
	32%	Q499_0114A	285

1.9 Aims

The overall objective of this work was to increase the understanding of how *Chlamydia* manipulates host cells through its T3SS effector proteins. For this we focused on *C. trachomatis* CteG and pursued the following specific aims:

- Test whether the CteG homologs within *Chlamydiaceae* that are type III secreted by *Yersinia* can be transported by *C. trachomatis* into the cytoplasm of infected cells;
- Based on the subcellular localization in infected cells of CteG homologs within *Chlamydiaceae*, deduce regions in CteG that may determine its localization at the Golgi and plasma membrane of host cells of ectopically expressed and *Chlamydia*-delivered CteG truncated proteins.

2

MATERIALS AND METHODS

2.1 Cell lines

HeLa 229 cells (from the European Collection of Authenticated Cell Cultures; ECACC) were maintained in Dulbecco's modified Eagle Medium (DMEM; Thermo Fisher Scientific) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific) at 37°C in a 5% (v/v) CO_2 incubator. Cells were checked for *Mycoplasma* by conventional PCR[34].

2.2 Plasmids and primers

Plasmids and primers used in this work are listed in Tables A.1 and A.2 in Annexes, respectively, as well as their relevant characteristics. Plasmids were generated using restriction enzymes or by PCR overlap reaction. Plasmids were constructed and purified using standard molecular biology procedures, using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific), Fast Digest restriction enzymes (Thermo Fisher Scientific), T4 DNA Ligase (Thermo Fisher Scientific), DreamTaq DNA polymerase (Thermo Fisher Scientific), NZYTaqII (NZYTech), DNA clean & concentrator™-5 kit, Zymoclean™ gel DNA recovery kit (Zymo Research), and GeneElute Plasmid Miniprep kit (Sigma-Aldrich) or NZYMidiprep kit (NZYTech) according to manufacturer's instructions. The accuracy of the nucleotide sequence of all the inserts in the constructed plasmids was confirmed by DNA sequencing.

2.3 Bacterial strains and growth conditions

Escherichia coli NEB 10β (New England Biolabs) was used for construction and purification of plasmids, and *E. coli* ER2925 (New England Biolabs) was used to purify plasmids for transformation of *C. trachomatis*. *E. coli* strains were grown at 37°C in liquid or solid lysogeny broth (LB) media (NZYTech) with the appropriate antibiotics and supplements. Plasmids were introduced into *E. coli* by electroporation.

2.4 *C. trachomatis* strains and their propagation and transformation

C. trachomatis serovar L2 prototype strain 434/Bu ACE051 (L2/434; obtained from Derek J. Fisher - wild type strain originally from Tony Maurelli's lab, University of Florida) was propagated in HeLa 229 cells using standard procedures[35]. *C. trachomatis* transformants were generated as described by Agaisse and Derré[36]. First, 6 µg of the plasmid DNA was mixed with 200 µl of $CaCl_2$ buffer (10 mM Tris, 50 mM $CaCl_2$, pH 7.4) by vortexing. Next, 25 µl of previously thawed and vortexed *C. trachomatis*, stored in sucrose-phosphate-glutamate buffer (SPG; 0.22 M sucrose, 17 mM Na_2HPO_4, 3 mM NaH_2PO_4, 5 mM L-glutamic acid) was added to the plasmid DNA/$CaCl_2$ mixture. The solution was homogenized by pipetting up and down and incubated for 30 minutes at room temperature. Then, 4×10^6 trypsinized HeLa cells were pelleted (240 x g, 5 min, room temperature), washed once with phosphate buffered saline (PBS; Lonza) and resuspended in 200 µl of $CaCl_2$ buffer. After this incubation, the resuspended cells were added to the *C. trachomatis*/DNA mix and incubated for an additional 20 minutes at room temperature with gentle mixing by pipetting up and down every 5 min. The whole mixture was equally distributed into 2 wells of a 6-well plate, containing 3 ml of DMEM supplemented with 10% (v/v) heat-inactivated FBS each, and incubated for 16 h at 37°C in a 5% (v/v) CO_2 incubator. Then, fresh DMEM supplemented with 10% (v/v) heat-inactivated FBS supplemented with the selective antibiotic (0.3 U/ml of penicillin G (Sigma)) was added. At 44 h post-infection, the medium was removed, and the cells were osmotically lysed with 500 µl of sterile H_2O. The lysate was added to 1 ml of 2x SPG (0.44 M sucrose, 34 mM Na_2HPO_4, 6 mM NaH_2PO_4, 10 mM L-glutamic acid) and centrifuged for 5 min at 150 x g, at room temperature. The supernatant was added to newly seeded HeLa cells ($4x10^6$ cells in a 25 cm^2 surface area flask), previously equilibrated in Hank's balanced salt solution (HBSS). The cells were incubated 1 h with the supernatant (at room temperature, with gentle rocking) after which the inoculum was removed,

and DMEM supplemented with 10% (v/v) heat-inactivated FBS, 1 µg/ml cycloheximide (Fluka), and the appropriate selective antibiotic was added. This same procedure was repeated one or two more times in the same conditions and selected using 1 U/ml penicillin G. When transformants were observed, one passage of the bacteria was performed in the presence of 10 U/ml penicillin G. At the indicated times post-infection, cells were collected and analyzed by immunoblotting or immunofluorescence microscopy.

2.5 Quantification of inclusion forming units (IFUs) in infected cells

To quantify infectious progeny, HeLa cells infected with *C. trachomatis* strains for 24 h were lysed by osmotic shock (15 min incubation in sterile H_2O). The lysed cells were vigorously resuspended by pipetting up and down and the suspension was added to 2x SPG. The lysates obtained were homogenized by vortexing, serial diluted in SPG and used to infect freshly seeded HeLa cells. The newly infected cells were fixed after 24 h, *Chlamydia* were immunolabelled, and the number of inclusion forming units (IFUs) was calculated as described by Scidmore[35].

2.6 Infection of HeLa 229 cells with *C. trachomatis*

For immunofluorescence analysis and immunoblotting, $1x10^5$ HeLa 229 cells were seeded per well in 24-well plates, the first one containing 13 mm glass coverslips. The day after seeding, media was replaced by HBSS and the cells were incubated ~15 min at 37°C in a 5% (v/v) CO_2 incubator, while the *C. trachomatis* inocula (previously titrated infectious particles) were prepared in SPG. The buffer was then removed and the *C. trachomatis* inocula were added at a multiplicity of infection (MOI) of 0.3 for immunofluorescence, and of 3 for immunoblotting, and incubated for 30 min at 37°C in a 5% (v/v) CO_2 incubator. At this point, the inocula were removed and replaced by DMEM supplemented with 10% (v/v) heat-inactivated FBS. If needed, gentamicin (10 µg/ml) and/or anhydrotetracycline (50 ng/ml) were added to synchronize the infection and kill extracellular *Chlamydia* or to induce gene expression, respectively. This was considered the time zero of infection.

2.7 Transfection of HeLa 229 cells

HeLa cells were transfected using the jetPEI™ reagent (Polyplus-Transfection) according to manufacturer's instructions. Briefly, $1x10^5$ HeLa 229 cells were seeded per well in 24-well plates. After 24 h, 250 ng of plasmid DNA was mixed with 50 µl of 150 mM NaCl, and 1.5 µl of jetPEI™ reagent was mixed with 50 µl of 150 mM NaCl. Both mixtures were vortexed and spun down. Then, 50 µl of the jetPEI™ solution was added to the DNA solution, the mixture was briefly vortexed and spun down, and incubated for 20 minutes at room temperature. After the incubation, 100 µl of the jetPEI™/DNA mix was added per well. The plate was centrifuged at 180 x g for 5 min at room temperature and then incubated at 37°C in a 5% (v/v) CO_2 incubator. After 4 h, media with jetPEI™ reagent was replaced by fresh media, to avoid cytotoxicity. At 24 h post-transfection, cells were collected for immunoblotting or fixed for immunofluorescence microscopy analysis.

2.8 Antibodies and fluorescent dyes

The following antibodies were used for immunoblotting: rat anti-HA (3F10; Roche; 1:1000), mouse anti-chlamydial Hsp60 (A57-B9; Thermo Fisher Scientific; 1:1000), mouse anti-α-tubulin (clone B-5-1-2; Sigma-Aldrich; 1:1000), goat anti-GFP (Abcam; 1:1000), followed by anti-rat, anti-mouse or anti-goat horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare and Jackson ImmunoResearch; 1:10000).

For immunofluorescence microscopy, the following antibodies were used: rat anti-HA (3F10; Roche; 1:200), rabbit anti-GM130 (Sigma Aldrich; 1:200), rabbit anti-Cap1 (kindly provided by Agathe Subtil[37]; 1:200), goat anti-*Chlamydia* major outer membrane protein (MOMP) (Abcam; 1:200), and goat anti-*C. trachomatis* FITC-conjugated antibody (Sigma-Aldrich, 1:150), followed by appropriate fluorophore-conjugated Rhodamine Red-X-conjugated anti-rat, AF488-conjugated anti-rabbit, DyLight 405-conjugated anti-goat antibodies (Jackson ImmunoResearch; 1:200), and DAPI (4',6-Diamidino-2-phenylindole; 1:30000).

2.9 Immunoblotting

Transfected and/or infected HeLa cells were washed with PBS and detached from plates by incubation with TrypLE Express (Thermo Fisher Scientific) for 5 min at 37°C in a 5% (v/v) CO_2

incubator. Cells were collected, centrifuged, and washed 2 times with ice-cold PBS. Then, cells were resuspended in an appropriate volume of SDS-PAGE loading buffer and stored as a pellet at -80ºC until use. Before running on SDS-PAGE, the cells were thawed and further denatured by an incubation of 5 min at 100 °C, followed by addition of benzonase (Novagen) to destroy DNA and reduce the viscosity of the samples. In all cases, samples were separated by 1% (v/v) SDS-PAGE and transferred onto 0.2 µm nitrocellulose membranes (Bio-Rad) using Trans-Blot Turbo Transfer System (BioRad). Immunoblotting detection was done with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and exposed to Amersham Hyperfilm ECL (GE Healthcare).

2.10 Immunofluorescence microscopy

For immunofluorescence microscopy analysis, transfected and/or infected HeLa 229 cells were fixed with 4% (w/v) paraformaldehyde (PFA) for 20 min at room temperature and permeabilized with PBS containing 0.1% (v/v) Triton X-100 (PBST). For quantification of IFUs, infected HeLa cells were fixed with freezing methanol (-20ºC) for 10 min. Immunostaining was performed with antibodies diluted in PBST or PBS containing 10% (v/v) horse serum. Cells were washed with PBS and H_2O before assembling the coverslips on microscopy glass slides using Aqua-poly/Mount (Polysciences). Samples were analyzed by fluorescence microscopy in a Axio Imager.D2 (Zeiss) upright microscope. Images were collected by an Axiocam MRm (Zeiss) camera and processed with Zeiss ZEN (Zeiss) software, Fiji software.

3

RESULTS

3.1 Analysis of the expression and localization in infected cells of CteG homologs from *Chlamydiaceae*

In a previous study, it was discovered that proteins from different *Chlamydia* species sharing significant identity with CteG (CteG homologs) were secreted by *Yersinia* in a type III secretion assay (Maria da Cunha *et al*, unpublished) (Table 1.2). In this work, we analyzed the localization of these proteins in host cells infected by *C. trachomatis*. Besides CteG from *C. trachomatis* as reference, the proteins analyzed were from *C. suis* (Q499_0113 and Q499_0114A), *C. pneumoniae* (Cpn_0404 and Cpn_0405), *C. muridarum* (TC_0381), *C. abortus* (CAB376), *C. caviae* (CCA_00389, CCA_00390, CCA_00297 and CCA_00298) and *C. pecorum* (G5S_0729 and G5S_0733) (Figure 3.1).

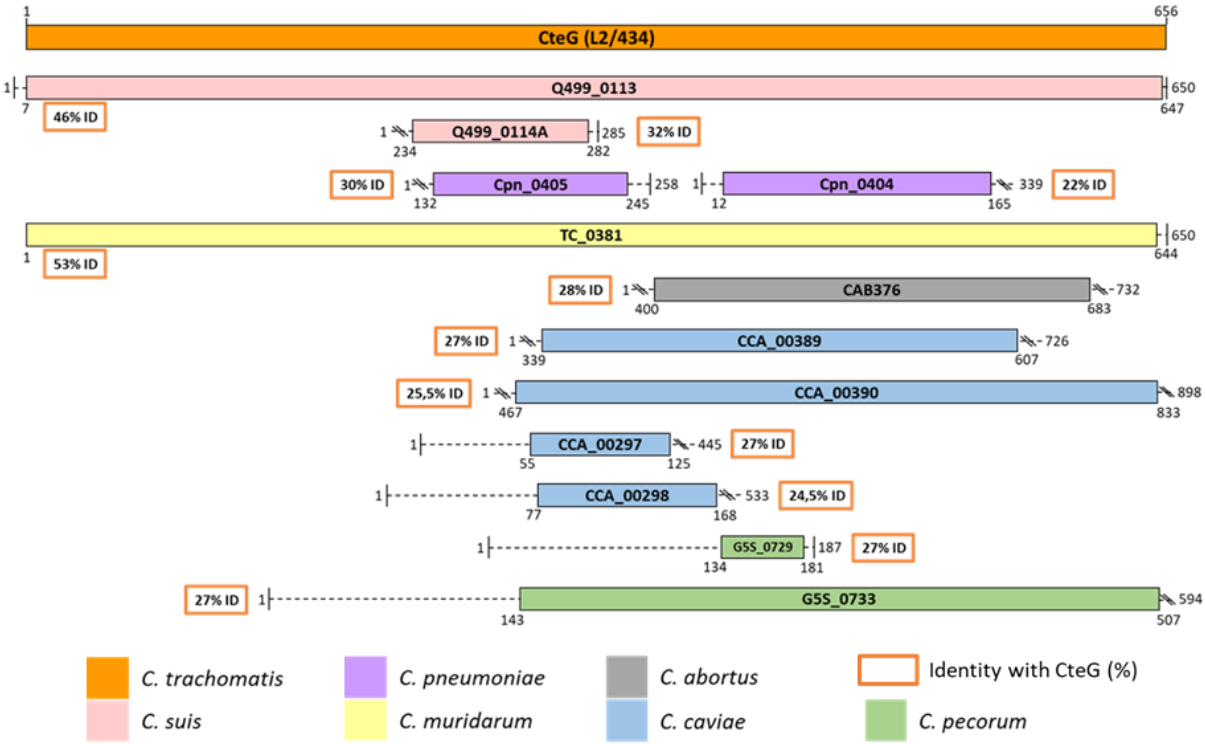

Figure 3.1 - Alignment of CteG homologs analyzed in this work. Alignment of proteins from different *Chlamydia* species that share significant identity with CteG and can be secreted by *Yersinia* in a type III secretion assay (Maria da Cunha *et al*, unpublished). The species represented are: *C. trachomatis* (orange), *C. suis* (pink), *C. pneumoniae* (purple), *C. muridarum* (yellow), *C. abortus* (grey), *C. caviae* (blue), *C. pecorum* (green). The percentage of identity with CteG (at amino acid level) is shown in orange boxes. The numbers below each protein represent the amino acids that delimitate the identical region.

3.1.1 Generation of *C. trachomatis* strains encoding CteG homologs

To generate *C. trachomatis* strains encoding CteG homologs from *Chlamydiaceae*, the *C. trachomatis cteG::aadA* strain (CteG-deficient) was transformed with plasmids encoding the CteG homologs with a C-terminal double haemagglutinin (2HA) tag, to enable the detection by immunoblotting and immunofluorescence. As the expression of CteG homologs could be toxic to *C. trachomatis cteG::aadA*, the gene encoding the CteG homologs was expressed from the tetracycline promoter (*Ptet*), which can be induced by the presence of anhydrotetracycline. The plasmids were constructed (together with Maria da Cunha; Table A.1 in Annexes) and *cteG::aadA*-derived strains were generated (together with Maria da Cunha; Table A.3 in Annexes), with the exception of the strain encoding CCA_00390-2HA (Figure 3.1), which after several transformation attempts was never possible to generate. The generated *C. trachomatis* strains were verified by PCR for the presence of the desired plasmid and controlled for being *Mycoplasma*-free.

3.1.2 Optimization of the timepoint for expression induction by anhydrotetracycline

The optimal timepoint post-infection to add anhydrotetracycline to induce the expression of *cteG* and its homologs was verified. For this, HeLa 229 cells were infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA (complemented strain) (Figure 3.2A), p*Ptet*-CteG-2HA without addition of anhydrotetracycline (control) (Figure 3.2B), or p*Ptet*-CteG-2HA with anhydrotetracycline added at different timepoints (0, 4, 8, 24 or 0 and 24 h post-infection). At 24, 30 and 46 h post-infection, cells were fixed and immunolabelled with antibodies against *C. trachomatis* major outer membrane protein (MOMP), HA and GM130 (a *cis*-Golgi protein), and appropriate fluorophore-conjugated secondary antibodies. The immunolabelled cells were then analyzed, and the fluorescence microscopy analysis revealed that when cells were fixed at 24 h post-infection, the optimal timepoint to add anhydrotetracycline was at 0 h post-infection, because when comparing to the addition at 4 or 8 h post-infection, there was no significant difference (Figures 3.2C and A.1 in Annexes). When infected cells were fixed at 46 h post-infection, the optimal timepoint to add anhydrotetracycline was at 0 and 24 h post-infection, because when comparing to the addition just at 0 or 24 h post-infection, the signal was significantly more perceptive (Figures 3.2D and A.1 in Annexes). Therefore, in the subsequent experiments anhydrotetracycline was added at time zero of infection to process cells at ~24 h post-infection, or at time zero and at 24 h post-infection to process cells at ~46 h post-infection.

