The role of human Ephrin receptor tyrosine kinase A2 (EphA2) in *Chlamydia trachomatis* infection

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Die Rolle der humanen Rezeptor-Tyrosinkinase EphrinA2 (EphA2) in der *Chlamydia trachomatis* Infektion

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I hereby confirm that the thesis entitled ***'The role of human Ephrin receptor tyrosine kinase A2 (EphA2) in Chlamydia trachomatis infection'*** is the result of my own work. I did not receive any help or support from any commercial consultants. All sources and/or materials applied are specified in the thesis.

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Hiermit erkläre ich an Eides statt, die Dissertation *'**Die Rolle der humanen Rezeptor-Tyrosinkinase EphrinA2 (EphA2) in der Chlamydia trachomatis Infektion**'* eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahen vorgelegen hat.

Würzburg,

Dedicated to my beloved Parents

Dad & Mom

M. Subbarayal & S. Dhanalakshmi

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I bow my knees before the Almighty God and Saviour for everything

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Prema Subbarayal

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III. ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µl	Microliter
AB	Aberrant body
AKAP1	A-kinase anchor protein1
APS	Ammonium persulfate
ATCC	American type culture collection
Bad	Bcl-2 antagonist, causing cell death
Bag-1	Bcl-2-associated athanogene -1
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-associated X protein
Bcl-2	B-cell lymphoma 2
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyltransferase
CHO	Chinese hamster ovary cells
CHX	Cycloheximide
CnBr	Cyanobromide
CPAF	Chlamydial protease-like activity factor
Ctr	*Chlamydia trachomatis*
DA	Dasatinib
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
Dpi	Days post infection
EB	Elementary body
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal growth factor receptor
eNOS	Nitric oxide synthase
Eph	Erythropoietin-producing human hepatocellular carcinoma line
EphA2	EphrinA2 receptor
EphA2ΔIC	EphA2 lacking intracellular cytoplasmic domain
ER	Endoplasmic reticulum
ERAL1	Era-like 12S mitochondrial rRNA chaperone 1
ERK	Extracellular signal regulated kinase
Erp72	Endoplasmic reticulum protein of 72 kDa
FACS	Flow cytometry
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FGF2	Fibroblast growth factor 2

FGFR	Fibroblast growth factor receptor
Fimb	Fimbriae of uterine tube epithelial cells
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
gH	glycoproteins H
gL	glycoproteins L
GLUTs	Glucose transporters
GP96	Glucose regulated protein96
GPI	Glycosylphosphotidylinositol
GPP130	Golgi phosphoprotein of 130 kDa
GSK3	Glycogen Synthase Kinase-3
h	hour(s)
HA	Hemagglutinin
Hcl	Hydrochloric acid
HDM2	Human form of murine double minute 2
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hex1	Hexokinase1
HHV-6	Human herpes virus-6
HI-*Ctr*	Heat inactivated-*Ctr*
Hpi	Hours post infection
Hsp70	Heat shock protein 70
HUVEC	Human umblical vein endothelial cells
i.e.	id est, that is
IF	Immunofluorescence
IFN-γ	Interferon gamma
IgG	Immunoglobulin
IKK	I-κB Kinase
IL-8	Interleukin-8
Inc	Inclusion membrane proteins
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Iso	Isotype
kDa	Kilo Dalton
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpes virus
L	Liter
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LGV	Lymphogranulomavenereum
M	Molar
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell lymphoma-1

MDM2	Mouse double minute 2 homolog
MEK	MAP ERK kinase
ml	Milliliter
mM	Milli molar
min	Minutes
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
Ms	Mouse
mTOR	Mammalian target of rapamycin
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
nM	Nano molar
OMPs	Outer membrane proteins
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffer saline
PDGFRβ	Platelet-derived growth factor receptor
PDI	Protein disulfide isomerase
PEI	Polyethylenimine
PHB	Prohibitin
PI3K	Phosphoinositol 3-Kinase
PID	Pelvic inflammatory disease
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKB	Protein Kinase-B
PTEN	Phosphatase and tensin
PTPMT1	Protein tyrosine phosphatase, mitochondrial 1
PVDF	Polyvinylidene difluoride
qRT PCR	Quantitative real time PCR
Rb	Rabbit
RB	Reticulate body
rhEphA2	recombinant human EphA2
RNA	Ribonucleic acid
RNAi	RNA interference
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RTKs	Receptor tyrosine kinases
SAM	Sterile alpha motif
Sam50	Sorting assembly machinery50
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamidegel electrophoresis
Sec	Second(s)
siRNA	Small interfering RNA

Sn	*Simkania negevensis*
SNARES	Soluble NSF attachment protein receptor
SPG	Sucrose phosphate glutamate
STS	Staurosporine
T3SS	Type III secretion system
TARP	Translocated actin recruiting phosphoprotein
TBS	Tris buffer saline
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TNFR1	Tumor necrosis factor receptor 1
TNF-α	Tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase
UN	Uninfected
v/v	Volume per volume
Vamp	Vesicle associated membrane proteins
VAPB	Vamp-associated protein B
w/v	weight per volume
WB	Western blot
µM	Micro molar

IV. ABSTRACT

Chlamydia trachomatis (*Ctr*), an obligate intracellular gram negative human pathogen, causes sexually transmitted diseases and acquired blindness in developing countries. The infectious elementary bodies (EB) of *Ctr* involved in adherence and invasion processes are critical for chlamydial infectivity and subsequent pathogenesis which requires cooperative interaction of several host cell factors. Few receptors have been known for this early event, yet the molecular mechanism of these receptors involvement throughout *Ctr* infection is not known.

Chlamydial inclusion membrane serves as a signaling platform that coordinates *Chlamydia*-host cell interaction which encouraged me to look for host cell factors that associates with the inclusion membrane, using proteome analysis. The role of these factors in chlamydial replication was analyzed by RNA interference (RNAi) (in collaboration with AG Thomas Meyer). Interestingly, EphrinA2 receptor (EphA2), a cell surface tyrosine kinase receptor, implicated in many cancers, was identified as one of the potential candidates. Due to the presence of EphA2 in the *Ctr* inclusion proteome data, I investigated the role of EphA2 in *Ctr* infection.

EphA2 was identified as a direct interacting receptor for adherence and entry of *C. trachomatis.* Pre-incubation of *Ctr*-EB with recombinant human EphA2, knockdown of EphA2 by siRNA, pretreatment of cells with anti-EphA2 antibodies or the tyrosine kinase inhibitor dasatinib significantly reduced *Ctr* infection. This marked reduction of *Ctr* infection was seen with both epithelial and endothelial cells used in this study. *Ctr* activates EphA2 upon infection and invades the cell together with the activated EphA2 receptor that interacts and activates PI3K survival signal, promoting chlamydial replication. EphA2 upregulation during infection is associated with *Ctr* inclusion membrane inside the cell and are prevented being translocated to the cell surface.

Ephrins are natural ligands for Ephrin receptors that repress the activation of the PI3K/Akt pathway in a process called reverse signaling. Purified Ephrin-A1, a ligand of EphA2, strongly interferes with chlamydial infection and normal development, supporting the central role of these receptors in *Chlamydia* infection. Overexpression of full length EphA2, but not the mutant form lacking the intracellular cytoplasmic domain, enhanced PI3K activation and *Ctr* infection. *Ctr* infection induces EphA2 upregulation and is mediated by activation of ERK signaling pathway. Interfering with EphA2 upregulation sensitizes *Ctr*-infected cells to apoptosis induced by tumor necrosis factor-alpha (TNF-α) suggesting the importance of intracellular EphA2 signaling.

Collectively, these results revealed the first Ephrin receptor "EphA2" that functions in promoting chlamydial infection. In addition, the engagement of a cell surface receptor at the inclusion membrane is a new mechanism how *Chlamydia* subverts the host cell and induces apoptosis resistance. By applying the natural ligand Ephrin-A1 and targeting EphA2 offers a promising new approach to interfere with *Chlamydia* infection. Thus, the work provides the evidence for a host cell surface tyrosine kinase receptor that is exploited for invasion as well as for receptor-mediated intracellular signaling to facilitate the chlamydial replication.

V. ZUSAMMENFASSUNG

Chlamydia trachomatis (Ctr) ist ein obligat intrazellulär lebendes Gram negatives Bakterium, das Geschlechtskrankheiten verursachen kann. In Entwicklungsländern führt es zudem häufig zu erworbener Blindheit. Die infektiösen Elementarkörper (EB) sind für die Anheftung an die Wirtszelle sowie die Aufnahme von *Ctr* in die Wirtzelle verantwortlich. Dies ist ein wichtiger Schritt, da nur so die sich anschließende Krankheitsentwicklung stattfinden kann. Diese ist auch abhängig vom engen Zusammenspiel der *Ctr* Proteine mit den Wirtszellfaktoren. Obgleich dieser Schritt so wichtig ist, wurden erst wenige Wirtszellrezeptoren gefunden und welche Rolle diese Rezeptoren im weiteren Verlauf der Infektion spielen, ist noch nicht richtig verstanden.

Die chlamydiale Inklusionsmembran fungiert als Signalplattform, die das Zusammenspiel von Chlamydien und Wirtszelle koordiniert. In dieser Arbeit wurden die Wirtszellproteine, die an der Inklusionsmembran lokalisiert sind, mit Hilfe einer Proteomanalyse identifiziert. Anschließend wurde die Rolle dieser Proteine bei der Chlamydienvermehrung in einem RNAi screen untersucht (in Zusammenarbeit mit der AG Thomas Meyer). Hier wurde überraschenderweise der EphrinA2 Rezeptor, eine sich auf der Oberfläche der Zellen befindliche Rezeptor Tyrosin Kinase, die vor allem mit Krebs in Verbindung gebracht wird, als ein potentieller Kandidat identifiziert. Da die Proteomdaten gezeigt haben, dass EphrinA2 an der Inklusionsmembran lokalisiert ist, wurde die Rolle von EphrinA2 während der *Ctr* Infektion hier näher untersucht.

Es konnte gezeigt werden, dass EphrinA2 ein direkter Rezeptor für *Ctr* ist, der sowohl die Adhärenz als auch die Aufnahme von *Ctr* in die Wirtszelle bewerkstelligt. Vorinkubation von *Ctr*-EB mit rekombinantem menschlichen EphrinA2, das herunterregulieren von EphrinA2 mit Hilfe einer siRNA oder das Vorinkubieren der menschlichen Zelle mit Antikörpern gegen EphrinA2 oder dem Tyrosinkinase Inhibitor Dasatinib, reduzierten die *Ctr* Infektion signifikant. Diese drastische Reduktion der *Ctr* Infektion wurde sowohl in Epithelzellen als auch in Endothelzellen

beobachtet. *Ctr* aktiviert EphrinA2 während der Infektion und invadiert die Wirtszelle zusammen mit dem aktivierten Rezeptor, dieser interagiert mit dem aktivierten PI3K Überlebenssignal, was die Replikation der Chlamydien ermöglicht. An der Inklusionsmembran akkumuliert EphrinA2, da der Transport von neuem Rezeptor zur Zellmembran unterbunden ist. Ephrine sind die natürlichen Liganden der Ephrinrezeptoren, sie unterdrücken die Aktivierung des PI3K/Akt Signalweges in einem Prozess, der reverse Signalübertragung genannt wird.

Aufgereinigtes Ephrin-A1, ein Ligand des EphrinA2 Rezeptors, verhindert eine normale Chlamydieninfektion, was eine zentrale Rolle dieses Rezeptors weiterhin bestätigt. Die Überexpression von EphrinA2, erhöhte die PI3K Aktivierung und *Ctr* Infektion. Dies war nicht der Fall, wenn eine Mutante, der die intrazelluläre Domäne fehlt, überexprimiert wurde. Eine *Ctr* Infektion induziert die Hochregulierung von EphrinA2, welche durch die Aktivierung des ERK Signalwegs bewerkstelligt wird. Wenn die Hochregulierung von EphrinA2 verhindert wird, werden *Ctr* infizierte Zellen sensitiver für Apoptose induziert durch tumor necrosis factor-alpha (TNF-α), was ein weiter Hinweis für die Bedeutung der intrazellulären EphrinA2 Signalübermittlung ist.

Insgesamt haben diese Ergebnisse den ersten Ephrin Rezeptor "EphA2" offenbart, der in der Förderung chlamydialer Infektionen fungiert. Hinzu kommt, dass die Bindung eines Oberflächenrezeptors an die Inklusionsmembran ein neuer Mechanismus ist, die Wirtszelle zu verändern und eine Apoptoseresistenz in der Zelle zu induzieren. Die Zugabe des natürlichen Liganden Ephrin-A1 eröffnet eine neue vielversprechende Möglichkeit Chlamydieninfektionen zu bekämpfen. Daher liefert diese Arbeit erste Hinweise, das eine Wirtszelltyrosinkinase, die sich an der Zelloberfläche befindet, notwendig ist für die Invasion und die intrazelluläre Signalübermittlung, welche für die chlamydiale Replikation notwendig ist, essentiell ist.

1. INTRODUCTION

1.1. *Chlamydiales*

Chlamydia, an obligate intracellular, gram-negative human pathogen, displays a unique biphasic developmental life cycle and infects a variety of organisms [1]. *Chlamydia* cause a wide range of severe human diseases, such as infections of the urogenital tract, the eye causing a preventable blindness called trachoma and also in the lung causing respiratory tract diseases [2]. *Chlamydia* interferes with several host signaling pathways and accomplish their intracellular lifestyle successfully by acquiring nutrients and signals from the host cell [3]. Therefore, a broad analysis of how *Chlamydia* makes their association and replication with and within the host have to be investigated which is of strong scientific interest.

1.1.1. Taxonomy of *Chlamydia*

The order Chlamydiales is subdivided into four families based on the taxonomic analysis of 16S and 23S rRNA genes including *Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae* [4, 5]. The family *Chlamydiaceae* is subdivided into two genera, *Chlamydophila* and *Chlamydia* encompassing nine species, which include the most important human pathogenic species especially *Chlamydia trachomatis,* the infectious agent associated with blindness and *Chlamydophila pneumoniae,* the major cause of pneumonia (Figure 1.1). Three new non *Chlamydiaceae* families like the *Parachlamydiaceae, Waddliaceae* and *Simkaniaceae* were also added as drawn below. Different species in the order Chlamydiales, their specific host and mode of entry are listed out in the table 1.1.

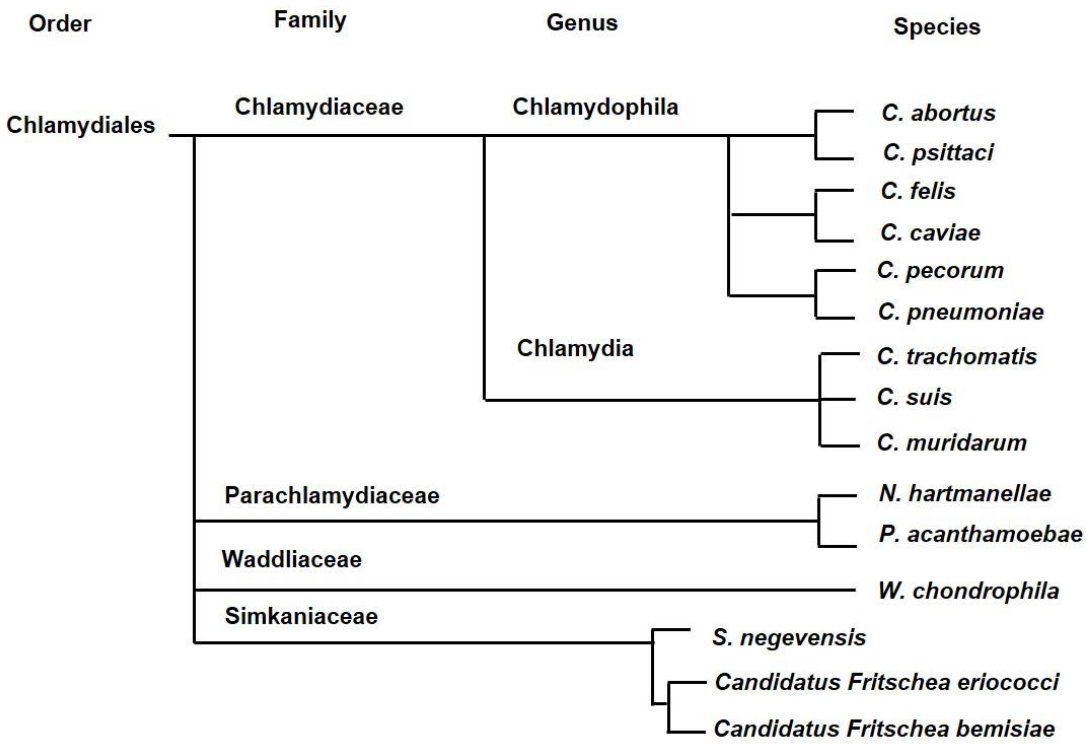

Figure 1.1. Taxonomy of the order *Chlamydiales* (adapted from [4]). The phylogenetic tree of the order Chlamydiales consisting of four families: *Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae*. The family *Chlamydiaceae* is divided into the two genera *Chlamydophila* and *Chlamydia*. The genus *Chlamydia* is divided into *C. trachomatis, C. suis* and *C. muridarum*. The lengths of the lines do not represent the actual phylogenetic distance.

Species	Host	Entry route
C. muridarum	Mouse, Hamster	Pharyngeal, genital
C. suis	Swine	Pharyngeal
C. trachomatis	Human	Pharyngeal, ocular, genital, rectal
C. pneumoniae	Human, Frog, Koala, Horse	Pharyngeal, ocular
C. caviae	Guinea pig	Pharyngeal, ocular, genital, urethal
C. psittaci	Birds	Pharyngeal, ocular, genital
C. abortus	Mammals	Oral, genital
C. felis	Cat	Pharyngeal, ocular, genital
C. pecorum	Mammals	Oral
S. negevensis	Ameoba, Human	Pharyngeal

Table 1.1. List of species in the genus *Chlamydia*, their specific hosts and mode of entry (adapted from [6]).

1.1.2. Pathology of *Chlamydia*

The two most important human pathogens of the order *Chlamydiales* are *C. trachomatis and C. pneumoniae.*

Chlamydia trachomatis (Ctr) is a leading cause of most common sexually transmitted diseases affecting about 90 million new cases each year worldwide [7], as well as potentially blinding trachoma affecting 50 million people with 1.3 million of them causing blindness [8]. Based on the immunogenic epitope analysis of the major outer membrane protein (MOMP) of *C. trachomatis* with polyclonal and monoclonal antibodies, *Ctr* is classified into 15 serologically distinguished serovars [9, 10]. These serovars can be divided into two biovars called trachoma and lymphogranuloma venereum (LGV). The trachoma serovars from A-C invades mucosal epithelia in the ocular tissue which can lead to blinding trachoma and are transmitted by direct contact with eye, nose or throat secretions from infected individuals. If not treated with antibiotics, chronic infection can lead to eyelid scarring and subsequent blindness causing severe inflammatory response [11]. The serovars from D-K infect mucosal epithelial cells of the urogenital tract causing pelvic inflammatory disease (PID), ectopic pregnancy and infertility [12-14]. The LGV serovars L1, L2 and L3 are more invasive and cause outbreaks of systemic infections in humans by invading lymph nodes thereby causing the sexually transmitted systemic syndrome lymphogranuloma venereum (LGV) [15, 16].

The species *C. pneumoniae* cause human respiratory tract diseases by infecting epithelial cells of the upper and lower respiratory tract [17, 18]. It is transmitted by droplet infection while in 70% of the cases the infection is asymptomatic [19, 20]. *C. pneumoniae* is also responsible for infections including pharyngitis, sinusitis, otitis, acute bronchitis, persistent cough, chronic obstructive pulmonary disease and Flu like syndrome [21]. The symptoms of an acute infection can range from a minor cold to a serious pneumonia while chronic infection are associated with asthma [22], atherosclerosis [23] or Alzheimer's disease [24].

If these *Chlamydia* infections are detected at proper time, they can be effectively treated with antibiotics including tetracyclines (doxycycline, tetracycline, etc), macrolides (azithromycin, clarithromycin, erythromycin, etc) and quinolones (Centers for Disease Control and Prevention, 2010, http://www.cdc.gov/std/treatment/2010/chlamydial-infections.htm). But as the infected people are often asymptomatic, the infections are not diagnosed which leads to such serious complications. Therefore, awareness should rise for education on having protected sexual intercourse as well as active screening for chlamydial infections [25].

1.1.3. Life cycle of *Chlamydia trachomatis*

The unique biphasic life cycle of *Chlamydia* includes a characteristic intracellular reproduction within a membrane-surrounded protective compartment termed “inclusion”. Electron microscope studies showed that two morphologically and structurally distinct development forms, the so-called elementary bodies (EB) and reticulate bodies (RB) exist [26] (Figure 1.2).

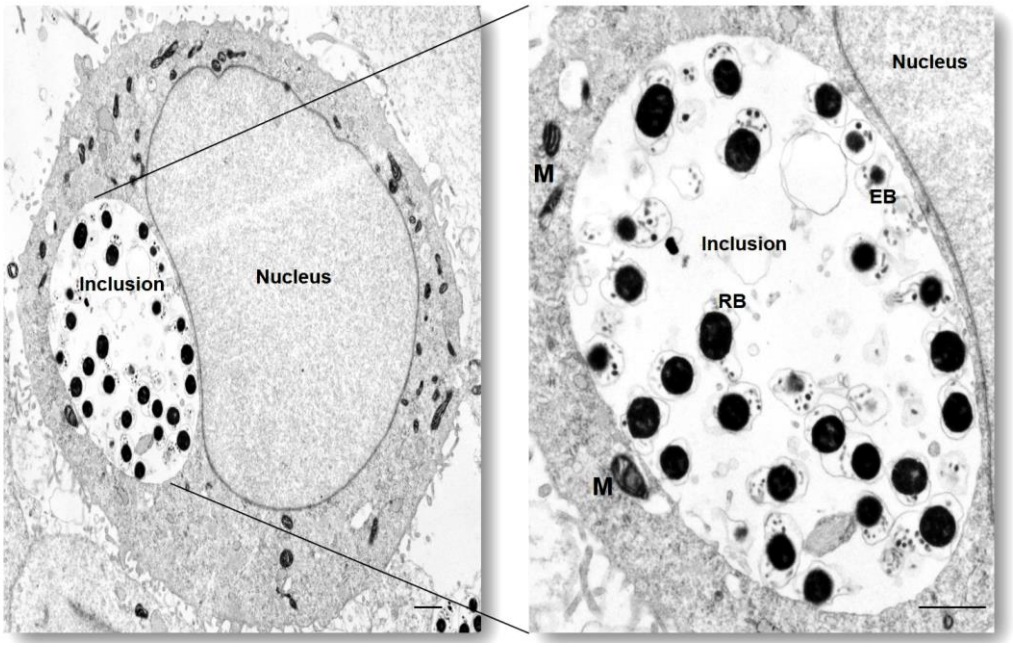

Figure 1.2. Transmission electron microscopic image of a HeLa cell-infected with *Chlamydia* MOI-1 for 24 h: The image of the inclusion with EB (Elementary body), RB (Reticulate body) and M (Mitochondria) inside the cell was magnified on the right hand side. Bars represent 2 µm at magnification 6000× (left image) and 8000× (right image).

The small metabolically inactive EB (0.3 µm) are osmotically stable and resistant to extracellular environmental stress. It binds and infects most of the cultured human cells [27]. Infection starts with the EB binding to the host cell membrane via electrostatic interaction with heparan sulphate-like glycosaminoglycans, followed by a more specific attachment to host cell receptor. Attachment is followed by uptake of the infectious particles inside the cell (endocytosis) (Figure 1.3). Within the cell and inside the inclusion, EB develops into the metabolically active, replicating but non-infectious RB with a size of approximately 1 µm [28]. To cause an acute infection, after numerous rounds of replication by binary fission, RB re-differentiate back into infectious EB that are released from the cell to infect new cells and thereby enter a new life cycle [1] (Figure 1.3). The signals inducing for the transition from EB to RB and later from RB to EB are not known. In reaction to external stimuli, such as interferon gamma (IFN-γ), penicillin, amino acid- or iron-starvation, the chlamydial development cycle can descend into persistence [29, 30]. In the persistence stage, the RB develop into aberrant bodies (AB), where the host cell is not lysed and only few infectious particles are produced [31]. In the absence of external stimuli, the EB converts back into RB which can lead to an acute infection.

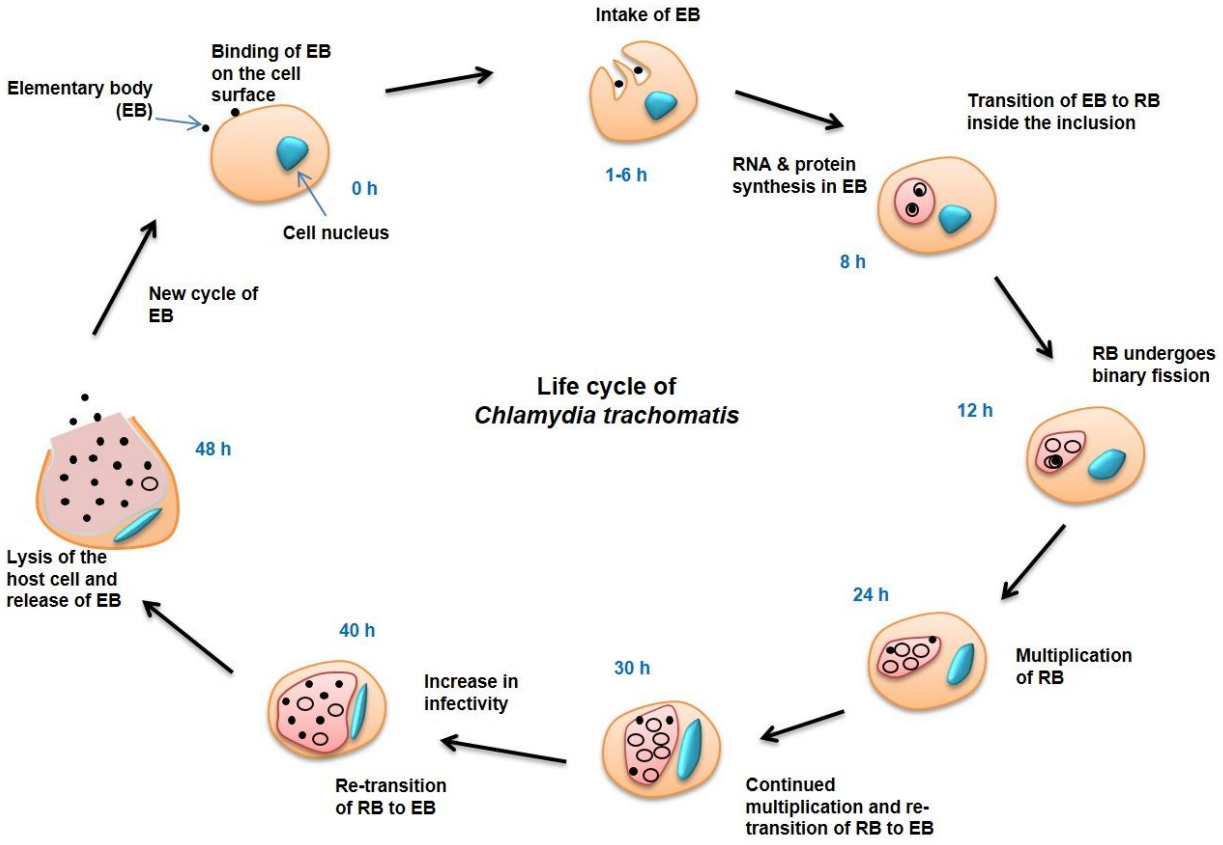

Figure 1.3. Developmental cycle of *Chlamydia*: The life cycle of *C. trachomatis* starts with the attachment of EB on the host cell surface receptors which is followed by invasion. After internalization, *Ctr* develops an inclusion followed by the differentiation of EB into RB. RB multiply inside the inclusion by binary fission followed by its re-differentiation into EB. After 48 h, the EB are released from the host cell though cell lysis in order to start a new round of infection (Modified from http://sahsrojas.pbworks.com/w/page/3695831/chlamidia).

1.2. Pathogen-Host interaction

1.2.1. *Chlamydia*-Host cell surface interaction

Chlamydia communication with the host is absolutely essential for their survival. Attachment of EB to the surface of the host cell initiates the infection. Like in other gram negative bacteria, the presence of a type III secretion system (T3SS) was confirmed by sequencing the chlamydial genomes [32]. The genomic arrangement of the T3SS genes differs in comparison to other gram-negative bacteria. *Chlamydia* entry into the cells is multifaceted process generally initiated

by the molecular interactions that occur between various *Chlamydia* factors secreted by T3SS or the outer membrane proteins which can interact with cell surface exposed receptor/co-receptor molecules and are fundamental to pathogenesis. Some of the chlamydial factors like major outer membrane protein [33], glycosaminoglycans [34], Hsp70 heat shock protein 70 [35], translocated actin recruiting phosphoprotein (TARP) [36, 37] and OmcB [38] whereas several host surface proteins like epithelial membrane protein 2 [39], the estrogen receptor complex [40] and its subunit protein disulfide isomerase (PDI) [40, 41] are known to be involved in the chlamydial adherence. In addition, receptor tyrosine kinases (RTKs) also play an important role in a wide range of signaling processes in development. Several host cell tyrosine kinases and their associated intracellular signaling cascades are known to play a pivotal role in facilitating *Chlamydia* adherence and regulating its infection. The host tyrosine kinase platelet-derived growth factor receptor (PDGFR) and Abl kinase are involved in the chlamydial uptake into non-phagocytic cells [37]. Another tyrosine kinase receptor, fibroblast growth factor receptor (FGFR), is activated upon *Chlamydia* infection and are recruited to the cell surface associated *Chlamydia* [42]. *Chlamydia* induce their uptake into non-phagocytic cells through small-GTPase dependent reorganization of the actin cytoskeleton [43].

1.2.2. Intracellular biology of chlamydial proteins and its interaction with host proteins

The T3SS of *Chlamydia* secretes several membrane proteins (Inc) that are anchored on its inclusion membrane which interact with host proteins in order to attain nutrients and lipids for the inclusion growth thereby ensuring its intracellular replication. Chlamydial inclusion proteins help RB to acquire iron and nutrients from the host [44]. Some of the well-studied Inc proteins are IncA, IncD and IncG. A snare motif is seen in the protein sequence of IncA which can form stable tetramers capable of fusing *Ctr* inclusions. The cytosolic portion of IncA interacts with IncA on other inclusion membranes enabling homotypic vesicle fusion [45]. Thus, IncA proteins

are important in eukaryotic compartment fusion and is able to recruit a subset of host SNARES like Vamp 8, Vamp 7 and Vamp 3 which leads to the selective recruitment of intracellular compartments around the inclusion thereby controlling the intracellular trafficking by mimicking SNAREs [46]. Apart from IncA, there is another Inc protein called *Ct*229 which can also perform such type of vesicle fusion [47].