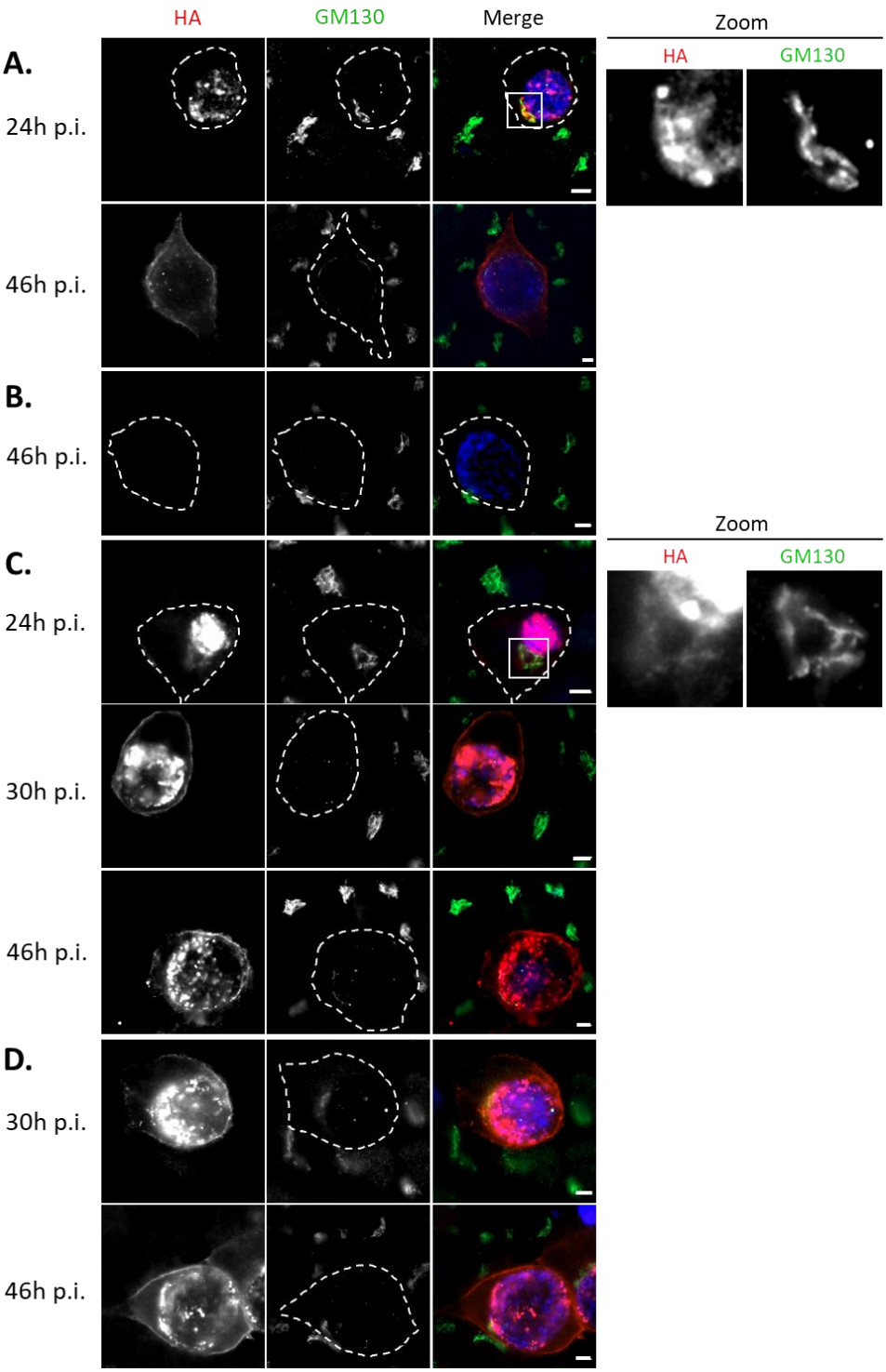

Figure 3.2 - Analysis of the optimal timepoint to add anhydrotetracycline to induce expression of *cteG*. HeLa 229 cells were infected, at a MOI of 0.3, with *C. trachomatis cteG::aadA* harboring (A) p*PcteG*-CteG-2HA (complemented strain), (B) p*Ptet*-CteG-2HA without anhydrotetracycline added (control), (C) p*Ptet*-CteG-2HA with anhydrotetracycline added at 0 h post-infection (p.i.) or (D) p*Ptet*-CteG-2HA with anhydrotetracycline added at 0 and 24 h p.i. At 24, 30 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against *C. trachomatis* MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies. The fixed cells were then analyzed by immunofluorescence microscopy. In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

3.1.3 Analysis of anhydrotetracycline-inducible expression of CteG homologs by immunoblotting

To analyze the anhydrotetracycline-inducible expression of CteG homologs by immunoblotting, HeLa 229 cells were infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-CteG-2HA, p*Ptet*-Q499_0113-2HA, p*Ptet*-Q499_0114A-2HA, p*Ptet*-TC_0381-2HA, p*Ptet*-CAB376-2HA, p*Ptet*-Cpn_0404-2HA, p*Ptet*-Cpn_0405-2HA, p*Ptet*-CCA_00389-2HA, p*Ptet*-CCA_00297-2HA, p*Ptet*-CCA_00298-2HA, p*Ptet*-G5S_0729-2HA or p*Ptet*-G5S_0733-2HA (Tables A.1 and A.3 in Annexes). At 24 and 46 h post-infection, whole cell extracts were prepared and then analyzed by immunoblotting with antibodies against HA, *C. trachomatis* Hsp60 (bacterial loading control) and human α-tubulin (HeLa cell loading control). The production of 2HA-tagged proteins of the expected molecular mass was confirmed in most of the cases (Figure 3.3). The exceptions were Q499_0114A (*C. suis*) and TC_0381 (*C. muridarum*) whose expression could not be detected (Figure 3.3). As CteG from *C. trachomatis*[31], the detection of expression of CteG homologs from *Chlamydiaceae* by immunoblotting revealed multiple bands besides the band corresponding to the predicted molecular mass (Figure 3.3). We therefore proceeded with the analysis of localization in infected cells of the CteG homologs whose expression could be detected by immunoblotting.

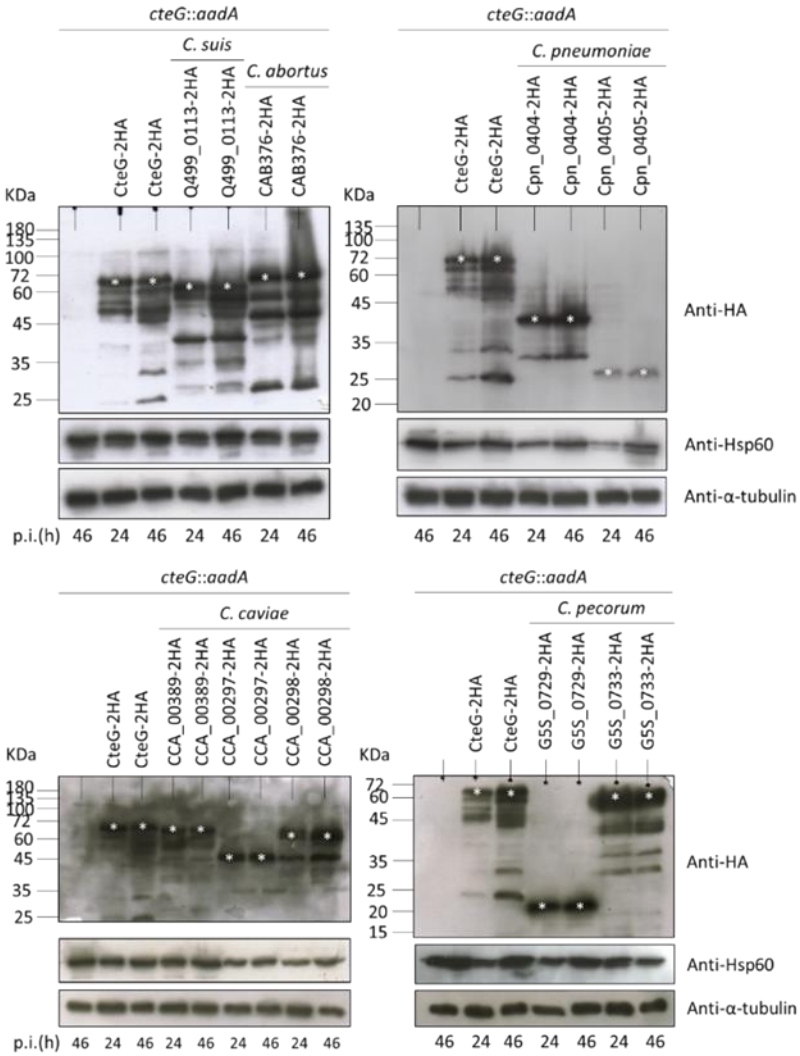

Figure 3.3 - Analysis of the anhydrotetracycline-inducible expression of CteG homologs by immunoblotting. HeLa 229 cells were infected, at a MOI of 3, with *C. trachomatis cteG::aadA* harboring p*Ptet*-CteG-2HA, p*Ptet*-Q499_0113-2HA, p*Ptet*-Q499_0114A-2HA, p*Ptet*-TC_0381-2HA, p*Ptet*-CAB376-2HA, p*Ptet*-Cpn_0404-2HA, p*Ptet*-Cpn_0405-2HA, p*Ptet*-CCA_00389-2HA, p*Ptet*-CCA_00297-2HA, p*Ptet*-CCA_00298-2HA, p*Ptet*-G5S_0729-2HA or p*Ptet*-G5S_0733-2HA. At 24 and 46 h p.i., whole cell extracts were prepared and then analyzed by immunoblotting with antibodies against HA, *C. trachomatis* Hsp60 (bacterial loading control) and human α-tubulin (HeLa cell loading control). The detection was made using SuperSignal West Pico detection kit (Thermo Fisher Scientific) to detect Hsp60 or α-tubulin, or SuperSignal West Femto detection kit (Thermo Fisher Scientific) to detect 2HA-tagged proteins. The band corresponding to the predictive molecular mass of the proteins is indicated with a white asterisk. Expression of Q499_0114A-2HA and TC_0381-2HA was not detected.

3.1.4 Analysis of the subcellular localization in infected CteG homologs by immunofluorescence microscopy

To analyze the subcellular localization of CteG homologs from *Chlamydiaceae* by immunofluorescence microscopy, HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-CteG-2HA, p*Ptet*-Q499_0113-2HA, p*Ptet*-Cpn_0404-2HA, p*Ptet*-Cpn_0405-2HA, p*Ptet*-CAB376-2HA, p*Ptet*-CCA_00389-2HA, p*Ptet*-CCA_00297-2HA, p*Ptet*-CCA_00298-

2HA, p*Ptet*-G5S_0729-2HA or p*Ptet*-G5S_0733-2HA (Tables A.1 and A.3 in Annexes) for 24 and 46 h. The cells were fixed at these times post-infection and then analyzed by indirect immunofluorescence microscopy. This was done in two steps. First, all cells infected with *C. trachomatis* strains expressing CteG homologs were immunolabelled with antibodies against Cap1 (a *C. trachomatis* protein localizing at the inclusion membrane)[38] and HA (and also stained with DAPI), followed by appropriate fluorophore-conjugated secondary antibodies. This enabled to analyze which proteins, at which fixation timepoints, were detected outside the inclusion membrane (translocated into host cells) (Figures 3.4.1 to 3.4.9). This revealed that the proteins Q499_0113 (from *C. suis*), CCA_00297 and CCA_00298 (from *C. caviae*) were translocated into host cells at both 24 and 46 h post-infection, as CteG, while CAB376 (from *C. abortus*), CCA_00389 (from *C. caviae*) and G5S_0733 (from *C. pecorum*) were translocated only at 46 h post-infection. The proteins Cpn_0404 and Cpn_0405 (from *C. pneumoniae*) and G5S_0729 (from *C. pecorum*) were not detected outside the inclusion membrane, suggesting that they may not recognize the *C. trachomatis* T3SS (Figures 3.4.1 and 3.4.9).

Then, cells infected with *C. trachomatis* expressing CteG homologs that were translocated into host cells were immunolabelled with antibodies against MOMP, GM130 and HA, and appropriate fluorophore-conjugated secondary antibodies. This allowed to see whether the proteins localized in the Golgi complex of the host cells (Figures 3.4.1 to 3.4.9).

The localization of the translocated CteG homologs was initially roughly compared with the one of CteG from *C. trachomatis* (Figure 3.4.1). In summary (Table 3.1):

- The protein Q499_0113 (from *C. suis*) showed a localization very similar to CteG, at the Golgi at 24 h post-infection and at the plasma membrane at 46 h post-infection (Figure 3.4.2).

- The protein CAB376 (from *C. abortus*) localized at the plasma membrane at 46 h post-infection (Figure 3.4.4).

- The proteins CCA_00389, CCA_00297 and CCA_00298 (from *C. caviae*) showed different localizations; CCA_00389 localized at the plasma membrane at 46 h post-infection (Figure 3.4.5); CCA_00297 localized at the Golgi both at 24 and 46 h post-infection (Figure 3.4.6); and CCA_00298 localized at the Golgi at 24 h post-infection and both at the plasma membrane and Golgi at 46 h post-infection (Figure 3.4.7).

- The protein G5S_0733 (from *C. pecorum*) localized at the Golgi complex at 46 h post-infection (Figure 3.4.9).

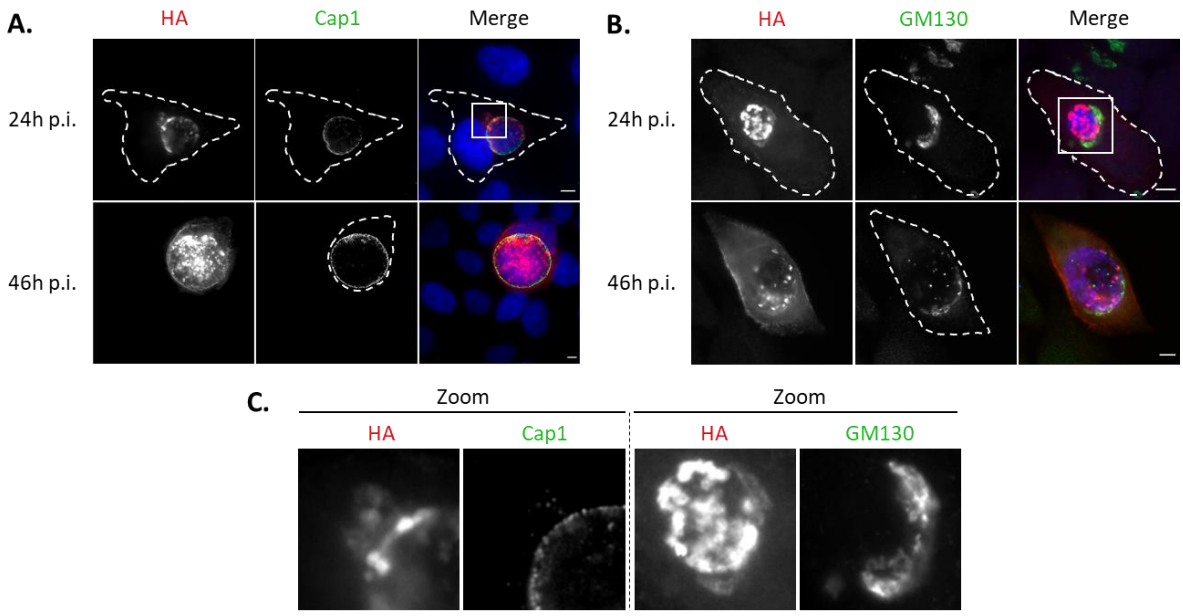

Figure 3.4.1 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG, upon induction of expression with anhydrotetracycline. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-CteG-2HA (*C. trachomatis*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. (C) In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

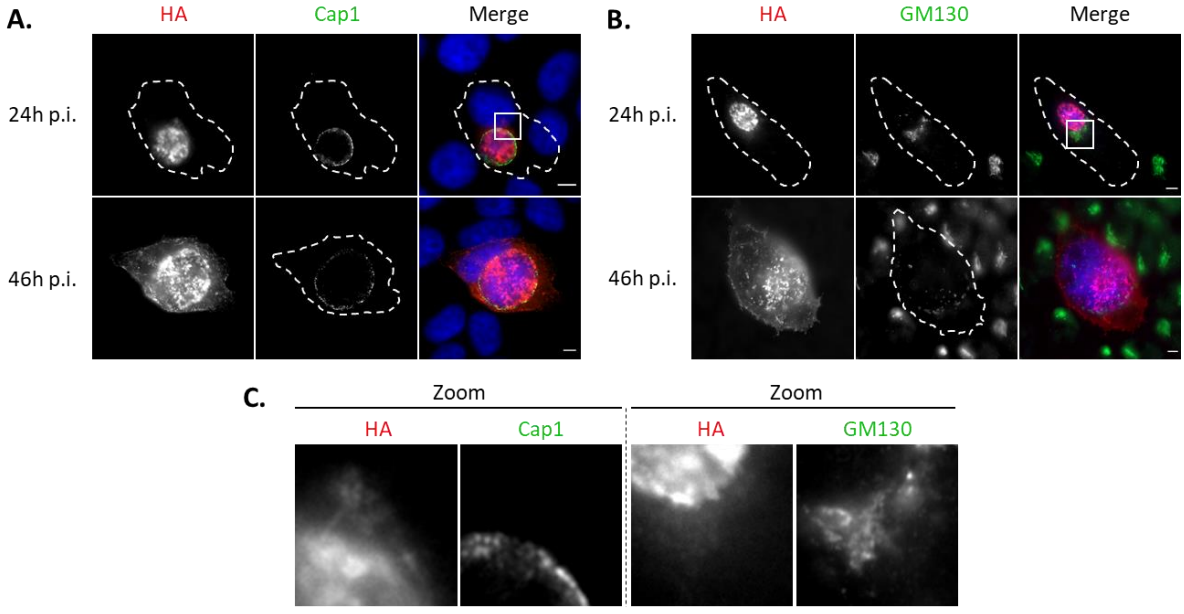

Figure 3.4.2 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG homolog Q499_0113 (*C. suis*), upon induction of expression with anhydrotetracycline. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-Q499_0113-2HA (*C. suis*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. (C) In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

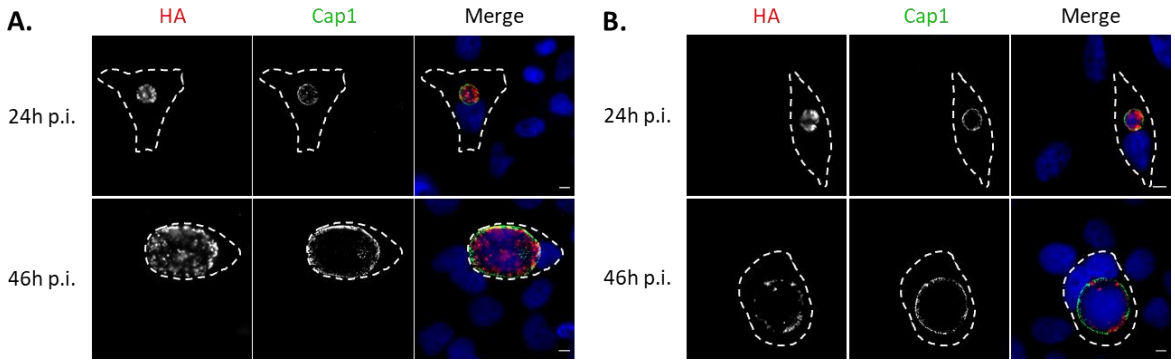

Figure 3.4.3 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG homologs Cpn_0404 and Cpn_0405 (*C. pneumoniae*), upon induction of expression with anhydrotetracycline. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring (A) p*Ptet*-Cpn_0404-2HA or (B) p*Ptet*-Cpn_0405-2HA (*C. pneumoniae*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against DAPI (blue), HA (red) and Cap1 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. Scale bars, 5 µm.

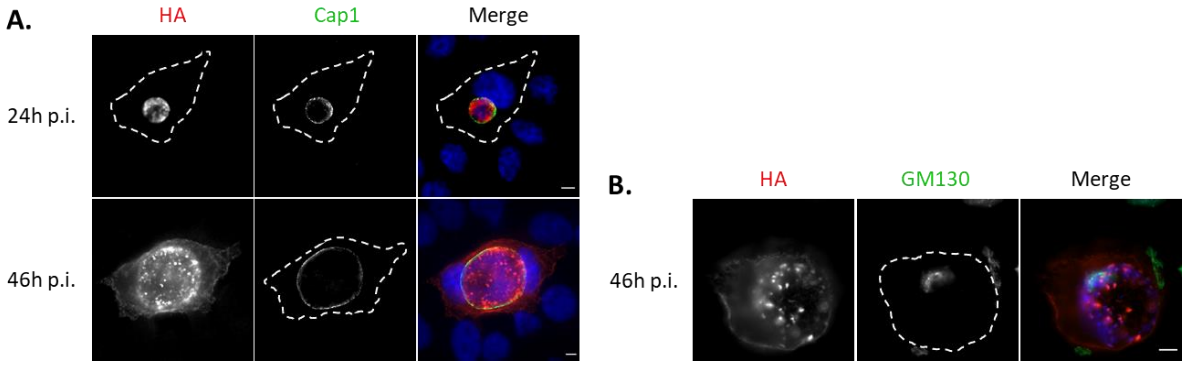

Figure 3.4.4 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG homolog CAB376 (*C. abortus*), upon induction of expression with anhydrotetracycline. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-CAB376-2HA (*C. abortus*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. Scale bars, 5 µm.

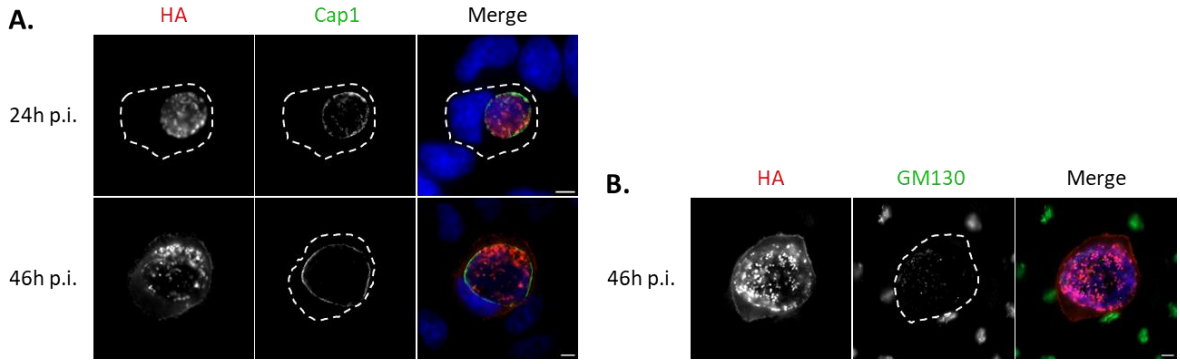

Figure 3.4.5 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG homolog CCA_00389 (*C. caviae*), upon induction of expression with anhydrotetracycline. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-CCA_00389-2HA (*C. caviae*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. Scale bars, 5 µm.

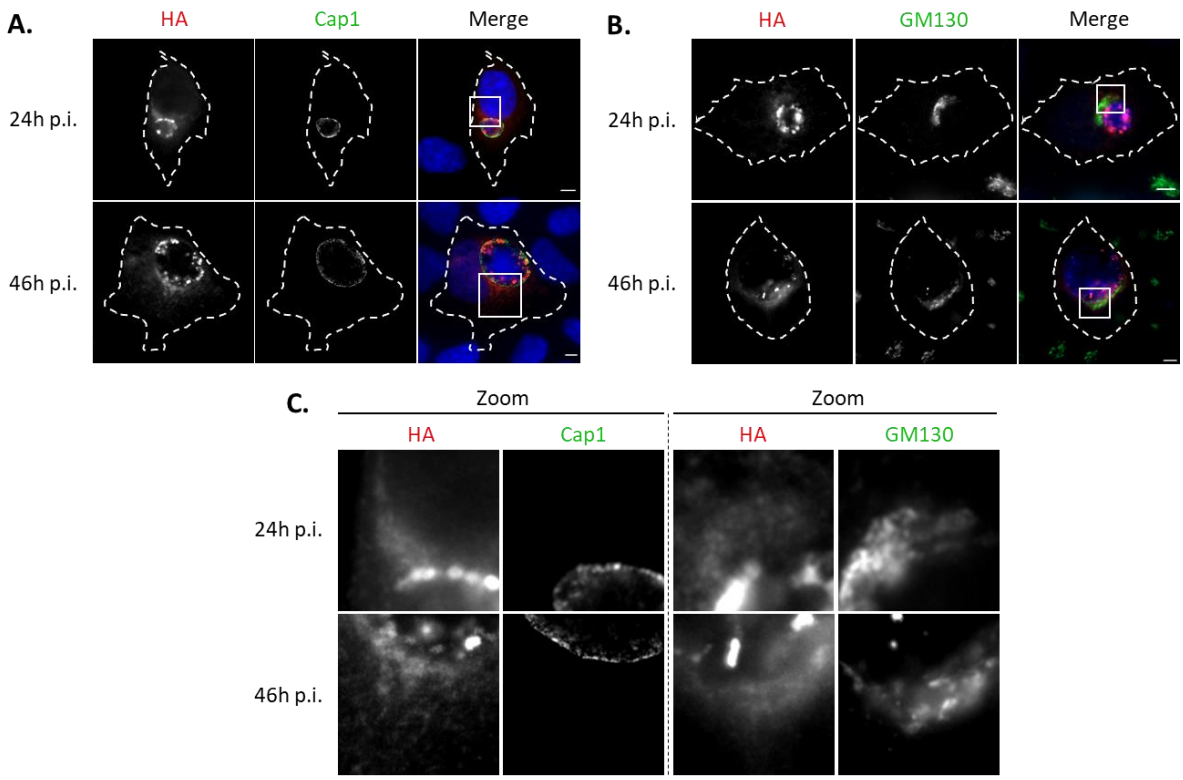

Figure 3.4.6 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG homolog CCA_00297 (*C. caviae*), upon induction of expression with anhydrotetracycline. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-CCA_00297-2HA (*C. caviae*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. (C) In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

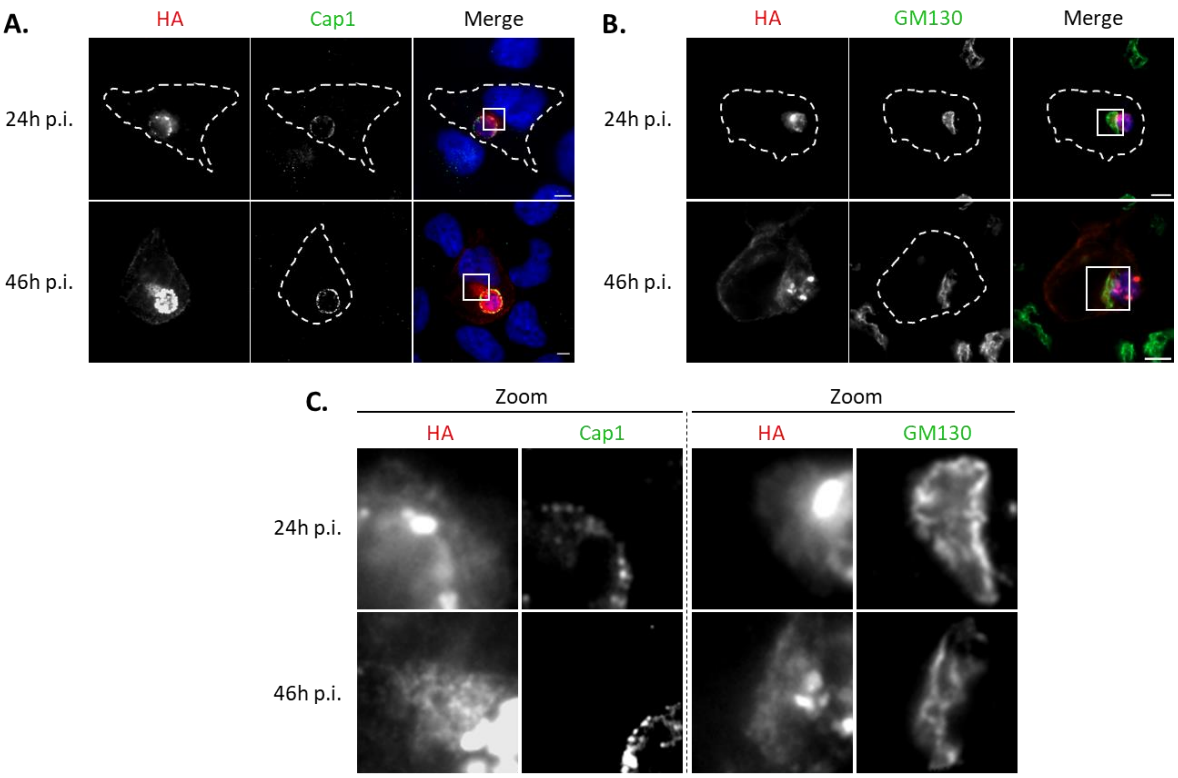

Figure 3.4.7 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG of CteG homolog CCA_00298 (*C. caviae*), upon induction of expression with anhydrotetracycline. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-CCA_00298-2HA (*C. caviae*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. (C) In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

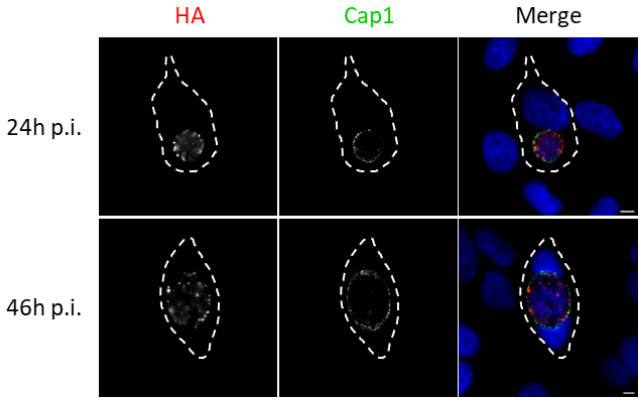

Figure 3.4.8 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG homolog G5S_0729 (*C. pecorum*), upon induction of expression with anhydrotetracycline. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-G5S_0729-2HA (*C. pecorum*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against DAPI (blue), HA (red) and Cap1 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. Scale bars, 5 µm.

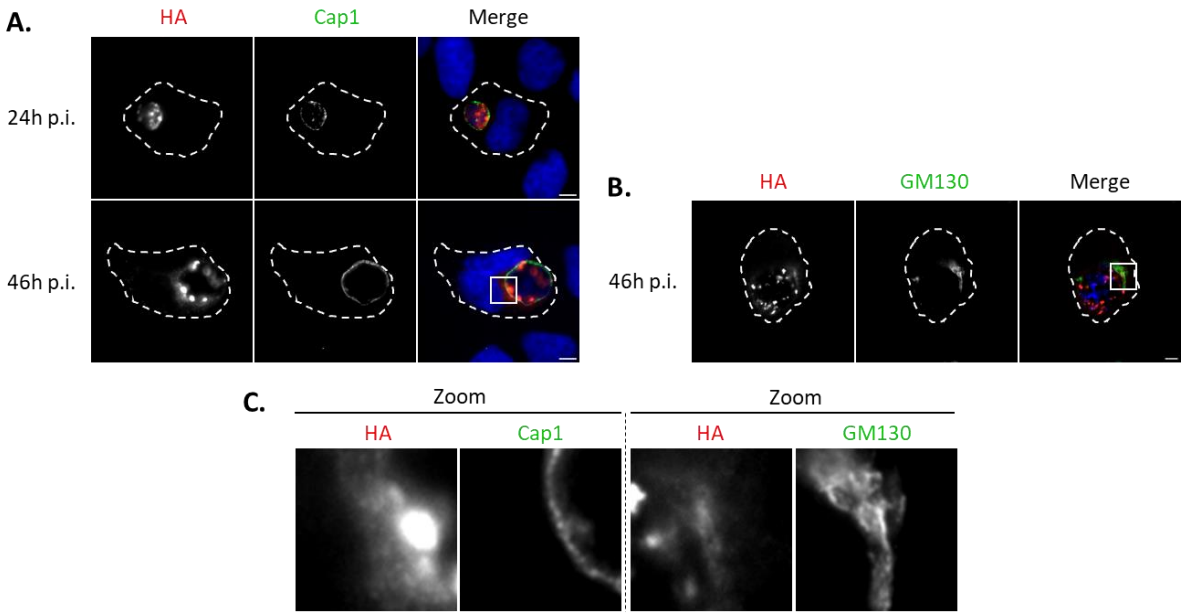

Figure 3.4.9 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG homolog G5S_0733 (*C. pecorum*), upon induction of expression with anhydrotetracycline. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-G5S_0733-2HA (*C. pecorum*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. (C) In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

To corroborate the initial analysis of the localizations of the CteG homologs from *Chlamydiaceae* in infected cells, the subcellular localization of the proteins at 24 and/or 46 h post-infection was systematically enumerated and quantified by fluorescence microscopy aiming to look at 50 infected cells for each case. Different locations of the host cell were considered: cytosol, plasma membrane (PM), Golgi, or both plasma membrane and Golgi (PM + Golgi) (Figure 3.5). The quantification corroborated the initial analysis, concluding that (Figure 3.5):

- The protein Q499_0113 (from *C. suis*) was the CteG homolog most identical to CteG, localizing at the Golgi at 24 h post-infection and at the plasma membrane of host cell at 46 h post-infection.

- The proteins CAB376 (form *C. abortus*) and CCA_00389 (from *C. caviae*) were translocated just at 46 h, but had a similar localization to CteG, at the plasma membrane.

- The proteins CCA_00297 and CCA_00298 (from *C. caviae*) had the same localization of CteG at 24 h post-infection at the Golgi, but at 46 h post-infection CCA_00297 localized at the Golgi and CCA_00298 both at the plasma membrane and Golgi.

- The protein G5S_0733 (from *C. pecorum*) was the CteG homolog with the most different behavior to CteG, being translocated just at 46 h and localizing at the Golgi.

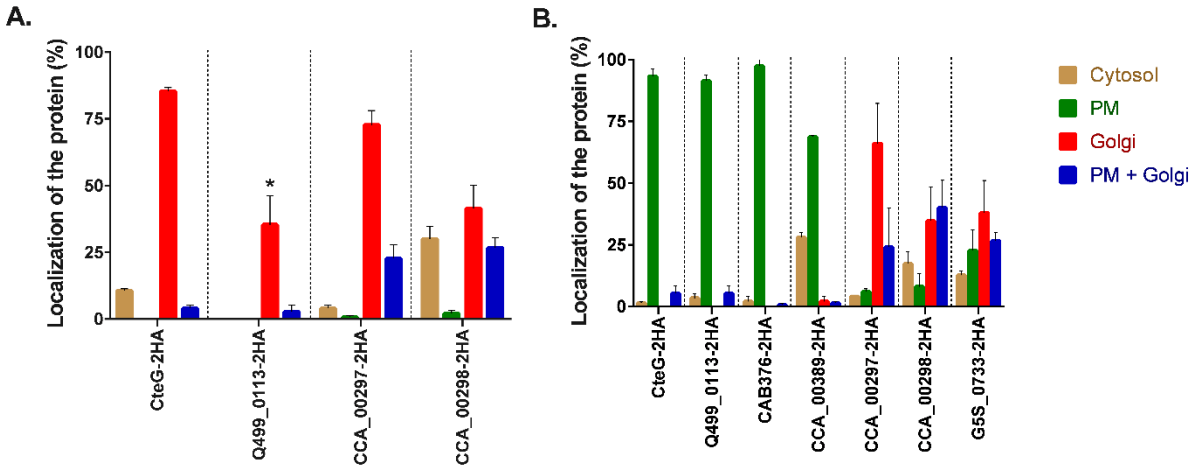

Figure 3.5 - Quantification of the different localizations in infected host cells of CteG homologs, upon induction of expression with anhydrotetracycline. Through immunofluorescence microscopy, the proteins detected outside the inclusion (translocated) were quantified in different locations of the host cell: Cytosol, Plasma Membrane (PM), Golgi or both Plasma Membrane and Golgi (PM + Golgi). (A) At 24 h p.i., 50 HeLa cells infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-CteG-2HA (*C. trachomatis*), p*Ptet*-Q499_0113-2HA (*C. suis*), p*Ptet*-CCA_00297-2HA or p*Ptet*-CCA_00298-2HA (*C. caviae*), were counted. Bars correspond to mean ± standard error of the mean (n=3). * means that protein translocation was only detected in 35% of the counted cells. (B) At 46 h p.i., 50 HeLa cells infected with *C. trachomatis cteG::aadA* harboring p*Ptet*-CteG-2HA (*C. trachomatis*), p*Ptet*-Q499_0113-2HA (*C. suis*), p*Ptet*-CAB376-2HA (*C. abortus*), p*Ptet*-CCA_00389-2HA, p*Ptet*-CCA_00297-2HA, p*Ptet*-CCA_00298-2HA (*C. caviae*) or p*Ptet*-G5S_0733-2HA (*C. pecorum*), were counted. Bars correspond to mean ± standard error of the mean (n=3).

3.1.5 Analysis of the expression and subcellular localization in infected cells of CteG homologs Q499_011A and TC_0381

As expression of the CteG homologs Q499_0114A (from *C. suis*) and TC_0381 (from *C. muridarum*) was not detected in strains where expression of their encoding genes is directed by *Ptet* (Figure 3.3), new plasmids were constructed where expression of genes encoding these two homologs was directed by *cteG* promoter (*PcteG*) (Table A.1 in Annexes). The plasmids encoding C-terminal 2HA-tagged Q499_0114A and TC_0381 were then used to transform *C. trachomatis cteG::aadA* strain (Table A.3 in Annexes). The generated strains were verified by PCR for the presence of the desired plasmid and confirmed to be *Mycoplasma*-free.