Some Inc proteins were identified to interact with Rab GTPases and thereby selectively recruiting them to the inclusion, e.g. Rab4, Rab11, Rab1, Rab14 and Rab6 [48, 49]. *Ctr* is able to intercept trafficking vesicles via the recruitment of Rab proteins, which are able to specifically snatch vesicles from the retrograde intra-Golgi trafficking or interact with host organelles, such as the endoplasmic reticulum (ER). Several Inc proteins also associate with active Src family kinases, thereby regulating the interaction with the microtubule network and the acquisition of sphingolipids [47, 50].

Eukaryotic protein 14-3-3β, whose functions are involved in the regulation of apoptosis, cell cycle and cell differentiation, is found to interact with the cytosplasmically exposed caroxy-terminal domain of the chlamydial protein IncG which is confirmed in infected HeLa cells by indirect immunofluorescence microscopy [51]. Also the interaction level is dependent on the phosphorylation state of IncG which is phosphorylated by host or chlamydial-derived kinases. Since IncG was phosphorylated by yeast kinases via yeast hybrid system, signifying that eukaryotic kinases are capable of phosphorylating IncG allowing the specific interaction with 14-3-3β. Ser-166 of IncG is essential for the interaction between IncG and 14-3-3β as the mutation of Ser-166 to Ala completely inhibits the binding to 14-3-3β. The group found large amounts of 14-3-3β localized into the inclusion membrane during chlamydial infection and so the levels in the cytoplasm are reduced possibly altering the host signal pathway [51]. The importance of 14-3-3β binding to inclusion in the pathogenesis of chlamydial infection remains to be established. By recruiting 14-3-3β, the ligands of 14-3-3β could be recruited to the inclusion thereby

transducing some unknown signal from the cytoplasm to the interior of the inclusion and vice versa.

Another *Ctr* inclusion membrane protein IncD interacts with ER to Golgi ceramide transfer protein called CERT which partly co-localize with an ER protein-Vamp-associated protein B (VAPB) thereby exploiting the host non-vesicular lipid transport machinery [52]. Other than the inclusion membrane proteins *Chlamydia* can also secrete cytosolic effector proteins like CT671, CADD and many others localized to the cytosol of the host cell thereby interacting with the eukaryotic factors [53, 54]. Thus, *Chlamydia* intercepts numerous host trafficking pathways during infection to get lipids, amino acids, nucleotides and other nutrients (reviewed in [3]). The membrane covering the inclusion of *Chlamydia* protects the pathogen and helps their interaction with the host cells to accomplish their biosynthesis, replication and differentiation within the inclusion.

1.2.3. *Chlamydia* and cell survival pathways

Chlamydia acquires nutrients and lipids, prevents lysosomal fusion and escapes from host immune response as well as from cell death by hijacking several host signaling pathways mainly mitogen-activated protein kinases (MAPK) and phosphoinositol 3-kinase (PI3K) as shown in figure 1.4. Both these pathways are important in modulating host cell apoptosis and cell survival during *C. trachomatis* infection [55-58].

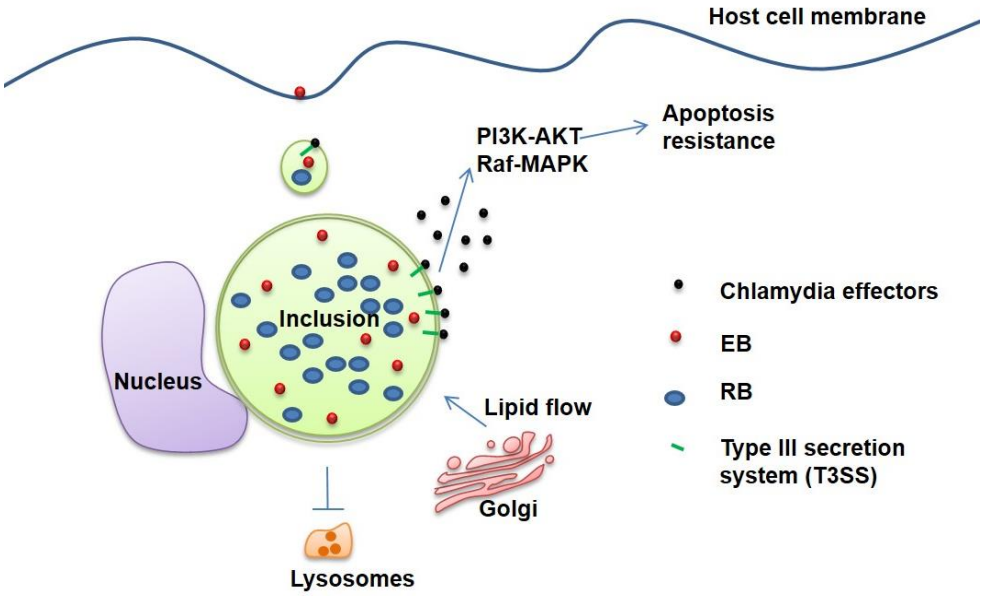

Figure 1.4. Exploitation of host cell by *Chlamydia*: *C. trachomatis* secretes inclusion membrane (Inc) proteins and other effector proteins though T3SS to interfere with several host cell pathways like PI3K-Akt and RAF-MAPK in order to acquire nutrients, overcome host defense and support the survival of the host cell.

1.2.3.1. MAPK pathway

MAPK are protein kinases that are important in regulating several cellular functions such as proliferation, gene expression, differentiation, mitosis, cell survival and apoptosis [59-61]. This pathway is activated by several events including a wide variety of receptors like receptor tyrosine kinases (RTKs), integrins and ion channels. A set of adaptors like Shc, GRB2 and Crk that links the receptor to a guanine nucleotide exchange factor and transduce the signal to small GTP-binding proteins like RAS and Rap1, which then activates cascade composed of a MAPKKK (RAF), a MAPKK (MEK1/2) and MAPK (ERK) (cell signaling pathway [62]). An activated ERK dimer translocate to the nucleus where it phosphorylates a variety of transcription factors regulating the gene expression [63] (Figure 1.5).

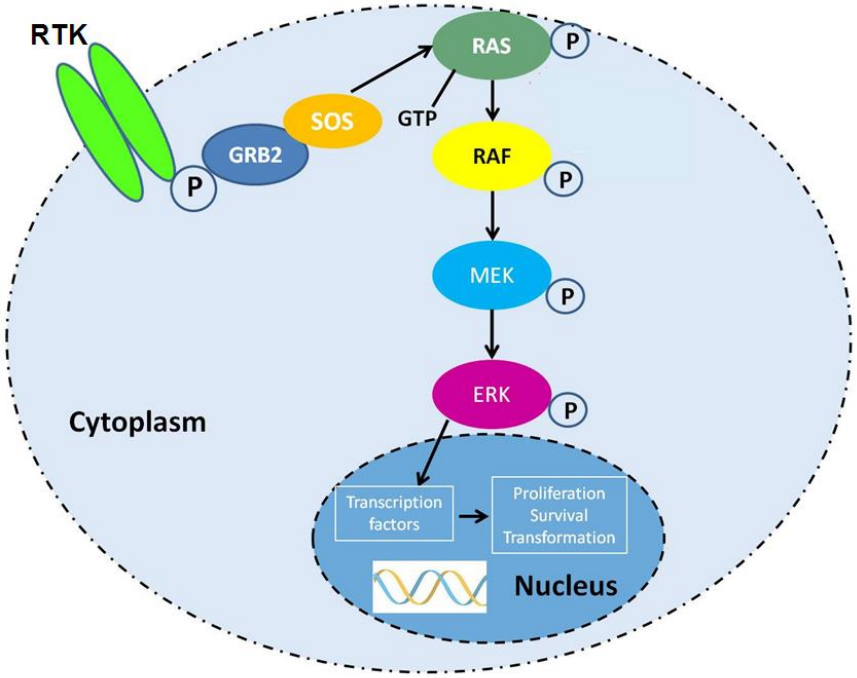

Figure 1.5. RAS/RAF/MEK/ERK pathway: The chronological phosphorylation of RAS-RAF-MEK-ERK, after dimerization of RTK upon ligand binding. The ERK cascade is constituted by RAS, RAF, MEK and ERK proteins. After the binding of the extracellular peptide with the RTK and its dimerization, it activates the RAS protein, which then activates the RAF family, MEK and, finally, ERK protein which regulates the gene expression by phosphorylating the transcription factors (modified-http://www.intechopen.com/books/).

Chlamydial efficient internalization and anti-apoptotic activity involves the activation of the MEK/ERK survival pathway. Inhibition of the pathway sensitized *Chlamydia*-infected cells to STS-mediated cell apoptosis [64]. The activation of ERK during *Chlamydia* infection is followed by the downstream regulation of several molecules like cPLA2, required for the chlamydial uptake of host glycerophospholipids [65] and also for regulating the intracellular immunity to *Chlamydia* [66]. *Ctr*-mediated ERK activation also leads to the time-dependent induction of interleukin-8 (IL-8) [67, 68], shedding of TNF receptor 1 (TNFR1) [69] and anti-apoptotic Mcl-1 stabilization [70]. Further, stimulation of fibroblast growth factor 2 (FGF2) [42] and induction of anti-apoptotic Bcl-2-associated athanogene-1 (Bag-1) [71] are also associated with ERK activation during *Ctr* infection which are necessary for the intracellular development.

1.2.3.2. PI3K pathway

Another pathway that plays a major role in protecting *Chlamydia* from apoptosis is PI3K pathway [58]. Akt or Protein Kinase-B (PKB), downstream of PI3K, is a serine/threonine kinase involved in mediating various biological responses like inhibition of apoptosis and stimulation of cell proliferation [72, 73]. Activation of Akt begins with the binding of a ligand to a receptor in the cell membrane. The ligands like growth factors, cytokines, mitogens, hormones, insulin and a variety of growth factors binds to RTK and causes autophosphorylation of tyrosine residues on the intracellular domain of the receptor. PI3K is therefore targeted to the inner cell membrane. PI3K is composed of a p110 catalytic subunit and a p85 regulatory subunit. Binding of p85 subunit of PI3K (p85-PI3K) to the phosphorylated RTK leads to conformational changes in the catalytic domain of PI3K (p110) and results in kinase activation. PI3K phosphorylates membrane bound PIP2 to PIP3 (Figure 1.6). Binding of PIP3 to the PH domain anchors Akt to the plasma membrane and allows its phosphorylation and subsequent activation [74-78]. The physiological roles of Akt include involvement in transcription factor regulation, metabolism, protein synthesis, cell cycle and apoptosis pathways [79, 80].

Akt applies its effects in the cell by phosphorylating a variety of downstream substrates most particularly Bcl-2 antagonist of cell death (BAD) [81], glucose transporters (GLUTs) [82], nitric oxide synthase (eNOS) [83], mammalian target of rapamycin (mTOR) [84], I-κB kinase (IKK), nuclear factor-κB (NF-κB) [85], Caspase 9 [86], MDM2 [87] and Glycogen Synthase Kinase-3 (GSK3) [84]. Briefly, Akt inhibits apoptosis by phosphorylating the BAD component of the Bcl-2 related protein long isoform complex (BAD/BclXL) which binds to 14-3-3 causing dissociation of the BAD/BclXL complex and allowing cell survival. Akt activates IKK, which leads to NF-κB activation leading to cell survival. Caspase9 is one of the notable substrates of Akt. Phosphorylation of Caspase9 at Ser136 by Akt decreases apoptosis by directly inhibiting the

protease activity. Akt also activates glycogen synthesis by phosphorylating and inactivating GSK3, which leads to the activation of glycogen synthase and CyclinD1. Akt phosphorylates human form of murine double minute 2 [MDM2]) (HDM2) allowing its entry into the nucleus where it targets the tumor suppressor protein p53 for degradation. The actions of Akt in the cell are abundant and diverse, nevertheless all results in anti-apoptosis or pro-cell proliferation effects.

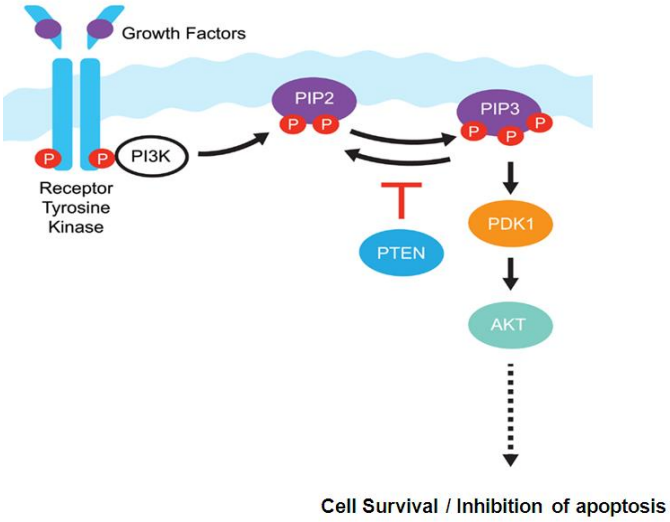

Figure 1.6. PI3K/Akt pathway: PI3K is activated after ligand binding to a receptor. The p110 subunit of PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to PIP3 which recruits Akt to the plasma membrane and results in the activation of PDK1 and Akt proteins which regulates a huge number of downstream targets. The phosphatase and tensin (PTEN) analog protein acts as a repressor by de-phosphorylating PIP3 to PIP2 and inhibiting the Akt activation (adapted from-[88]).

Chlamydia infection induces PI3K activation which is important for the subsequent phosphorylation and activation of downstream effector Akt. Activation of Akt leads to phosphorylation of Bad which is sequestrated by 14-3-3β on the surface of chlamydial inclusion (Figure 1.7). *Chlamydia* infection mediates the degradation of most of the unphosphorylated Bad but remaining Bad is sequestrated away from mitochondria by 14-3-3β to the *Chlamydia* inclusion membrane protein IncG. Thus, expression of IncG on *Chlamydia* inclusion and binding of 14-3-3β by IncG allows the inclusion to recruit phosphorylated Bad preventing the ability of

remaining bad to translocate to the mitochondria and induce host cell death [51, 89]. Further, *Chlamydia* infection leads to the MEK-dependent up-regulation of Mcl-1 transcription and PI3K-dependent stabilization of Mcl-1 protein levels. Interfering with Mcl-1 levels sensitizes infected cells to apoptosis [70]. Recently, it was found that chlamydial infection downregulates host p53 and this reduction is mediated by the PI3K/Akt signaling pathway which activates HDM2 and subsequent proteasomal degradation of p53 [90]. p53 inhibits glucose 6 phosphate dehydrogenase activity, the first rate limiting step in pentose phosphate pathway [91]. Thus, p53 stabilization impairs chlamydial development due to lack of metabolic support, as *Chlamydia* entirely depend on the uptake of amino acids and nucleotides from the host [92].

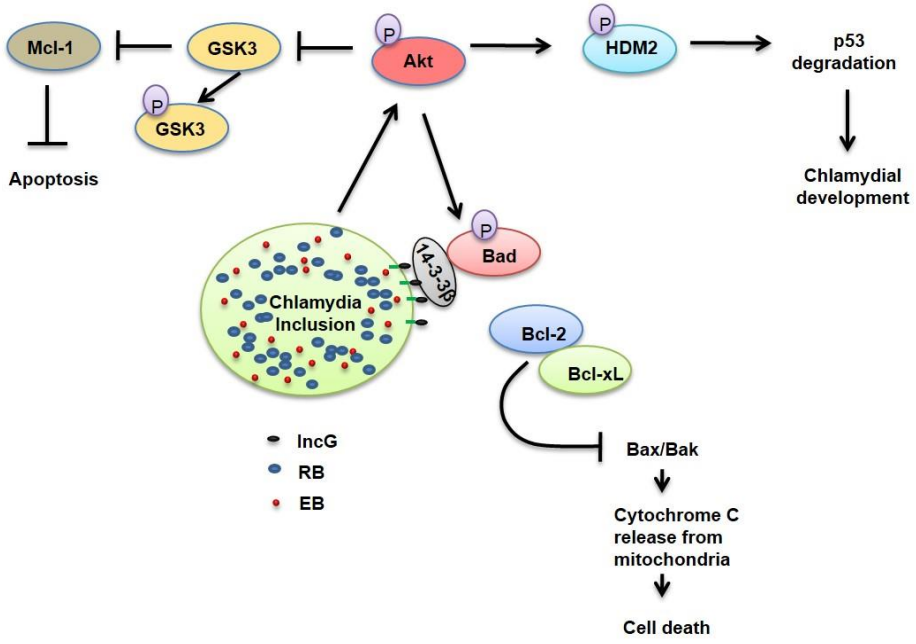

Figure 1.7. Schematic demonstration on the effects of Akt activation during *Chlamydia* infection. Dephosphorylated Bad forms heterodimer complex with Bcl-2/Bcl-xL inactivating them and allowing Bax/Bak triggered apoptosis under stress conditions. During *Chlamydia* infection, Bad is phosphorylated by activated Akt and the phosphorylated Bad gets dissociated from Bcl-2/Bcl-xL complex releasing them. Phosphorylated Bad is recruited to the *Chlamydia* inclusion by 14-3-3β. The released Bcl-2/Bcl-xL inhibits Bax/Bak triggered intrinsic apoptosis. Akt activation during chlamydial infection inactivate GSK3 by phosphorylation which prevents the inhibition of Mcl-1 that is necessary for resisting apoptosis. Further, HDM2 is phosphorylated by activated Akt which degrades p53 through preoteosomal degradation necessary for the chlamydial development.

1.3. EphA2 receptor

1.3.1. Structure of Eph receptors and Ephrin ligands

Eph receptors (Erythropoietin-producing human hepatocellular carcinoma line) are the largest subfamily of RTKs having 16 members. They can be divided into two subclasses, EphA and EphB which are encoded by the genetic loci designated *EPHA* and *EPHB* respectively [93]. They are activated in response to binding to Ephrin ligands or Eph family receptor interacting proteins which are membrane-bound and interact with an Eph receptor upon cell-to-cell contact [94]. Out of the 16 receptors, humans are known to express nine EphA (EphA1-8 and EphA10) and five EphB (EphB1-4 and EphB6) [95]. EphA/Ephrin-A interaction occur with higher affinity than EphB/Ephrin-B interactions as the former requires only little conformational change whereas the latter utilize greater amount of energy to alter the conformation of EphB to bind to Ephrin-B [96]. Like other receptor tyrosine kinases, Eph receptors also share a similar structure with an extracellular, transmembrane and cytoplasmic portion. The extracellular portion starts with Ephrin-binding (ligand binding) globular domain, followed by a cysteine-rich region and two fibronectin-type III repeats [96]. The intracellular or the cytoplasmic portion of the receptor consists of a juxtamembrane region with two autophosphorylation tyrosine sites, a highly conserved tyrosine kinase domain, SAM (sterile alpha motif) and PDZ binding motif [97] (Figure 1.8). The Ephrin-A and Ephrin-B receptor families, their functions and tissue specificities are reviewed in [98].

The structure of Ephrin is different between Ephrin-A and Ephrin-B. Ephrin-A ligands are membrane anchored proteins which do not have a transmembrane region and are linked to the membrane by a short glycosylphosphotidylinositol (GPI) anchor. Ephrin-B ligands possess a transmembrane region, a cytoplasmic portion containing a phosphorylation site and PDZ binding domain. The glycosylphosphatidylinositol-linked Ephrin-A ligands binds to EphA

receptors and the transmembrane-bound Ephrin-B ligands binds to EphB receptors [99]. Binding of an Ephrin ligand to the extracellular globular domain of an Eph receptor causes phosphorylation at the tyrosine and serine residues in the juxtamembrane region of the Eph receptor [100] followed by the activation of intracellular tyrosine kinase and consequently activate or repress downstream signaling cascades [101].

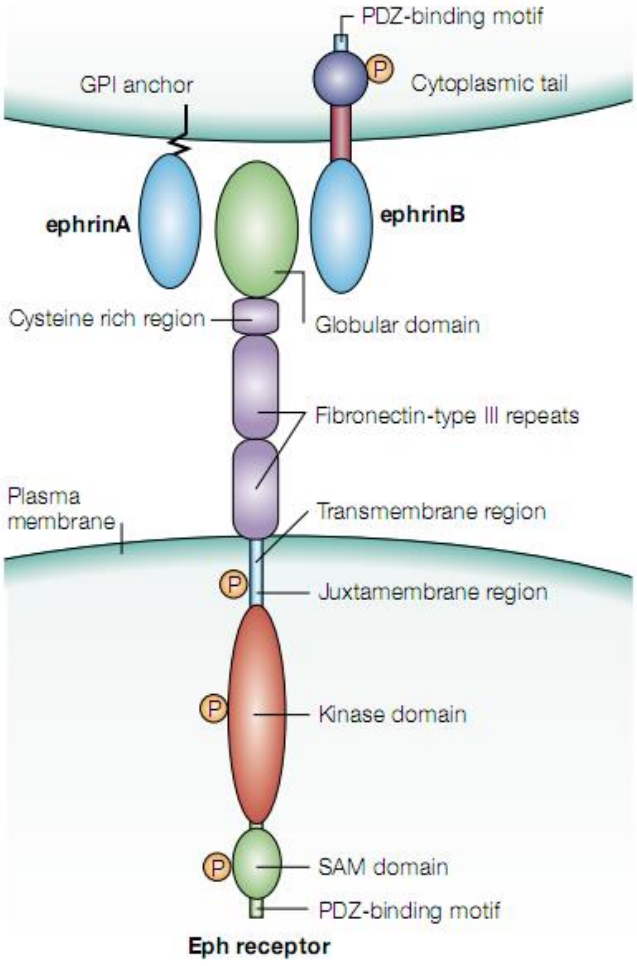

Figure 1.8. Schematic representation of Eph receptors and respective ligands [102]. Eph receptor possess a highly conserved N-terminal glycosylated extracellular ligand-binding domain, a cysteine-rich region, and two fibronectin type III repeats on the extracellular portion. The intracellular region is comprised of a juxtamembrane domain, a kinase domain, SAM and PDZ binding motif. Ephrin-A ligands are linked to the membrane by a short GPI anchor. Ephrin-B ligands possess a transmembrane region and cytoplasmic portion.

1.3.2. Eph receptor signaling

Eph/Ephrin undergoes bidirectional forward and reverse signaling which controls diverse signaling pathways and involves crosstalk with other signaling systems like Src family kinases, RAS/Rho family GTPases, PI3K/Akt, MEK/ERK and integrins along with the crosstalk of other receptors [e.g., Epidermal growth factor receptor (EGFR)]. The forward signaling is mediated by the receptor expressing cells whereas reverse signaling is initiated within ligand-expressing cells both often mediate opposite effects [103].

EphrinA2 receptor (EphA2) gene is located on chromosome 1p36. EphA2 is transcribed and translated into a 976-amino acid transmembrane glycoprotein (~130 kDa). Ephrin-A1 is a TNF-α early-inducible gene product located within chromosomal region 1q21-q22 with the molecular mass of 22 kDa and 205-amino acids. In the presence of Ephrin-A1, the receptor EphA2 tyrosine gets phosphorylated which counteracts the growth factor signaling by inhibiting activation of RAF/MEK/ERK and PI3K/Akt and this inhibition is necessary for the maintenance of epithelial homeostasis in case of normal conditions as well as during stress conditions [104, 105]. In the absence of Ephrin-A1, unligated EphA2 signaling takes place and can serve as a substrate of Akt where serine residue of EphA2 gets phosphorylated and is incapable of suppressing RAS/ERK pathway [106] (Figure 1.9). In addition, the association of EphA2 with activated PI3K was extensively studied before. The p85 subunit of PI3K interacts with phosphorylated Tyr^{734} in the kinase domain and Tyr^{929} in the SAM domain of EphA2 [107]. Further, inhibition of EphA2 affects the activation of PI3K/Akt [105, 108, 109].

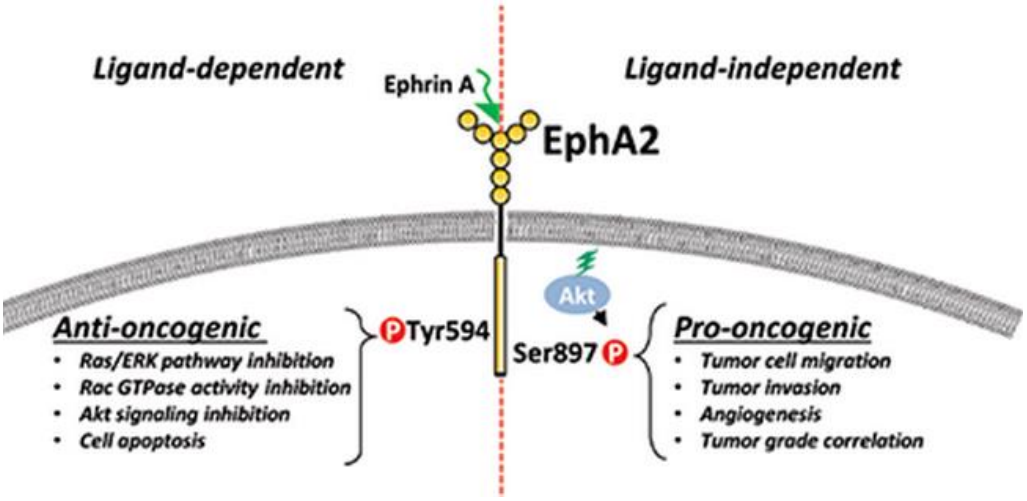

Figure 1.9. Ligand dependent and independent EphA2 signaling: (adapted from-http://www.cellapplications.com/epha2-antibody.php). Ephrin-A1 ligand-mediated activation of EphA2 signaling inhibits both RAF/MEK/ERK and PI3K/Akt pathways whereas ligand independent EphA2 signaling is important to promote those pathways [105, 110, 111]. ([105]-Bidirectional signaling).

1.3.3. EphA2 role in physiology and cancer

The role of Eph receptors was extensively studied in the developing nervous system as Eph-Ephrin bidirectional signaling is important for the communication between neurons and glial cells regulating the formation of synaptic connections [112]. Outside of nervous system, Eph-Ephrin interaction has been shown to play an important role in the immune system (thymocytes differentiation), glucose homeostasis and diabetes (insulin secretion by β cells), repair after injury, bone maintenance and remodeling (osteoblast differentiation) and intestinal homeostasis (epithelial cell differentiation and migration) (reviewed in [113]). Thus, Eph/Ephrin signaling regulates multiple host cell processes most notably involving embryonic axon guidance, angiogenesis, cell death, migration and differentiation in development and cancer [113-118].

Of all Eph receptors, EphA2 is the most studied receptor and has generated greatest interest in the recent years due to its increased expression (both mRNA and protein) levels seen in a variety of human malignancies like ovary [119, 120], breast [121], cervix [122], kidney [123],

prostate [124, 125], bladder [126], lung [127], esophagus [128], colon [129], stomach [130], and pancreas [131]. High expression of EphA2 is also detected in melanomas [132] and glioblastomas [133-135] with poor diagnosis and decreased survival [123, 126, 136, 137]. Additionally, expression of Ephrin-A1 ligand was suppressed by the activation of oncogenic signaling induced by EphA2 overexpression [138] which leads to the disruption of balance of EphA2 bi-directional signaling important to maintain the cell homeostasis (Figure 1.10).

Soluble Ephrin-A1 induces the activation of EphA2 receptor which leads to the suppression of RAS-MAPK signaling and therefore inhibits tumor cell growth as well as motility [103]. EphA2 when overexpressed exhibits low Ephrin-A1 [138, 139]. In contrast, cells that express high levels of Ephrin-A1 exhibit low EphA2 and the condition is reversed by siRNA mediated knockdown of Ephrin-A1 [138]. EphA2 is a transcriptional target of MAPK pathway and this pathway is also involved in the down regulation of Ephrin-A1 [138].

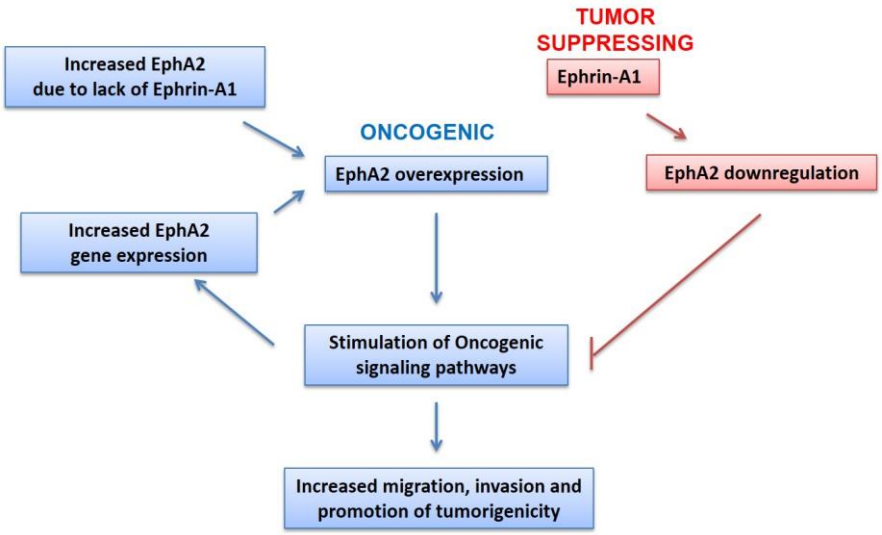

Figure 1.10. Role of EphA2 and Ephrin-A1 signaling in solid tumor cells (adapted from [140]): In the absence of Ephrin-A1, EphA2 becomes overexpressed due to increased gene expression. Overexpressed EphA2 stimulates migration, invasion and therefore the promotion of tumorigenicity. In contrast, the ligand Ephrin-A1 causes EphA2 downregulation contributing to the tumor-suppressing effects [103].

1.3.4. EphA2 receptor expression and regulation during pathogenic infection

More has been known concerning EphA2 function in cancer field and very less is known about its role in infection. EphA2 and EGFR were proposed as a cellular factors for Hepatitis C Virus (HCV) which mediate the viral entry. This demonstrated the importance of RTKs as the host factors for HCV entry and possible targets for antiviral therapy [141]. Hepatitis C, caused by the HCV, is an infectious disease affecting primarily the liver causing liver cirrhosis and hepatocellular carcinoma. Using functional RNAi screen, the group found both EGFR and EphA2 as key components due to their high expression in liver. The antibodies specific for amino terminal domain of EphA2, EphA2 surrogate ligands and EphA2 inhibitor dasatinib impaired HCV entry and infection. Both EphA2 and EGFR are involved in viral glycoprotein-dependent membrane fusion, cell to cell transmission and viral dissemination.

EphA2 was also proposed to be a cellular receptor for Kaposi's sarcoma-associated herpes virus (KSHV) glycoproteins H and L [142]. KSHV causes Kaposi's sarcoma (KS) [143], a tumor originating from lymphatic endothelial cells [144, 145]. EphA2 is co-precipitated with the glycoprotein dimeric complex H and L (gH and gL) of KSHV [142] which is required for the entry of herpesvirus into the host cells [146]. Binding of gH and gL of KSHV with EphA2 triggered EphA2 activation. Moreover, antibodies or siRNA against EphA2, pretreatment of KSHV with soluble EphA2 or pretreating the cells with EphA2 ligand inhibited the infection of KSHV, whereas EphA2 overexpression enhanced the infection. Ligand binding of EphA2 results in receptor clustering and endocytosis. Thus, KSHV may profit through this mechanism by using the ligand binding domain of EphA2 to enter the cell.

Human dermal endothelial cells infection with KSHV interacts with EphA2 and integrins in the lipid raft region followed by the viral entry by EphA2 by co-ordinating integrin-c-Cbl associated signaling. Further, EphA2 shRNA transduction reduced the phosphorylation of Src and PI3K in

KSHV infected endothelial cells which lead to reduced viral entry and infection. Thus, EphA2 acts as a master assembly regulator of KSHV entry which induces signal molecules and regulates KSHV endothelial cell entry [109, 147]. But in human foreskin fibroblast cells, KSHV enters by lipid draft independent clathrin mediated endocytosis. During its entry, KSHV induces the association of integrins with EphA2 in the non-lipid raft regions and activates EphA2 which induces the association with several downstream signaling complexes. Further, c-Cbl directed polyubiquitination of EphA2 helps interaction with accessory proteins. These interactions promote activation, recruitment and assembly of clathrin to the formation of clathrin coated pits and these events trigger the internalization of KSHV. Inhibition of EphA2 reduces the induction of these signaling complexes thereby reducing the viral entry and infection [147].

EphA2 role is also implicated in *Mycobacterium tuberculosis* whose infection interferes with host immune response by inducing EphA2. EphA2 knockout mice displayed greater pathology, higher accumulation of T cells and dendritic cells and also higher levels of proinflammatory cytokines, efficient migration of T cells than the wildtype mice. Whereas wildtype mice infection showed higher expression of EphA1, EphA2 as well as its ligand indicating that ligand dependent signaling turns to be an important mechanism for the survival of *Mycobacterium tuberculosis* during chronic phase of infection. EphA2 signaling appears to vary depending on the cell specific environment. Thus, induction of EphA2 is engaged in modulating immune response and enhancing the bacterial survival [148]. Another sexually transmitted pathogen*, Neisseria gonorrhoeae,* also induces the expression of Eph receptors like EphA2 and EphA3 in epithelial cells upon infection [140], however, the role of these receptors in *N. gonorrhoea*e infection was not examined.

2. AIM OF THE WORK

The current concept on how *Ctr* communicates with host cell during bacterial replication is based on the identification of host proteins that interacts with bacteria. *Ctr* enter the host cell by the molecular interactions that occur between *Ctr* effectors and cell surface exposed receptor molecules. Inside the cell, *Ctr* releases their virulence factors targeting and hijacking multiple host trafficking pathways in order to acquire nutrients and to prevent cell death ensuring its intracellular replication. Though few mechanisms on how *Chlamydia* interferes with certain signaling components are identified, but still it seems to be a tricky pathogen to explore. Identification and analysis of the host molecular complexes that associate with *Ctr* inclusion would facilitate to understand the mechanisms by which the pathogen effectively manipulates the host signaling pathways and escape from the host cell death pathway. Therefore, it is of utmost importance to have a global knowledge by investigating the host interacting proteins with chlamydial inclusion thereby understanding the crosstalk between *Chlamydia* and host during infection.

In the first part of my work, intact *Ctr* inclusions will be isolated from the infected cells and the host proteins that were enriched or depleted in the inclusion of *Chlamydia* will be identified *via* mass spectrometry analysis. Further, the role of those host proteins in chlamydial replication will be analyzed by RNAi (in collaboration with AG Thomas Meyer). As a validation study, the protein expression pattern of the selected hits will be analyzed by immunoblot studies in time course *Ctr* infection in order to choose a host candidate for further experimental studies to identify its crucial role in *Ctr* infection. Therefore, in the second part of my work, I aimed to investigate the role of the chosen host candidate in early infection to find out if the candidate plays any potential role in *Ctr* entry process. Further studies will be conducted, by combination of molecular approaches, to investigate the underlying signaling mechanism of the host

candidate in mid-phase *Ctr* infection and the reason behind its enrichment found in inclusion proteome data. This work shall give an idea on the role of an unidentified host factor necessary for the chlamydial development and infection.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Cell line

Cell line	Feature	Origin	Source
HeLa 229	Cervical adenocarcinoma	Human	ATCC no. CCL-2.1

Table 3.1. Human tissue culture cell line used in the study.

3.1.2. Primary cells

Cells	Tissue	Origin
HUVEC (Human Umblical Vein Endothelial cells)	Umbilical vein/vascular endothelium	Human
Fimb (Fimbriae of Uterine tube Epithelial cells)	Ovary epithelial cells	Human

Table 3.2. Primary cells used in the study.

3.1.3. Bacterial strains

Bacteria	Serovars	Source
Chlamydia trachomatis	LGV L2 (434)	ATCC no. VR-902B
Chlamydia trachomatis	D	ATCC no. VR-885
Chlamydia trachomatis	A	ATCC no. VR-571B
Chlamydia trachomatis pGFP::SW2	LGV L2 (434)	Dr. Adrian Mehlitz
Chlamydia trachomatis pIncA-flag	LGV L2 (434)	This work, Prema Subbarayal
Chlamydia pneumoniae	CWL-029	ATCC no. VR-1310

Table 3.3. Bacterial strains used in the study.

3.1.4. Cell culture medium and buffers

Type	Medium
Hela229 cells	1x RPMI 1640+ L-Glutamax +25 mM HEPES (GIBCO, Germany),10% (v/v) heat inactivated FCS (Biochem)
HUVEC cells	RPMI (GIBCO) with 10% FBS and HUVECs cultured in medium 200 supplemented with low serum growth supplement (LSGS, Gibco BRL)
Fimb cells	1x RPMI 1640+ L-Glutamax +25 mM HEPES, 10% (v/v) heat inactivated FCS
Bacterial culture media	1x RPMI 1640+ L-Glutamax +25 mM HEPES, 10% (v/v) heat inactivated FCS
Freezing media	1x RPMI 1640+ L-Glutamax +25 mM HEPES, 10% (v/v) heat inactivated FCS, 10 % (v/v) DMSO
Transfection media	Cell culture medium + 20% (v/v) Optimem (GIBCO)
SPG buffer	0.22 M sucrose, 10 mM Na_2HPO_4, 3.8 KH_2PO_4, 5 mM glutamate
PEI stock solution	Dissolve PEI powder to a concentration of 2 mg/ml in water which has been heated to 80°C. Allow solution to cool to room temperature (RT). Adjust pH to 7.0 with 5M HCl. Filter sterilize. Freeze aliquots at -80°C

Table 3.4. List of cell culture medium and buffers used in this study.

3.1.5. Buffer and Solutions

3.1.5.1. Buffer for FACS

Buffers	Ingredients
PBS (1x) (/L)	8 g NaCl, 0.2 g KCl, 2.68 g Na_2HPO_4 (1x H_2O), 0.24g KH_2PO_4, adjust to pH 7.4
Fixing buffer (/L)	1x PBS + 4% (w/v) PFA, adjust to pH 7.2
Permeabilisation buffer	1x PBS + 0.2% (v/v) Triton X 100
Blocking buffer	1x PBS + 10% (v/v) FCS

Table 3.5. Buffers and components used for FACS analysis.

3.1.5.2. Buffer for Western blot

Buffers	Ingredients
10 x SDS buffer (/L)	30.275 g Tris, 144 g glycine, 10 g SDS
10x Semi dry buffer (/L)	24 g Tris, 113 g glycine, 2 g SDS
1x Semi dry transfer buffer	1x semi dry buffer + 20% (v/v) methanol
10x TBS (/L)	60.5 g Tris, 87.6 g NaCl, adjust to pH 7.5 with HCl
TBST20	1 x TBS + 0.5% (v/v) Tween20
Blocking solution	TBST20 + 5% (w/v) dry milk powder or BSA
Stripping buffer (500 ml)	Prod#46430 Thermo Scientific
2x Laemmli buffer	100 mM Tris/HCl [pH 6.8], 20% (v/v) glycerol, 4% (w/v) SDS, 1.5% (v/v) 2-mercaptoethanol, bromophenol blue
10% SDS lower gel solution	For 10 ml: 4.15 ml H_2O, 3.38 ml 30% (v/v) acrylamid : bisacrylamid mix (37.5:1), 2.5 ml 10% (w/v) SDS, 2.5 ml, 1.5 M Tris/HCl [pH 8.8], 0.1 ml 10% APS, 4 µl TEMED
SDS upper gel solution	For 5 ml: 3.4 ml H_2O, 0.83 ml 30% (v/v) acrylamid : bisacrylamid mix (37.5:1), 0.63 ml 1.0 M Tris/HCl [pH 6.8], 0.1 ml 10% (w/v) SDS, 0.05 ml 10% APS, 5 µl TEMED

Table 3.6. Buffers and components used for western blot analysis.

3.1.5.3. Buffer for Immunofluorescence

Buffers	Ingredients
Fixing buffer (/L)	1x PBS + 4% (w/v) PFA, adjust to pH 7.2
Permeabilisation buffer	1x PBS + 0.2% (v/v) Triton X 100
Blocking buffer	1x PBS + 10% (v/v) FCS
Mowiol mounting medium	2.4 g Mowiol 488, 6 g glycerol, 6 ml H_2O, 12 ml 0.2 M Tris/HCl adjust to pH 8.5

Table 3.7. Buffers and components used for immunofluorescence analysis.

3.1.6. Commercial kits used

Name	Purpose	Company
TUNNEL Kit	Cell death detection kit	Roche
Plasma membrane protein extraction kit	Isolation of plasma membrane proteins	Abcam

Table 3.8. List of commercial kits used in this study.

3.1.7. Inhibitors and Inducers

Name	Purpose	Company
TNF-α	Apoptosis inducer	BD Pharmingen
LY294002	PI3 kinase inhibitor	Cell signaling
UO126	ERK inhibitor	Cell signaling
Dasatinib	RTK inhibitor	Cell signaling

Table 3.9. List of inducers and inhibitors used in the study.

3.1.8. Fine chemicals

Compound	Manufacturer
Complete protease inhibitor	Roche
Phosphatase inhibitor	Roche
DRAQ5	BioStatus Limited, UK.
ECL immunoblotting substrate	Thermo
Phalloidin	Invitrogen
LipofectamineTM 2000	Invitrogen

Table 3.10. List of fine chemicals used in the study.

3.1.9. Antibodies

Name (primary)	Source	Company	Product#	Dilution	Application
Actin	Ms	Sigma	A5441	1:3000	WB
IncA	Rb	This work	--	1:300	WB
Pan-cadherin	Ms	Santa Cruz	59876	1:1000	WB
Total Akt	Rb	Cell signaling	4685	1:1000	WB
Total ERK	Rb	Cell signaling	9108	1:1000	WB
EphA2 (D4A2)	Rb	Cell signaling	6997	1:1000/1:200	WB/IF, FACS
EphA2	Ms	R&D	MAB3035	1:200	FACS, IF
PI3 Kinase p85	Rb	Cell signaling	4292	1:300	WB, IP, IF
PARP	Rb	Santa Cruz	H-250	1:1000	WB
Hsp60	Ms	Santa Cruz	57840	1:1000, 1:200	WB, IF
pAkt	Rb	Cell signaling	4060, 9275	1:500	WB
pERK	Ms	Cell signaling	9106	1:500	WB
Ephrin-A1	Ms	Santa Cruz	377362	1:100	WB
pEphA2 Ser897	Rb	Cell signaling	6347	1:500	WB
pEphA2 Tyr594	Rb	Cell signaling	6347	1:500	WB
EphA2	Rb	Santa Cruz	924	1:1000	WB
GP96	Ms	Santa Cruz	53929	1:1000	WB
PDI	Ms	Pierce (Thermo)	MA3-019	1:1000	WB
PDGFRβ	Goat	R&D	AF385	1:1000	WB
EphB4	Rb	R&D	AF3038	1:1000	WB

Ms=Mouse, Rb=Rabbit

Secondary Antibody	Source	Company	Dilution	Application
ECL α-mouse IgG HP linked	Goat/Donkey	GE healthcare	1:2500	WB
ECL α-rabbit IgG HP linked	Sheep /Donkey	GE healthcare	1:2500	WB
α-mouse Cy2/Cy3 linked	Goat	Dianova	1:200	IF
α-rabbit Cy2/Cy3 linked	Donkey/Goat	Dianova	1:200	IF

Table 3.11. List of primary and secondary antibodies used in the study.

3.1.10. Recombinant proteins

Recombinant human EphA2 (rhEphA2) and recombinant human Ephrin-A1 were purchased from R&D systems. Recombinant IgG1-Fc was bought from Life Technologies.

3.1.11. Technical equipment and software used

Equipments like Hera Cell 150 incubator (Thermo), Hera Cell sterile bench (Thermo), Rotanta 460R centrifuge (Hettich), AvantiTM J-25I centrifuge (Beckman Coulter), cold centrifuge 5417R (Eppendorf), Owl Hep semidry electro-blotting system (Thermo), PerfectBlueTM Dual Gel Twin PAGE chambers (Peqlab Biotechnology), DM1500 confocal microscope (Leica), confocal Leica TCS SP5, DMR epifluorescence microscope (Leica), DMIL light microscope (Leica), Thermo-mixer comfort (Eppendorf), 2720 Thermal cyclers (Applied Biosystems), Nano drop 1000 spectrophotometer (Peqlab Biotechnlogy) were used.

Software like Windows XP, Windows 7, Windows 8.1, EndNote X6 & X7, Microsoft PowerPoint 2010/2013, LAS-AF confocal microscopy, Scaffold_4.3.2, Image J were used.

3.2. METHODS

3.2.1. Cell Culture and bacterial infections

HeLa 229 cells were cultured in RPMI (GIBCO) with 10% FCS and primary uterine tube epithelial cells (Fimb) were cultured in RPMI (GIBCO) with 10% FCS on collagen coated plates. HUVEC cells were cultured in medium-200 supplemented with low serum growth supplement. Cells were grown at 37°C and 5% CO_2. The propagation of *Ctr*-LGV-L2 434/Bu, *Ctr*-serovar D, *Ctr*-serovar A, *C. pneumoniae* and the recombinant *C. trachomatis* strains [*Ctr*-pGFP::SW2, *Ctr*-pIncA-flag] in HeLa cells were made as previously described [149]. Infected cells were then mechanically detached using a cell scraper (Sarstedt). The bacteria were released using ~5 mm glass beads (Carl Roth GmbH + Co. KG). Low-speed supernatant (5 min at 4000 x g and 4°C) was carried out to high-speed centrifugation (25 min at 40,000 x g and 4°C) in order to pellet the bacteria. Bacteria were washed twice with 10 ml SPG, aliquoted and stored at -80°C in SPG buffer and freshly thawed for each experiment. Cells were infected with *Ctr* with respective medium in the presence of 5% FCS and grown at 35°C with 5% CO_2.

3.2.2. Isolation of *C. trachomatis* intact inclusions

Isolation and purification of the inclusions were performed as previously done [150] with modifications as below. HeLa cells were infected with *C. trachomatis* in T175 flasks (Corning) by adding a suspension of EB at a multiplicity of infection (MOI) 1 for 24 h. After washing both the uninfected and infected cells twice in the phosphate-buffered saline of Dulbecco lacking Ca and Mg (Life technologies), cells were detached using trypsin (Life technologies). RPMI containing 5% FCS was added to the trypsinized cells and spinned down at 500 x g for 5 min at 4°C. The pelleted cells were washed thrice with PBS and centrifuged at 500 x g at 4°C. Cells were then re-suspended in sterile filtered swelling buffer containing 10 ml of 0.25 M sucrose-EDTA Tris buffer solution (1.5 mM EDTA in 10 mM Tris-HcL buffer, 5mM NaCl pH 7.4) and kept in ice for

90 min. To ensure better swelling, cells were mixed up and down in between with the pipette. The re-suspended cells were transferred to a glass dounce (10-15ml) and homogenized gently for 20 strokes. The homogenate was taken in a 10-ml plastic syringe and filtered the suspension though an 11 micron nylon filter (Millipore) having two filters in one cartridge on both sides of the rubber ring. This step can be repeated twice depending upon the cell status. The filtered cell suspensions were centrifuged at 500 x g for 5 min at 4°C. The supernatant was passed through a second glass membrane filter (Millipore Cat No: AP2001300). The collected supernatant was centrifuged at 4°C for 10 min at 1500 x g and washed twice with sterile filtered sucrose-Tris buffer (0.25 M sucrose in 10 mM Tris-HcL buffer). To the final washing step, sterile filtered Sucrose-Tris buffer containing 5% BSA was added to the pellet which were mixed gently and centrifuged at 1500 x g for 10 min at 4°C. (This step is not necessary if the protein lysate has to be prepared immediately in a denaturing condition). The purification procedure was monitored by light microscopy and confocal microscopy. The cell status was analyzed at each stages of purification. Pure inclusions were seen abundantly at the final stage of purification.

3.2.3. Electron microscopy

HeLa cells were infected with *Ctr* for 24 h. The infected cells were washed twice with PBS and fixed with 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 6.8) at 4°C for 1 h. The fixed cells were washed with cacodylate buffer and taken for preparation of the sample. Samples were transferred to the electron microscopy department (group of Prof. Dr. Georg Krohne) for further preparation.

3.2.4. Preparation of intact *Ctr* inclusions for LC-MS/MS analysis

Inclusion isolation procedure was performed for uninfected HeLa cells and *C. trachomatis* or *C. trachomatis*-GFP infected HeLa cells separately. For LC-MS/MS analysis, 2x Laemmli buffer

was added to break the inclusions. Purified materials from four biological replicates were prepared and sent to Greifswald proteome platform in a pack with dry ice.

3.2.5. LC-MS/MS data analysis

The following LC-MS/MS analysis was performed by AG Doerte Becher at Griefswald, Germany. Tryptic digests of the samples were subjected to liquid chromatography performed on an EASYnLC (Proxeon, Odense, Denmark) with in house packed columns (Luna 3µ C18(2) 100Å, Phenomenex, Germany) in a one- column setup. Following loading/ desalting at a flow of 700 nL/min at a maximum of 220 bar with water in 0.1% acetic acid, separation of the peptides was achieved by the application of a binary non-linear 70 min gradient from 5-50% acetonitrile in 0.1% acetic acid at a flow rate of 300nl/min. The LC was coupled online to an LTQ- Orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany) at a spray voltage of 2.4kV. After a survey scan in the orbitrap (R = 30,000) MS/MS data were recorded for the five most intensive precursor ions in the linear ion trap. Singly charged ions were not taken into account for MS/MS analysis. The lock mass option was enabled throughout all analyses.

After mass spectrometric measurement, MS-data was subjected to database searching via Sorcerer using Sequest (SageN, Milptas, CA, USA.) (Thermo Fisher Scientific, San Jose, CA, USA; version 27, revision 11) without charge state deconvolution and deisotoping performed. For database searching, a concatenated fasta database of *Chlamydia trachomatis* and a human database were used. Scaffold (version Scaffold_4.3.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Sequest identifications required at least deltaCn scores of greater than 0.10 and XCorr scores of greater than, 2.2, 3.3 and 3.8 for doubly, triply and quadruply charged peptides. Protein identifications were accepted if they contained at least 2 identified peptides. Proteins that contained similar

peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Purified materials from four biological replicates were analyzed by mass spectrometry and the Scaffold 4.3.2 program which displays a complete, experiment-wide view of the proteins (http://www.proteomesoftware.com/products/scaffold/).

3.2.6. *Chlamydia*-IncA antibody production and purification

The cytoplasmic domain of IncA of *Ctr* L2 strain was inserted into pGex4T3 vector (pGex4T3-IncA-CP plasmid) which provides the inserted gene an N-terminal GST tag and an inducible lac-promoter. The gene was cloned in frame into the pGex4T3 vector system with the restriction enzyme pair BamHI/ XhoI (cloning work done by *Miriam Sturm*). The sequenced vectors were eventually transformed into chemo-competent *E. coli* BL21 star and selected with ampicillin.

In order to check the over-expression of the desired protein IncA-CP, the lac-operon of the pGex4T3 vector was induced with IPTG for 2 h. The bacteria was then pelleted and resuspended in Laemmli buffer for SDS- page analysis. For immunization, the purification was performed with 2 liters of induced culture. The collected pellet was then resuspended in ice-cold lysis buffer with lyzozyme and sarcosyl. Then the bacterial cells were disrupted by 2x 90 seconds of sonification (50% intensity; 50% duty cycle at output control 4 sonifier (Branson, Sonifier B-12). Afterwards 200 ml of detergent buffer was added to the lysate and centrifuged for 10 min at 5000 x g and 4°C (Beckman Avanti, rotor JA-10). To the supernatant, triton-X 100 was added and eluted using the elution buffer (50 mM Tris-HCl pH 8, 10 mM reduced glutathione, 100 µg/ml Lysozyme) for 20 min at 25°C in a thermomixer at 700 x g. The protein concentration was measured by Bradford assay and the eluted purified recombinant protein (referred as antigen) was sent to Gramsch laboratories for immunization to obtain the IncA polyclonal antibody serum.

The obtained polyclonal antibody serum was affinity purified to remove the unspecific antibodies obtained after immunization. The affinity purification was performed using antigen columns. The Cyanobromide (CnBr) beads (0.4 g) were swollen for 10 min in 1 mM HCl followed by one washing step using 0.2 M carbonate buffer. The buffer of the antigen was exchanged to 0.2 M carbonate buffer. The activated CnBr beads were immediately mixed with 300 µg antigen and incubated for 1 h at RT followed by 4°C overnight. Beads were then washed twice with 0.2 M carbonate buffer. The remaining coupling sites were blocked with 100 mM Ethanolamine for 1 h at RT. The beads were washed thrice with 0.2 M carbonate buffer followed by equilibration in 500 mM NaCl in PBS and mixed with 10-20 ml serum. Affinity matrix and antibody serum were incubated at 4°C overnight on a rotary shaker. Beads were harvested by centrifugation at 500 x g for 5 min and 4°C and washed twice with 20 ml of 500 mM NaCl in PBS. The beads were transferred into a column and continuously washed using 150 ml of 500 mM NaCl in PBS. Antigen-specific antibodies were eluted under low pH with 0.2 M Glycine pH 2.5 and immediately neutralized by addition of 200 µl/ml of 1 M Tris HCl/pH 7.5. Fractions of the antibody elution were combined and concentrated using Amicon ultra centrifugation filter units with a molecular weight cut off of 10 kDa. Antibody fraction was finally mixed with one volume of 87% glycerol and stored at -20°C.

3.2.7. Adherence assay

Ctr adherence assay was performed by pretreating the cells with rhEphrin-A1 (5 µg/ml) or with control (Fc) for 1 h at 37°C followed by infection with purified-EB for 1 h at 4°C in HBSS media (Life Technologies). Otherwise required MOI of EB were treated with rhEphA2 or with control in a serum free media containing 0.1% BSA for 1 h at 37°C and the mixture was added to the cells and incubated for 1 h at 4°C. Cell surface EphA2 was blocked using control (IgG) or antibodies against specific proteins (10 µg/ml) for 1 h at 4°C followed by infection with EB for 1 h at 4°C.

Before fixing the cells for immunostaining, cells were washed three times with PBS. Co-localization of EB with EphA2 was determined manually by quantifying from 8 different fields of higher magnification view in 3 independent experiments where the samples were blinded.

3.2.8. Pull-down assay

To prove the direct interaction of EphA2 with *Ctr*-EB, pull-down assay was performed. *Ctr*-EB (L2) were purified as described before [151]. Purified *Ctr*-EB was incubated with rhEphA2 (10 µg/ml) or with rhEphrin-A1 (10 µg/ml) in a serum free RPMI media containing 0.1% BSA for 45 min at 37°C in a rotary shaker. Additionally, rhEphrin-A1-preincubated rhEphA2 was incubated with EB as above and the mixture was centrifuged at 5000 x g for 4 min at 4°C. The pellet was gently washed three times with ice cold-PBS by centrifugation at 5000 x g for 4 min at 4°C and subjected to WB analysis.

3.2.9. Invasion assay

Ctr invasion assay was performed by transfecting the cells with siRNA against luciferase gene (Luci) or EphA2 gene for 40 h at 37°C or treating the cells with IgG or α-EphA2 antibody for 1 h at 4°C as mentioned in the adherence assay. The bacteria of required MOI were incubated with cells for 4-6 h at 35°C. The cells were fixed with 4% ice cold PFA for 30 min at 4°C. The fixed cells were taken for immunostaining after permeabilisation. Invasion assay in EphA2 overexpressed cells was performed after 40 h of EphA2-pcDNA3 transfection for flow cytometry analysis to determine the relative invasion rate of *Ctr* comparing to control empty-pcDNA3-transfected cells under permeabilised condition.

3.2.10. Infectivity assay

Transfection and infection in HeLa cells were performed as described above. Supernatant from the primary infected cells was removed and the cells were washed with PBS. Cells from one set

of experiment were lysed with glass beads and the lysate was passed through a pipette several times. The suspensions were transferred to HeLa cells (1:200 dilution) for secondary infection. The next day, cells were fixed with 4% PFA followed by immunostaining. Draq5 (DNA) was used for counterstaining of nuclei and Hsp60 antibody was used to stain the *Ctr* inclusion. Number of the inclusions was counted by analyzing on five random fields under the microscope and size of the inclusion was determined by ImageJ software (http://imagej.nih.gov/ij).

3.2.11. Generation of *Ctr* expressing recombinant-IncA (*Ctr*-pIncA-flag)

Full length IncA was fused with tag sequences (HA and Flag) by overlap PCR and inserted into pGFP::SW2 replacing GFP:CAT. The transformation of *Ctr*-L2 was performed as previously described [152]. The plasmid DNA (6 µg) and *Ctr* $1x10^7$ IFU (transfection mixture) were mixed in $CaCl_2$ buffer and incubated for 30 min at RT. The transfection mixture was added to McCoy cells and incubated for another 20 min followed by culturing in a T75 flask with fresh RPMI supplemented with 10% FCS. After 48 h, cells were lysed by sterile glass beads and the supernatant was used to infect the fresh cells which were cultured in the presence of 2 U/ml penicillin G and 2 µg/ml CHX and passaged every 48 h. When productive inclusions were visible, HeLa cells were used for infection and the concentration of penicillin was increased up to 50-70 U/ml to prepare the stock.

3.2.12. Plasmids and transfection

Expression plasmid EphA2 (full length ref NP_004422.2) was generated by PCR amplification using cDNA of pDONR223-EphA2 from Addgene (ID:23926). The resulted amplicon was inserted into the pcDNA3 plasmid (Invitrogen). Mutant EphA2 without cytoplasmic domain (EphA2ΔIC) was created by inserting the region of human EphA2 encoding amino acids 1-560 into pcDNA3. Empty pcDNA3 was used as a control. p85-PI3K expression plasmid was purchased from Addgene (ID:11499). Transfection was performed using 1 µg/ml plasmid per

well in 12 well plate using Polyethyleniminde (PEI) and OptiMEM transfection medium (Gibco). Transfection medium was changed after 5 h. Infection was performed at different time points after 20 h of transfection.

3.2.13. siRNA and transfection

siRNA smart pool targeting EphA2 (siGENOME Human EphA2, 5 nmol, M-003116-02-0005) was purchased from Dharmacon. Pooled siRNA against PDI and PDGFRβ were bought from Santa Cruz. Cells at ~60% confluent were transfected at with siRNA (50 pmol/ml) using Lipofectamine 2000 (Invitrogen) and optimum. 8 h post transfection, medium was exchanged and 40 h post transfection, cells were infected with *Ctr* for different time points. siRNA targeting luciferase gene was used as a control, siLuci (Control): AACUUACGCUGAGUACUUCGA.

3.2.14. Inhibitor assay

Cells were pre-treated with DMSO control or dasatinib for 1 h at 37°C followed by infection with *Ctr*. Or 14 h infected cells were treated with DA for 10 h. After 24 h of total infection, protein lysates were subjected to WB analysis or immunostaining. Uninfected or infected HUVEC cells were treated with DMSO or PI3K inhibitor LY294002 or MAPK inhibitor UO126 for 10 h. EphA2 plasmid-transfected HeLa cells were left uninfected or infected with *Ctr* for 24 h and treated with DMSO or UO126 for 3 h or 6 h.

3.2.15. Flow Cytometry (FACS)

For staining the surface exposed N-terminal EphA2, cells were grown in 6-well plates. After the respective time points of infection, cells were washed 3 times in ice cold PBS and detached using 5 mM EDTA at RT for 10-15 min. The cells were collected in Eppendorf tubes and washed 3 times in ice cold PBS containing 0.5% BSA (blocking buffer) by centrifugation at 350 x g for 4-5 min at 4°C. Cells were fixed with 4% paraformaldehyde (PFA) for 20 min at 4°C

followed by incubation with 2% FCS for blocking to prevent unspecific binding of the antibody to the cell surface proteins. Antibodies directed against EphA2 (R&D systems), were diluted 1:200 in blocking solution and added to cells for 1 h at 4°C in the dark followed by a washing step in blocking buffer. Secondary anti mouse-Cy5 antibody (GE healthcare, 1:200) was incubated for 1 h followed by two washing steps in PBS and then taken for flow cytometry analysis. Via FSC-A and SSC-A, population of intact cells was determined and 10,000 events per sample were analyzed using “FACS AriaIII” and the “BD FACS Diva” software for all experiments. “FCSalyzer 0.9.3” software was used to create the overlay histograms. For surface EphA2, cells were not permeabilised whereas for detecting the total EphA2, cells were permeabilised using 0.2% Triton-X-100 in PBS. For analyzing the invasion rate of *Ctr* (invasion assay) in EphA2 overexpressing cells, *Ctr*-GFP was used. GFP was excited at 488 nm and Cy5 at 647 nm, respectively.