To analyze the expression of Q499_0114A-2HA and TC_0381-2HA by immunoblotting, HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA, p*PcteG*-TC_0381-2HA or p*PcteG*-Q499_0114A-2HA. At 24 and 46 h post-infection, whole cell extracts were prepared and then analyzed by immunoblotting with antibodies against HA, *C. trachomatis* Hsp60 (bacterial loading control) and human α-tubulin (HeLa cell loading control). The production of 2HA-tagged proteins of the expected molecular mass was confirmed in both cases (Figure 3.6).

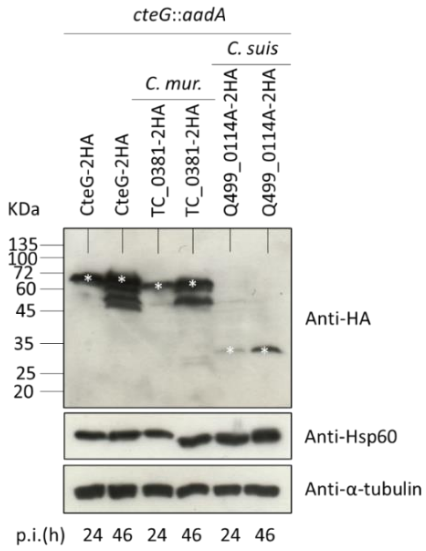

Figure 3.6 - Immunoblotting analysis of CteG homologs expressed from the *cteG* promoter. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA, p*PcteG*-TC_0381-2HA or p*PcteG*-Q499_0114A-2HA, at a MOI of 3. At 24 and 46 h p.i., whole cell extracts were prepared and then analyzed by immunoblotting with antibodies against HA, *C. trachomatis* Hsp60 (bacterial loading control) and human α-tubulin (HeLa cell loading control). The detection was made using SuperSignal West Pico detection kit (Thermo Fisher Scientific) to detect Hsp60 or α-tubulin, or SuperSignal West Femto detection kit (Thermo Fisher Scientific) to detect 2HA-tagged proteins. The band corresponding to the predictive molecular mass of the proteins is indicated with a white asterisk.

Then, the subcellular localization of Q499_0114A-2HA and TC_0381-2HA in infected cells was analyzed by immunofluorescence microscopy. For this, HeLa cells were infected for 24 and 46 h with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA, p*PcteG*-TC_0381-2HA or p*PcteG*-Q499_0114A-2HA and then fixed. Once again, the infected cells were first immunolabelled with antibodies against Cap1 and HA (and stained with DAPI), and appropriate fluorophore-conjugated secondary antibodies. Fluorescence microscopy revealed that translocation of TC_0381-2HA could be detected (Figure 3.7.1 and Table 3.1), while Q499_0114A-2HA could only be detected within the inclusion (Figure 3.7.2 and Table 3.1).

Cells infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA or p*PcteG*-TC_0381-2HA were then immunolabelled with antibodies against GM130 and HA, and appropriate fluorophore-conjugated secondary antibodies (Figures 3.2A and 3.7.1).

As before, by immunofluorescence microscopy analysis the localization of TC_0381-2HA was initially roughly compared with CteG-2HA (Figure 3.2A). This indicated that localization of TC_0381 (from *C. muridarum*) in infected cells was very similar to CteG at the Golgi at 24 h post-infection, but both at the plasma membrane and Golgi at 46 h post-infection (Figure 3.7.1).

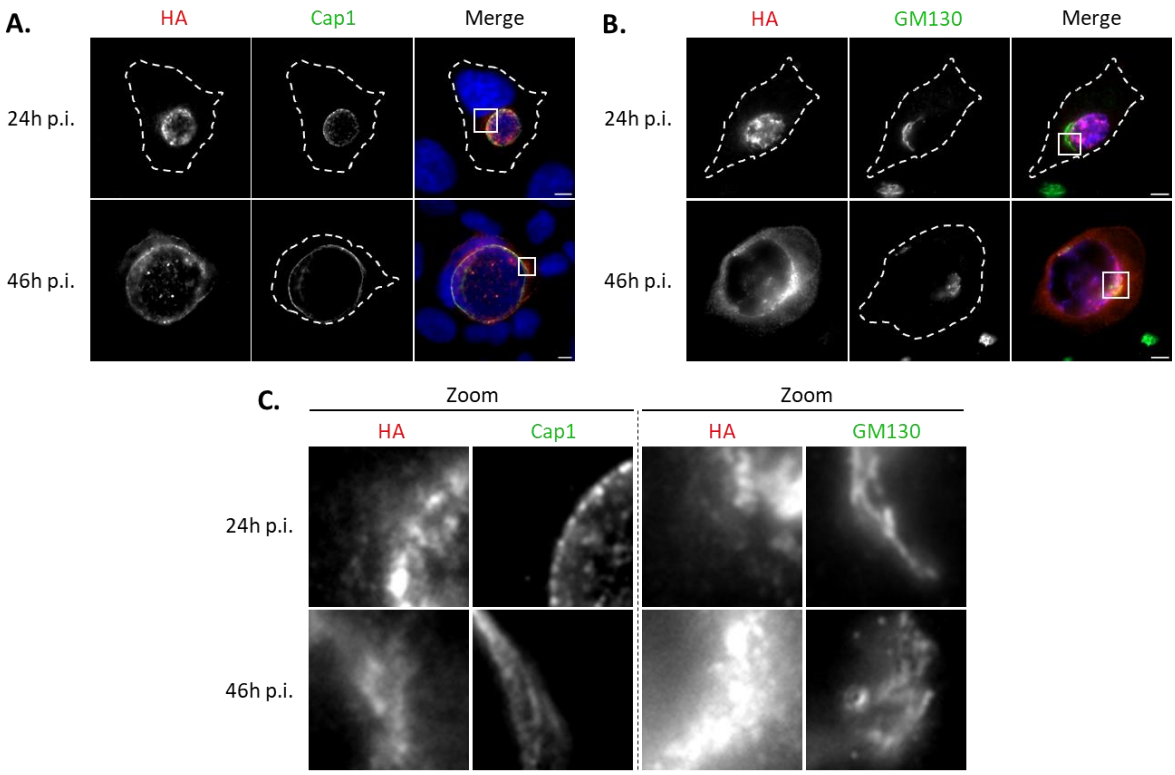

Figure 3.7.1 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG homolog TC_0381 (*C. muridarum*) after expression from the *cteG* promoter. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-TC_0381-2HA (*C. muridarum*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. (C) In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

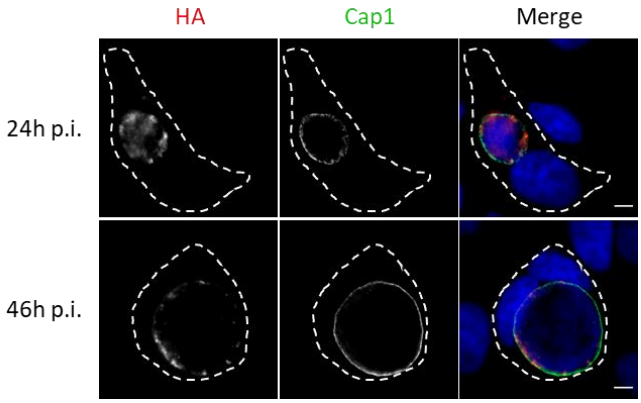

Figure 3.7.2 - Immunofluorescence microscopy analysis of the translocation and localization in infected host cells of CteG homolog Q499_0114A (*C. suis*) after expression from the *cteG* promoter. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-Q499_0114A-2HA (*C. suis*), at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against DAPI (blue), HA (red) and Cap1 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. Scale bars, 5 µm.

As to corroborate the initial analysis of the localization of TC_0381-2HA in infected cells, the subcellular localization of the protein at 24 and 46 h post-infection was systematically

enumerated and quantified by fluorescence microscopy aiming to look at 50 infected cells for each case. The same locations of the host cell were considered: cytosol, plasma membrane (PM), Golgi, or both plasma membrane and Golgi (PM + Golgi) (Figure 3.8).

Overall, this corroborated the initial analysis and confirmed that TC_0381 shows a localization in infected cells that is very similar to CteG. However, at 46 h post-infection, while CteG most concentrates at the plasma membrane, TC_0381 distributes between the plasma membrane and Golgi (Figure 3.8).

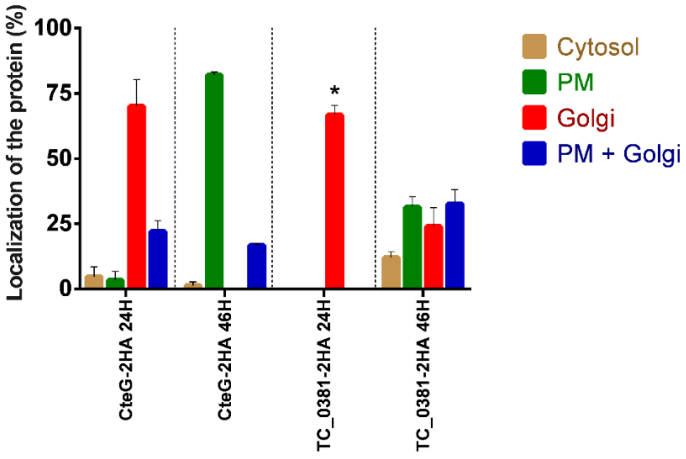

Figure 3.8 - Quantification of the different localizations in infected host cells of CteG homologs expressed from the *cteG* promoter. Through immunofluorescence microscopy, the proteins detected outside the inclusion (translocated) were quantified in different locations of the host cell: Cytosol, Plasma Membrane (PM), Golgi or both Plasma Membrane and Golgi (PM + Golgi). At 24 and 46 h p.i., 50 HeLa cells infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA (*C. trachomatis*) or p*PcteG*-TC_0381-2HA (*C. muridarum*), were counted. Bars correspond to mean ± standard error of the mean (n=3). * means that protein translocation was only detected in 67% of the counted cells.

Table 3.1 — Localization of CteG homologs in infected host cells.

CteG homologs	Cap1 Labelling (translocation)	GM130 Labelling (localization)
C. suis Q499_0113 (p*Ptet*)	24 h p.i. – Translocated 46 h p.i. – Translocated	24 h p.i. – Golgi 46 h p.i. – PM
C. suis Q499_0114A (p*PcteG*)	24 h p.i. – Not translocated 46 h p.i. – Not translocated	–
C. pneum. Cpn_0404 (p*Ptet*)	24 h p.i. – Not translocated 46 h p.i. – Not translocated	–
C. pneum. Cpn_0405 (p*Ptet*)		
C. mur. TC_0381 (p*PcteG*)	24 h p.i. – Translocated 46 h p.i. – Translocated	24 h p.i. – Golgi 46 h p.i. – PM + Golgi
C. abortus CAB376 (p*Ptet*)	24 h p.i. – Not translocated 46 h p.i. – Translocated	46 h p.i. – PM

C. caviae CCA_00389 (p*Ptet*)	24 h p.i. – Not translocated 46 h p.i. – Translocated	46 h p.i. – PM
C. caviae CCA_00297 (p*Ptet*)	24 h p.i. – Translocated 46 h p.i. – Translocated	24 h p.i. – Golgi 46 h p.i. – Golgi
C. caviae CCA_00298 (p*Ptet*)	24 h p.i.– Translocated 46 h p.i. – Translocated	24 h p.i. – Golgi 46 h p.i. – PM + Golgi
C. pecor. G5S_0729 (p*Ptet*)	24 h p.i. – Not translocated 46 h p.i. – Not translocated	–
C. pecor. G5S_0733 (p*Ptet*)	24 h p.i. – Not translocated 46 h p.i. – Translocated	46 h p.i. – Golgi

3.2 Analysis of determinants of the subcellular localization of CteG in host cells

Based on the information collected about the localization of each CteG homolog from *Chlamydiaceae* in infected host cells (Table 3.1), we deduced regions of CteG that could be responsible for its localization at the Golgi complex or at the plasma membrane of the host cell (Figure 3.9).

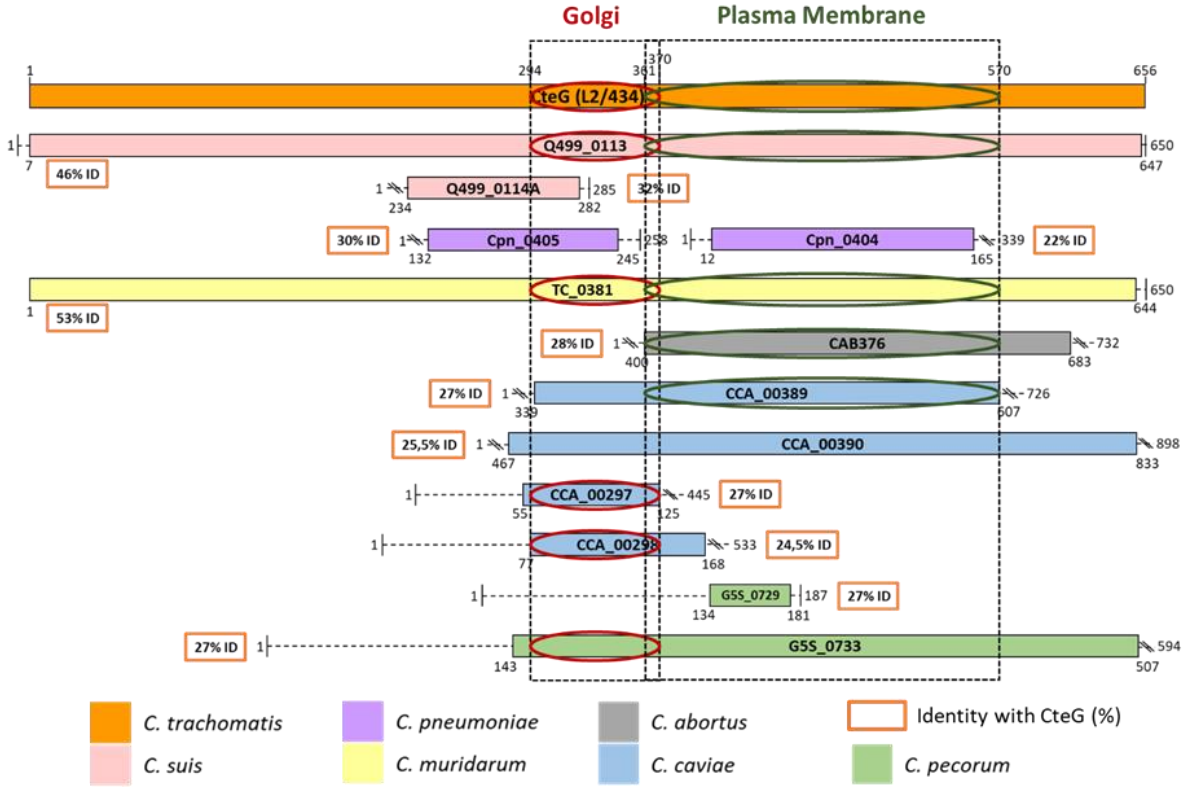

Figure 3.9 - Regions deduced as possible determinants of the localization of CteG in the host cell. Through the localization of each CteG homolog in infected host cells, it was possible to design a possible region responsible for the localization of CteG at the Golgi complex or at the plasma membrane of the host cell.

Based on this analysis and considering the predicted secondary structure of CteG (deduced using JPred4[39]), we designed the generation of CteG mutant proteins truncated of specific regions whose localization could be subsequently analyzed after their ectopic expression (by transfection) in HeLa cells or after infection by *C. trachomatis*. This considering that in a previous study was shown that ectopically expressed CteG is also directed to the Golgi and plasma membrane of HeLa cells[31], although the localization of CteG in transfected cells is quite heterogenous.

Regarding determinants of localization at the Golgi, plasmids encoding four mutant proteins ($CteG_{\Delta 298-358}$, $CteG_{\Delta 308-317}$, $CteG_{1-298}$ and $CteG_{1-358}$) were constructed enabling transfection (as EGFP fusion proteins) and transformation of *C. trachomatis*. Regarding determinants of localization at the plasma membrane, plasmids encoding two mutant proteins ($CteG_{\Delta 444-455}$ and $CteG_{\Delta 514-534}$) were constructed enabling transfection (as EGFP fusion proteins) (Table A.1 in Annexes).

3.2.1 Analysis of expression and localization of CteG mutant proteins after transfection of HeLa cells

HeLa cells were transfected with plasmids encoding EGFP, EGFP-CteG, EGFP-$CteG_{\Delta 298-358}$, EGFP-$CteG_{\Delta 308-317}$, EGFP-$CteG_{1-298}$, EGFP-$CteG_{1-358}$, EGFP-$CteG_{\Delta 444-455}$ or EGFP-$CteG_{\Delta 514-534}$. Whole cell extracts were prepared and then analyzed by immunoblotting with antibodies against GFP and human α-tubulin (HeLa cell loading control). The production of proteins of the expected molecular mass was confirmed, although EGFP-$CteG_{1-358}$, EGFP-$CteG_{\Delta 444-455}$ and EGFP-$CteG_{\Delta 514-534}$ were produced at significantly lower levels than the remaining proteins (Figure 3.10).

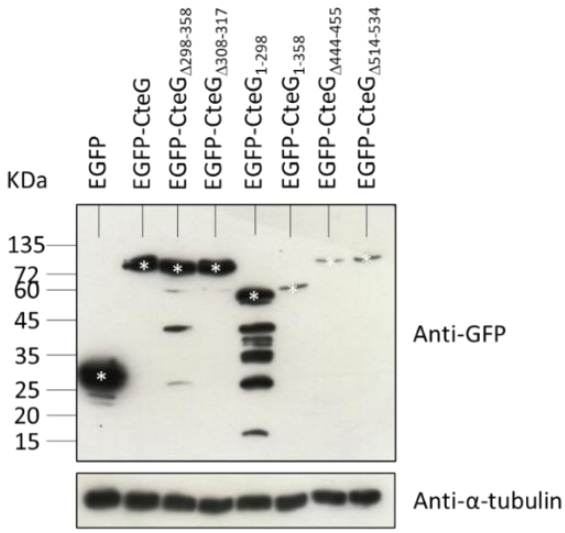

Figure 3.10 - Immunoblotting analysis of the ectopic expression of CteG mutant proteins in HeLa cells. HeLa cells were transfected using jetPEI® (Polyplus-transfection) with plasmids encoding EGFP, EGFP-CteG, EGFP-CteG$_{\Delta 298-358}$, EGFP-CteG$_{\Delta 308-317}$, EGFP-CteG$_{1-298}$, EGFP-CteG$_{1-358}$, EGFP-CteG$_{\Delta 444-455}$ or EGFP-CteG$_{\Delta 514-534}$. Whole cell extracts were prepared and then analyzed by immunoblotting with antibodies against GFP and human α-tubulin (HeLa cell loading control). The detection was made using SuperSignal West Pico detection kit (Thermo Fisher Scientific) to detect GFP or α-tubulin. The band corresponding to the predictive molecular mass of the proteins is indicated with a white asterisk.