3.2.16. Apoptosis sensitization assay

Transfection of the cells with siLuci or siEphA2 was performed as described above. Uninfected and *Ctr*-infected cells (MOI-1, 15-16 h) were induced to apoptosis with 50 ng/ml of human recombinant TNF-α and 5 µg/ml of cycloheximide (CHX) (Sigma) for 5-6 h. After the respective time post induction, the stimulated and control cells were fixed for immunostaining using TUNEL kit or subjected to WB analysis to determine the PARP, cleaved PARP (C-PARP), Hsp60 and Actin.

3.2.17. Re-infection assay

HeLa cells transfected with EphA2-pcDNA3 were first infected with *Ctr* (MOI-1) for 24 h followed by re-infection using EB (MOI-15) for 4 h. The cells were washed to remove the unbound EB and fixed for immunostaining against EphA2 (EphA2, red), *Ctr* (Hsp60, green) and Actin filaments (Phalloidin, blue). The total number of re-infected bacteria (both adherent and invaded

new EB) in EphA2 overexpressed and neighboring untransfected-infected cells were counted separately for quantification.

3.2.18. Plasma membrane isolation

Plasma membrane proteins were isolated using the plasma membrane protein extraction kit from Abcam. Cells were grown in 150 cm^2 dishes and infected for different time points. The cells were scraped down and washed twice with ice cold PBS and resuspended in homogenizing buffer and homogenized for 30-50 strokes using a dounce homogenizer. 5% of the homogenate was collected as input. The homogenate was centrifuged to obtain total cellular membrane proteins in the pellet. The total cellular membrane proteins were subjected to further purification as described by the protocol of the supplier to obtain 10-100 µg of plasma membrane proteins in the pellet.

3.2.19. TCA Precipitation

Supernatants of the uninfected (UN) and time course infected samples were collected. Around 250 µl of 72% TCA and 12.5 µl of 1% Na-deoxycholate were added to final volume of 1 ml and incubated on ice for 30 min followed by centrifugation for 30 min at 14,000 x g in cold centrifuge. The pellet was washed with 400 µl of ice-cold acetone followed by another centrifugation for 10 min and then dried at 37°C for 5 min which was then resuspended by shaking for 15 min at 65°C followed by WB analysis.

3.2.20. Immunofluorescence (IF) microscopy

The 4% PFA fixed cells were permeabilised using 0.2% Triton-X-100 for 30 min and blocked with 2% FCS for 1 h at RT. The cells were incubated with respective primary antibodies for 2 h at RT. After washing with PBS, cells were secondary stained with phalloidin (Cy5) and Cy2 linked anti-mouse to stain the cell membrane and bacteria respectively for 1 h at RT. For

staining the adhesive bacteria on the cell surface, the PFA fixed cells were blocked with 2% FCS and stained for extracellular bacteria without permeabilisation. Samples were washed as above and mounted with Mowiol. The exposure time was same for all images in each set of experiments.

3.2.21. Immunoprecipitation and Western blotting

For immunoprecipitation, UN or *Ctr*-infected cells were washed once with PBS and lysed in 1% NP-40, 150 mM NaCl, 2.5 mM EDTA/EGTA, 20 mM HEPES pH 7.4. The lysates were cleared by centrifugation at 20,000 x g and supernatants were incubated with α-EphA2 or α-p85-PI3K antibody for 1.5 h and precipitated using magnetic beads. All the steps and buffers used during immunoprecipitation were carried out at 4°C with phosphatase and protease inhibitor (Roche). The precipitated complexes were subjected to WB and were resolved by 8-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Roche Diagnostics GmbH) and blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% bovine serum albumin. Primary antibodies were incubated overnight at 4°C. Proteins were detected with peroxidase-coupled secondary antibodies using the ECL system (Pierce) and an Intas Chem HR 16-3200 reader. The signal bands were quantified by ImageJ software.

3.2.22. Statistical analysis

For statistical calculations and histograms, Excel (Microsoft) was used. Statistical significance was calculated using the Student's T test. Two-tailed T test was performed to calculate the P-value.

4. RESULTS

4.1. Identification of host interacting proteins present in the isolates of *Ctr* inclusions by mass spectrometry

4.1.1. Isolation of intact chlamydial inclusions from the infected cells

Several host trafficking pathways are targeted and manipulated efficiently by the virulence factors of *Ctr* to acquire nutrients for their successful intracellular replication. The knowledge of how this pathogen can effectively control the signaling pathways and escape from the host cell death pathway depends on the identification and analysis of the host molecular factors that interacts with the *Ctr* inclusion. To study that, a methodology for the native *Ctr* inclusions isolation was established (described briefly in methods 3.2.2). Purification of the inclusions was performed by double filtrations and further centrifugations including washing steps with appropriate concentration of buffers in order to get the intact chlamydial inclusions.

Phase contrast-light microscopy images that were taken during the process of inclusion isolation were shown in figure 4.1. Image 1 (Figure 4.1A) was taken when the cells were kept in the swelling buffer for 60 min in ice which leads them to swollen. The inclusions were clearly seen as bright bodies in the central region of the cytoplasm. Image 2 (Figure 4.1A) was obtained after strokes of homogenization followed by double filtration. Some debris were visible near to the inclusion which was removed by further washing and centrifugation steps. Finally, intact inclusions were obtained with some cell organelles attaching to the membrane of the inclusion which could not be avoided during *invitro* purification (Image 3 in Figure 4.1A). Therefore, uninfected cells were also purified similarly to eliminate the contaminating proteins. To further prove that the purified inclusions were still intact, GFP-positive *Chlamydia* (*Ctr*-GFP) was used [152]. As shown in figure 4.1B, it was clear that most of the inclusions were still intact (green arrows) with some disturbed inclusions which have left over RB (indicated in white arrows).

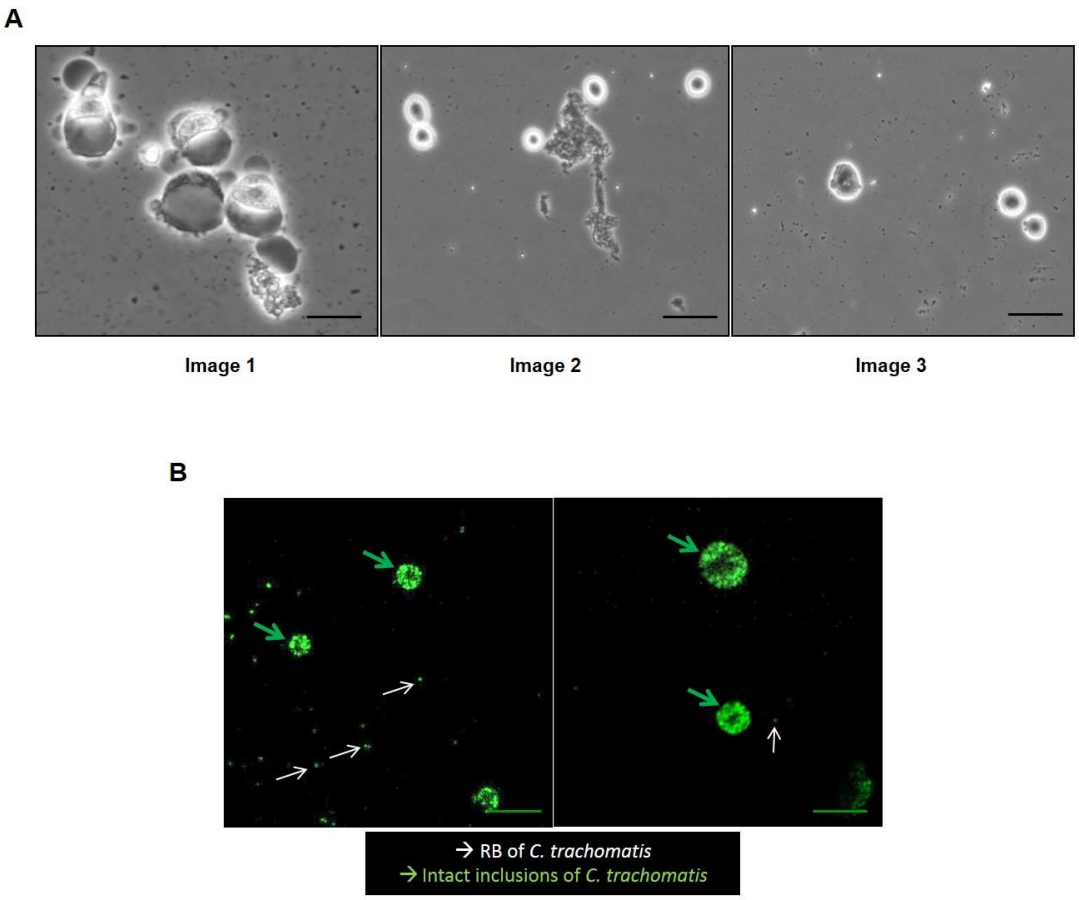

Figure 4.1. Monitoring the isolated chlamydial inclusions: (A) HeLa cells were infected with *Ctr* at MOI-1 for 24 h and the chlamydial inclusion purification was performed as described in methods. The purification procedure was monitored at each stage by phase contrast microscopy. Image 1- 1 h incubation in swelling buffer, Image 2- Filtration, Image 3- Final step. Scale bar, 10 µm. (B) *Ctr*-GFP inclusions were monitored by confocal microscopy showing the intact inclusions (green arrows) and disturbed inclusions with left-over RB (white arrows). Scale bar, 10 µm.

Purity of the isolated inclusions was analyzed by immunoblot studies using the organelle markers. The total cell lysates of uninfected and *Ctr*-GFP-infected cells shows the presence of proteins of respective cell organelles as well as the chlamydial markers Hsp60 and IncA in the infected sample (Figure 4.2). After purification, chlamydial markers (Hsp60 and IncA) seen in the infected sample were similar to total cell infected lysates comparing to other organelle markers which shows the enrichment of the purified inclusions (Figure 4.2).

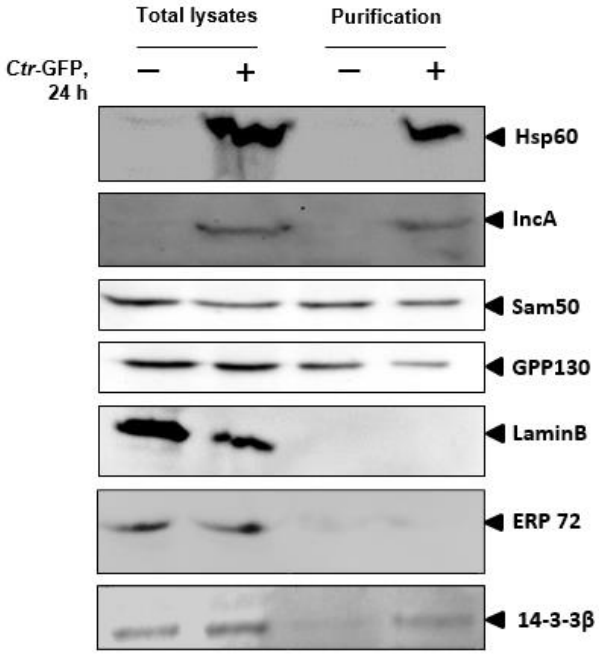

Figure 4.2. Immunoblot analysis of total cell lysates and lysates after purification of uninfected and infected HeLa cells: Markers for different organelles have been detected (Sam50-mitochondrial marker, GPP130-Golgi marker, LaminB-Nuclear envelope marker, ERP72-Endoplasmic reticulum marker). The host adaptor protein 14-3-3β has been shown to interact with the chlamydial inclusion [51] and was therefore used as a marker for chlamydial inclusion.

The organelle markers were still present in the purified sample which could not be avoided in such type of filtration purification. Therefore, uninfected sample was also purified similar to infected sample to avoid the contaminating proteins that passes though the filter. To further investigate the purity of the inclusions, electron microscopy analysis was performed. The purified samples were prefixed using 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 6.8) and the post fixation was done by the core facility (Electron microscopy-Methods).

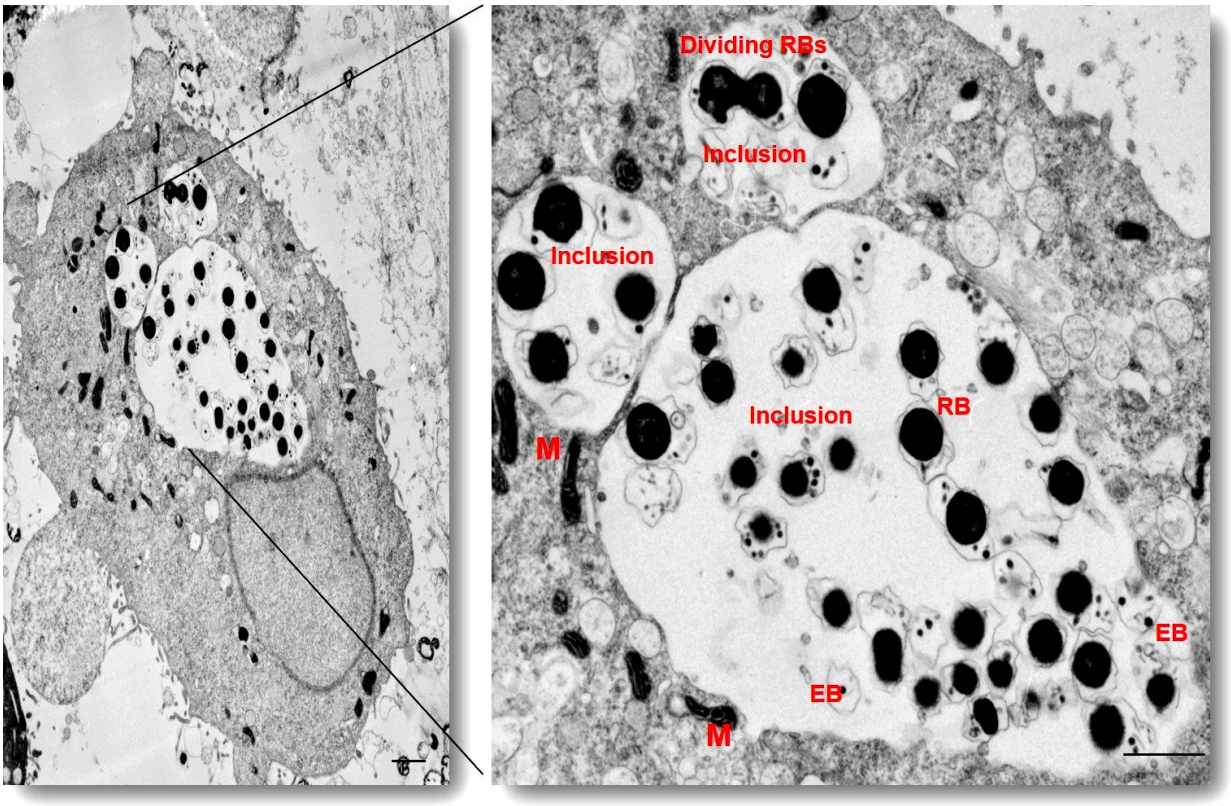

Figure 4.3. Electron microscopic analysis of pre-isolated inclusions: The image of the infected cells kept in swelling buffer. The membranes of the RB and EB inside the inclusion were swollen. RB-Reticulate body, EB-Elementary body, M-Mitochondria. Bars represent 2 µm at magnification 6000× (left image) and 8000× (right image).

The image of the infected cells kept in swelling buffer was shown in figure 4.3. The inclusion membrane, its association with mitochondria and *Chlamydia*-EB and -RB inside the inclusion was visible. Due to the cells incubation in swelling buffer, the membranes of both EB and RB were also swollen inside the inclusion (Figure 4.3). The inclusion membrane of the purified inclusions was completely broken off with left over EB and RB after processing the purified inclusions for electron microscopy analysis (Figure 4.4).

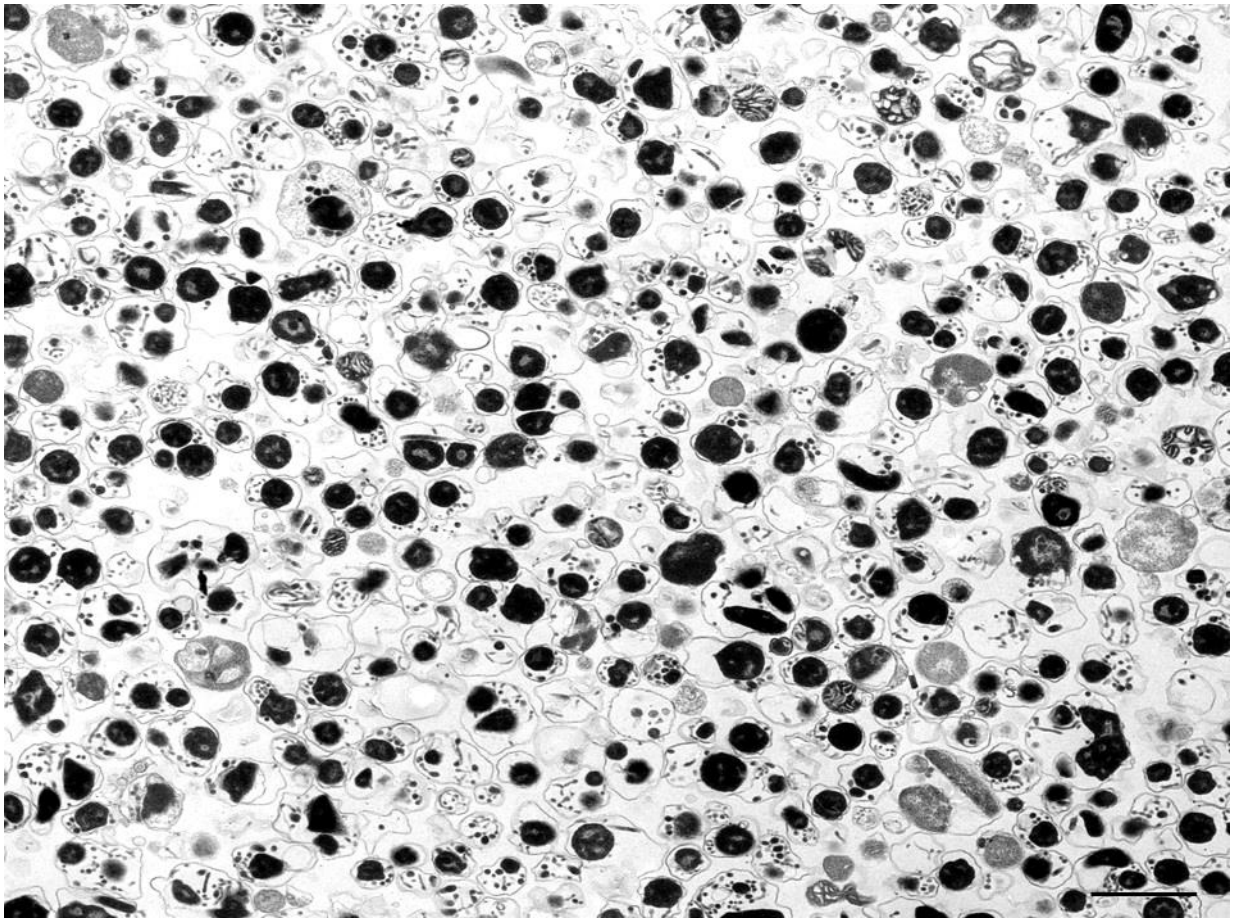

Figure 4.4. Electron microscopic analysis of post-isolated inclusions: The image was taken after purification showing only the membrane ruptured inclusions with enriched elementary and reticulate bodies that occurred due to the processing of pre- and post-fixation procedures for the electron microscopy analysis. Bars represent 6 µm at magnification 10,000×.

The reason behind the damage of the inclusion could be the post fixation procedures for electron microscopy. However, the inclusions were intact immediately after the isolation procedure (Figure 4.1) and the rupture of the inclusion membrane happened only during electron microscopy processing. Therefore, purified inclusions were solved in Laemmli buffer and the proteome was analyzed by mass spectrometry.

4.1.2. Mass spectrometry analysis of the enriched-isolated *Ctr* inclusions

The experimental setup for the mass spectrometric analysis was described briefly in methods (3.2.4 and 3.2.5) and in figure 4.5.

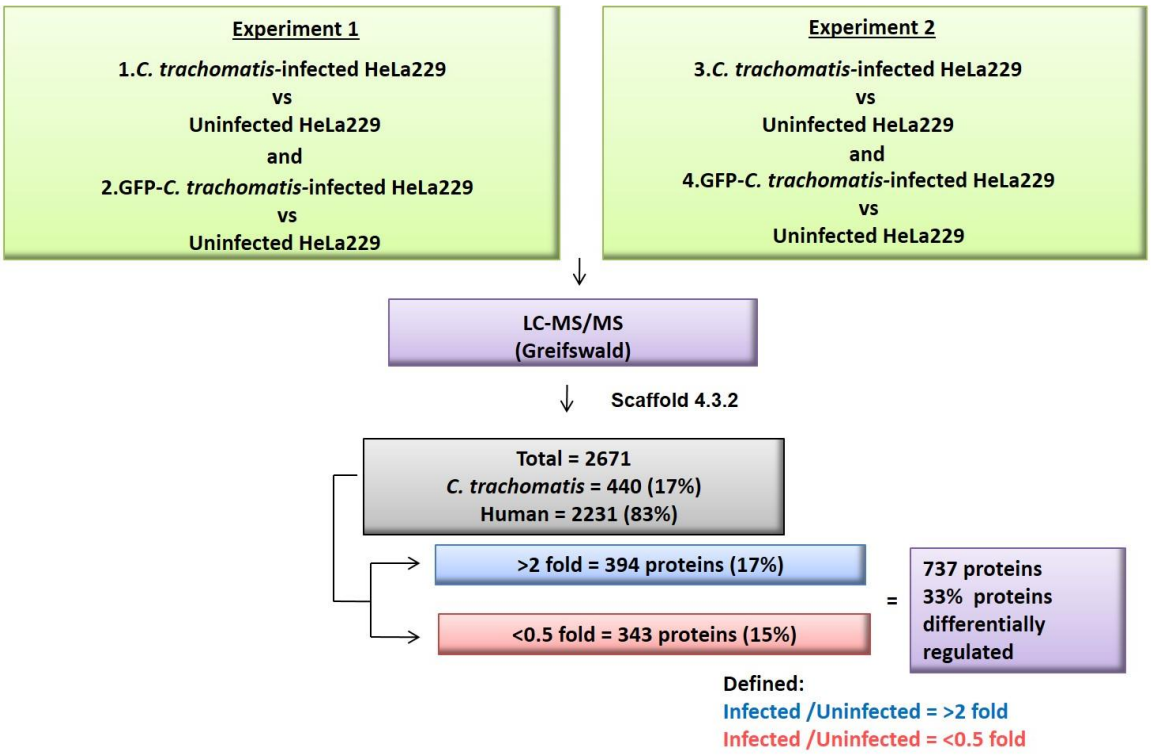

Figure 4.5. LC-MS/MS analysis of enriched *Ctr* inclusions: Samples from four biological replicates were analyzed by mass spectrometry and the Scaffold 4.3.2 program which displays a complete, experiment-wide view of the proteins.

In total, 2671 proteins were identified of which 2231 were host proteins and 440 were *Ctr* proteins. The host proteins that are associated with the chlamydial inclusion were identified via mass spectrometry analysis after determining the fold change. Out of 2231 host proteins, 394 proteins were enriched as defined >2 fold and 343 proteins were depleted as defined <0.5 fold in the membranes of the infected samples (data not shown). Accordingly, 737 differentially regulated proteins (enriched proteins + depleted proteins in the membrane of the inclusions) were identified (data not shown). Among them, 102 proteins (36 proteins were enriched and 66 proteins were depleted) were reproducibly identified in all the samples. To further characterize

the role of these host cell proteins in the infection process, whole genome RNAi was performed [in collaboration with AG Thomas Meyer, MPI, Berlin (data not shown)] which helped to short list the host candidates for further analysis.

4.1.3. Validation of identified host proteins associated with *Ctr* inclusions

Protein targets that play a crucial role in the regulation of host cell metabolism and apoptosis were chosen for the validation studies because the important prerequisite for successful *Ctr* infection involves energy acquisition form host cell and inhibiting cell death. Five hits namely EphrinA2 receptor (EphA2), Protein tyrosine phosphatase mitochondrial 1 (PTPMT1), Hexokinase 1 (Hex1), A-kinase anchor protein 1 (AKAP1) and Era-like 12S mitochondrial rRNA chaperone 1 (ERAL1) were selected after comparing *Ctr* inclusion proteome data with RNAi data.

EphrinA2 (EphA2) is a receptor tyrosine kinase whose expression is found in most cancers including lung, breast, ovary, prostate, colorectal, skin and esophagus [153]. Inhibiting EphA2 by siRNA induces apoptosis and attenuates tumor growth in malignant mesothelioma [154]. PTPMT1, a protein tyrosine phosphatase, is a regulator of cardiolipin biosynthesis [155]. Cardiolipin is an important component of the mitochondrial inner membrane where it constitutes about 20% of the total lipid composition. Loss of PTPMT1 leads to dramatic diminution of cardiolipin. It also mediates dephosphorylation of mitochondrial proteins, thereby playing an essential role in ATP production [156]. Hexokinase 1 is an enzyme that phosphorylate glucose to produce glucose-6-phosphate, the first step in most glucose metabolism pathways. Hex1 interacts directly with VDAC to induce channel closure and prevent the release of cytochrome c thereby inhibiting intrinsic cell death [157]. AKAP1 binds to type I and type II regulatory subunits of protein kinase A and anchors them to the cytoplasmic face of the mitochondrial outer membrane [158]. Protein kinase A has several functions in the cell, including regulation of

glycogen, sugar and lipid metabolism [Voet, Voet & Pratt (2006). Fundamentals of Biochemistry. Wiley. Pg 492]. ERAL1 is a GTPase that plays role in the mitochondrial ribosomal small subunit assembly. Depletion of ERAL1 leads to apoptosis, cell death occurs due to loss of mitochondrial protein synthesis or reduction in the stability of mitochondrial mRNA [159]. As a preliminary analysis, the protein expression of five host candidates during time course *Ctr* infection was checked by WB analysis (Figure 4.6).

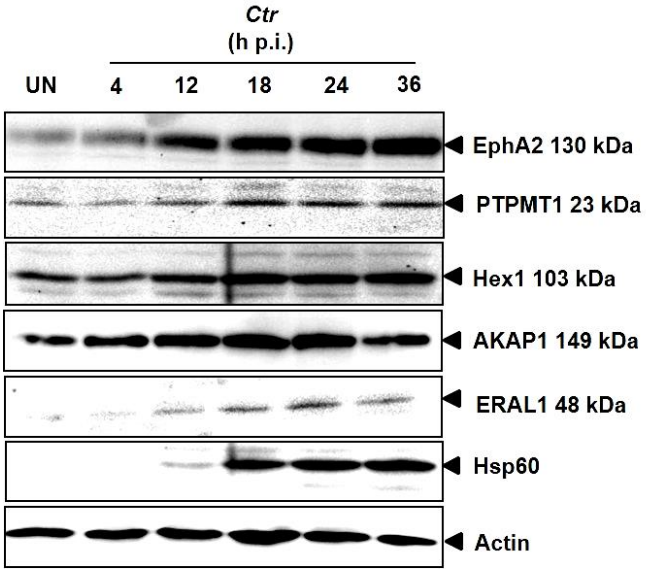

Figure 4.6. Protein expression analysis of the identified hits during *Ctr* infection: HeLa cells were uninfected (UN) or infected with *Ctr* at MOI-2 for different time points and the expression level of 5 hits were analyzed by WB using respective antibodies. Hsp60 acts as a marker for *Ctr* infection and actin serves as a loading control.

All the five host proteins were upregulated during time course *Ctr* infection. Of all the hits identified, EphA2, a cell surface tyrosine kinase receptor was found to be an attractive host target due to its increased expression level with increased time frame of *Ctr* infection as well as being a cell surface receptor. Therefore, further studies were designed to identify the importance of upregulated EphA2 during chlamydial infection.

4.2. Role of EphA2 in early *Ctr* infection

4.2.1. EphA2 is an adherence receptor for *Ctr*

4.2.1.1. *Ctr*-EB interacts with EphA2 in an Ephrin-A1 sensitive manner

Since EphA2 is a cell surface receptor, it was first investigated if EphA2 acts as a cellular receptor for *Ctr*. Therefore, adherence assay was performed at 4°C to prevent the uptake of adherent EB as previously described ([160]; methods 3.2.7). First, I tested if EB co-localize with EphA2 and if Ephrin-A1, a known ligand of EphA2 may interfere with EB-EphA2 co-localization.

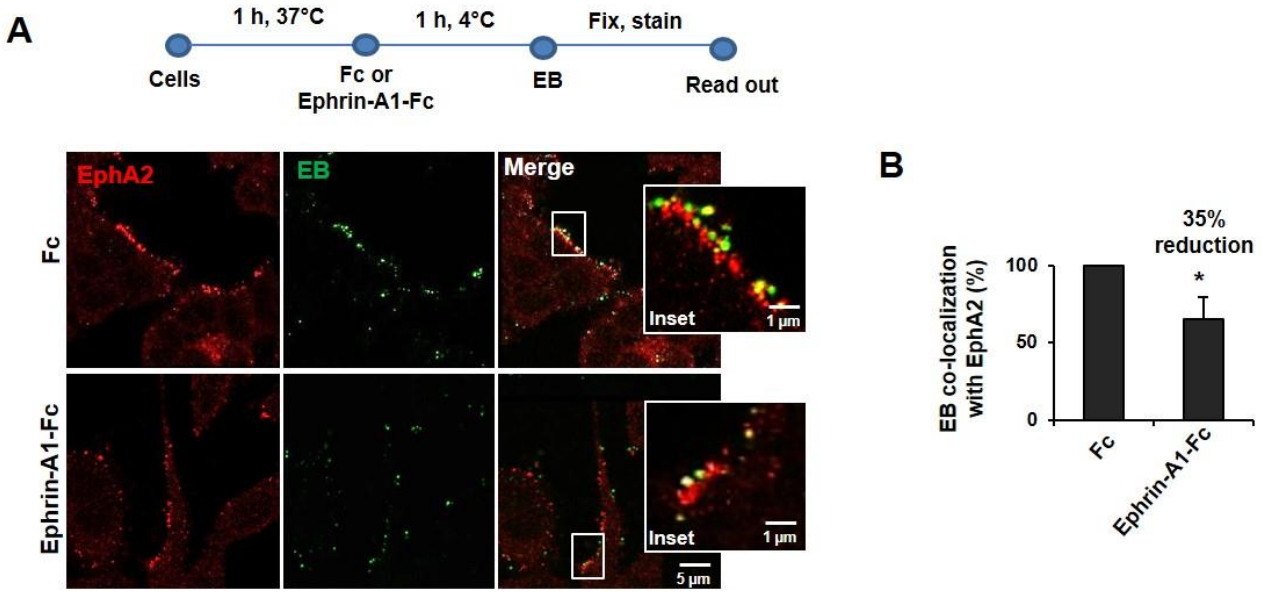

Figure 4.7. Interaction of EphA2 with *Ctr*-EB: (A, B) HeLa cells were pre-treated with control-Fc (purified Fc fragment) or Ephrin-A1-Fc (rhEphrin-A1) (5 µg/ml) for 1 h at 37°C and infected with *Ctr* (MOI-100) for 1 h at 4°C. Cells were fixed and immunostained for *Ctr* (Hsp60, green) and EphA2 (EphA2, red). (A) Images show EphA2 or EB or the co-localization of EB with EphA2 (yellow) on the cell surface. (B) Co-localization of EB with EphA2 was quantified from 8 different fields of view in 3 independent experiments. The data are expressed as a mean percentage of EphA2-associated EB (± SD) compared to total EB. *P<0.05. Error bars show mean ± SD.

Adherence assay performed confirmed the co-localization of EB with cell surface EphA2. The EB-EphA2 co-localization was reduced by 35% upon pre-treating the cells with the recombinant ligand Ephrin-A1-Fc (Figure 4.7A inset; Figure 4.7B). Binding of EphrinA1-Fc to the ligand

binding domain of EphA2 reduced the interaction of EB with extracellular domain of EphA2. These results suggested that EphA2 and EB interact on the surface of host cells in an Ephrin-A1-sensitive manner.

4.2.1.2. *Ctr* induces EphA2 activation upon binding on the cell surface

In order to examine whether *Ctr* induces EphA2 activation upon infection, cells were transfected with EphA2 siRNA and the knockdown efficiency was monitored by WB analysis before and after infection (Figure 4.8A). Phosphorylation of EphA2 at Ser897 (pEphA2), indicative of receptor activation, was detected comparing to the uninfected control (Figure 4.8A). EphA2 activation was detected close to adhering EB during the entry phase of chlamydial infection (Figure 4.8B), suggesting that interaction of EB with EphA2 induced receptor activation. As shown in figure 4.8C, approximately 23% EB co-localized with pEphA2. Cross-reaction of the pEphA2-specific antibody could be excluded since no signal was detected upon knockdown of EphA2 expression. Therefore, these results concluded that *Ctr* infection induces EphA2 activation on the cell surface.

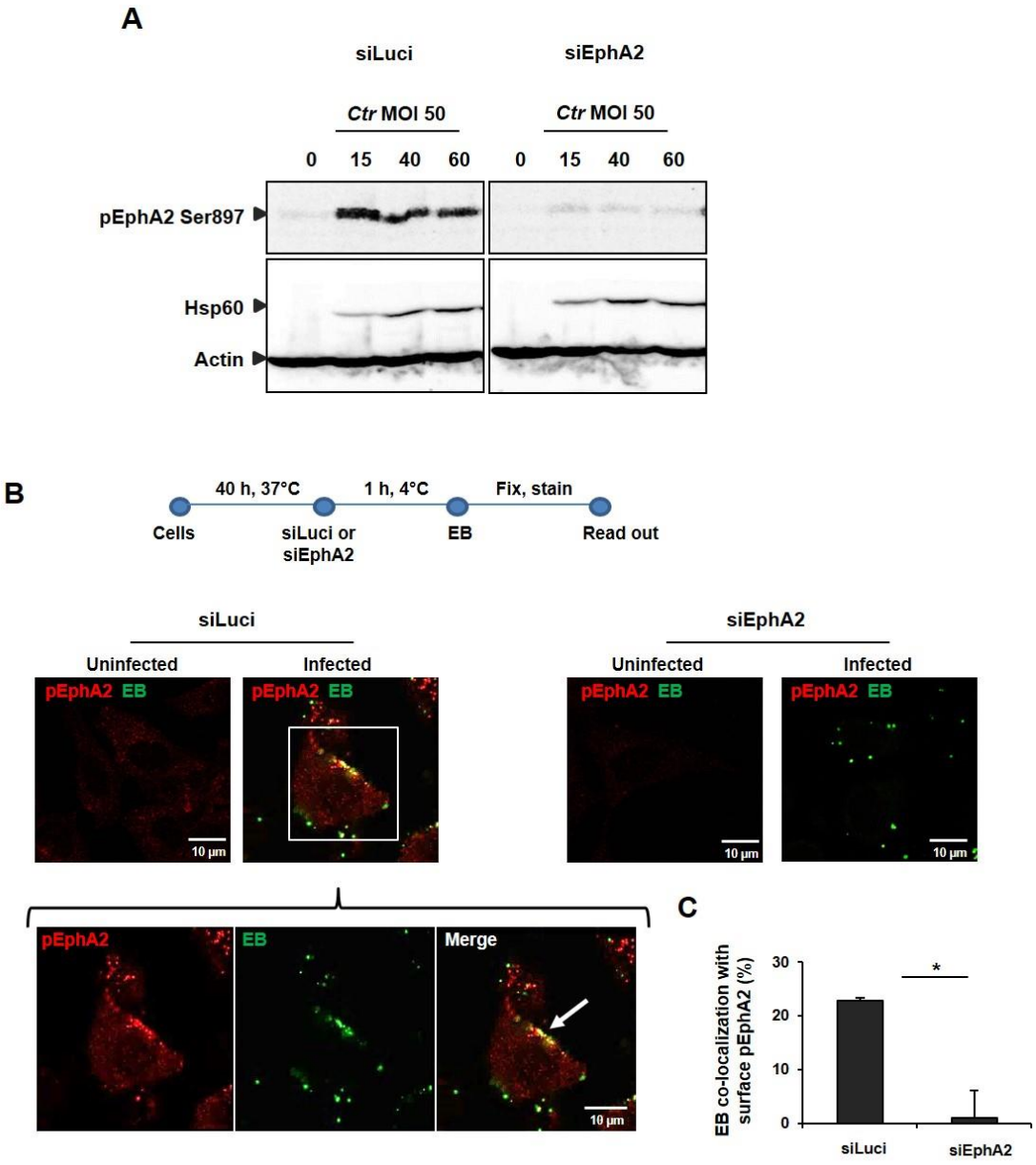

Figure 4.8. EphA2 activated upon early infection co-localizes with *Ctr*: (A) HeLa cells were untransfected (control) or transfected with siRNA against luciferase gene (siLuci) or EphA2 gene (siEphA2) for 40 h at 37°C. The transfected cells were uninfected or infected with *Ctr* for 15 min, 40 min and 60 min. The knockdown efficiency was monitored by WB analysis. (B) Adherence assay: HeLa cells were transfected with siRNA against luciferase gene (siLuci) or EphA2 gene (siEphA2) for 40 h at 37°C. The transfected cells were infected with *Ctr* (MOI-100) for 1 h at 4°C followed by immunostaining *Ctr*-EB (Hsp60, green) and pEphA2 (phospho EphA2 Ser897, red). (C) Co-localization of EB with pEphA2 was quantified. The graph was made similar to figure 4.7B. The data are expressed as a mean percentage of pEphA2-associated EB (± SD) compared to total EB. *P<0.05. Error bars show mean ± SD.

4.2.1.3. Direct interaction of cell surface EphA2 with *Ctr*-EB

To test the direct interaction of EphA2 with EB, recombinant human EphA2 (rhEphA2) was incubated with purified EB in the absence of host cells. EB and rhEphA2 co-localized in this cell-free assay (Figure 4.9A) and the co-localization was reduced when rhEphA2 was pre-incubated with the EphA2-ligand rhEphrin-A1 prior to incubation with EB (Figure 4.9B). Thus, the results confirmed the interaction of EB with EphA2. Direct interaction was further confirmed in a pull-down experiment showing that purified EB interacts with purified rhEphA2 in a rhEphrin-A1-sensitive manner (Figure 4.9C). These experiments clearly conclude the direct interaction of EphA2 with EB.

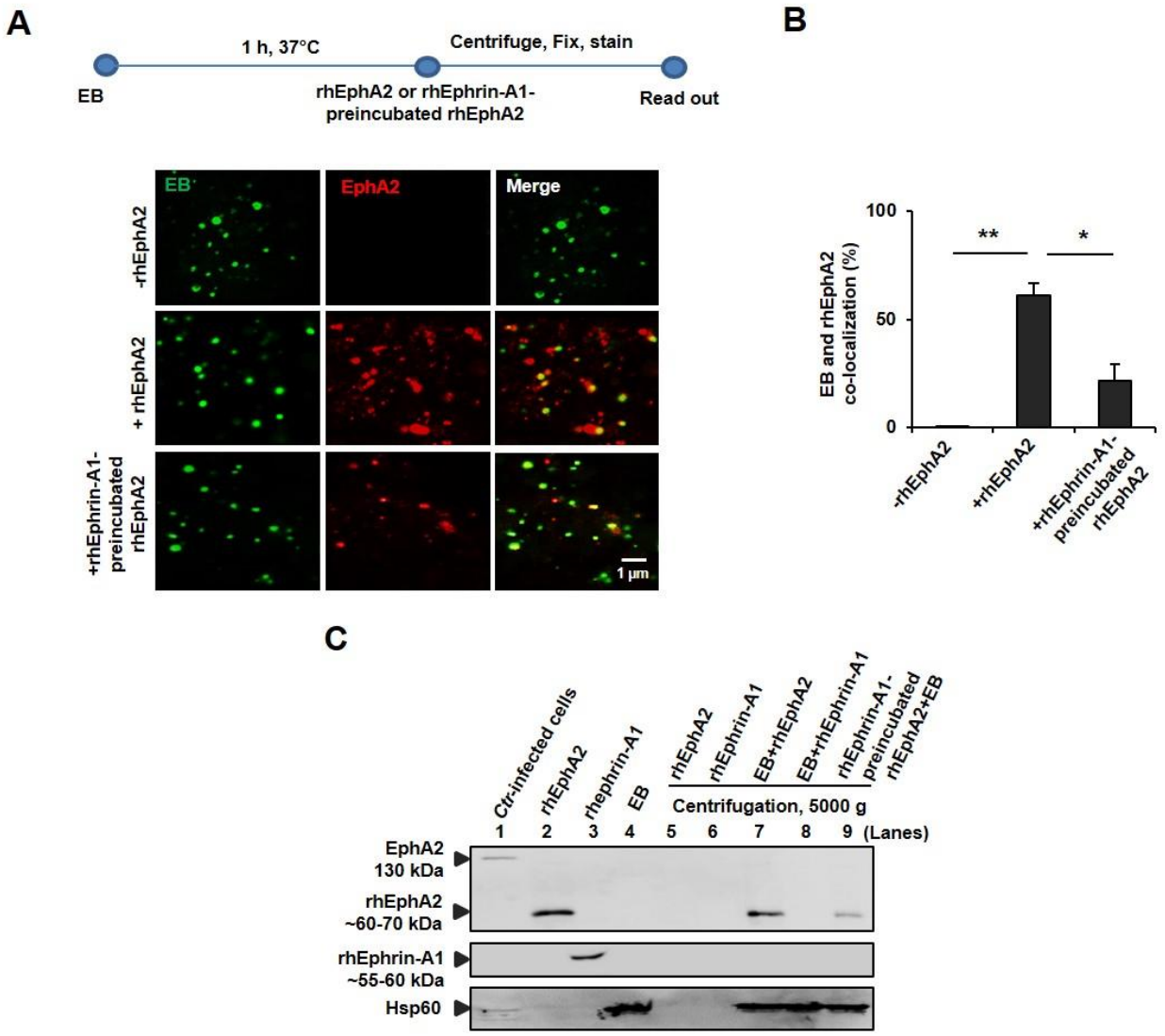

Figure 4.9. *Ctr*-EB directly interacts with cell surface EphA2: (A, B) EB were incubated with or without rhEphA2 (5 µg/ml) in a serum free RPMI media containing 0.1% BSA for 1 h at 37°C. Additionally, rhEphrin-A1-preincubated rhEphA2 was incubated with EB as above. The mixture was centrifuged onto coverslips, washed 3 times with ice cold-PBS, stained and quantified as Figure 4.7B. The data are expressed as a mean percentage of EphA2-associated EB (± SD) compared to total EB. *P<0.05, **P<0.01. Error bars show mean ± SD. (C) Pull-down assay: Purified-EB were incubated with rhEphA2 or with rhEphrin-A1 each 10 µg/ml in a serum free RPMI media containing 0.1% BSA for 45 min at 37°C in a rotary shaker. Additionally, rhEphrin-A1-preincubated rhEphA2 was incubated with EB as above and the mixture was centrifuged at 5000 x g for 4 min at 4°C. The pellet was gently washed 3 times with ice cold-PBS. *Ctr*-infected cells act as a positive control for full length EphA2 expression. The samples were subjected to WB analysis to determine the full length endogenous EphA2 and rhEphA2 using N-terminal EphA2 specific antibody. Hsp60 acts as a control for EB.

Further, pre-incubation of purified EB with rhEphA2 reduced the adherence of the EB to the cell surface by 36% compared to EB pre-treated with an unrelated His-tagged control protein, rhPHB (PHB-His) (Figure 4.10A). Prohibitin (PHB) is a mitochondrial inner membrane protein. rhPHB acts as a control for His-tagged rhEphA2. These results suggested that EB adhere to epithelial cells via a bacterial ligand that interacts with EphA2, possibly via the ligand-binding domain of EphA2. Indeed, blocking the ligand-binding domain of EphA2 with an antibody directed against the N-terminus of EphA2 reduced adherence of EB to HeLa cells by 33% confirming a role of the ligand-binding domain in the EB-EphA2 interaction (Figure 4.10B).

It was previously shown that the cell surface protein disulphide isomerase (PDI) enzymatic activity is required for chlamydial entry into cells [161]. *Chlamydia* do not appear to utilize plasma membrane–associated PDI as a receptor, suggesting that *Chlamydia* binds to a cell surface protein that requires structural association with PDI. Further, blocking cell surface PDI using PDI antibody did not affect the *Ctr* attachment [161]. Therefore, as a known control, PDI was blocked and found no reduction in *Ctr* adherence (Figure 4.10B). In addition, blocking an unrelated EphB4 receptor [142] had no effect on adherence of *Ctr* (Figure 4.10B). Thus, from the results, either blocking the extracellular domain of EphA2 by rhEphrinA1 or EphA2 antibody significantly reduced *Ctr* infection suggesting that EB directly interacts with EphA2 on the cell surface upon infection.

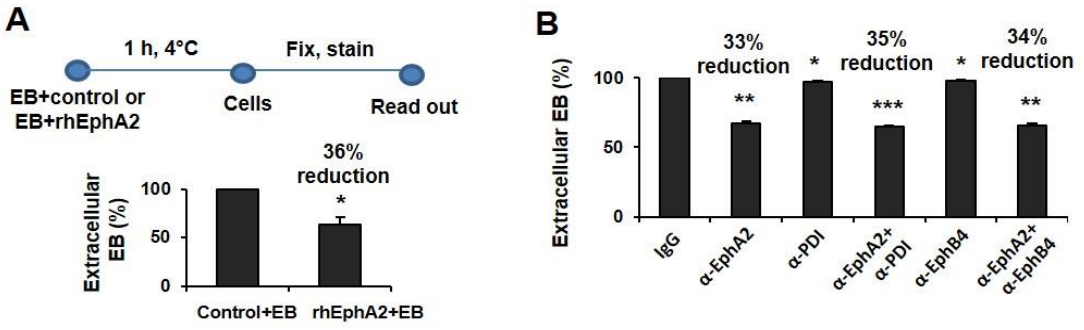

Figure 4.10. EphA2 is required for chlamydial adherence: (A) EB pre-incubated with rhPHB (PHB-His) or with rhEphA2 (EphA2-His) each 5 µg/ml for 1 h at 4°C were added to HeLa cells for 1 h at 4°C. Cells were fixed and immunostained for extracellular EB and actin filaments. The number of extracellular EB was counted randomly from 40 different cells. Data are expressed as percentage of extracellular EB relative to control. Shown is the mean ± SD of three independent experiments normalized to control+EB. *P<0.05. Error bars show mean ± SD. (B) HeLa cells were incubated with antibody against N-terminal-EphA2 or -PDI or -EphB4 each 10 µg/ml for 1 h at 4°C and were infected with GFP-expressing *Ctr* for 1 h at 4°C. Cells were fixed and immunostained against actin filaments (phalloidin). Graph was made similar to (A). Shown is the mean ± SD of two independent experiments normalized to IgG control. *P<0.05, **P<0.01, ***P<0.001.

4.2.2. EphA2 is involved in host cell invasion of *Ctr*

4.2.2.1. Cell surface EphA2 inhibition affects the invasion of *Ctr*-EB

The role of EphA2 was then investigated for the uptake of *Ctr* by HeLa cells (referred to 'invasion'). Invasion was analyzed by infecting cells for 4-6 h at 35°C to allow the uptake of the EB by the host cell (Figure 4.11).

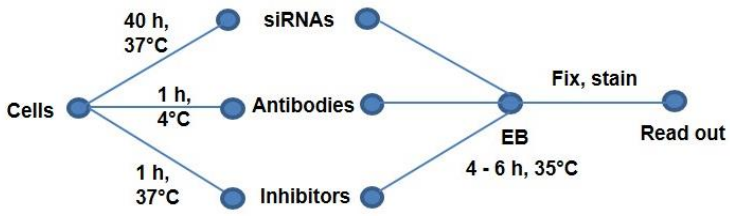

Figure 4.11. Representation for invasion assay: Cells transfected with siRNAs or preincubated with antibodies or inhibitors were infected with EB at 35°C for 4-6 h.

At this time period, EB transform to RB and thus the bacteria do not replicate at this stage of the developmental cycle. After silencing total EphA2 expression by siRNA transfection (Figure 4.12A), *Ctr* invasion was reduced by 30% (Figure 4.12B). It was previously shown that PDI enzymatic activity is required for *Ctr* entry [161]. Silencing of PDI (Figure 4.12A) reduced the invasion rate of *Ctr* by 65% and by 87% if both EphA2 and PDI were silenced together (Figure 4.12B) confirming a role of both proteins in the uptake of EB.

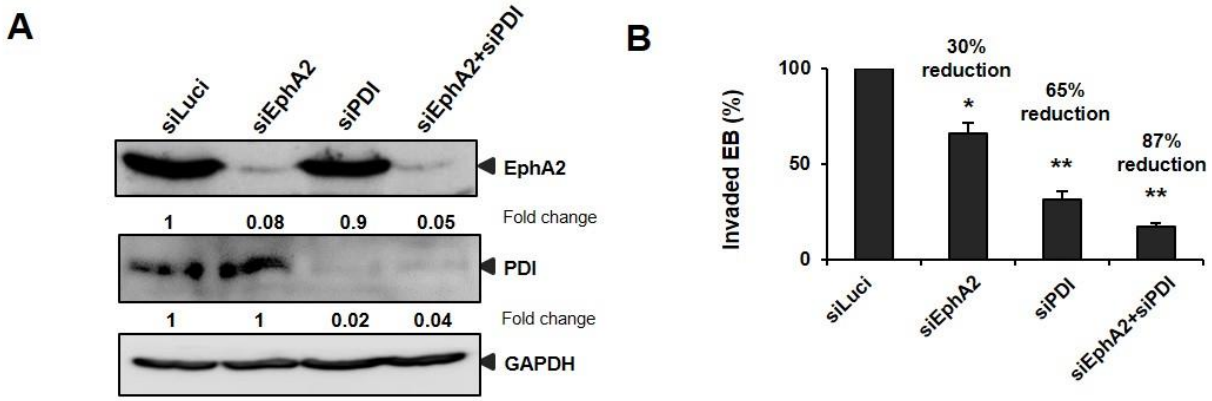

Figure 4.12. EphA2 knockdown affects the invasion of *Ctr*-EB: (A) HeLa cells were transfected with siRNA against luciferease or EphA2 or PDI or EphA2 and PDI for 40 h at 37°C. The knockdown efficiency was monitored by WB analysis against total EphA2, PDI and GAPDH. (B) Transfected cells of A) were infected with *Ctr* (MOI 15-20) for 4 h at 35°C. The cells were immunostained against EB and actin filaments. Number of EB invaded the cell were counted from 10 separate fields of view. Shown is the mean ± SD of three independent experiments normalized to siLuci-infected cells. *P<0.05, **P<0.01. Error bars show mean ± SD.

The ligand-binding domain of EphA2 was blocked with an antibody directed against the N-terminus of EphA2. This EphA2 antibody blocking experiment reduced the invasion of *Ctr* by 30% in a biopsy-derived human fallopian tube epithelial cells (Fimb) which are the primary target cells of *Ctr* genital infections (Figure 4.13A and 4.13B), demonstrating that this is not a cell line specific effect. Thus, EphA2 is required for both, efficient adherence as well as invasion of EB, suggesting that it directly interacts with *Ctr* on the cell surface and this interaction is followed by the induced uptake of EB by the host cell.

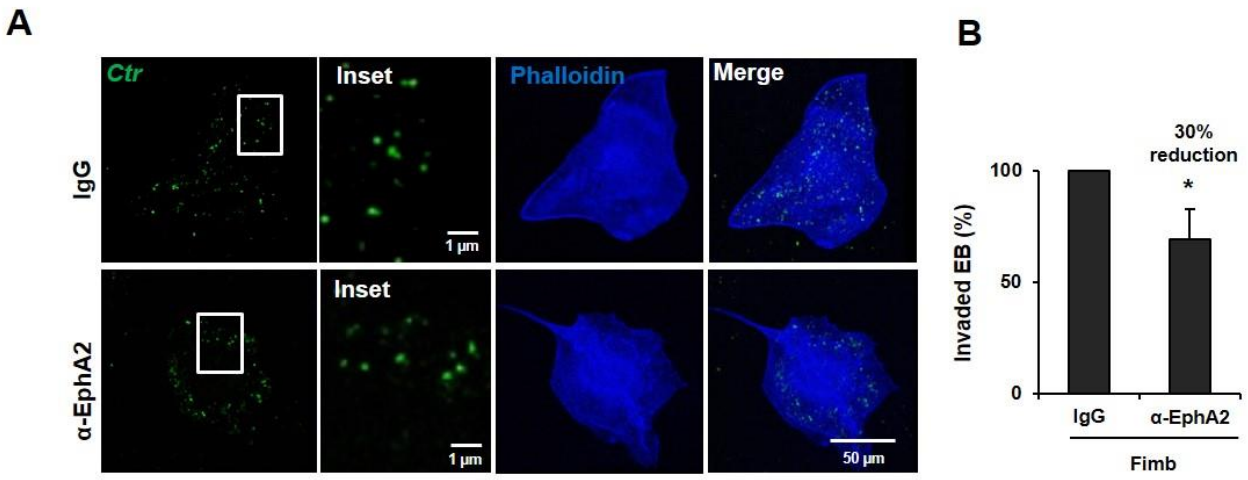

Figure 4.13. Blocking EphA2 affects the invasion of *Ctr*-EB: (A, B) Fimb cells were pre-treated with control IgG or N-terminal specific antibody against EphA2 for 1 h at 4°C and then infected with *Ctr* (MOI-50) for 4 h. (A) Cells were immunostained against *Ctr*-EB (Hsp60) and actin filaments (Phalloidin) and (B) quantified by counting the invaded EB out of 30 different cells. Shown is the mean ± SD of two independent experiments normalized to IgG control. *P<0.05. Error bars show mean ± SD.

4.2.2.2. EphA2 tyrosine kinase inhibitor "Dasatinib" affects *Ctr* invasion

Induced uptake of receptor tyrosine kinases frequently requires the kinase function of the receptor [162]. To get a first hint whether the kinase activity of EphA2 is required for *Ctr* entry, a clinically approved small molecule EphA2 tyrosine kinase inhibitor, dasatinib (DA), was used [141, 163]. In comparison to the control treatment (DMSO), pretreatment of cells with 2.5 or 5 µM DA significantly reduced the entry of EB by 73% or 97%, respectively (Figure 4.14A and 4.14B). Although we cannot exclude a role of other DA-sensitive kinases like Src family kinases, BCR-ABL, KIT (a cytokine receptor) and PDGFR [164, 165], the drastic reduction in *Ctr* invasion after DA treatment demonstrates the functional relevance of RTKs for *Ctr* invasion. This result concluded the kinase activity of EphA2 is required for the *Ctr* entry.

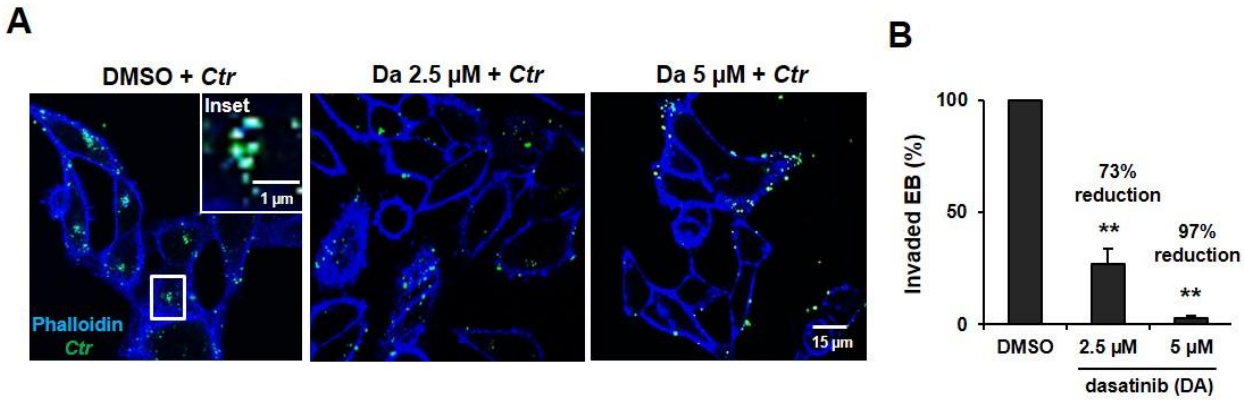

Figure 4.14. Dasatinib affects the invasion of *Ctr*-EB: (A, B) HUVEC cells pre-treated with DMSO control and with 2.5 µM or 5 µM DA, respectively, for 1 h at 37°C were infected with *Ctr* (MOI-20) for 6 h. (A) Cells were fixed and immunostained as (Figure 4.13A). (B) Invaded *Ctr* was counted randomly from 20 different cells under the microscope. Shown is the mean ± SD of three independent experiments normalized to DMSO-treated infected cells. **P<0.01. Error bars show mean ± SD.

4.2.3. *Ctr* Infection induces rapid EphA2 activation and quantitative receptor internalization

4.2.3.1. Internalized EphA2 remains active inside the cell and co-localizes with *Ctr*

Having found that the cell surface EphA2 is required for *Ctr* adherence and invasion into the cells, I next investigated whether the surface EphA2 upon binding with *Ctr* directly mediates the entry of *Ctr* into the cells. Activated EphA2 is located in the infected cell after receptor endocytosis. Interestingly, the internalized active EphA2 receptor localized directly to the invaded EB, but also accumulated close to the bacteria at 4-5 h post infection (p.i.) (Figure 4.15). Thus, the internalized EphA2 is stabilized and continues to signal after endocytosis in *Ctr*-infected cells.

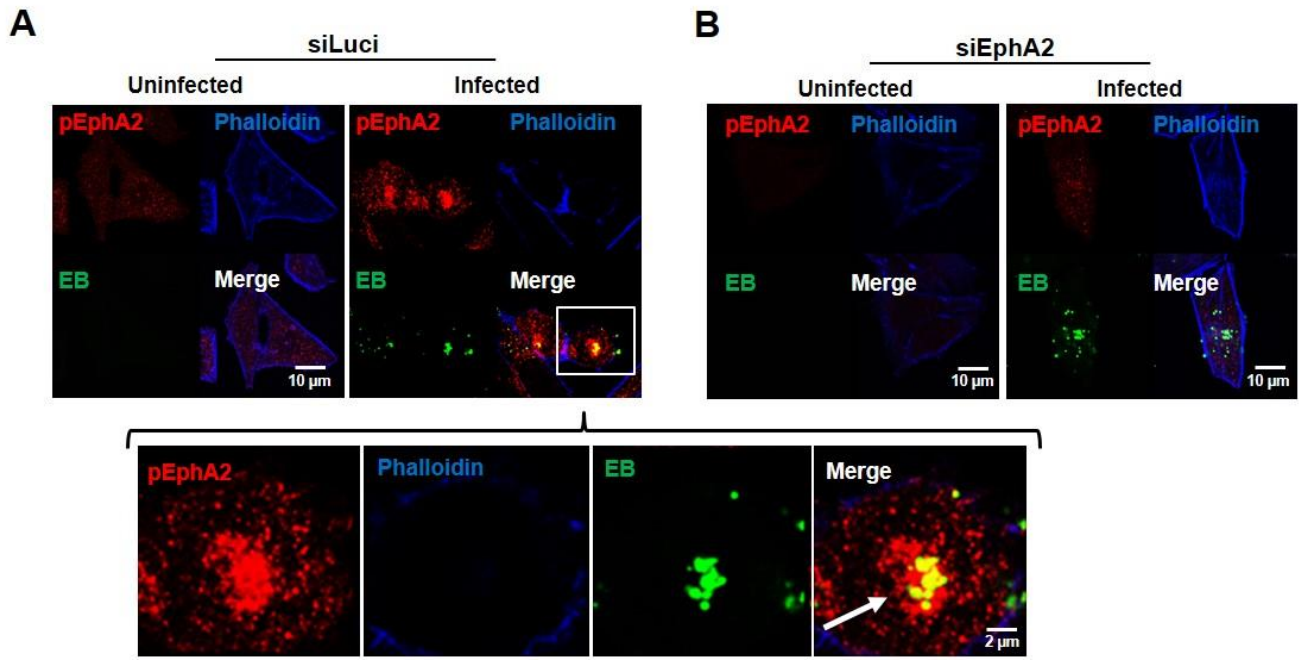

Figure 4.15. Internalized EphA2 is active and co-localizes with *Ctr*: Invasion assay: Cells were transfected with siRNA against Luci gene (A) and EphA2 gene (B). The transfected cells were infected with *Ctr* (MOI-20-25) for 4-5 h at 35°C followed by immunostaining against phospho EphA2 (pEphA2), *Ctr*-EB (Hsp60) and actin filaments (Phalloidin). Arrows indicate the co-localization of *Ctr* with pEphA2 (yellow).