To analyze the localization of CteG truncated proteins in transfected cells, HeLa cells were transfected as for immunoblotting, but were subsequently fixed and immunolabelled with an antibody against GM130 and an appropriate fluorophore-conjugated secondary antibody, and then analyzed by fluorescence microscopy. Through this analysis, GFP-tagged proteins (EGFP, EGFP-CteG, EGFP-CteG$_{\Delta 298-358}$, EGFP-CteG$_{\Delta 308-317}$, EGFP-CteG$_{1-298}$, EGFP-CteG$_{1-358}$, EGFP-CteG$_{\Delta 444-455}$ or EGFP-CteG$_{\Delta 514-534}$) were visually quantified in different locations of the cell: cytosol, plasma membrane (PM), Golgi or both plasma membrane and Golgi (PM + Golgi). We aimed to count 50 transfected cells in each case (Figure 3.12). As mentioned before, the localization of EGFP-CteG in transfected cells is heterogenous and was used as comparison to analyze the localization of the mutant proteins (Figure 3.11).

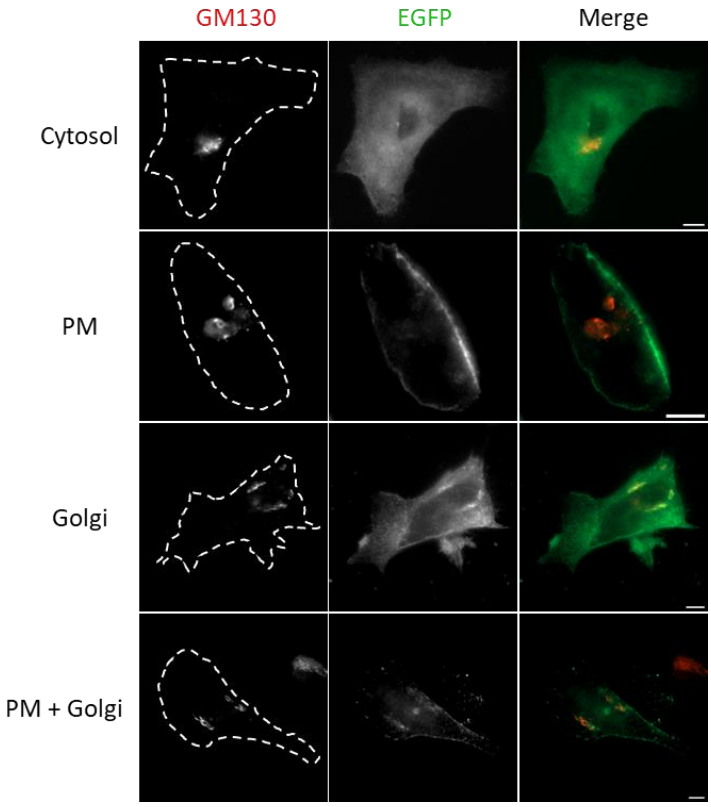

Figure 3.11 - Immunofluorescence microscopy analysis of the localization of ectopically expressed CteG or CteG mutant proteins in transfected HeLa cells. HeLa cells were transfected using jetPEI® (Polyplus-transfection) with plasmids encoding EGFP, EGFP-CteG, EGFP-CteG$_{\Delta 298\text{-}358}$, EGFP-CteG$_{\Delta 308\text{-}317}$, EGFP-CteG$_{1\text{-}298}$, EGFP-CteG$_{1\text{-}358}$, EGFP-CteG$_{\Delta 444\text{-}455}$ or EGFP-CteG$_{\Delta 514\text{-}534}$. Transfected cells were fixed with 4% (w/v) PFA and immunolabelled with antibody against GM130 (red), and appropriate fluorophore-conjugated secondary antibody, and analyzed by immunofluorescence microscopy. The images depict examples of the variable localization of EGFP-CteG in transfected cells. Scale bars, 5 µm.

EGFP-CteG$_{\Delta 308\text{-}317}$ showed a very similar localization to CteG, mostly at the plasma membrane, indicating that the deletion did not make a significant difference in the localization of CteG (Figure 3.12). On the other hand, EGFP-CteG$_{\Delta 298\text{-}358}$ localized both at the plasma membrane and Golgi, suggesting that the deletion affected the localization of the protein at the plasma membrane (Figure 3.12). The proteins EGFP-CteG$_{1\text{-}298}$, EGFP-CteG$_{1\text{-}358}$, EGFP-CteG$_{\Delta 444\text{-}455}$ and EGFP-CteG$_{\Delta 514\text{-}534}$ showed a marked localization mostly at the Golgi complex of the cells, suggesting that in transfected cells the C-terminal region of CteG mediates its localization at the plasma membrane of host cells (Figures 3.12 and 3.13).

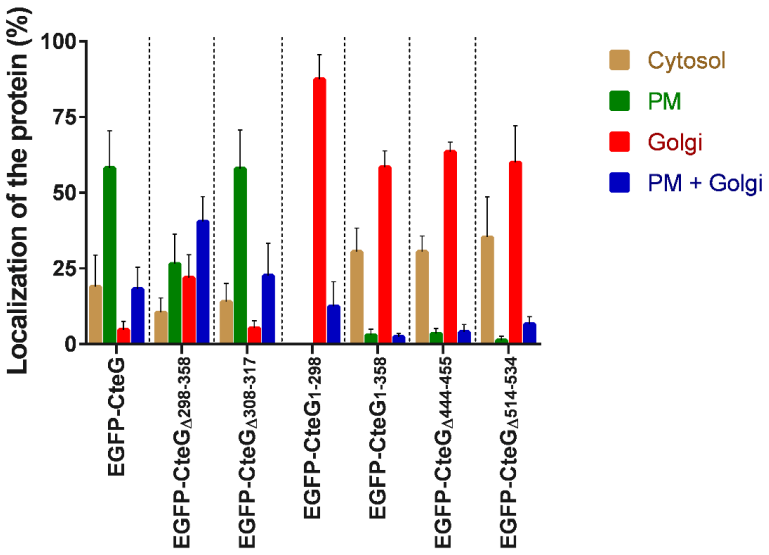

Figure 3.12 - Quantification of the different localization in transfected cells of CteG mutant proteins ectopically expressed in HeLa cells. Through immunofluorescence microscopy, GFP was visually quantified in different locations of the cell: Cytosol, Plasma Membrane (PM), Golgi or both Plasma Membrane and Golgi (PM + Golgi). 50 HeLa cells transfected with plasmids encoding EGFP, EGFP-CteG, EGFP-CteG$_{\Delta 298-358}$, EGFP-CteG$_{\Delta 308-317}$, EGFP-CteG$_{1-298}$, EGFP-CteG$_{1-358}$, EGFP-CteG$_{\Delta 444-455}$ or EGFP-CteG$_{\Delta 514-534}$, were counted. Bars correspond to mean ± standard error of the mean (n=3).

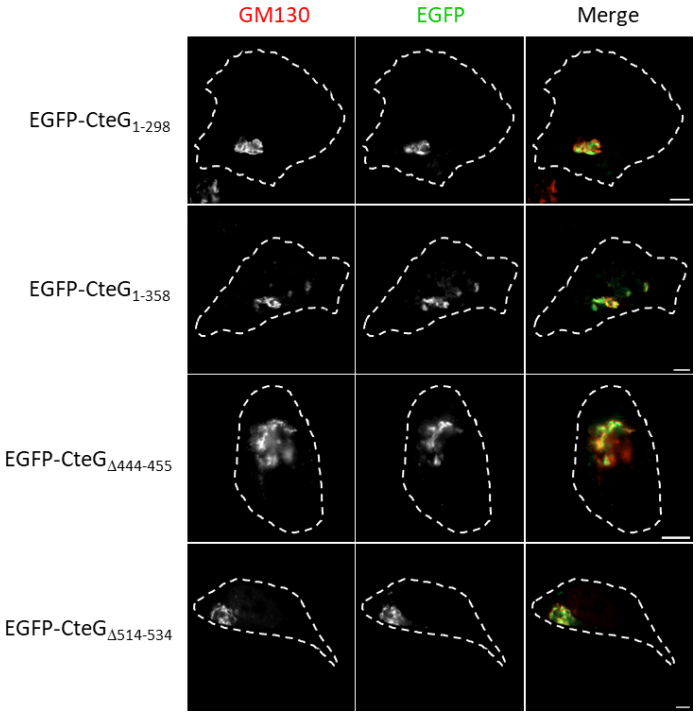

Figure 3.13 - Immunofluorescence microscopy analysis of the localization of ectopically expressed CteG mutant proteins that concentrate at the Golgi in transfected HeLa cells. HeLa cells were transfected using jetPEI® (Polyplus-transfection) with plasmids encoding EGFP, EGFP-CteG, EGFP-CteG$_{\Delta 298-358}$, EGFP-CteG$_{\Delta 308-317}$, EGFP-CteG$_{1-298}$, EGFP-CteG$_{1-358}$, EGFP-CteG$_{\Delta 444-455}$ or EGFP-CteG$_{\Delta 514-534}$. Transfected cells were fixed with 4% (w/v) PFA and immunolabelled with antibody against GM130 (red), and appropriate fluorophore-conjugated secondary antibody, and analyzed by immunofluorescence microscopy. The images depict the localization of EGFP-CteG$_{1-298}$, EGFP-CteG$_{1-358}$, EGFP-CteG$_{\Delta 444-455}$ or EGFP-CteG$_{\Delta 514-534}$ in transfected cells. Scale bars, 5 µm.

3.2.2 Analysis of expression and localization of CteG mutant proteins after *C. trachomatis* infection of HeLa cells

C. trachomatis cteG::aadA-derived strains were generated by transformation with plasmids encoding $CteG_{\Delta 298\text{-}358}$-2HA, $CteG_{\Delta 308\text{-}317}$-2HA, $CteG_{1\text{-}298}$-2HA and $CteG_{1\text{-}358}$-2HA (Tables A.1 and A.3 in Annexes). In all cases the genes encoding the mutant proteins were expressed from the *cteG* promoter. The generated strains were verified by PCR for the presence of the desired plasmid and confirmed to be *Mycoplasma*-free. HeLa cells were then infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA, p*PcteG*-$CteG_{\Delta 298\text{-}358}$-2HA, p*PcteG*-$CteG_{\Delta 308\text{-}317}$-2HA, p*PcteG*-$CteG_{1\text{-}298}$-2HA and p*PcteG*-$CteG_{1\text{-}358}$-2HA. At 24 and 46 h post-infection, whole cell extracts were prepared and then analyzed by immunoblotting with antibodies against HA, *C. trachomatis* Hsp60 (bacterial loading control) and human α-tubulin (HeLa cell loading control). The production of 2HA-tagged proteins of the expected molecular mass was confirmed for $CteG_{\Delta 298\text{-}358}$-2HA and $CteG_{\Delta 308\text{-}317}$-2HA, but not for $CteG_{1\text{-}298}$-2HA and $CteG_{1\text{-}358}$-2HA (Figure 3.14). However, even if the signal was very weak, $CteG_{1\text{-}298}$-2HA and $CteG_{1\text{-}358}$-2HA could be detected by immunofluorescence microscopy. Therefore, in subsequent experiments the subcellular localization of the four CteG mutant proteins (encoding $CteG_{\Delta 298\text{-}358}$-2HA, $CteG_{\Delta 308\text{-}317}$-2HA, $CteG_{1\text{-}298}$-2HA, $CteG_{1\text{-}358}$-2HA) was analyzed.

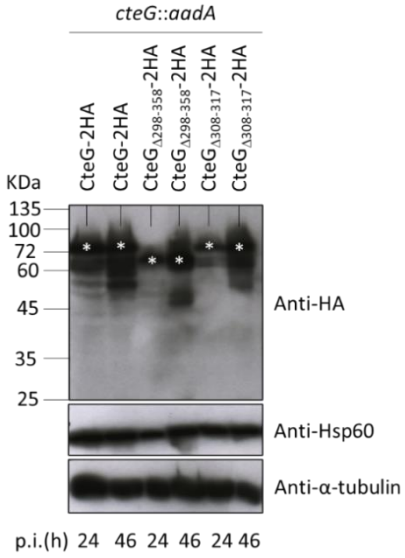

Figure 3.14 - Immunoblotting analysis of CteG mutants expressed in *C. trachomatis*. HeLa cells were infected, at a MOI of 3, with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA, p*PcteG*-$CteG_{\Delta 298\text{-}358}$-2HA, p*PcteG*-$CteG_{\Delta 308\text{-}317}$-2HA, p*PcteG*-$CteG_{1\text{-}298}$-2HA, p*PcteG*-$CteG_{1\text{-}358}$-2HA. At 24 and 46 h p.i., whole cell extracts were prepared and then analyzed by immunoblotting with antibodies against HA, *C. trachomatis* Hsp60 (bacterial loading control) and human α-tubulin (HeLa cell loading control). The detection was made using SuperSignal West Pico detection kit (Thermo Fisher Scientific) to detect Hsp60 or α-tubulin, or SuperSignal West Femto detection kit (Thermo Fisher Scientific) to detect 2HA-tagged proteins. The band corresponding to the predictive molecular mass of the proteins is indicated with a white asterisk. Expression of $CteG_{1\text{-}298}$-2HA and $CteG_{1\text{-}358}$-2HA was not detected.

To analyze the subcellular localization of CteG mutant proteins in infected cells, HeLa cells were infected for 24 and 46 h with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA, p*PcteG*-CteG$_{\Delta 298\text{-}358}$-2HA, p*PcteG*-CteG$_{\Delta 308\text{-}317}$-2HA, p*PcteG*-CteG$_{1\text{-}298}$-2HA or p*PcteG*-CteG$_{1\text{-}358}$-2HA, and then fixed. First, the infected cells were immunolabelled with antibodies against HA and Cap1 (and stained with DAPI), and appropriate fluorophore-conjugated secondary antibodies, to analyze which proteins, at which fixation timepoints, were translocated (Figures 3.15.1 to 3.15.4). This revealed that all the four mutant proteins were translocated into infected cells, although translocation of CteG$_{1\text{-}358}$-2HA was only detected at 46 h post-infection (Figures 3.15.1 to 3.15.4).

The infected cells were then immunolabelled with antibodies against MOMP, HA and GM130, and appropriate fluorophore-conjugated secondary antibodies, to see whether the proteins localized at the Golgi complex of the host cells (Figures 3.15.1 to 3.15.4).

As before for CteG homologs, by fluorescence microscopy analysis, the localization of CteG$_{\Delta 298\text{-}358}$-2HA, CteG$_{\Delta 308\text{-}317}$-2HA, CteG$_{1\text{-}298}$-2HA and CteG$_{1\text{-}358}$-2HA was initially roughly compared with CteG-2HA (Figures 3.2A). This indicated that:

- The proteins CteG$_{\Delta 298\text{-}358}$ and CteG$_{\Delta 308\text{-}317}$ showed a very similar localization at the Golgi complex at 24 h post-infection, but at 46 h post-infection they localized both at the plasma membrane and Golgi (Figure 3.15.1 and 3.15.2).

- The proteins CteG$_{1\text{-}298}$ and CteG$_{1\text{-}358}$ were very difficult to analyze, because of the low expression, but localized at the Golgi complex of the infected host cells both at 24 and 46 h post-infection (Figure 3.15.3 and 3.15.4).

About cells infected with *C. trachomatis* expressing CteG$_{1\text{-}358}$-2HA, it should be noted that, at 46 h post-infection, the inclusion was not homogeneously filled with bacteria, leaving "dark spaces" (Figure 3.15.4B).