4.2.3.2 EphA2 overexpressing cells increases the invasion rate of *Ctr*

EphA2 was overexpressed in cells to further substantiate its invasion supportive function and its association with invaded *Ctr*. The invasion rate of *Ctr* 6 h p.i. and total EphA2 was determined by FACS under permeabilized conditions to determine the total EphA2 levels. Increased EphA2 expression was confirmed in EphA2-pcDNA3-transfected compared to the control-transfected cells (Figure 4.16A and 4.16C). Invasion of *Ctr* increased about 2-fold if EphA2 was overexpressed (Figure 4.16B and 4.16C). Consistent with the previous observation (Figure 4.15), invaded *Ctr* co-localized with EphA2 inside the cell (Figure 4.16C, indicated by arrows). These results confirm that EB bind and activate surface EphA2, resulting in *Ctr* and receptor internalization.

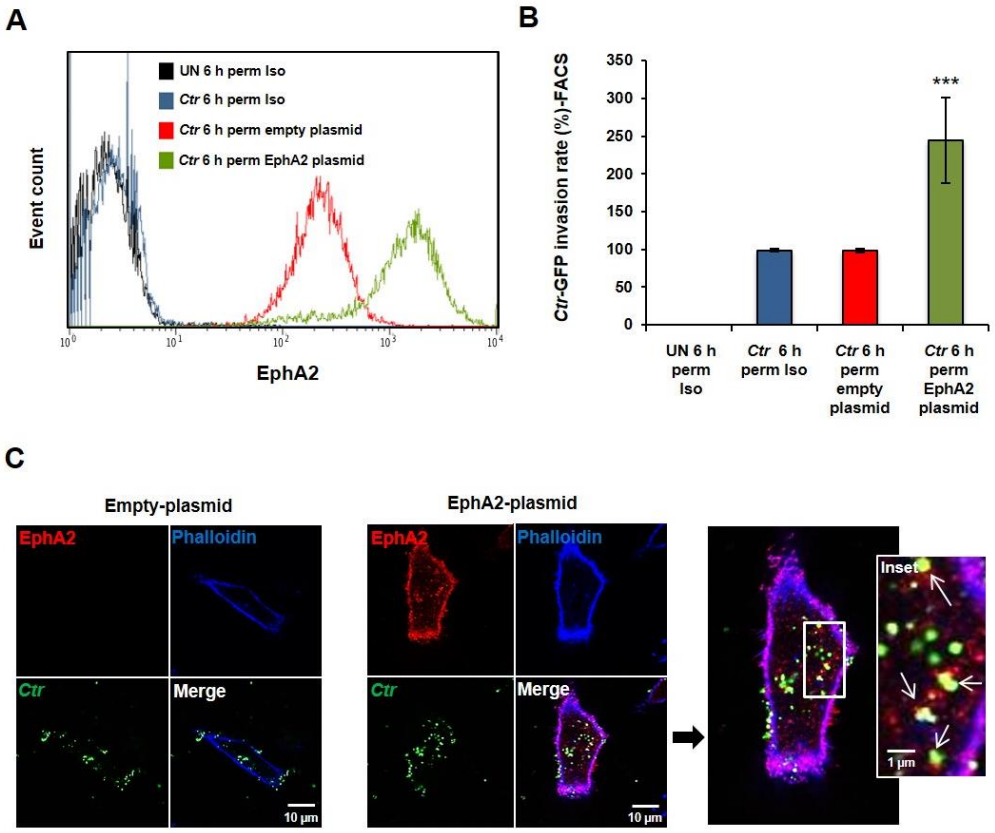

Figure 4.16. EphA2 overexpression enhances the invasion rate of *Ctr*-GFP: (A, B) HeLa cells were left untransfected or transfected with empty-pcDNA3 or EphA2-pcDNA3 for 40 h followed by infection with *Ctr*-GFP (MOI-50) for 6 h. EphA2 expression (A) and invaded *Ctr*-GFP (B) were checked by FACS under permeabilised condition. The graph (B) shows the mean fluorescence value of the uninfected (UN) or infected cells under permeabilised condition compared to the respective controls. Shown is the mean± SD of three experiments.***P<0.001). (A, B) UN: uninfected, Iso: isotype and perm: permeabilised. (C) Empty-pcDNA3 or EphA2-pcDNA3 transfected HeLa cells were infected with *Ctr* for 4 h (MOI-20). Cells were fixed for immunostaining against EphA2 (EphA2, red), Actin (Phalloidin) and *Ctr* (Hsp60). Arrows were drawn to indicate the co-localization of invaded *Ctr* with EphA2 (yellow).

4.2.3.3. Surface expression of EphA2 rapidly changes upon early *Ctr* infection

The results so far demonstrated that the surface EphA2 is required for *Ctr* adherence and invasion and that the kinase activity of EphA2 may be involved in the uptake of EB. If invasion of EB changes surface display of EphA2 during *Ctr* entry. Unexpectedly, surface display of EphA2 increased as early as 30 min p.i. but returned to basal levels by 3 h p.i. as was determined by

FACS under non-permeabilized condition measuring surface exposed EphA2 only (Figure 4.17A, B-curve chart and 4.17C-bar chart). This raised the question whether the rapid downregulation of surface EphA2 that follows the initial increase results from receptor internalization. FACS analysis under permeabilized conditions to detect surface exposed and internalized proteins showed an increased EphA2 signal at 3 h p.i. (Figure 4.17A, B-curve chart and 4.17C-bar chart). Since EphA2 in the supernatant of cells at this time-point could not be detected (Figure 4.17D), it was concluded that EphA2 was internalized into the cell.

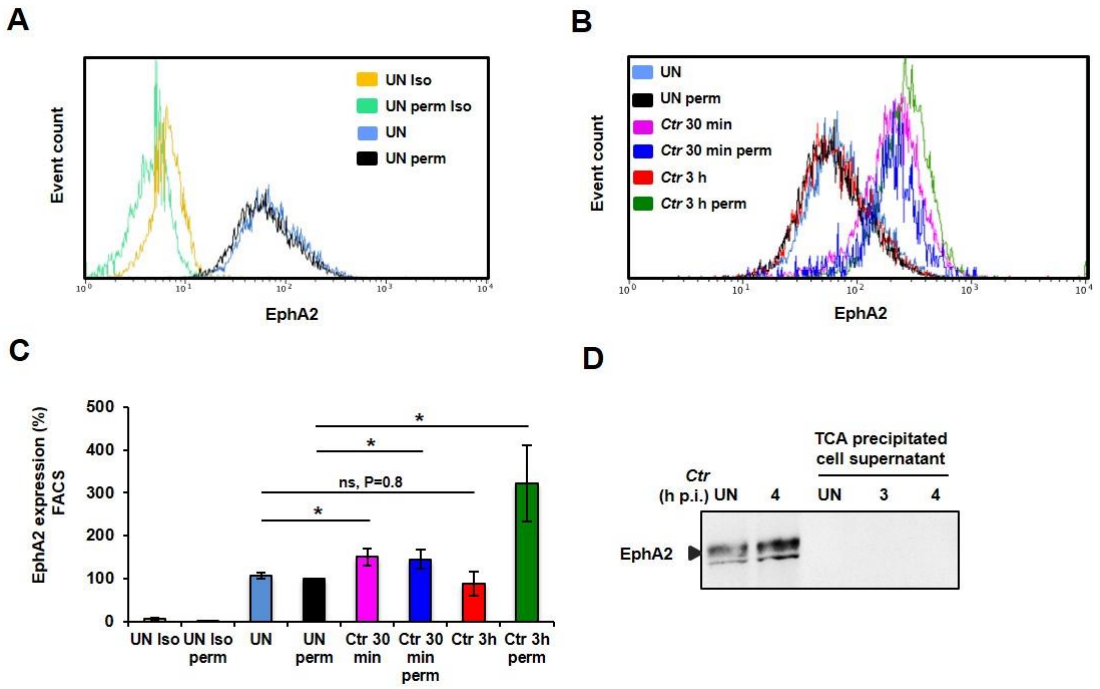

Figure 4.17. *Ctr*-induces receptor internalization during early infection: (A, B) HeLa cells uninfected (UN) or infected with *Ctr* (MOI-50-75) for 30 min or 3 h were analyzed via FACS for surface EphA2 under non permeabilised or total EphA2 under permeabilised condition. Controls were indicated on the left curve chart (A) and corresponding samples on the right curve chart of the same experiment (B). (UN: uninfected, Iso: isotype and perm: permeabilised). (C) Result of A,B were indicated in bar chart for better understanding. Shown is the mean ± SD of three independent experiments normalized to UN perm. *P<0.05, ns: non significant. Error bars show mean ± SD. (D) Culture medium of the UN as well as time course (as indicated) *Ctr*-infected cells were collected and TCA precipitated. The precipitated lysates were subjected to WB analysis against total EphA2.

4.2.3.4. Unlike Ephrin-A1-induced EphA2 degradation, EphA2 is rapidly activated and not degraded during *Ctr* infection

Internalization is probably mediated by binding of EB to the ligand binding domain of EphA2, since Ephrin-A1 ligand-induced aggregation of EphA2 on the cell surface has previously been shown to function as the internalization signal [166]. This ligand-induced activation of EphA2 results in auto-phosphorylation followed by rapid degradation. In line with receptor activation by *Ctr* infection, increased EphA2 phosphorylation was observed, reaching a 9-fold increase as early as 15 min p.i. (Figure 4.18A and also 4.8A). Interestingly, in contrast to ligand-induced EphA2 degradation ([166] and Figure 4.18B), EphA2 was not degraded in the cell during *Ctr* infection. Total levels of EphA2 rather started to increase around 4 h p.i. (Figure 4.18A), implying that EphA2 signaling induced by Ephrin-A1 and EB interaction differs. In addition, the rapid increase and decline in cell surface exposed EphA2 during early infection time-points is independent of changes in total EphA2.

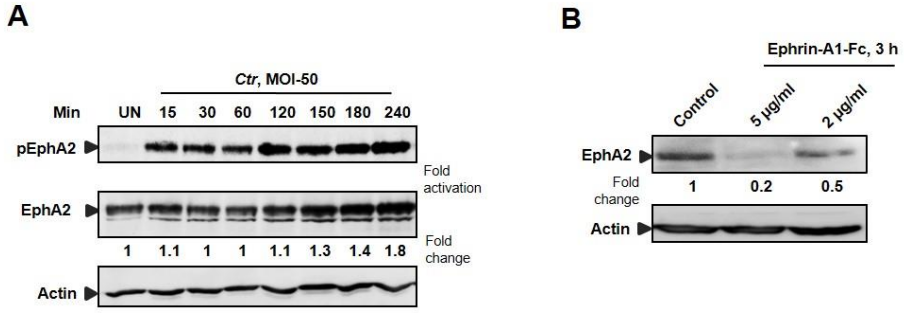

Figure 4.18. *Ctr*-induces rapid EphA2 activation: (A) HeLa cells were UN or infected with *Ctr* (MOI-50) for the indicated time points. The cells were immunoblotted against pEphA2 and Actin. The blot was stripped and reprobed for total EphA2. (B) HeLa cells were treated with control-Fc or Ephrin-A1-Fc for 3 h and were harvested for WB analysis.

4.2.4. EphA2 activated by *Ctr* infection recruits and activates PI3 Kinase

Infection with *Ctr* activates the RAF/MEK/ERK and PI3K/Akt pathways that support normal chlamydial development and keep the infected cell in an apoptosis resistant state [70]. The

pathway that leads to PI3K activation has not been identified so far in *Ctr* infection. Several studies in recent years have shown an association of activated EphA2 with PI3K [107, 109].

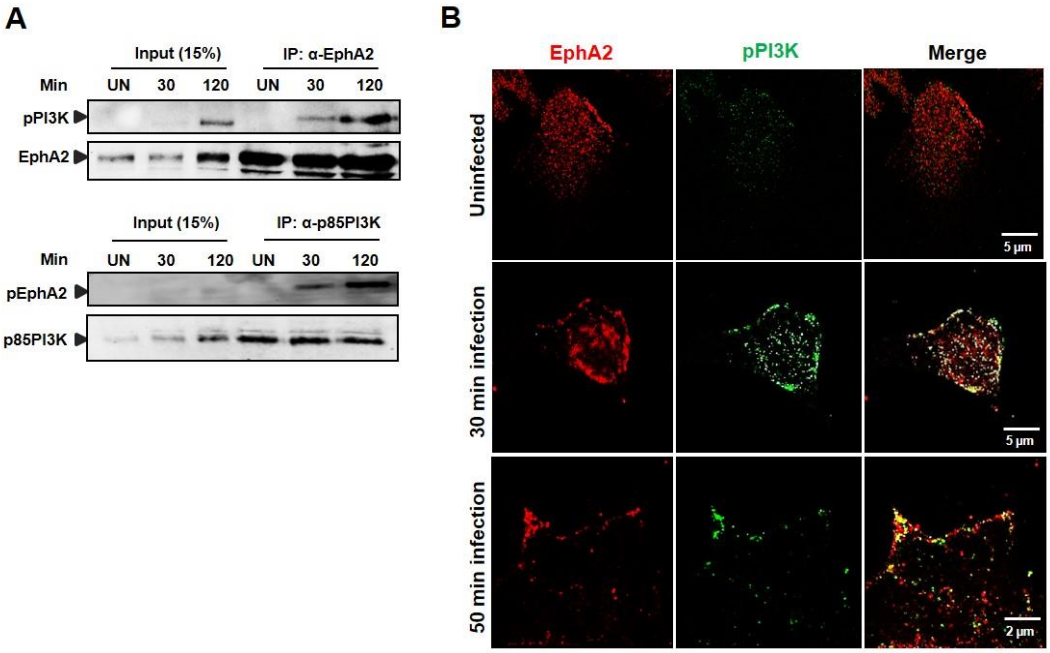

Figure 4.19. EphA2 associates with pPI3K during early infection: (A) HeLa cells were UN or infected with EB for the indicated time points and immunoprecipitated (IP) with α-EphA2 or α-p85-PI3K antibodies. The IP material was solved in 40 µl Laemmli (100%) and loaded 20 µl for WB studies (50%) or (B) immunostained using α-EphA2 and α-pPI3K for 2 h at RT followed by secondary staining with anti-mouse Alexa fluor 488 and anti-rabbit Alexa fluor 647 for the microscopic analysis, respectively.

To reveal whether the activated EphA2 induced by *Ctr* infection interacts with PI3K, endogenous EphA2 or the endogenous p85 regulatory subunit of PI3K (p85-PI3K) was immunoprecipitated from the lysates of uninfected or *Ctr*-infected cells. Activated PI3K co-immunoprecipitated with EphA2 and activated EphA2 co-immunoprecipitated with p85-PI3K at 30 min p.i. and the activation of both proteins were increased when immunoprecipitated from cells infected for 2 h (Figure 4.19A). The interaction was further verified by microscopy showing the co-localization of EphA2 with activated PI3K after *Ctr* infection (Figure 4.19B). These results strongly suggested that EphA2 activated upon *Ctr* infection recruits and activates PI3K during early infection.

4.3. Role of EphA2 in mid-phase *Ctr* infection

4.3.1. Intracellular EphA2 associates with *Ctr* inclusion during mid-phase of the *Ctr* developmental cycle and is prevented from re-translocating to the cell surface

4.3.1.1. EphA2 upregulated and activated throughout the *Ctr* infection is intracellular

To this point, all data on the role of EphA2 during chlamydial infection were obtained during the early phase of infection and prior to EB-RB transformation. However, the proteomics data (Figure 4.6) suggested that EphA2 is also associated with the inclusion during the mid-phase of infection (24 h p.i.) when RB fully replicate. Therefore the role of EphA2 during mid-phase *Ctr* infection was investigated.

EphA2 is activated and upregulated not only in HeLa cell line (Figure 4.20) but also in primary cells like HUVEC and Fimb cells (Figure 4.20A-HUVEC and 4.20B-Fimb) upon infection, demonstrating that this is not a cell line specific effect. Further, the expression of PDGFRβ was tested, which has been shown to be a receptor for *C. muridarum* [37] and of PDI required for *Ctr* invasion [161] as well as of EphB4, which is not required for *Ctr* adherence (Figure 4.10B). In contrast to EphA2, PDGFRβ was downregulated during *Ctr* infection and no significant changes were observed for PDI or EphB4 expression (Figure 4.20C). The amount and phosphorylation of EphA2 increased during viable *Ctr* infection in HeLa, but not heat-killed bacteria (Figure 4.20D). These results strongly conclude that EphA2 is specifically upregulated and activated during *Ctr* infection.

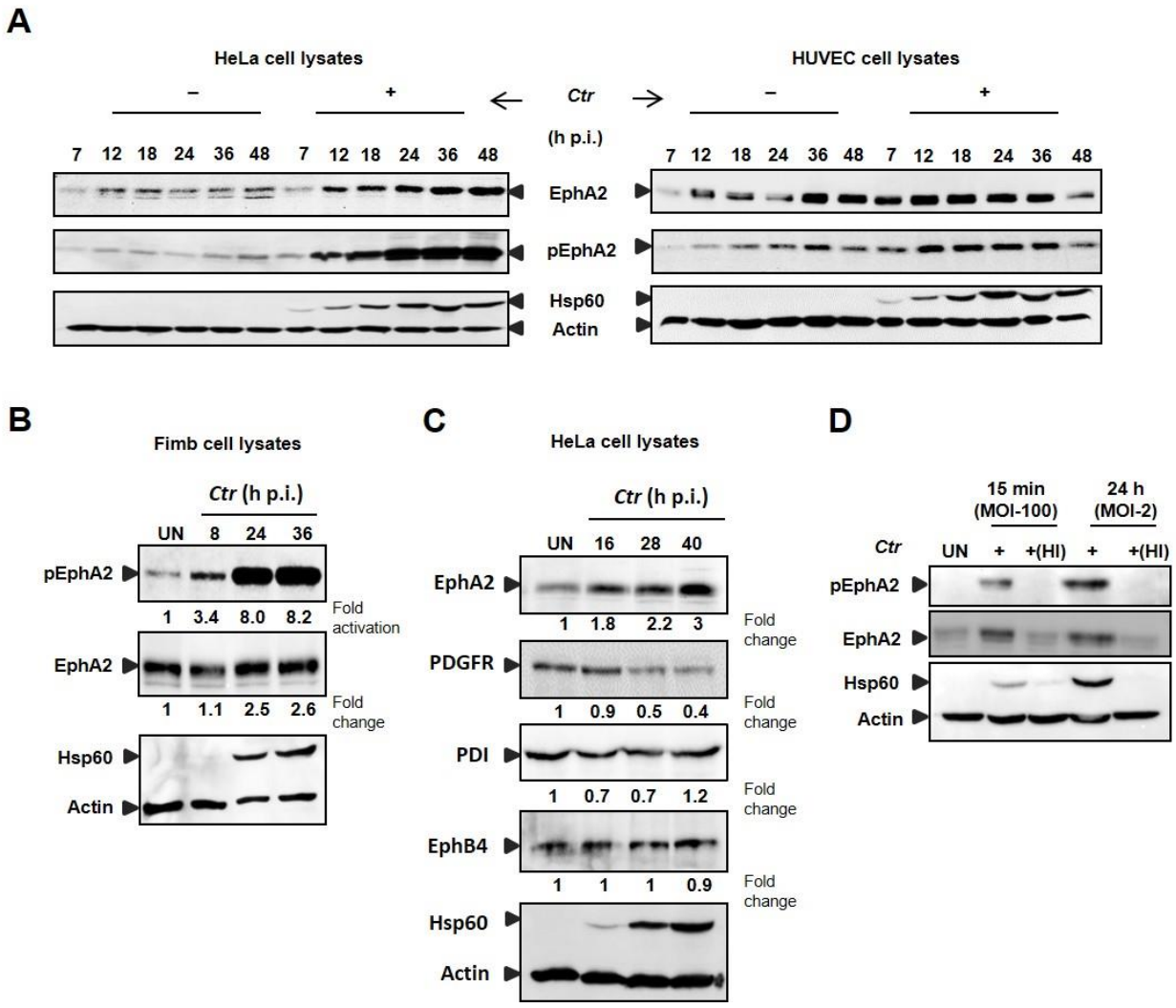

Figure 4.20. Viable *Ctr* infection induces the expression and activation of intracellular EphA2 in different cell lysates: (A, B, C) HeLa or HUVEC or Fimb cells were UN or infected with *Ctr* for different time points and subjected to WB analysis to determine the expression of the proteins indicated. (D) Cells were UN or infected with viable *Ctr* or heat-inactivated *Ctr* (HI) (65°C, 30 min) at MOI-100 for 15 min or with MOI-2 for 24 h. Lysed cells were immunoblotted against pEphA2 and Actin. The blot was stripped and reprobed for total EphA2. Increased levels of total EphA2 upon 15 min p.i. depend on the high MOI of 100 used in this experiment.

In order to determine where the overexpressed and activated EphA2 is located in the infected cell during the mid-phase of *Ctr* infection, plasma membrane protein isolation was performed. Plasma membrane isolated from infected cells at those time points contained constant levels of

total and activated EphA2, suggesting that the basal levels of EphA2 and activated EphA2 at these time points were unchanged at the cell surface (Figure 4.21A and 4.21B).

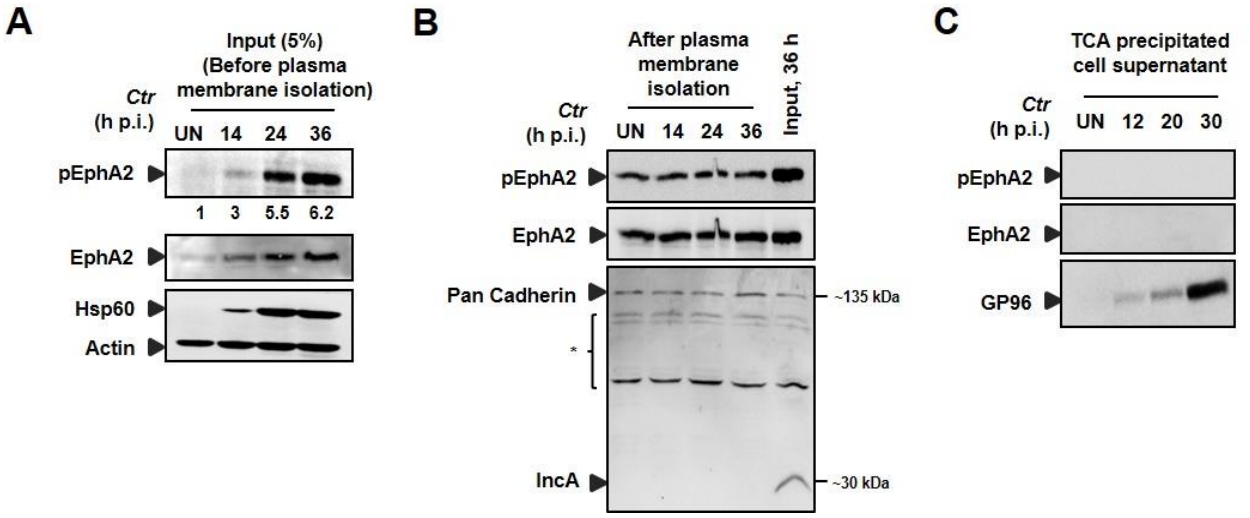

Figure 4.21. *Ctr*-induced EphA2 expression is intracellular: (A) HeLa cells were infected with *Ctr*-EB for 14, 24 and 36 h. Input (5%) was taken after homogenization step (before plasma membrane protein isolation) and subjected to WB analysis to determine pEphA2, Hsp60, and Actin. The blot was stripped and reprobed for total EphA2. Numbers under the blot represents fold activation for pEphA2 with respect to total EphA2. (B) Plasma membrane protein was isolated from UN or *Ctr*-infected cells at different time points and subjected to WB analysis. Pan Cadherin was used as a control for plasma membrane. IncA was used as a marker to test the quality of the isolated plasma membrane. * Unspecific bands detected by IncA polyclonal serum (C) Culture medium of the UN as well as time course *Ctr*-infected cells (as indicated) was collected and TCA precipitated. The precipitated lysates were subjected to WB analysis against pEphA2, total EphA2 and GP96.

Further, the supernatants of the infected cells were precipitated to test if surface EphA2 is shed into the culture medium during infection as previously demonstrated for GP96 (Karunakaran et al., submitted). EphA2 was not detectable in the culture supernatant (Figure 4.21C), confirming that the increase in levels of EphA2 and activated EphA2 is restricted to the intracellular space. The levels of EphA2 on the cell surface as well as inside the cell during mid-phase *Ctr* infection were also determined by FACS analysis under permeabilised and non-permeablised condition. Surface EphA2 expression was similar at three different time points of *Ctr* infection (14, 20 and 26 h p.i.) whereas permeabilised-infected cells (20 and 26 h p.i.) displayed a clear increase of

intracellular EphA2 levels (Figure 4.22). Thus, EphA2 upregulated during *Ctr* infection is intracellular and is prevented being translocated to the cell surface.

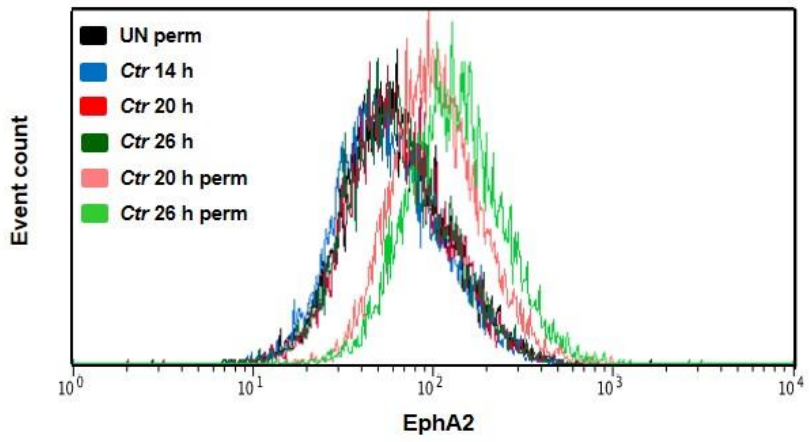

Figure 4.22. Analysis of surface and total EphA2 expression during mid-phase *Ctr* infection: HeLa cells were infected with *Ctr*-EB (MOI-1) for 14, 20 and 26 h and cells were analyzed via FACS as Figure 4.17A. (UN: uninfected; perm: permeabilised).

4.3.1.2. EphA2 is recruited to the inclusion membrane of *Ctr*

To define the intracellular localization of EphA2, cells were first transfected with EphA2-pcDNA3 and infected for different time intervals with *Ctr*. Overexpressed EphA2 was detected in the vicinity (6 and 16 h p.i.) and in association (24 and 40 h p.i.) with the inclusion (Figure 4.23A). Interestingly, the inclusion size appeared bigger in EphA2 overexpressing cells comparing to neighboring untranfected cells (Figure 4.23B), which shows the importance of EphA2 intracellular signaling. However, it is not clear whether the internalized EphA2 is recruited by any inclusion specific membrane protein.

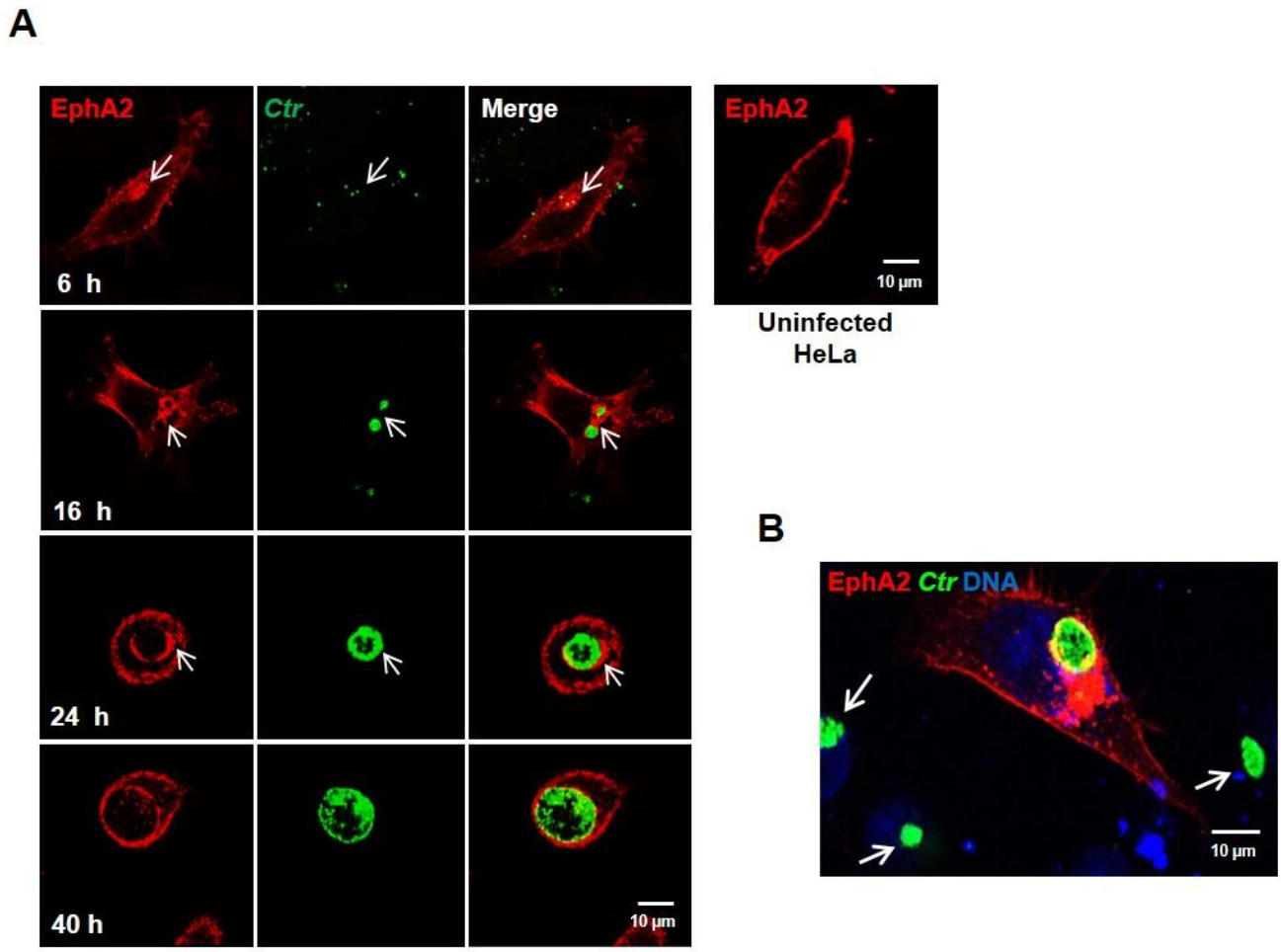

Figure 4.23. EphA2 associates with *Ctr* inclusion: (A) Cells were transfected with EphA2-pcDNA3 and left uninfected (UN) or infected with *Ctr* (MOI-1) for different times as indicated. Cells were immunostained against EphA2 (red) and Hsp60 (*Ctr*, green). (B) Cells transfected with EphA2-pcDNA3 were infected with *Ctr* (MOI-1) and immunostained against EphA2 (red), Hsp60 (*Ctr*, green) and DNA (Draq5, blue). *Ctr* inclusion of the untransfected cells (indicated with white arrows) were smaller than the inclusions of EphA2-transfected cells.