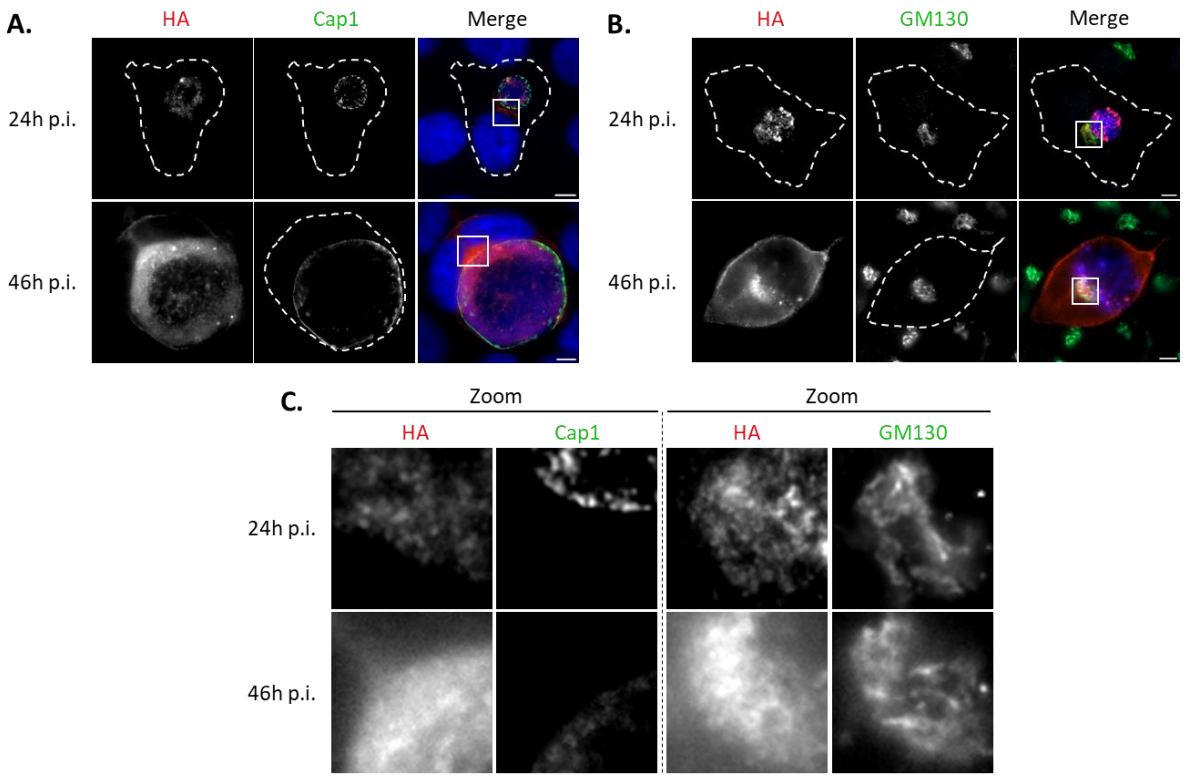

Figure 3.15.1 - Immunofluorescence microscopy analysis of the translocation and localization of CteG mutant CteG$_{\Delta 298\text{-}358}$ in infected host cells. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG$_{\Delta 298\text{-}358}$-2HA, at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. (C) In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

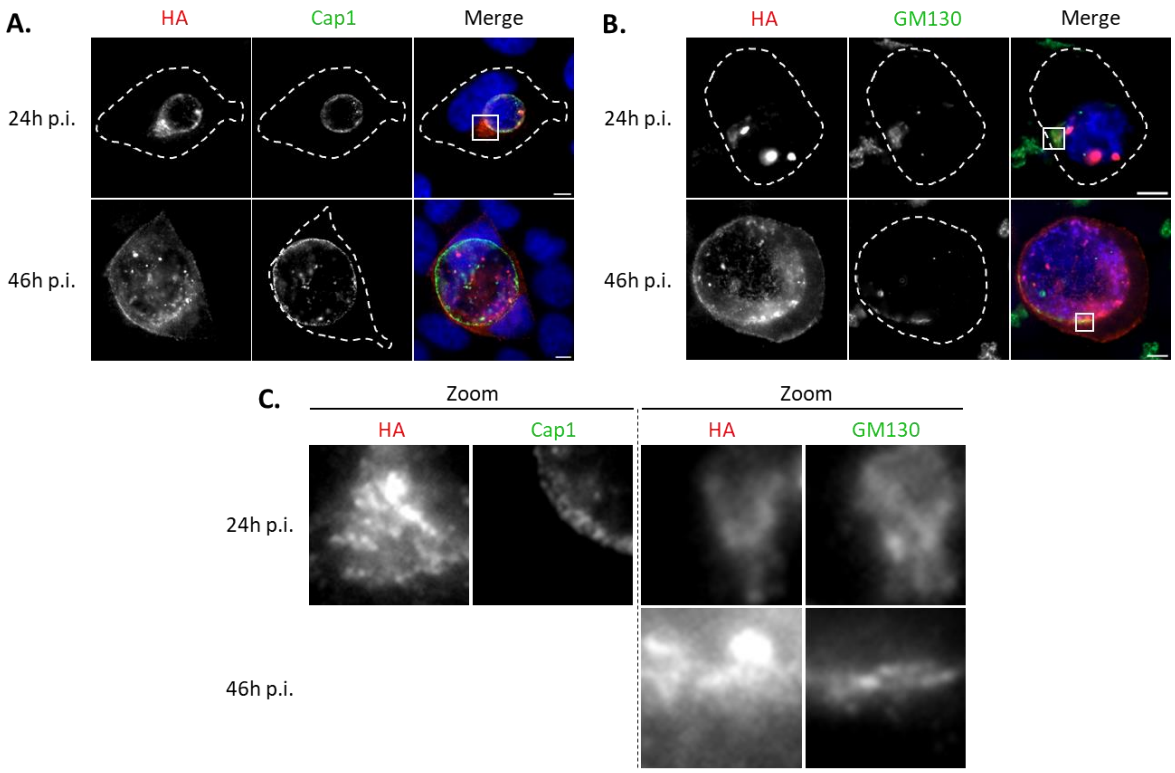

Figure 3.15.2 - Immunofluorescence microscopy analysis of the translocation and localization of CteG mutant CteG$_{\Delta 308\text{-}317}$ in infected host cells. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG$_{\Delta 308\text{-}317}$-2HA, at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. (C) In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

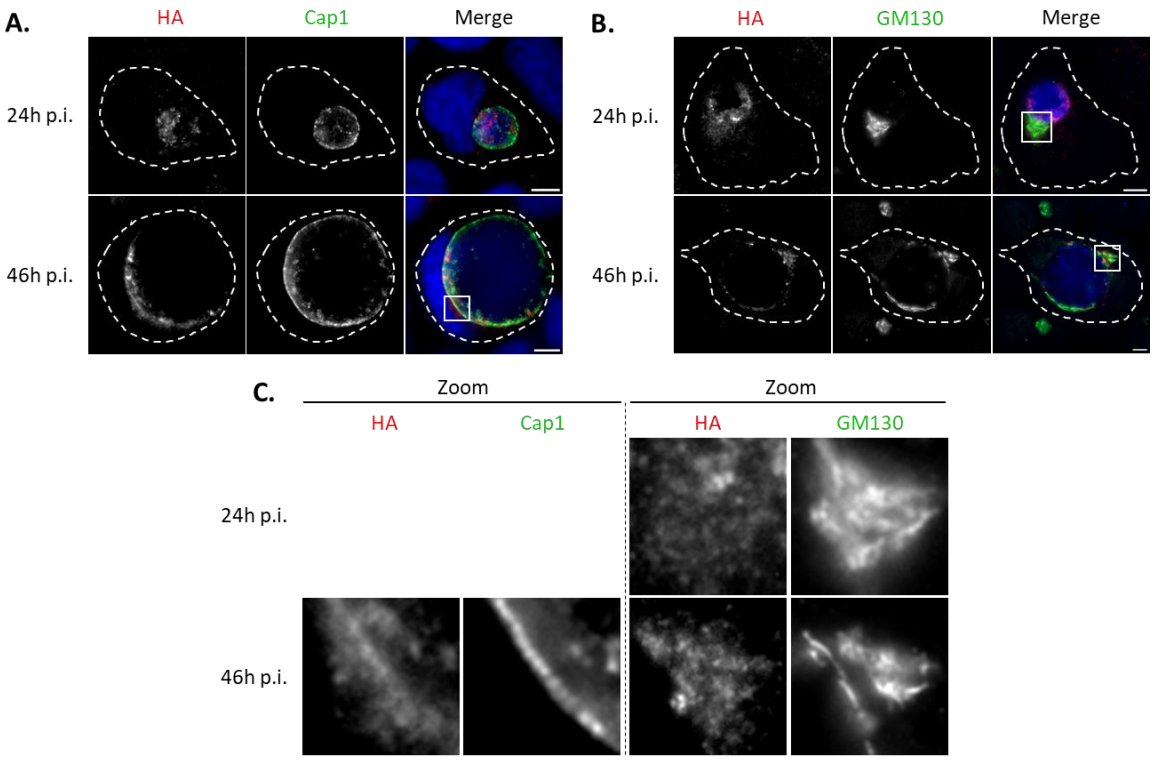

Figure 3.15.3 - Immunofluorescence microscopy analysis of the translocation and localization of CteG mutant CteG$_{1-298}$ in infected host cells. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG$_{1-298}$-2HA, at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. (C) In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

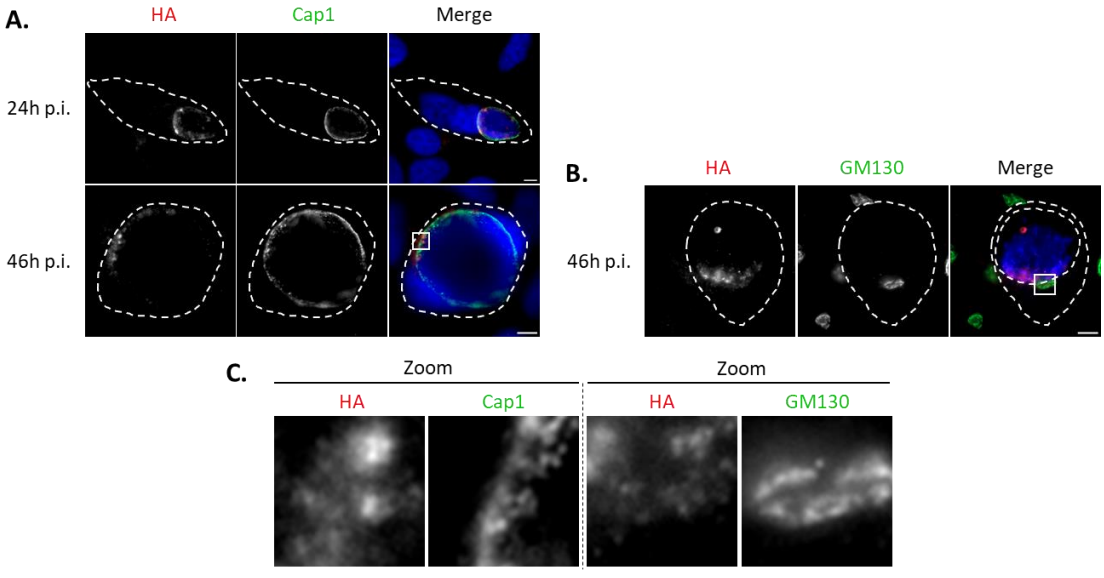

Figure 3.15.4 - Immunofluorescence microscopy analysis of the translocation and localization of CteG mutant CteG$_{1-358}$ in infected host cells. HeLa cells were infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG$_{1-358}$-2HA, at a MOI of 0.3. At 24 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against (A) DAPI (blue), HA (red) and Cap1 (green) or (B) MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies, and analyzed by immunofluorescence microscopy. (C) In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.

As before, to corroborate the initial analysis of the localization of CteG$_{\Delta 298\text{-}358}$-2HA, CteG$_{\Delta 308\text{-}317}$-2HA, CteG$_{1\text{-}298}$-2HA and CteG$_{1\text{-}358}$-2HA in infected cells, the subcellular localization of the protein at 24 and/or 46 h post-infection was systematically enumerated and quantified by fluorescence microscopy aiming to look at 50 infected cells for each case. The same locations of the host cell were considered: cytosol, plasma membrane (PM), Golgi, or both plasma membrane and Golgi (PM + Golgi) (Figure 3.16).

This quantification corroborated the initial analysis, concluding that (Figure 3.16):

- At 24 h post-infection, the proteins CteG$_{\Delta 298\text{-}358}$-2HA, CteG$_{\Delta 308\text{-}317}$-2HA and CteG$_{1\text{-}298}$-2HA had a very similar localization to CteG, at the Golgi complex of the host cells.

- At 46 h post-infection, the four CteG mutant proteins had a very different localization to CteG. The proteins CteG$_{\Delta 298\text{-}358}$-2HA and CteG$_{\Delta 308\text{-}317}$-2HA localized both at the plasma membrane and Golgi, and CteG$_{1\text{-}298}$-2HA and CteG$_{1\text{-}358}$-2HA localized at the Golgi complex.

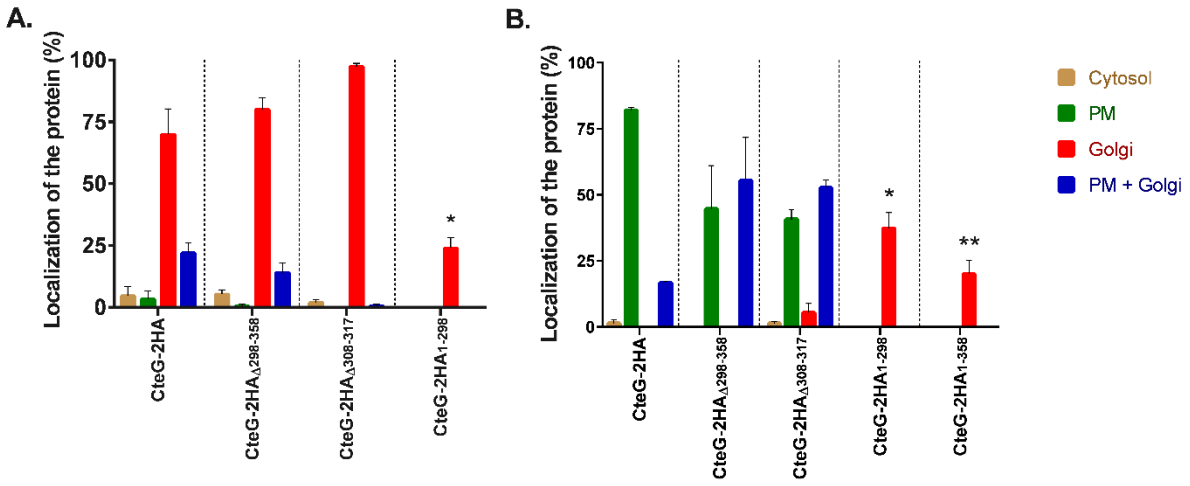

Figure 3.16 - Quantification of the different localization of CteG mutant proteins in infected host cells. Through immunofluorescence microscopy, the proteins detected outside the inclusion (translocated) were quantified in different locations of the host cell: Cytosol, Plasma Membrane (PM), Golgi or both Plasma Membrane and Golgi (PM + Golgi). (A) At 24 h p.i., 50 HeLa cells infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA, p*PcteG*-CteG$_{\Delta 298\text{-}358}$-2HA, p*PcteG*-CteG$_{\Delta 308\text{-}317}$-2HA or p*PcteG*-CteG$_{1\text{-}298}$-2HA, were counted. Bars correspond to mean ± standard error of the mean (n=3). * means that protein translocation was only detected in 24% of the counted cells. (B) At 46 h p.i., 50 HeLa cells infected with *C. trachomatis cteG::aadA* harboring p*PcteG*-CteG-2HA, p*PcteG*-CteG$_{\Delta 298\text{-}358}$-2HA, p*PcteG*-CteG$_{\Delta 308\text{-}317}$-2HA, p*PcteG*-CteG$_{1\text{-}298}$-2HA or p*PcteG*-CteG$_{1\text{-}358}$-2HA, were counted. Bars correspond to mean ± standard error of the mean (n=3). * means that protein translocation was only detected in 37% of the counted cells. ** means that protein translocated was only detected in 20% of the counted cells.

These results suggest that the deletions did not have a significant effect in CteG localization at 24 h post-infection, but very much did at 46 h post-infection (Figure 3.16). However, the analysis of CteG$_{1\text{-}298}$-2HA and CteG$_{1\text{-}358}$-2HA is hampered, and not totally reliable, by the reduced levels of expression of the proteins (undetected by immunoblotting). In the case of CteG$_{\Delta 298\text{-}358}$-2HA and CteG$_{\Delta 308\text{-}317}$-2HA, their localization at 46 h post-infection was slightly different relative to CteG-2HA. However, an analysis of chlamydial growth was not

performed, so it is presently unknown whether this corresponds to a subcellular targeting defect or to a slower developmental cycle of the strains producing CteG$_{\Delta 298\text{-}358}$-2HA and CteG$_{\Delta 308\text{-}317}$-2HA relative to the strain producing CteG-2HA.

4

DISCUSSION

Understanding how *C. trachomatis* manipulates host cells through type III secretion system (T3SS) effector proteins is an important step forward to understand how *Chlamydia* causes infections. This work focused on the *C. trachomatis* effector associated with the Golgi (CteG), a T3SS effector non-Inc protein recently discovered that alters the normal functioning of host cells. For instance, CteG promotes chlamydial host cell lytic exit[32] and, its interaction with centrin-2 may impact the centrosome amplification, which is important for cell division[40]. Beyond that, CteG shows an interesting change of localization during the developmental cycle of *Chlamydia*[31,32]. CteG initially localizes at the Golgi complex, from ~20 h post-infection, and then starts localizing at the host plasma membrane, from ~30 h post-infection, being this its predominant localization at the end of the developmental cycle[31].

This work had its starting point in previous results from the host lab where potential CteG homologs from other *Chlamydia* spp.[31] were found to be type III secreted by *Yersinia* (da Cunha *et al*, unpublished). This revealed twelve proteins from different *Chlamydia* species: Q499_ 0113 and Q499_0114A from *C. suis*, Cpn_0404 and Cpn_0405 from *C. pneumoniae*, TC_0381 from *C. muridarum*, CAB376 from *C. abortus*, CCA_00389, CCA_00390, CCA_00297 and CCA_00298 from *C. caviae*, and G5S_0729 and G5S_0733 from *C. pecorum*. This kind of analysis of *Chlamydia* effector proteins homologs through a type III secretion assay in other bacteria has been already described, for example for DUF582 proteins[41], but we wanted to test whether the CteG homologs could be transported specifically by *C. trachomatis* into the cytoplasm of infected cells. With this extent, this was the first time such analysis was done for homologs of *Chlamydia* effector proteins. For this, a CteG-deficient strain was transformed to generate *C. trachomatis* strains carrying the genes encoding the CteG homologs expressed from tetracycline promoter. While nine strains were generated by this procedure, the strain encoding

CCA_00390 (from *C. caviae*) could not be transformed, even after several transformation attempts, and the expression of Q499_0114A (from *C. suis*) and TC_0381 (from *C. muridarum*) could not be detected by immunoblotting. About CCA_00390, we can hypothesize that somehow, even without the addition of anhydrotetracycline during the transformation process, the protein was being expressed and inhibiting chlamydial growth. As for Q499_0114A and TC_0381 we went through another strategy to analyze these CteG homologs. Therefore, the CteG-deficient strain was transformed to generate *C. trachomatis* strains carrying the genes encoding Q499_0114A and TC_0381 expressed from *cteG* promoter.

The detection of the expression of CteG homologs by immunoblotting revealed multiple bands besides the band corresponding to the predicted molecular mass, corroborating the hypothesis thought for CteG of possible degradation or processing during the *C. trachomatis* developmental cycle[31,32].

Then, the subcellular localization of CteG homologs was analyzed and quantified in two steps. The first immunofluorescence microscopy analysis was to identify which CteG homologs were detected outside the inclusion membrane (i.e., translocated into host cells) and at which times post-infection. The overall conclusion of this first analysis was that most of CteG homologs were translocated, except for proteins Q499_0114A (from *C. suis*), Cpn_0404 and Cpn_0405 (from *C. pneumoniae*) and G5S_0729 (from *C. pecorum*); beyond that, within the seven translocated homologs, the proteins Q499_0113 (from *C. suis*), TC_0381 (from *C. muridarum*), and CCA_00297 and CCA_00298 (from *C. caviae*) were translocated into host cells both at 24 and 46 h post-infection, while CAB376 (from *C. abortus*), CCA_00389 (from *C. caviae*) and G5S_0733 (from *C. pecorum*) were translocated only at 46 h post-infection. This brings to discussion the possibility of not translocated CteG homologs do not recognize the *C. trachomatis* T3SS or need a specific chaperone that is absent in *C. trachomatis*. T3SS chaperones have a crucial role in the secretion of some effector proteins into host cells, by delivering the substrates to the T3SS and/or preventing their degradation[42]. Also, we do not know if these CteG homologs maintained the same function as effector proteins or evolved a different role within the *Chlamydiae*.