To obtain the proof for the localization and activation of EphA2 around the inclusion membrane, the inclusion membrane-associated chlamydial IncA protein was fused to a HA and Flag tag in the plasmid pGFP::SW2 [152], replacing the GFP:CAT (Figure 4.24A). The *Ctr* strain expressing recombinant IncA-HA-Flag (*Ctr*-pIncA-flag) was confirmed by WB analysis (Figure 4.24B). In cells infected with *Ctr*-pIncA-flag, EphA2 was active (Figure 4.24C) and co-localized (Figure

4.24D) at the chlamydial inclusion membrane concluding that the inclusion-associated EphA2 receptor is active.

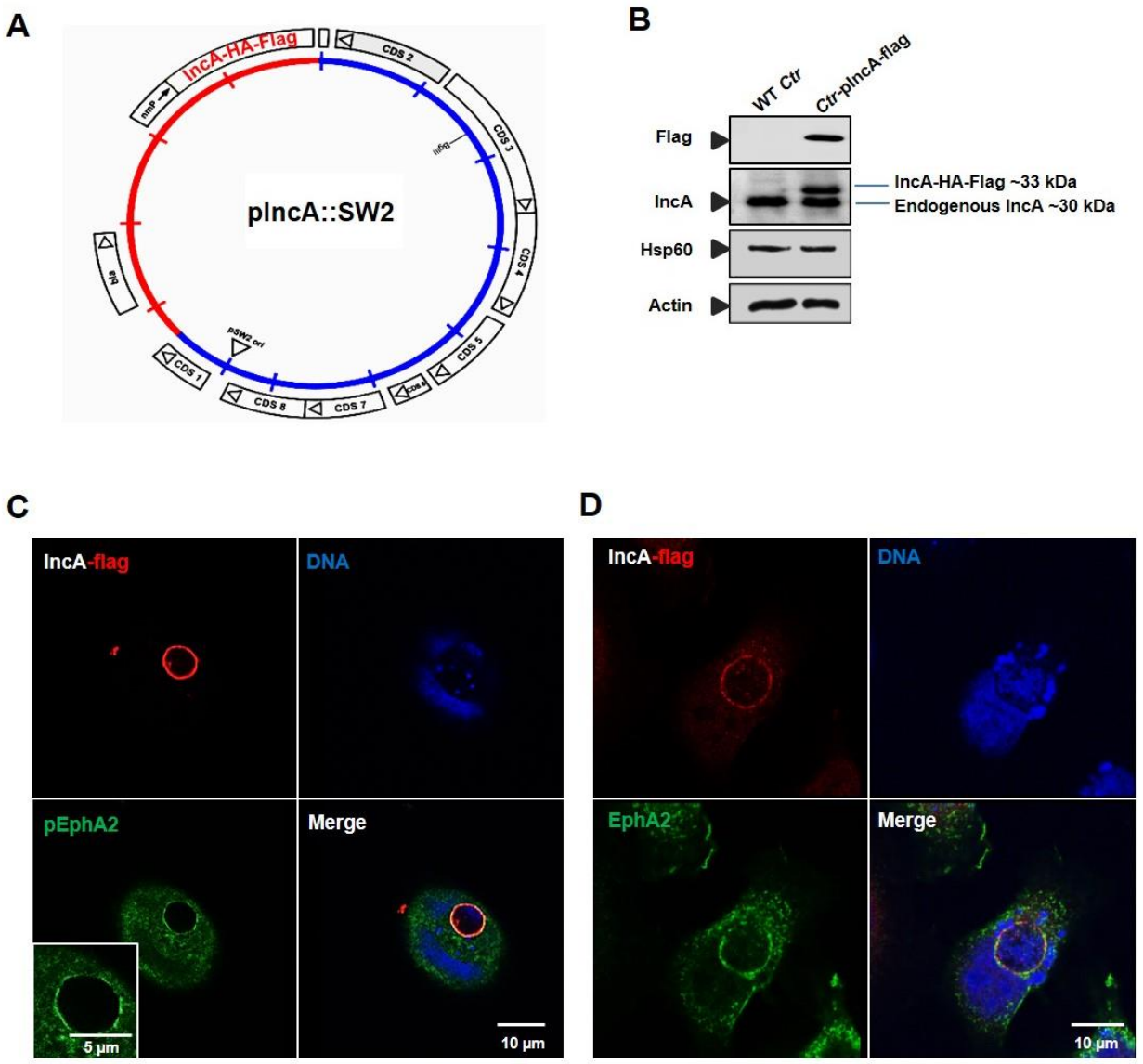

Figure 4.24. Association of EphA2 with *Ctr* inclusion membrane: (A) The vector map of pIncA::SW2 modified from the plasmid pGFP::SW2 [152] by replacing GFP:CAT with IncA-HA-Flag. (B) HeLa cells were infected with *Ctr* wild type (WT) or *Ctr*-pIncA-flag for 24 h. Cells were lysed and the indicated proteins were detected by immunoblotting after separation on a 17% SDS PAGE gel to separate the endogenous IncA from the *Ctr*-pIncA-Flag expressing recombinant IncA. (C, D) HeLa cells were transfected with EphA2-pcDNA3 at 37°C and the cells were infected with *Ctr*-pIncA-flag for 24 h at 35°C. (C) Infected were fixed and stained against Flag (red), EphA2 (green) and DNA (blue). (D) Infected were fixed and stained against Flag (red), pEphA2 (green) and DNA (blue).

4.3.2. Ephrin-A1 independent signaling takes place during chlamydial infection

4.3.2.1. Chlamydial infection induces ligand independent signaling

Ephrin-A1 ligand-mediated activation of EphA2 signaling inhibits both RAF/MEK/ERK and PI3K/Akt pathways whereas ligand-independent EphA2 signaling is important to promote those pathways [105, 110, 111]. Both ways of signaling are balanced in uninfected cells to maintain the cell homeostasis. To investigate whether Ephrin-A1-mediated EphA2 signaling takes place in *Ctr* infection, *Ctr*-infected lysates at different time points were collected and checked for the levels of Ephrin-A1. Levels of Ephrin-A1 were reduced in *Ctr*-infected HeLa cells and in primary HUVECs (Figure 4.25) at the indicated time points. Ephrin-A1 ligation with EphA2 induces phosphorylation of EphA2 at Tyr594 [167] and de-phosphorylation of EphA2 at Ser897 [104], as well as downregulation of EphA2 expression [168, 169]. In contrast, the data presented here shows that the EphA2 protein levels and phosphorylation at Ser897 were increased during *Ctr* infection in HeLa, HUVEC and also in Fimb cells (Figure 4.25). Thus, *Ctr* infection induces ligand-independent EphA2 signaling.

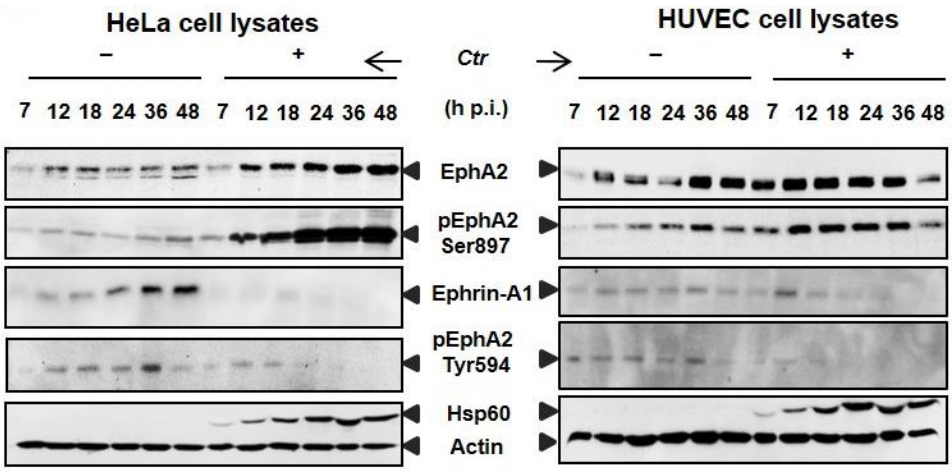

Figure 4.25. Ligand independent EphA2 signaling takes place during *Ctr* infection: HeLa or HUVEC cells were uninfected or infected with *Ctr* at a MOI-1 for different time points and expression of different proteins were analyzed by WB.

4.3.2.2. Ephrin-A1-mediated EphA2 signaling affects *Ctr* infection

To test whether Ephrin-A1-mediated EphA2 signaling affects *Ctr* infection or not, rhEphrinA1 was used. Comparing to the untreated infected cell, rhEphrin-A1 treatment reduced the EphA2 levels and subsequently reduced the primary and progeny infection (Figure 4.26A). The reduced *Ctr* primary infection in rhEphrin-A1 pre-treated cells was because of less availability of surface EphA2 for *Ctr* to invade the cell by considering the previous result (Figure 4.7A and 4.7B). From figure 4.21B and 4.22, the basal surface EphA2 levels in infected cells was detected similar to uninfected cells. Therefore, the rhEphrin-A1 presented in the culture medium might bind to the basal surface EphA2 and activates the reverse signal leading to the inhibition of pAkt and pERK survival pathways which further affects the invaded *Ctr* infection (Figure 4.26A and 4.26B).

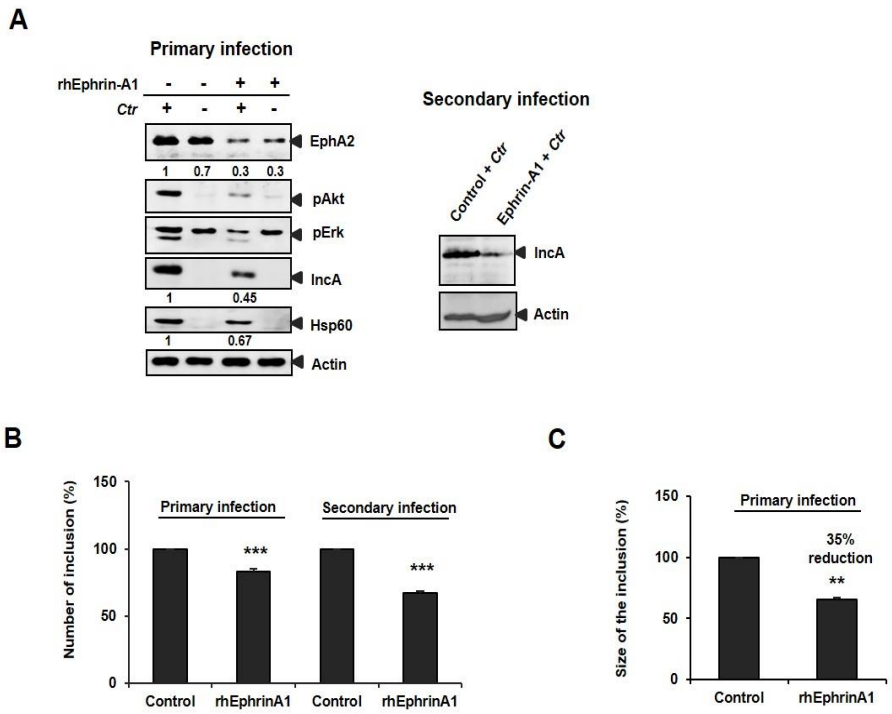

Figure 4.26. rhEphrin-A1 pretreatment affects *Ctr* infection: (A) Cells were pre-treated with Fc control or rhEphrin-A1-Fc (15 µg/ml) for 2 h at 37°C and then uninfected or infected with *Ctr* (MOI-1) for 28 h. Primary infected cell lysates were taken to infect the fresh cells for secondary infection (Infectivity assay). Cells were harvested to determine respective proteins by WB analysis. (B, C) Number and size of the inclusion per cell (%) for the infectivity assay were determined by Image J analysis. The graph was made after counting inclusions from 5 different microscopic fields in 3 independent experiment whereas size of the inclusion was measured using cells out of four microscopic fields from 2 independent experiments (Error bars represent ± SD. ***P <0.001, **P<0.01).

The interference of Ephrin-A1 signaling with *Ctr* infection was quantified. The number and size of the inclusions in HeLa cells were significantly reduced in rhEphrin-A1-treated cells comparing to the control-infected cells (Figure 4.26B and 4.26C). Thus, the high expression of EphA2 and low expression of Ephrin-A1 ligand could represent an advantage for *Ctr* infection. Hence the results provided the indication that Ephrin-A1-mediated EphA2 signaling acts negative for *Ctr* infection by preventing EphA2 from binding to its endogenous signaling ligands thereby suppressing the crucial signaling pathways required for *Ctr* infection. Thus, Ephrin-A1-mediated EphA2 signaling interferes with infection-induced PI3K signaling and *Ctr* infection.

4.3.3. EphA2 is required for long-lasting PI3K activation

The rapid and strong activation of EphA2 by chlamydial infection and the association of EphA2 with inclusions during the replicative phase of the developmental cycle suggested that EphA2 is required for chlamydial development, probably by signaling via PI3K. It is important to note that experiments conducted to investigate the role of EphA2 during the mid-phase of the chlamydial cycle were performed with a low MOI of 1-2 (in contrast to MOI of 50-100 for adherence and MOI of 20-50 for invasion experiments) to ensure similar initial infection irrespective of the EphA2 status (Infectivity assay-Figure 4.27 and methods 3.2.10).

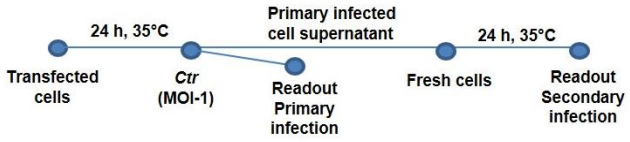

Figure 4.27. Infectivity assay: The transfected cells infected with *Ctr* (MOI-1) for 24 h at 35°C was referred as primary infection. The supernatant of the primary infected cells was taken to infect the fresh cells to determine the secondary infection.

4.3.3.1. Interfering with EphA2 influences PI3K activation and *Ctr* infection

To determine if changes in the expression of EphA2 influence Akt activation, EphA2 overexpression by an expression plasmid and EphA2 knockdown by siRNA were performed. Indeed, EphA2 overexpression resulted in increased pAkt levels in uninfected cells which further increased upon *Ctr* infection [Figure 4.28(1)A]. In line with a role of EphA2 in PI3K activation also at the mid-phase of the chlamydial cycle, Akt activation was reduced upon knockdown of EphA2 in *Ctr*-infected cells [Figure 4.28(1)B]. Interfering with pAkt signaling by modulating EphA2 levels also influenced *Ctr* primary and progeny infection [Figure 4.28(1)A-E]. EphA2 overexpression or EphA2 silencing changed the progeny infection by approximately 48% (increase during overexpression) and 37% (decrease during knockdown), respectively [Figure 4.28(1)C and 4.28(1)D]. Number of inclusions in both primary (18% reduction) and secondary

infection [43% reduction, Figure 4.28(1)E] as well as the size of the inclusion in primary infection [40% reduction, Figure 4.28(2)B] were significantly affected upon silencing EphA2 expression. Further, silencing of PDGFRβ had no effect on PI3K activation and *Ctr* infection [Figure 4.28(2)A and 4.28(2)B], demonstrating a specific role of EphA2 in *Ctr* infection. These results suggested that EphA2 activates the PI3K pathway and thereby supports chlamydial development.

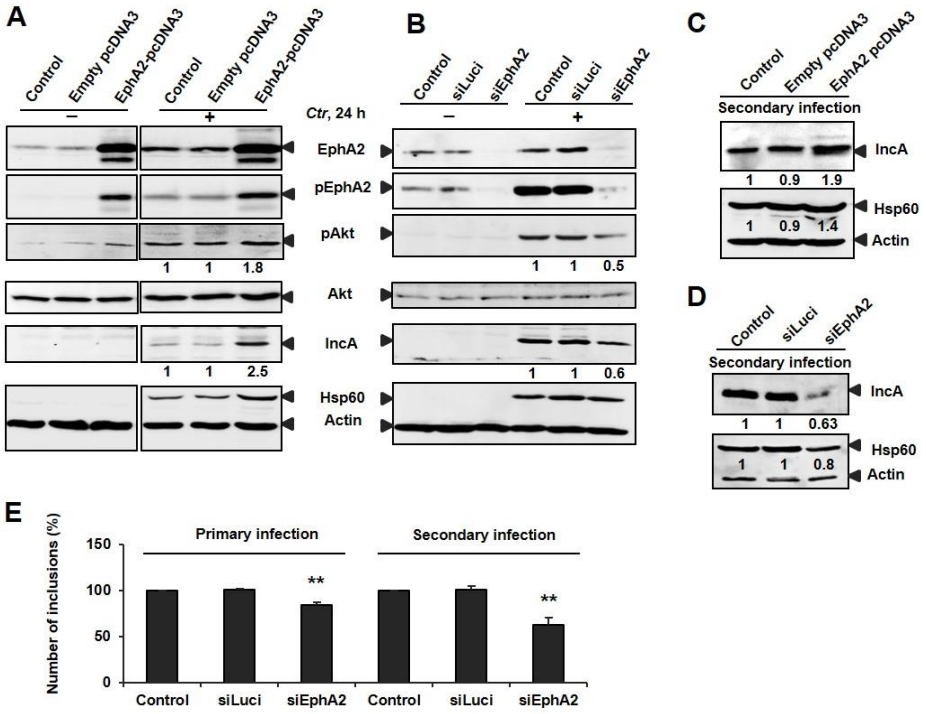

Figure 4.28(1). EphA2 overexpression and knockdown influences Akt activation and *Ctr* infection: (A) HeLa cells were transfected with empty pcDNA3 or EphA2-pcDNA3 each 1µg/ml and then left UN or infected with *Ctr* (MOI-1) for 24 h. Cells were harvested for WB analysis. (B) HeLa cells were untransfected (control) or transfected with siRNA against luciferase gene (siLuci) or EphA2 gene (siEphA2) for 40 h at 37°C and then left UN or infected with *Ctr* (MOI-1) for another 24 h. Cells were harvested for WB analysis. (C, D) Primary infected cell lysates of (B) and (C) were taken to infect the fresh cells for secondary infection (Infectivity assay). Numbers under the blot represents fold activation for phospho specific proteins with respect to total proteins and fold change for the total proteins. (E) Number of the inclusion per cell (%) for the infectivity assay was determined by counting inclusions on 10 independent fields. (Shown is the mean ± SD of three independent experiments normalized to control. **P<0.01).

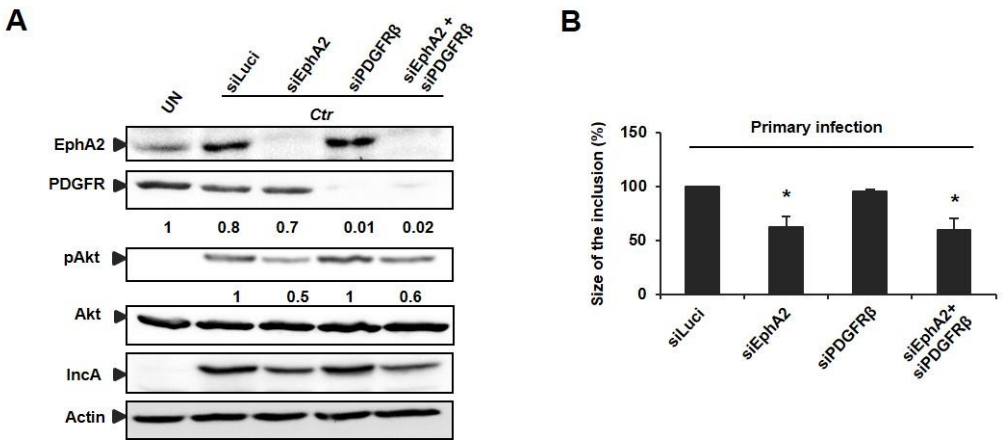

Figure 4.28(2). Specificity of EphA2 on Akt activation and *Ctr* infection: (A) Cells were transfected with siRNA against luciferase (siLuci) or EphA2 (siEphA2) or PDGFRβ (siPDGFRβ) or both together for 40 h at 37°C and then infected with *Ctr* for another 20 h. UN: uninfected cells. Cells were harvested to determine the respective proteins by WB analysis. Numbers under the blot represents fold activation for phospho specific proteins with respect to total proteins and fold change for the total proteins. (B) Size of the inclusion per cell (%) for (A) was determined by ImageJ analysis by measuring cells out of four microscopic fields (Shown is the mean ± SD of two independent experiments normalized to siLuci. *P<0.05).

4.3.3.2. EphA2 interacts with PI3K and the complex associates with *Ctr* infection

As increased Akt activation was found in EphA2–overexpressing-infected cells [Figure 4.28(1)A] and interaction of activated PI3K with EphA2 during early infection (Figure 4.19A and 4.19B), it was next determined whether increased total EphA2 during late *Ctr* infection also associates with activated PI3K. Co-immunoprecipitation experiment confirmed the presence of increased endogenous levels of activated PI3K with the immunoprecipitated endogenous EphA2 at 18 and 24 h p.i, when *Ctr* has reached the replicative state (Figure 4.29A). The interaction was further verified by confocal microscopy showing the co-localization of EphA2 with activated PI3K after *Ctr* infection (Figure 4.29B), suggesting that PI3K activation is an important function of intracellular EphA2 in *Ctr* infection.

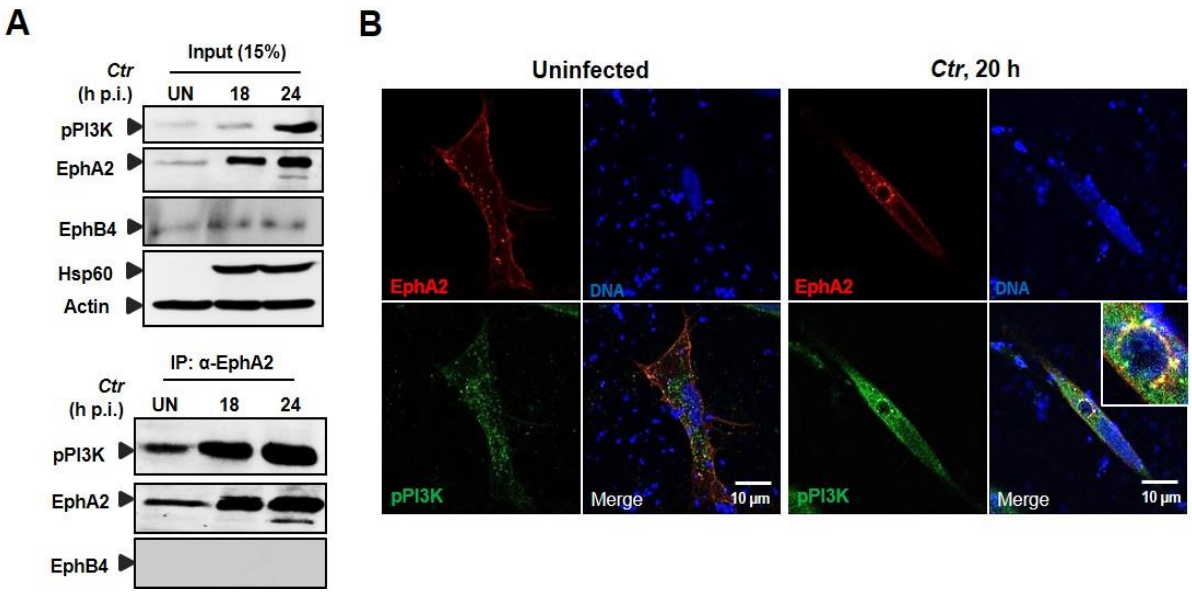

Figure 4.29. Interaction of EphA2 with pPI3K during mid-phase *Ctr* infection: (A) HeLa cells were left uninfected (UN) or infected with *Ctr* for the indicated period of times and immunoprecipitated using α-EphA2 antibody. The IP material was solved in 40 µl Laemmli (100%) and loaded 20 µl for WB studies (50%). (B) Cells were transfected with EphA2-pcDNA3 and p85-PI3K-expression plasmid together for 20 h followed by *Ctr* infection for 20 h. Cells were stained against EphA2 (red), DNA (blue) and pPI3K (green).

4.3.4. EphA2 intracellular cytoplasmic domain function is essential for chlamydial development

4.3.4.1. Mutant EphA2 without the intracellular cytoplasmic domain affects PI3K activation and *Ctr* infection

To identify whether the intracellular domain function of EphA2 is required for supporting *Ctr* development and PI3K activation, EphA2 deletion mutant lacking the cytoplasmic domain including the kinase domain (EphA2ΔIC) was constructed (Figure 4.30A).

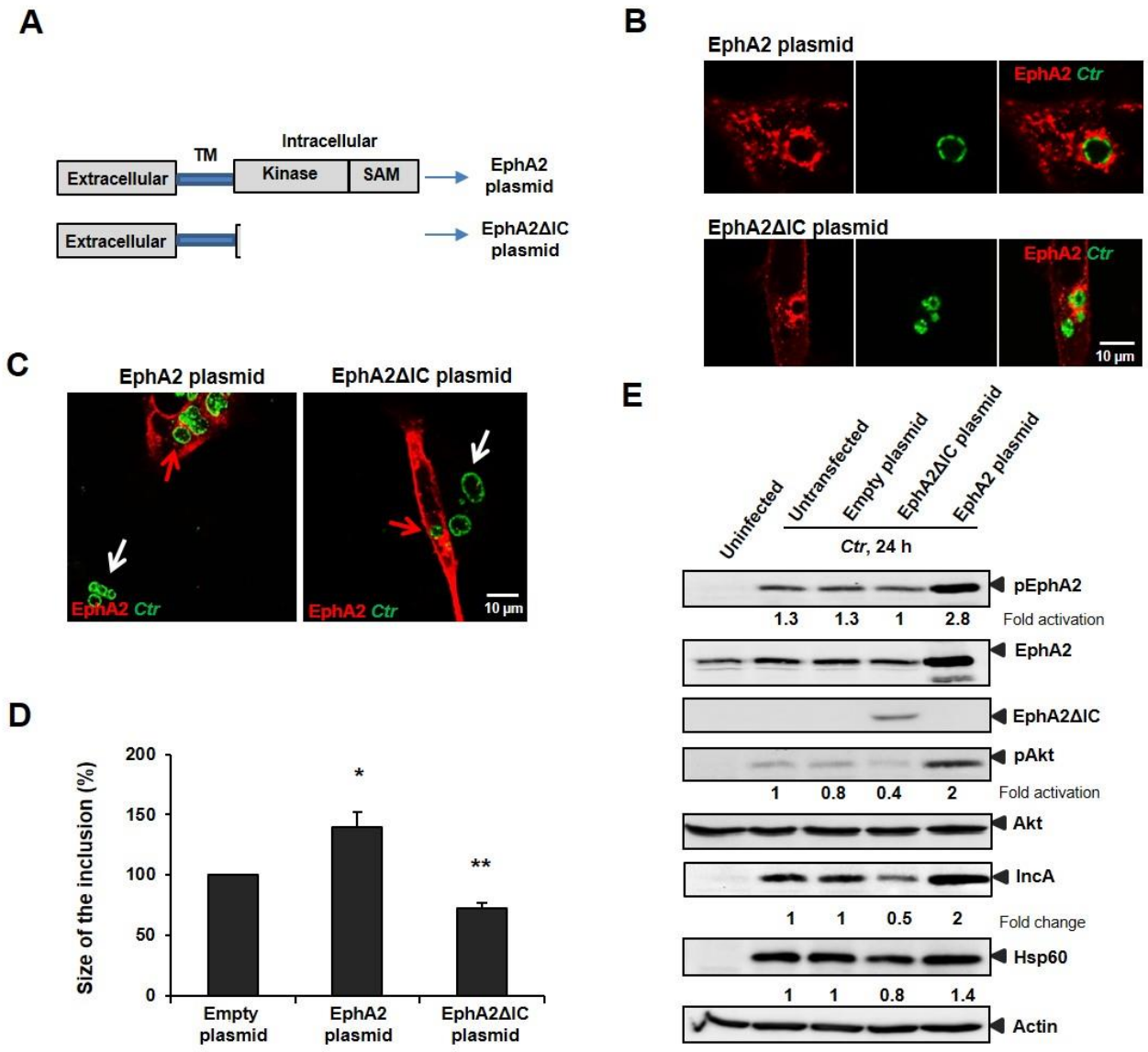

Figure 4.30. EphA2 Kinase domain is crucial for *Ctr* infection: (A) Schematic representation of full length EphA2 plasmid and EphA2ΔIC plasmid which do not possess intracellular cytoplasmic domain. TM: transmembrane domain. (B) The plasmids having full length EphA2 or EphA2ΔIC were transfected and these cells were infected with *Ctr* for another 24 h. Cells were fixed and stained against α-EphA2 antibody (red) and *Ctr* using α-Hsp60 (green). (C) Arrows were marked to illustrate the difference between the size of the inclusion of untransfected (white arrows) and transfected (red arrows) cells. (D) Size of the inclusion was determined by ImageJ analysis. Empty-plasmid transfected cells act as a control. (Shown is the mean ± SD of two independent experiments normalized to empty-plasmid transfected cells. *P<0.05, **P<0.01). (E) The cells after transfection followed by infection were subjected to WB analysis to analyze the proteins as indicated.

EphA2ΔIC localized to inclusions like the wildtype form and was expected to function in a dominant-negative way by competing with the endogenous EphA2 receptors for binding to EB without activating downstream signaling pathways (Figure 4.30B). Transient overexpression of either EphA2 or EphA2ΔIC had a profound effect on the inclusion size. In cells transfected with full length EphA2, the size of the inclusion appeared bigger compared to the untransfected neighboring cells (Figure 4.30C and 4.30D). In contrast, the inclusion size appeared smaller in EphA2ΔIC transfected cells compared to untransfected cells (Figure 4.30C and 4.30D). These results clearly demonstrate the importance of EphA2 kinase domain for *Ctr* infection. Interestingly, levels of activated Akt were reduced in EphA2ΔIC transfected cells (Figure 4.30E) suggesting that EphA2ΔIC interferes with signaling of endogenous EphA2 in a dominant negative manner, e.g. by preventing functional dimer formation.