The second immunofluorescence microscopy analysis was to see if translocated CteG homologs localized at the Golgi complex of host cells. The overall conclusion of this second analysis was that some CteG homologs localized at the Golgi at 24 h post-infection and at the plasma membrane at 46 h post-infection, similarly to CteG (Q499_0113 from *C. suis*), others localized only at the Golgi (CCA_00297 from *C. caviae* and G5S_0733 from *C. pecorum*), others localized only at the plasma membrane (CAB376 from *C. abortus* and CCA_00389 from *C.*

caviae) and others both at the plasma membrane and Golgi (TC_0381 from *C. muridarum* and CCA_00298 from *C. caviae*). We were expecting that Q499_0113 and TC_0381 had the most similar localization to CteG, since they share 46% and 53% of identity with CteG, respectively (Figure 3.1).

Based on these conclusions about the subcellular localization of CteG homologs and the secondary structure of CteG, we could deduce regions in CteG that may determine its localization at the Golgi and plasma membrane of host cells. To test this, molecular biology procedures were used to generate plasmids encoding truncated CteG proteins ($CteG_{\Delta 298\text{-}358}$, $CteG_{\Delta 308\text{-}317}$, $CteG_{1\text{-}298}$ and $CteG_{1\text{-}358}$) whose localization in host cells was then analyzed after their ectopic expression in HeLa cells or after T3SS delivery by *C. trachomatis*.

The analysis in transfected cells indicated that EGFP-$CteG_{\Delta 298\text{-}358}$ localized both at the plasma membrane and Golgi, EGFP-$CteG_{\Delta 308\text{-}317}$ localized at the plasma membrane, and EGFP-$CteG_{1\text{-}298}$, EGFP-$CteG_{1\text{-}358}$, EGFP-$CteG_{\Delta 444\text{-}455}$ and EGFP-$CteG_{\Delta 514\text{-}534}$ localized at the Golgi. We were expecting, based on the alignment of CteG homologs (Figure 3.9), that EGFP-$CteG_{\Delta 298\text{-}358}$ and EGFP-$CteG_{\Delta 308\text{-}317}$ would not localize at the Golgi, and EGFP-$CteG_{1\text{-}298}$, EGFP-$CteG_{1\text{-}358}$, EGFP-$CteG_{\Delta 444\text{-}455}$ and EGFP-$CteG_{\Delta 514\text{-}534}$ would not localize at the plasma membrane. Therefore, this analysis indicated that the C-terminal region of CteG (from residue 359) is required for the localization of the protein at the plasma membrane in transfected host cells.

Previous studies identified a Golgi-targeting region within the first 100 amino acids of CteG[31], and further studies revealed specific residues within the first 20 amino acids of CteG that are essential for targeting a EGFP-$CteG_{1\text{-}100}$ fusion proteins to the Golgi complex in transfected cells (Pereira and Mota, unpublished data). However, the subcellular localization of full-length CteG with those specific residues altered is only minimally altered both in infected and in transfected cells (Pereira and Mota, unpublished data). For this reason, we aimed to understand how CteG is driven to the Golgi. However, the truncations designed to affect the localization of CteG at the Golgi in transfected cells also showed only a minor impact in the localization of the ectopically produced proteins, and additional studies are required to address this issue.

The analysis in infected cells indicated that all the four mutant proteins were translocated into infected cells, although translocation of $CteG_{1\text{-}358}$ was only detected at 46 h post-infection. Beyond that, $CteG_{\Delta 298\text{-}358}$ and $CteG_{\Delta 308\text{-}317}$ localized at the Golgi at 24 h post-infection and both at the plasma membrane and Golgi at 46 h post-infection, and $CteG_{1\text{-}298}$ and $CteG_{1\text{-}358}$ localized only at the Golgi. We were expecting, based on the alignment of CteG homologs (Figure 3.9), that $CteG_{\Delta 298\text{-}358}$ and $CteG_{\Delta 308\text{-}317}$ would not localize at the Golgi, and $CteG_{1\text{-}298}$ and $CteG_{1\text{-}358}$

would not localize at the plasma membrane. However, it is important to notice that the localization of CteG$_{\Delta 298\text{-}358}$ and CteG$_{\Delta 308\text{-}317}$ both at the plasma membrane and Golgi at 46 h post-infection is similar to the localization of CteG in infected cells at 30 h post-infection[31], emerging the hypothesis that these deletions may delay the *C. trachomatis* developmental cycle. Also, we cannot jump to conclusions too quickly about CteG$_{1\text{-}298}$ and CteG$_{1\text{-}358}$ since this analysis was very difficult to do, because of the low expression, and these truncated proteins were not detected by immunoblotting. Beyond that, a peculiar phenomenon observed was that the inclusion did not appear homogeneously filled with bacteria, leaving "dark spaces" (Figure 3.14.4B). This may be because these strains were not purified.

These results suggest that the deletions did not have a major effect in CteG localization at 24 h post-infection, but very much did at 46 h post-infection. However, the low expression of CteG$_{1\text{-}298}$ and CteG$_{1\text{-}358}$ and the fact that the generated strains were not analyzed for chlamydial growth raises doubts whether the differences seen correspond to subcellular targeting defects of CteG.

In future studies on the analysis of the determinants of the subcellular localization of CteG, we should consider the tertiary structure of CteG, as predicted by AlphaFold[43] (Figure 4.1).

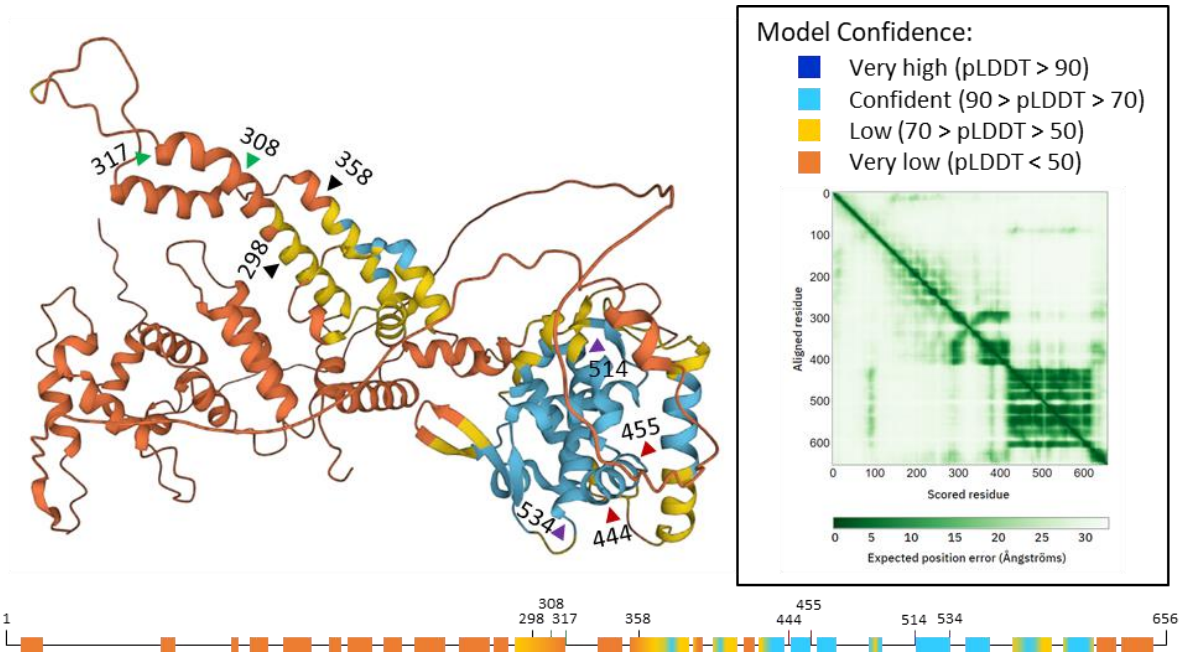

Figure 4.1 - Schematic model of the predicted tertiary structure of CteG. Tertiary structure of CteG predicted by AlphaFold[43]. The model of confidence and the predicted aligned error are also shown. The black arrow heads delimitate the deleted region between amino acids 298 and 358. The green arrow heads delimitate the deleted region between amino acids 308 and 317. The red arrow heads delimitate the deleted region between amino acids 444 and 455. The purple arrow heads delimitate the deleted region between amino acids 514 and 534. CteG is also represented linearly with the respective helices, to be visually more perceptive.

In fact, through the predicted tertiary structure of CteG, it can be observed that the amino acids 298, 308 and 358 are inserted in the middle of helices (Figure 4.1), which may disrupt the folding of the CteG truncated proteins $CteG_{\Delta 298\text{-}358}$, $CteG_{\Delta 308\text{-}317}$, $CteG_{1\text{-}298}$ and $CteG_{1\text{-}358}$. Taking this to consideration, for future work, new CteG truncated proteins should be constructed considering not only the alignment of CteG homologs and their subcellular localization in infected cells, and the predicted secondary structure of CteG, but also its predicted tertiary structure. For instance, some suggestions could be mutant proteins with deletions between amino acids 287 and 318, or 335 and 349, or 353 and 375, comprising whole helices, to seek any shred of evidence of the implication of these helices in CteG localization at the Golgi. Another aspect that should be analyzed is the subcellular localization of proteins $CteG_{\Delta 444\text{-}455}$ and $CteG_{\Delta 514\text{-}534}$ in infected host cells, to see if these helices in C-terminal region of CteG affect the localization of CteG at the plasma membrane in infected cells.

BIBLIOGRAPHY

1. Nunes, A. & Gomes, J. P. Evolution, phylogeny, and molecular epidemiology of *Chlamydia*. *Infect. Genet. Evol.* **23**, 49–64 (2014).
2. Taylor-Brown, A., Vaughan, L., Greub, G., Timms, P. & Polkinghorne, A. Twenty years of research into *Chlamydia*-like organisms: A revolution in our understanding of the biology and pathogenicity of members of the phylum *Chlamydiae*. *Pathog. Dis.* **73**, 1–15 (2015).
3. Dugan, J., Andersen, A. A. & Rockey, D. D. Functional characterization of IScs605, an insertion element carried by tetracycline-resistant *Chlamydia suis*. *Microbiology* **153**, 71–79 (2007).
4. Roulis, E., Polkinghorne, A. & Timms, P. *Chlamydia pneumoniae*: Modern insights into an ancient pathogen. *Trends Microbiol.* **21**, 120–128 (2013).
5. Perfettini, J. L. *et al.* Role of proapoptotic BAX in propagation of *Chlamydia muridarum* (the mouse pneumonitis strain of *Chlamydia trachomatis*) and the host inflammatory response. *J. Biol. Chem.* **278**, 9496–9502 (2003).
6. Sachse, K. *et al.* Recent developments in the laboratory diagnosis of chlamydial infections. *Vet. Microbiol.* **135**, 2–21 (2009).
7. Frazer, L. C. *et al.* Plasmid-Cured *Chlamydia caviae* Activates TLR2-Dependent Signaling and Retains Virulence in the Guinea Pig Model of Genital Tract Infection. *PLoS One* **7**, e30747 (2012).
8. Walker, E., Lee, E. J., Timms, P. & Polkinghorne, A. *Chlamydia pecorum* infections in sheep and cattle: A common and under-recognised infectious disease with significant impact on animal health. *Vet. J.* **206**, 252–260 (2015).
9. Mpiga, P. & Ravaoarinoro, M. *Chlamydia trachomatis* persistence: An update. *Microbiol. Res.* **161**, 9–19 (2006).
10. Taylor, H. R., Burton, M. J., Haddad, D., West, S. & Wright, H. Trachoma. *Lancet* **384**, 2142–2152 (2014).
11. Lijek, R. S., Helble, J. D., Olive, A. J., Seiger, K. W. & Starnbach, M. N. Pathology after *Chlamydia trachomatis* infection is driven by nonprotective immune cells that are distinct from protective populations. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 2216–2221 (2018).
12. Mabey, D. & Peeling, R. W. Lymphogranuloma venereum. *Sex. Transm. Infect.* **78**, 90–92 (2002).
13. Wang, S. A. *et al.* Evaluation of Antimicrobial Resistance and Treatment Failures for *Chlamydia trachomatis*: A Meeting Report. *J. Infect. Dis.* **191**, 917–923 (2005).

14. Brunham, R. C. & Rappuoli, R. *Chlamydia trachomatis* control requires a vaccine. *Vaccine* **31**, 1892–1897 (2013).
15. Wang, Y. *et al.* Development of a Transformation System for *Chlamydia trachomatis*: Restoration of Glycogen Biosynthesis by Acquisition of a Plasmid Shuttle Vector. *PLOS Pathog.* **7**, e1002258 (2011).
16. Hooppaw, A. J. & Fisher, D. J. A Coming of Age Story: *Chlamydia* in the Post-Genetic Era. *Infect. Immun.* **84**, 612–621 (2015).
17. AbdelRahman, Y. M. & Belland, R. J. The chlamydial developmental cycle. *FEMS Microbiol. Rev.* **29**, 949–959 (2005).
18. Galán, J. E., Lara-Tejero, M., Marlovits, T. C. & Wagner, S. Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. *Annu. Rev. Microbiol.* **68**, 415–438 (2014).
19. Mirrashidi, K. M. *et al.* Global Mapping of the Inc-Human Interactome Reveals that Retromer Restricts *Chlamydia* Infection. *Cell Host Microbe* **18**, 109–121 (2015).
20. Dehoux, P., Flores, R., Dauga, C., Zhong, G. & Subtil, A. Multi-genome identification and characterization of *chlamydiae*-specific type III secretion substrates: the Inc proteins. *BMC Genomics* **12**, 109 (2011).
21. Stanhope, R., Flora, E., Bayne, C. & Derré, I. IncV, a FFAT motif-containing *Chlamydia* protein, tethers the endoplasmic reticulum to the pathogen-containing vacuole. *Proc. Natl. Acad. Sci.* **114**, 12039–12044 (2017).
22. Nguyen, P. H., Lutter, E. I. & Hackstadt, T. *Chlamydia trachomatis* inclusion membrane protein MrcA interacts with the inositol 1,4,5-trisphosphate receptor type 3 (ITPR3) to regulate extrusion formation. *PLOS Pathog.* **14**, e1006911 (2018).
23. Agaisse, H. & Derré, I. Expression of the effector protein IncD in *Chlamydia trachomatis* mediates recruitment of the lipid transfer protein CERT and the endoplasmic reticulum-resident protein VAPB to the inclusion membrane. *Infect. Immun.* **82**, 2037–2047 (2014).
24. Hackstadt, T., Scidmore-Carlson, M. A., Shaw, E. I. & Fischer, E. R. The *Chlamydia trachomatis* IncA protein is required for homotypic vesicle fusion. *Cell. Microbiol.* **1**, 119–130 (1999).
25. Weber, M. M. *et al.* Absence of Specific *Chlamydia trachomatis* Inclusion Membrane Proteins Triggers Premature Inclusion Membrane Lysis and Host Cell Death. *Cell Rep.* **19**, 1406–1417 (2017).
26. Mehlitz, A. *et al.* Tarp regulates early *Chlamydia*-induced host cell survival through interactions with the human adaptor protein SHC1. *J. Cell Biol.* **190**, 143–157 (2010).
27. McKuen, M. J., Mueller, K. E., Bae, Y. S. & Fields, K. A. Fluorescence-Reported Allelic Exchange Mutagenesis Reveals a Role for *Chlamydia trachomatis* TmeA in Invasion That Is Independent of Host AHNAK. *Infect. Immun.* **85**, (2017).
28. Kumar, Y., Cocchiaro, J. & Valdivia, R. H. The Obligate Intracellular Pathogen *Chlamydia trachomatis* Targets Host Lipid Droplets. *Curr. Biol.* **16**, 1646–1651 (2006).
29. Pennini, M. E., Perrinet, S., Dautry-Varsat, A. & Subtil, A. Histone Methylation by NUE, a Novel Nuclear Effector of the Intracellular Pathogen *Chlamydia trachomatis*. *PLOS Pathog.* **6**, e1000995 (2010).
30. Rockey, D. D., Scidmore, M. A., Bannantine, J. P. & Brown, W. J. Proteins in the chlamydial inclusion membrane. *Microbes Infect.* **4**, 333–340 (2002).
31. Pais, S. V. *et al.* CteG is a *Chlamydia trachomatis* effector protein that associates with the

Golgi complex of infected host cells. *Sci. Rep.* **9**, 1–15 (2019).
32. Pereira, I. S. *et al.* The Type III Secretion Effector CteG Mediates Host Cell Lytic Exit of *Chlamydia trachomatis*. *Frontiers in Cellular and Infection Microbiology* **12**, (2022).
33. da Cunha, M. *et al.* Identification of type III secretion substrates of *Chlamydia trachomatis* using *Yersinia enterocolitica* as a heterologous system. *BMC Microbiol.* **14**, 40 (2014).
34. Uphoff, C. C. & Drexler, H. G. Detecting mycoplasma contamination in cell cultures by polymerase chain reaction. in *Cancer Cell Culture* 93–103 (Springer, 2011).
35. Scidmore, M. A. Cultivation and Laboratory Maintenance of *Chlamydia trachomatis*. *Curr. Protoc. Microbiol.* **00**, 11A.1.1-11A.1.25 (2006).
36. Agaisse, H. & Derré, I. A *C. trachomatis* Cloning Vector and the Generation of *C. trachomatis* Strains Expressing Fluorescent Proteins under the Control of a *C. trachomatis* Promoter. *PLoS One* **8**, (2013).
37. Gehre, L. *et al.* Sequestration of host metabolism by an intracellular pathogen. *Elife* **5**, e12552 (2016).
38. Fling, S. P. *et al.* CD8+ T cells recognize an inclusion associatecl protein from the pathogen *Chlamydia trachomatis*. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 1160–1165 (2001).
39. Drozdetskiy, A., Cole, C., Procter, J. & Barton, G. J. JPred4: a protein secondary structure prediction server. *Nucleic Acids Res.* **43**, W389–W394 (2015).
40. Steiert, B. *et al.* The *Chlamydia trachomatis* type III secreted effector protein CteG induces centrosome amplification through interactions with centrin-2. *bioRxiv* (2022).
41. Subtil, A. *et al.* A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates. *Mol. Microbiol.* **56**, 1636–1647 (2005).
42. Feldman, M. F. & Cornelis, G. R. The multitalented type III chaperones: all you can do with 15 kDa. *FEMS Microbiol. Lett.* **219**, 151–158 (2003).
43. Jumper, J. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583–589 (2021).

A

ANNEXES

Table A.1 - Plasmids used in this work.

Plasmid	Description	Source/Reference
pSVP247	Derivative of p2TK2-SW2 (Agaisse *et al.*, 2013) for expression of proteins with a C-terminal double HA (2HA) tag. Contains the terminator of the *incDEFG* operon (T*incD*) of *C. trachomatis* L2/434 (Amp^R).	(da Cunha *et al.*, 2017)
pSVP264	Expresses CteG-2HA under the control of the predicted *cteG* promoter. A DNA fragment containing *cteG* and its endogenous promoter was amplified from *C. trachomatis* L2/434 chromosomal DNA using primers 1680 and 1552. The resulting DNA product was digested with KpnI and NotI and inserted into those sites of pSVP247 (Amp^R).	(Pais, 2018)
pALT4	Transfection vector encoding mEGFP-CteG. A DNA fragment containing *cteG* was amplified from *C. trachomatis* L2/434 chromosomal DNA using primers 652 and 653. The resulting DNA product was digested with KpnI and SalI and inserted into those sites of pmEGFP-C1 (Km^R).	(Pais, 2018)
pMC114	Derivative of pSVP247 with the inducible tetracycline promoter (*Ptet*). *Ptet* was amplified from pMC85 using primer 2792 and 2793. The resulting DNA product was digested with KpnI and NdeI and inserted into those sites of pSVP247 (Amp^R).	Maria da Cunha
pMC115	Derivative of pMC114. Encodes *C. suis* Q499_0113-2HA under the control of *Ptet*. A DNA fragment containing *Q499_0113* was amplified from pMC103 using primers 2374 and 2794. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha

pMC116	Derivative of pMC114. Encodes *C. suis* Q499_0114A-2HA under the control of *Ptet*. A DNA fragment containing *Q499_0114A* was amplified from pMC104 using primers 2372 and 2795. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha
pMC117	Derivative of pMC114. Encodes *C. pneumoniae* Cpn_0404-2HA under the control of *Ptet*. A DNA fragment containing *Cpn_0404* was amplified from pMM1 using primers 28 and 2808. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha
pMC118	Derivative of pMC114. Encodes *C. pneumoniae* Cpn_0405-2HA under the control of *Ptet*. A DNA fragment containing *Cpn_0405* was amplified from pMM2 using primers 2368 and 2796. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha
pMC119	Derivative of pMC114. Encodes *C. muridarum* TC_0381-2HA under the control of *Ptet*. A DNA fragment containing *TC_0381* was amplified from pMM3 using primers 2370 and 2797. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha
pMC120	Derivative of pMC114. Encodes *C. abortus* CAB376-2HA under the control of *Ptet*. A DNA fragment containing *CAB376* was amplified from pMC105 using primers 2726 and 2798. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha
pMC121	Derivative of pMC114. Encodes *C. caviae* CCA_00389-2HA under the control of *Ptet*. A DNA fragment containing *CCA_00389* was amplified from pMC106 using primers 2727 and 2799. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha
pMC122	Derivative of pMC114. Encodes *C. caviae* CCA_00390-2HA under the control of *Ptet*. A DNA fragment containing *CCA_00390* was amplified from pMC107 using primers 2728 and 2800. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha
pMC123	Derivative of pMC114. Encodes *C. caviae* CCA_00297-2HA under the control of *Ptet*. A DNA fragment containing *CCA_00297* was amplified from	Maria da Cunha

	pMC108 using primers 2731 and 2801. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	
pMC124	Derivative of pMC114. Encodes *C. caviae* CCA_00298-2HA under the control of *Ptet*. A DNA fragment containing *CCA_00298* was amplified from pMC109 using primers 2717 and 2802. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha
pMC125	Derivative of pMC114. Encodes *C. pecorum* G5S_0729-2HA under the control of *Ptet*. A DNA fragment containing *G5S_0729* was amplified from pMC110 using primers 2718 and 2803. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha
pMC126	Derivative of pMC114. Encodes *C. pecorum* G5S_0733-2HA under the control of *Ptet*. A DNA fragment containing *G5S_0733* was amplified from pMC112 using primers 2724 and 2804. The resulting DNA product was digested with NdeI and NotI and inserted into those sites of pMC114 (Amp^R).	Maria da Cunha
pIL1	Expresses Q499_0114A-2HA under the control of *cteG* promoter. A DNA fragment containing *Q499_011A* was amplified from pMC116 and a DNA fragment containing *cteG* promoter was amplified from pSVP264. The resulting DNA product was fused to pSVP247 (Amp^R).	This work
pIL2	Expresses Cpn_0405-2HA under the control of *cteG* promoter. A DNA fragment containing *Cpn_0405* was amplified from pMC118 and a DNA fragment containing *cteG* promoter was amplified from pSVP264. The resulting DNA product was fused to pSVP247 (Amp^R).	This work
pIL3	Expresses TC_0381-2HA under the control of *cteG* promoter. A DNA fragment containing *TC_0381* was amplified from pMC119 and a DNA fragment containing *cteG* promoter was amplified from pSVP264. The resulting DNA product was fused to pSVP247 (Amp^R).	This work
pIL4	Derivative of pSVP264 encoding $CteG_{\Delta 298\text{-}358}$ with a double hemagglutinin epitope tag (2HA) at its C-terminus. A DNA fragment encoding CteG without the amino acid residues 298 to 358 was amplified from pSVP264 using primers 1680, 2853, 2854 and 1552. The resulting DNA product was digested with	This work

	KpnI and NotI and inserted into those sites of pSVP264 (Amp^R).	
pIL5	Derivative of pSVP264 encoding $CteG_{\Delta 308-317}$ with a double hemagglutinin epitope tag (2HA) at its C-terminus. A DNA fragment encoding CteG without the amino acid residues 308 to 317 was amplified from pSVP264 using primers 1680, 2855, 2856 and 1552. The resulting DNA product was digested with KpnI and NotI and inserted into those sites of pSVP264 (Amp^R).	This work
pIL6	Derivative of pSVP264 encoding $CteG_{1-298}$ with a double hemagglutinin epitope tag (2HA) at its C-terminus. A DNA fragment encoding CteG without its first 298 amino acid residues was amplified from pSVP264 using primers 1680 and 2875. The resulting DNA product was digested with KpnI and NotI and inserted into those sites of pSVP247 (Amp^R).	This work
pIL7	Derivative of pSVP264 encoding $CteG_{1-358}$ with a double hemagglutinin epitope tag (2HA) at its C-terminus. A DNA fragment encoding CteG without its first 358 amino acid residues was amplified from pSVP264 using primers 1680 and 2876. The resulting DNA product was digested with KpnI and NotI and inserted into those sites of pSVP247 (Amp^R).	This work
pIL8	Transfection vector encoding mEGFP-$CteG_{\Delta 298-358}$. A DNA fragment encoding CteG without the amino acid residues 298 to 358 was amplified from pALT4 using primers 652, 2853, 2854 and 653. The resulting DNA product was digested with SalI and KpnI and inserted into those sites of pALT4 (Km^R).	This work
pIL9	Transfection vector encoding mEGFP-$CteG_{\Delta 308-317}$. A DNA fragment encoding CteG without the amino acid residues 308 to 317 was amplified from pALT4 using primers 652, 2855, 2856 and 653. The resulting DNA product was digested with SalI and KpnI and inserted into those sites of pALT4 (Km^R).	This work
pIL10	Transfection vector encoding mEGFP-$CteG_{1-298}$. A DNA fragment encoding CteG without its first 298 amino acid residues was amplified from pALT4 using primers 652 and 2859. The resulting DNA product was digested with SalI and KpnI and inserted into those sites of pALT4 (Km^R).	This work
pIL11	Transfection vector encoding mEGFP-$CteG_{1-358}$. A DNA fragment encoding CteG without its first 358 amino acid residues was amplified from pALT4 using	This work

	primers 652 and 2860. The resulting DNA product was digested with SalI and KpnI and inserted into those sites of pALT4 (Km^R).	
pIL12	Transfection vector encoding mEGFP-CteG$_{\Delta 444\text{-}455}$. A DNA fragment encoding CteG without the amino acid residues 444 to 455 was amplified from pALT4 using primers 652, 2861, 2862 and 653. The resulting DNA product was digested with SalI and KpnI and inserted into those sites of pALT4 (Km^R).	This work
pIL13	Transfection vector encoding mEGFP-CteG$_{\Delta 514\text{-}534}$. A DNA fragment encoding CteG without the amino acid residues 514 to 534 was amplified from pALT4 using primers 652, 2863, 2864 and 653. The resulting DNA product was digested with SalI and KpnI and inserted into those sites of pALT4 (Km^R).	This work

Table A.2 - Primers used in this work.

Number	Description	Sequence (5′ → 3′)	Restriction enzyme
652	Forward primer to construct pALT4, pIL8, pIL9, pIL10, pIL11, pIL12, pIL13	GATCGATCGTCGACTCATTTGGTATTGGTAGTGC	SalI
653	Reverse primer to construct pALT4, pIL8, pIL9, pIL12, pIL13	GATCGGTACCCTAGATAGAGGAGCTTTGCACACC	KpnI
1552	Reverse primer to construct pSVP264, pIL4, pIL5	GATCGCGGCCGCGGATAGAGGAGCTTTGCACACC	NotI
1680	Forward primer to construct pSVP264, pIL3, pIL4, pIL5, pIL6, pIL7	GATCGGTACCTTCTTTATTATTGAGAAACG	KpnI
2795	Reverse primer to construct pIL1	GATCGCGGCCGCGGCCCAGCCAAGGGTACCC	NotI
2797	Reverse primer to construct pIL3	GATCGCGGCCGCGAGCCTCAGTAACCTTTACCAC	NotI

2836	Used to construct pIL1 by PCR overlap reaction	GAATCCGGGAGTTAAAGGTATGTCTGCACTAGGTAGC	-
2837	Forward primer to construct pIL1	GATCCATATGGGTACCTTCTTTATTATTGAGAAACG	NdeI
2838	Used to construct pIL1 by PCR overlap reaction	GCTACCTAGTGCAGACATACCTTTAACTCCCGGATTC	-
2841	Used to construct pIL3 by PCR overlap reaction	CCACTAATACCTAACGACATACCTTTAACTCCCGGATTC	-
2842	Used to construct pIL3 by PCR overlap reaction	GAATCCGGGAGTTAAAGGTATGTCGTTAGGTATTAGTGG	-
2853	Forward primer to construct pIL4, pIL8	ATCACCATGGGCAAATTTGGAAGAATATGC	NcoI
2854	Reverse primer to construct pIL4, pIL8	ATCACCATGGGCTTTGCGCAGCTAAC	NcoI
2855	Forward primer to construct pIL5, pIL9	ATCACCATGGGCAAGTGCGTGTGG	NcoI
2856	Reverse primer to construct pIL5, pIL9	ATCACCATGGACACAATAACGCGC	NcoI
2859	Reverse primer to construct pIL10	ATGGTACCGCTTTGCGCAGCTAAC	KpnI
2860	Reverse primer to construct pIL11	ATGGTACCTGCTTCTTCTGCAGG	KpnI
2861	Forward primer to construct pIL12	ATCACCATGGGCAGGCGGTACACAT	NcoI
2862	Reverse primer to construct pIL12	ATCACCATGGGGGGTTATCTAAAAT	NcoI
2863	Forward primer to construct pIL13	ATCACCATGGGGAAATGATCGCAGA	NcoI
2864	Reverse primer to construct pIL13	ATCACCATGGATTTAGCCCTTGACC	NcoI
2875	Reverse primer to construct pIL6	ATATGCGGCCGCAGCTTTGCGCAGCTAACATC	NotI
2876	Reverse primer to construct pIL7	ATATGCGGCCGCATGCTTCTTCTGCAGGAGAC	NotI

The underlined nucleotides represent the ones recognized by the restriction enzyme.

Table A.3 - *C. trachomatis* strains used and constructed in this work.

Strains	Description	Source/Reference
cteG::aadA	Derivative of L2/434/Bu ACE051 with *cteG* inactivated.	Pais *et al*, 2019
cteG::aadA (pSVP264)	Derivative of *cteG::aadA* carrying plasmid pSVP264.	Pais *et al*, 2019
cteG::aadA (pMC114)	Derivative of *cteG::aadA* carrying plasmid pMC114.	Maria da Cunha
cteG::aadA (pMC115)	Derivative of *cteG::aadA* carrying plasmid pMC115.	This work
cteG::aadA (pMC116)	Derivative of *cteG::aadA* carrying plasmid pMC116.	This work
cteG::aadA (pMC117)	Derivative of *cteG::aadA* carrying plasmid pMC117.	This work
cteG::aadA (pMC118)	Derivative of *cteG::aadA* carrying plasmid pMC118.	Maria da Cunha
cteG::aadA (pMC119)	Derivative of *cteG::aadA* carrying plasmid pMC119.	Maria da Cunha
cteG::aadA (pMC120)	Derivative of *cteG::aadA* carrying plasmid pMC120.	Maria da Cunha
cteG::aadA (pMC121)	Derivative of *cteG::aadA* carrying plasmid pMC121.	Maria da Cunha
cteG::aadA (pMC123)	Derivative of *cteG::aadA* carrying plasmid pMC123.	Maria da Cunha
cteG::aadA (pMC124)	Derivative of *cteG::aadA* carrying plasmid pMC124.	This work
cteG::aadA (pMC125)	Derivative of *cteG::aadA* carrying plasmid pMC125.	This work
cteG::aadA (pMC126)	Derivative of *cteG::aadA* carrying plasmid pMC126.	This work
cteG::aadA (pIL1)	Derivative of *cteG::aadA* carrying plasmid pIL1.	This work
cteG::aadA (pIL3)	Derivative of *cteG::aadA* carrying plasmid pIL3.	This work
cteG::aadA (pIL4)	Derivative of *cteG::aadA* carrying plasmid pIL4.	This work
cteG::aadA (pIL5)	Derivative of *cteG::aadA* carrying plasmid pIL5.	This work
cteG::aadA (pIL6)	Derivative of *cteG::aadA* carrying plasmid pIL6.	This work
cteG::aadA (pIL7)	Derivative of *cteG::aadA* carrying plasmid pIL7.	This work

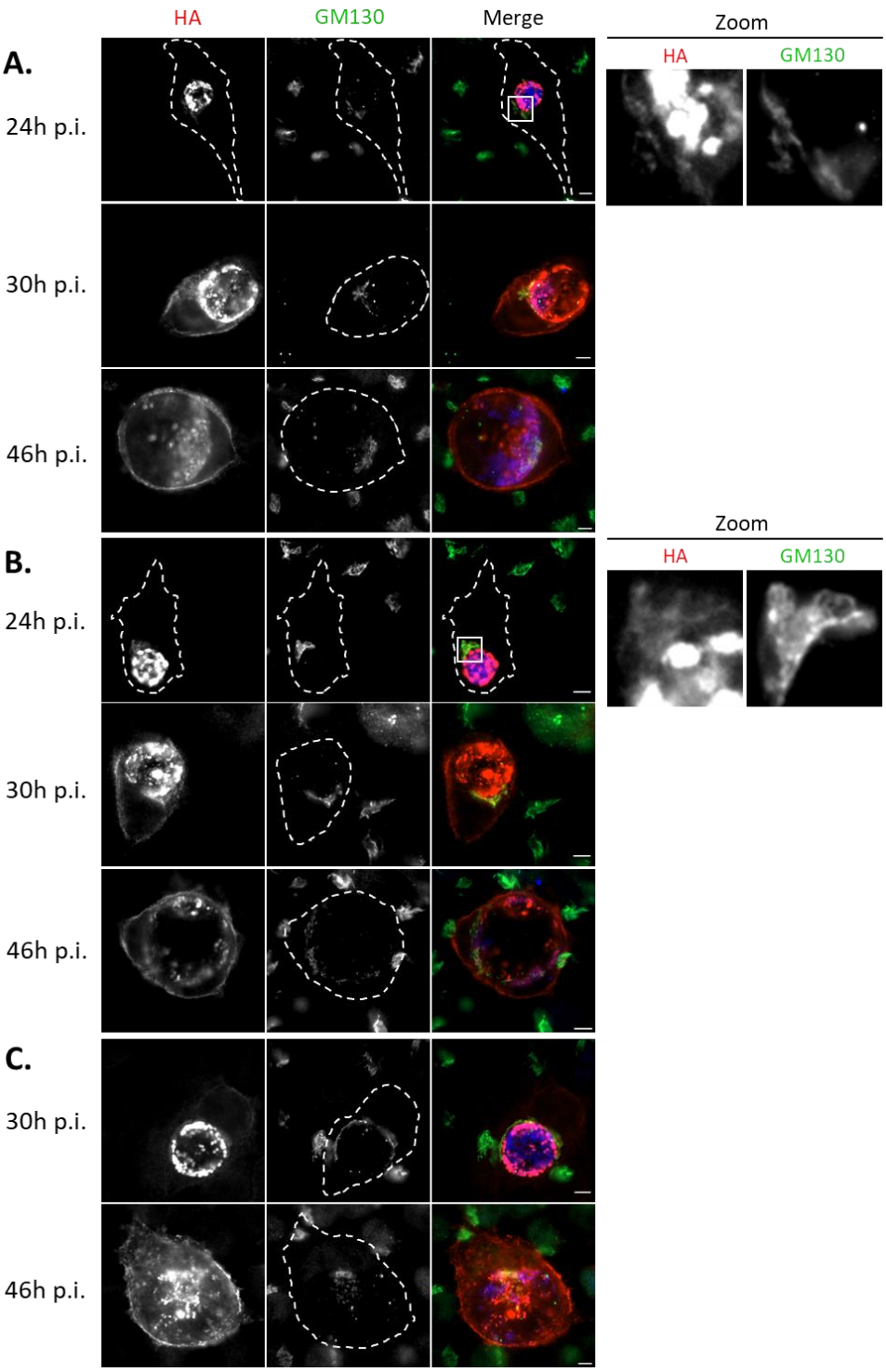

Figure A.1 - Analysis of other timepoints to add anhydrotetracycline to induce expression of *cteG*. HeLa 229 cells were infected, at a MOI of 0.3, with *C. trachomatis cteG::aadA* harboring p*Ptet*-CteG-2HA with anhydrotetracycline added at (A) 4 h post-infection (p.i.), (B) 8 h p.i. or (C) 24 h p.i. At 24, 30 and 46 h p.i., infected cells were fixed with 4% (w/v) PFA and immunolabelled with antibodies against *C. trachomatis* MOMP (blue), HA (red) and GM130 (green), and appropriate fluorophore-conjugated secondary antibodies. The fixed cells were then analyzed by immunofluorescence microscopy. In the area delimited by a white square, images were zoomed. Scale bars, 5 µm.