4.3.4.2. EphA2 tyrosine kinase inhibitor "Dasatinib" affects mid-phase *Ctr* infection

The RTK inhibitor dasatinib (DA) was used to further investigate the role of the kinase activity of endogenous EphA2 for *Ctr* infection. Pretreatment of cells with DA for 1 h followed by 24 h of infection inhibited EphA2 activation (Figure 4.31A) and strongly affected *Ctr* infection (Figure 4.31B and 4.31C). DA may also inhibit other RTKs like PDGFRβ which has previously been shown to be required for *C. muridarum* entry [37]. However, PDGFRβ is rather downregulated during *Ctr* infection and additional silencing of PDGFRβ had no effect on *Ctr*-induced PI3K activation [Figure 4.28(2)A] or inclusion size [Figure 4.28(2)B]. Also, co-silencing EphA2 together with PDGFRβ had no additive effect [Figure 4.28(2)], suggesting that *Ctr* does not employ the PDGFRβ during mid-phase infection.

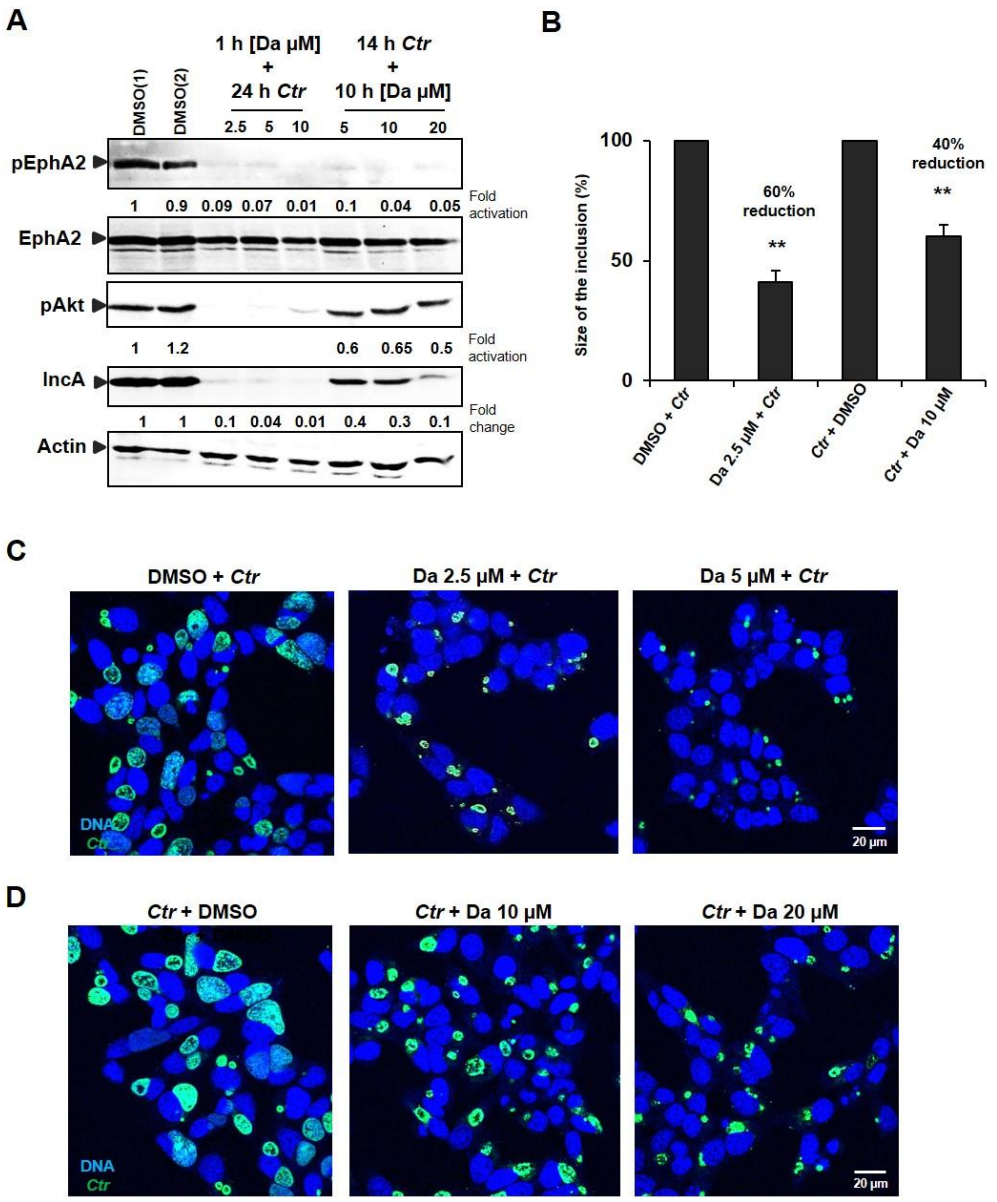

Figure 4.31. Small molecule EphA2 inhibitor dasatinib affects *Ctr* infection: (A) HUVEC cells were pre-treated with DMSO(1) control or with 2.5 µM, 5 µM and 10 µM of DA, respectively, for 1 h at 37°C and were infected with *Ctr* (MOI-1) for 24 h or the cells were first infected with *Ctr* for 14 h and then treated with DMSO(2) control or with 5 µM, 10 µM and 20 µM DA, respectively, for 10 h. Cells were harvested and immunoblotted against pEphA2 (Ser897) and IncA. The blot was stripped and reprobed for total EphA2 and Actin. (B, C, D) Experiments were performed as (A) and immunostained for *Ctr* inclusion (Hsp60, green) and DNA (Draq5, blue). (B) The size of the inclusion was measured by ImageJ analysis. Shown is the mean ± SD of two independent experiments normalized to control. **P<0.01.

To further substantiate a role of the EphA2 kinase activity for chlamydial replication and development, cells were infected with *Ctr* for 14 h followed by 10 h treatment with DA. These conditions allowed normal inclusion formation and EB to RB transition prior to the inhibition of EphA2 kinase during the replicative phase of *Ctr* development. This strategy did not only inhibit the *Ctr*-induced EphA2 activation (Figure 4.31A) but also reduced the *Ctr* infection in a dose-dependent manner (Figure 4.31B and 4.31D). Akt phosphorylation was only mildly affected when DA was added at 14 h p.i. despite a reduction in *Ctr* infection (Figure 4.31A). Hence, these results demonstrate the importance of the kinase domain and activity of EphA2 for *Ctr* development.

4.3.5. *Ctr*-induced EphA2 upregulation is mediated by ERK signaling

The strong and long-lasting upregulation of EphA2 expression leading to its accumulation around the inclusion implied that infection-induced signaling pathways are involved. Therefore the role of the MAPK and PI3K pathways in upregulation of EphA2 during *Ctr* infection was investigated. Cells were left uninfected or infected for 14 h followed by treatment with MEK-1 inhibitor U0126 (which blocks ERK activation) or the PI3K inhibitor LY294002 (which blocks Akt activation) to allow inclusion formation prior to application of chemical inhibitors. LY294002 treatment after 14 h of *Ctr* infection did not affect total EphA2 levels or its activation whereas treatment with U0126 strongly reduced total and activated EphA2 levels (Figure 4.32A) and *Ctr* infection (see IncA in Figure 4.32A). This shows that MEK pathway plays a major role in EphA2 expression during chlamydial infection.

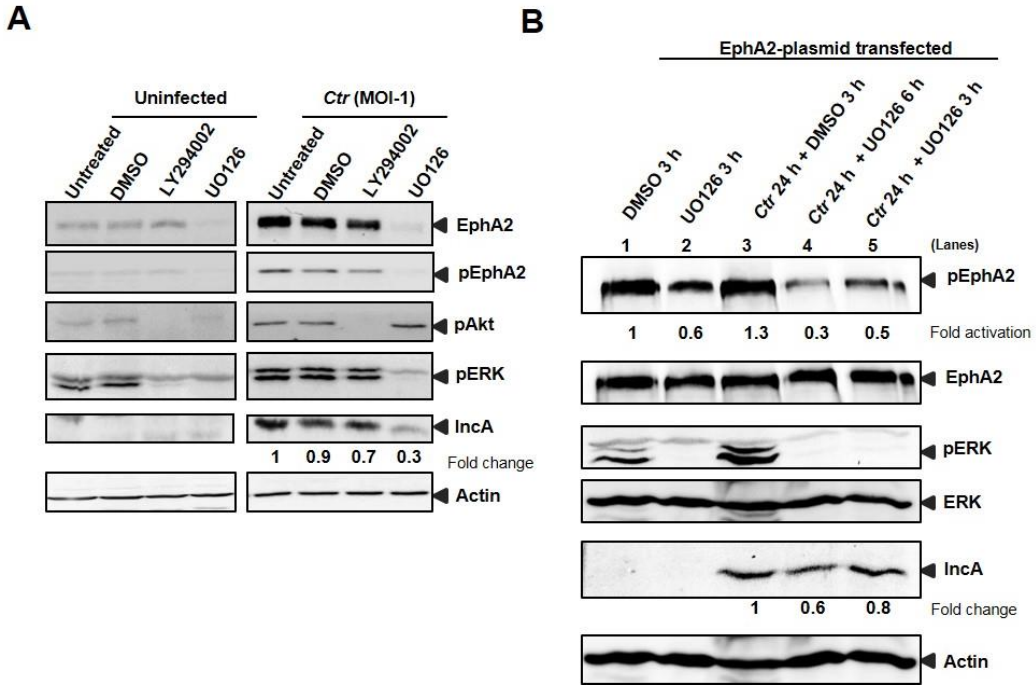

Figure 4.32. *Ctr*-mediated EphA2 regulation is ERK dependent: (A) HUVEC cells were left uninfected or infected with *Ctr* (MOI-1) for 14 h followed by treatment with DMSO or PI3K inhibitor LY294002 (15 μM) or MAPK inhibitor UO126 (15 μM) for 10 h. After 24 h of total infection, cells were subjected to WB analysis against respective proteins. (B) EphA2 plasmid-transfected HeLa cells were left uninfected or infected with *Ctr* for 24 h as mentioned above the lanes. The uninfected or infected cells were treated with DMSO or UO126 (30 μM) for 3 h or 6 h as described above the lanes and subjected to WB analysis to determine the respective proteins as indicated.

To discriminate if *Ctr* mediated-ERK activation is also essential for EphA2 activation (and not only for increased expression), EphA2 was overexpressed under the control of CMV promoter (pcDNA3 plasmid) and the effect of ERK inhibition before and after 24 h p.i., was investigated (Figure 4.32B). EphA2 overexpressed by transfection of an expression construct was not affected by ERK inhibition in both uninfected and *Ctr*-infected cells (Figure 4.32B). But interestingly, after ERK inhibition by UO126, the level of activated EphA2 was affected in both EphA2 transfected-uninfected as well as in 24 h *Ctr*-infected cells (lanes 2, 4 and 5 in Figure 4.32B). These data suggests that *Ctr* mediated-ERK activation is necessary for the increased EphA2 expression and also for the activation of EphA2 during infection.

4.3.6. EphA2 is required to inhibit apoptosis signaling in infected cells

Ctr infection is known to interfere with apoptosis signaling induced by various stimuli like staurosporine, etoposide, TNF-α, FAS antibody and granzyme B/perforin [53, 70]. Since EphA2 plays a role in anti-apoptosis signaling [154, 170] and ERK pathway is required for *Ctr*-induced apoptosis inhibition [70] and EphA2 activation (Figures 4.32A and 4.32B), the role of EphA2 in apoptosis inhibition of infected cells was tested. Akt activation is dependent on EphA2 during mid-phase (16 to 24 h) but not late-phase *Ctr* infection (48 h) (Figure 4.33A), in line with its role in anti-apoptosis signaling during the replicative phase. Consistently, silencing of EphA2 sensitized cells that had been infected for 16 h to apoptosis induced by TNF-α/CHX determined by PARP cleavage and apoptotic cell count (Figure 4.33B and 4.33C). Thus, EphA2-mediated signaling also supports apoptosis resistance of *Ctr*-infected cells, demonstrating its crucial intracellular role in *Ctr* infection.

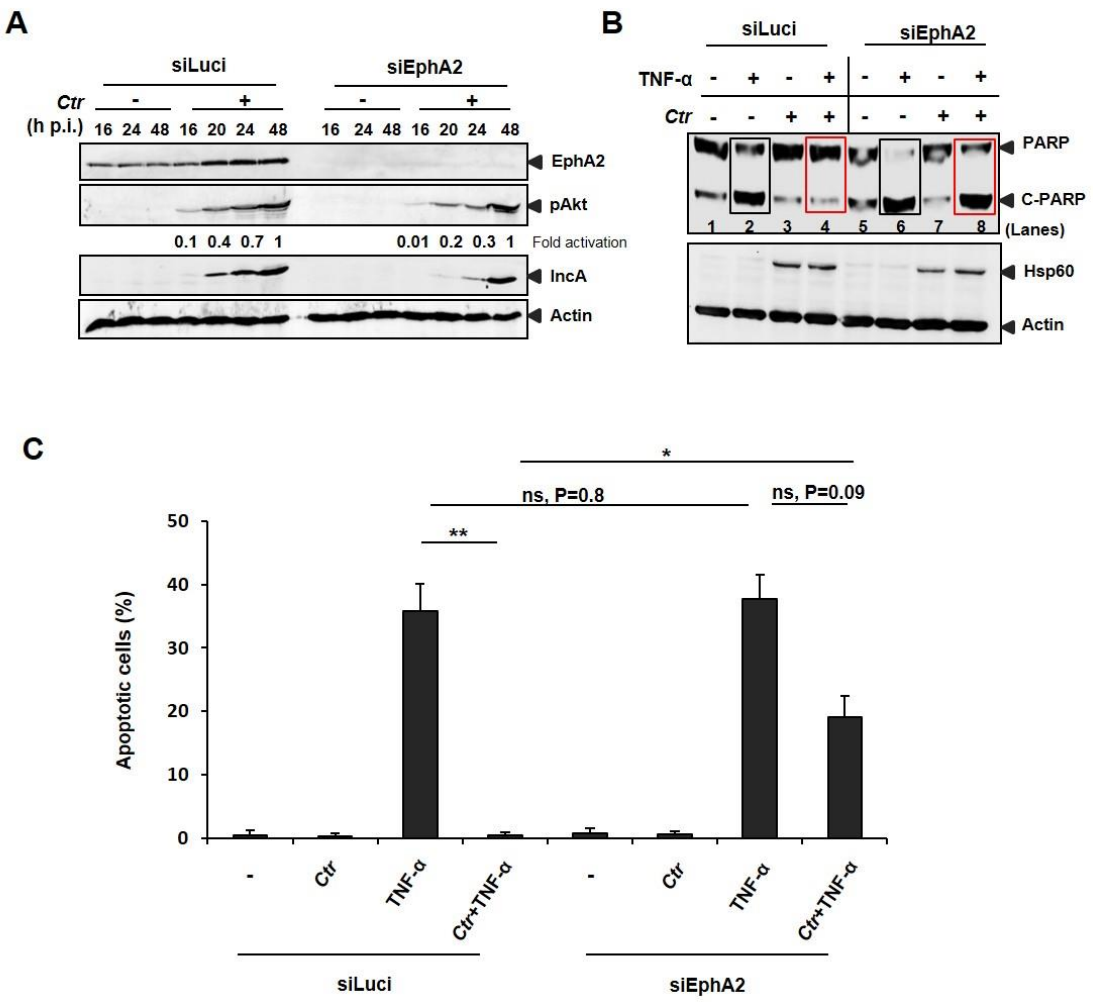

Figure 4.33. *Ctr*-infected cells were sensitized to TNF-α induced apoptosis upon EphA2 knockdown: (A) The transfection efficiency of siRNA directed against EphA2 was monitored by WB analysis against total EphA2. In addition, levels of pAkt, IncA and Actin were verified. (B) EphA2 knockdown followed by 16 h-infected cells were induced to apoptosis by TNF-α (50 ng/ml)/CHX (5 µg/ml) for 5-6 h. Processing of PARP, Hsp60 and Actin was monitored by WB analysis. Rectangle boxes (black: before infection) or (red: after infection) denotes the difference in PARP cleavage after TNF-α induction in siLuci and siEphA2 transfected cells. (C) For quantification, TUNEL positive cells from each sample were counted from ten different fields. (Shown is the mean ± SD of two independent experiments. **P<0.01, *P<0.05, ns: non-significant).

4.3.7. Increased EphA2 expression during *Ctr* infection is prevented translocating to the cell surface to protect the intracellular niche

It is not clear on how *Chlamydia* protects its host cell from competing re-infection. Our group found that *Ctr* prevents chlamydial re-infection in order to protect their intracellular niche

(Karunakaran et al., submitted), probably to avoid competition for the nutrients. In order to examine the reason behind the prevention of EphA2 translocation to the cell surface of the infected cell, re-infection assay was performed. Re-infection assay performed in EphA2 overexpressing cells showed 2.5 fold increased re-infection of new *Ctr*-EB in 24 h infected cells comparing to the untransfected infected cells (Figure 4.34A and 4.34B). Thus, to prevent the entry of new bacteria and to protect the intracellular niche, *Ctr*-infected cells might prevent EphA2 translocation to the cell surface apart from gaining EphA2 intracellular signals. The other reason might be to prevent EphA2 contact with its ligand Ephrin-A1 on the surface of neighboring cell which reverses EphA2 signaling of its own infected cell.

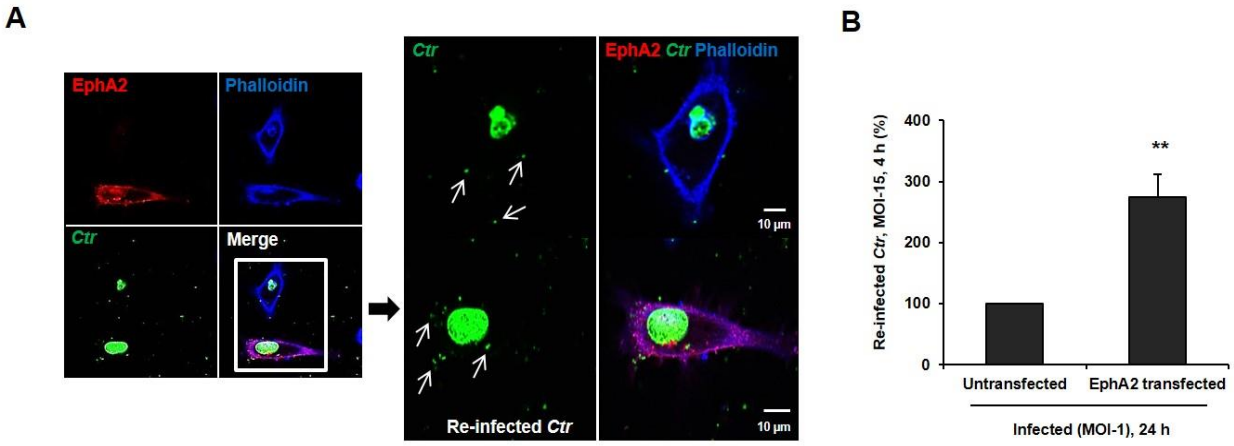

Figure 4.34. EphA2 overexpressed infected cells allow re-infection in 24 h *Ctr*-infected cells. (A) Re-infection assay: Cells transfected with EphA2-pcDNA3 were infected with *Ctr* (MOI-1) for 24 h followed by re-infection using EB (MOI-15) for 4 h. Cells were washed thrice with PBS to remove the unbound bacteria and immunostained against EphA2 (red), *Ctr* (Hsp60, green) and Actin filaments (Phalloidin, blue). Microscopic view was made focusing on the newly re-infected *Ctr* (arrows). Nearby untransfected and EphA2 transfected cells (red) were shown in the same image with zoomed in white boxes for better magnification of invaded bacteria in EphA2 transfected cells comparing to the untransfected cells. Arrows were drawn to indicate the newly adhered or invaded *Ctr.* (B) The total number of re-infected bacteria (both adhered and invaded new EB) in EphA2 overexpressed and untransfected-infected cells were counted for maximum of 30 cells. Shown is the mean ± SD of three independent experiments normalized to untransfected infected cells. **P<0.01. Error bars show mean ± SD.

4.3.8. EphA2 expression and activation in other chlamydial strains

I next investigated whether EphA2 is also upregulated in other important chlamydial species like *Chlamydia pneumoniae* and in other *Ctr*-serovars like urogenital serovar D and ocular tract serovar A. Our preliminary analysis showed that EphA2 was upregulated and activated upon *Ctr*-serovar A, *C. pneumoniae* and *Ctr*-serovar D infected HeLa cells (Figure 4.35) like that of *Ctr*-L2.

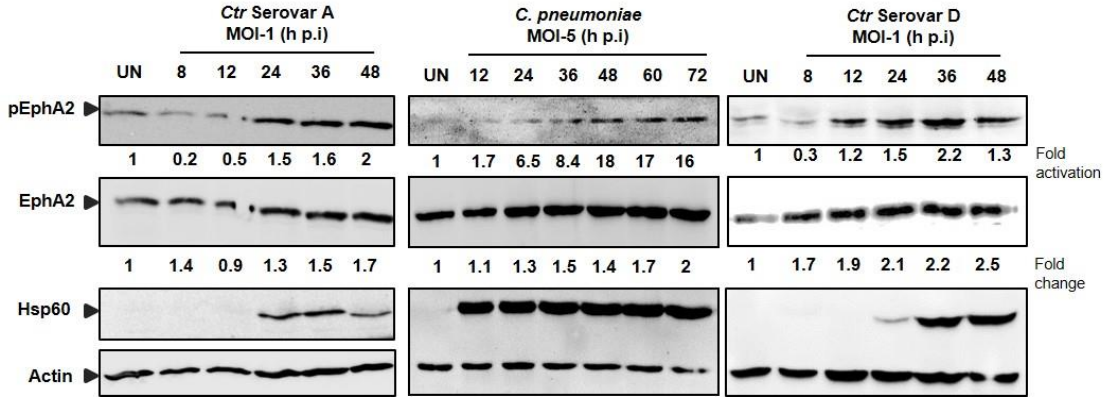

Figure 4.35. EphA2 expression and activation in *Ctr*-serovar A and *C. pneumoniae*-infected HeLa cells: HeLa cells were left UN or infected with *Ctr*-serovar A or *C. pneumoniae* or *Ctr*-serovar D and centrifuged at 910 x g for 30 min and allowed to infect for the indicated time points. The cells were harvested and subjected to WB analysis against pEphA2, Hsp60 and Actin. The blot was stripped and reprobed for total EphA2.

4.3.9. EphA2 is an invasion and apoptosis resistance signaling receptor also for *C. trachomatis*-serovar D

It was further tested if EphA2 can enhance *Ctr*-serovar D binding and invasion to HeLa cells like that of *Ctr*-L2. After silencing total EphA2 expression by siRNA transfection, *Ctr*-serovar D binding and invasion were reduced by 28% and 30% respectively (Figure 4.36A). Interestingly, like *Ctr*-L2, FACS analysis under non-permeabilised condition revealed an increased expression of surface EphA2 as early as 30 min p.i., but returned to basal levels by 3 h p.i., (Figure 4.36B).

FACS analysis under permeabilised conditions showed an increased total EphA2 signal at 3 h p.i., (Figure 4.36B). Thus, *Ctr*-serovar D also utilizes EphA2 to invade the cell.

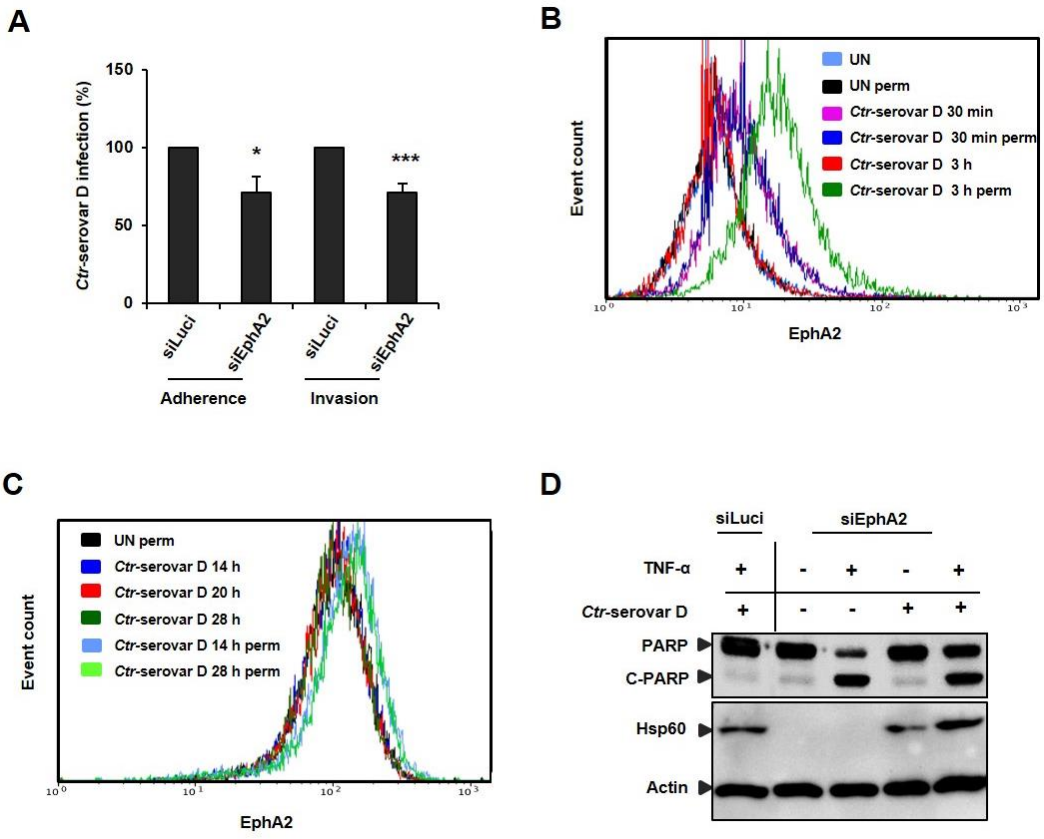

Figure 4.36. *Ctr*-serovar D utilize EphA2 for invading the cells and to prevent apoptosis induced by TNF-α: (A) HeLa cells were transfected with siRNA against luciferase gene (siLuci) or EphA2 gene (siEphA2) for 40 h at 37°C. Adherence assay: The transfected cells were infected with *Ctr*-serovar D for 1 h at 4°C. Cells were immunostained against EB and actin filaments. Number of extracellular EB was counted randomly from 30 different cells. Data are expressed as percentage of extracellular EB relative to siLuci. Shown is the mean ± SD of three independent experiments normalized to siLuci. *P<0.05. Error bars show mean ± SD. Invasion assay: The transfected cells were infected with *Ctr*-serovar D for 4-5 h at 35°C. Cells were immunostained against EB and actin filaments. Number of invaded EB was counted randomly from 30 different cells. Data are expressed as percentage of invaded EB relative to siLuci. Shown is the mean ± SD of three independent experiments normalized to siLuci. *P<0.001. Error bars show mean ± SD. (B) FACS analysis was performed with *Ctr*-serovar D infected cells as Figure 4.17A and 4.17B. (UN: uninfected, Iso: isotype and perm: permeabilised). (C) HeLa cells were left UN or infected with *Ctr*-serovar D (MOI-1) for 14, 20 and 28 h and cells were analyzed via FACS as Figure 4.22. (D) EphA2 knockdown followed by 16 h *Ctr*-serovar D infected cells were induced to apoptosis by TNF-α (50 ng/ml)/CHX (5 µg/ml) for 5-6 h. Processing of PARP, Hsp60 and Actin were monitored by WB analysis.

To determine the surface and total levels of EphA2, FACS analysis was performed during mid-phase *Ctr*-serovar D infection. Like *Ctr*-L2, the surface EphA2 expression was similar at three different time points of *Ctr*-serovar D infection (14, 20 and 28 h p.i.) whereas permeabilised-infected cells (14 and 28 h p.i.) displayed an increase of intracellular EphA2 levels (Figure 4.36C). Further, to find out the importance of internalized EphA2 in these cells, apoptosis sensitization assay was performed. The EphA2 silenced cells upon 16 h p.i. sensitized to TNF-α/CHX induced apoptosis determined by PARP cleavage (Figure 4.36D). Thus, EphA2-mediated signaling is essential for invasion and apoptosis resistance of *Ctr*-serovar D-infected cells, suggesting its crucial role as an entry and intracellular signaling receptor also in urogenital serovar of *Ctr*.

5. DISCUSSION

First identified in a cancer cell line called erythropoietin-producing hepatocellular [171], numerous Eph receptors were subsequently detected, exhibiting diverse functions in vascular development, cancer and virus infection. This work reveals the identification of EphA2 as a key factor of *Ctr* entry and development using an unbiased approach, based on the compilation of data from hypothesis-free proteomics and RNA interference approaches. Interestingly no EphA receptors other than EphA2 were found in the proteomics approach (data not shown). Further, out of 14 Eph receptor genes validated in the initial RNAi screen, only silencing of EphA2 revealed a strong inhibitory effect on infectivity (data not shown).

5.1. EphA2 serves as an adherence and entry receptor for *Ctr*

The receptors for *Chlamydia* are widespread on the surface of the epithelial cells. Several tyrosine kinase receptors like PDGFR and FGFR have been previously shown to be the receptor for *C. muridarum,* a mouse chlamydial pathogen. Further, interfering with epithelial membrane protein 2, a 4-transmembrane protein, affects *C. muridarum* infectivity [39]. The protein disulfide isomerase (PDI) [161] is required for *C. trachomatis* entry into cells and the mannose 6-phosphate receptor [172] influences *C. pneumoniae* attachment. But no receptors have been specifically found to be the direct interacting receptor for *C. trachomatis* on the cell surface. This study shows that the EphA2 receptor acts as a direct cellular receptor for *Ctr* and is activated upon infection. The adherence assay demonstrated that *Ctr* could bind to the ligand binding domain of EphA2 as blocking this domain by N-terminal specific EphA2 antibody or recombinant Ephrin-A1 affects *Ctr* adherence with the cell surface EphA2.

Ephrin-A1 glycosylation is known to play an important role in binding and activating EphA2 [173]. It was previously proposed that the outer membrane protein of *Ctr* is glycosylated [174] which further implicates that EphA2 may bind with high avidity to the glycoprotein complex of *Ctr*

and gets activated during the infection. Co-localization of EB with rhEphA2 was reduced upon incubating EB with rhEphrin-A1 pre-treated rhEphA2. This effect was further proven by *invitro* binding assay where the *invitro* enrichment of rhEphA2 precipitated together with EB. Additionally, the enrichment of rhEphA2 with EB was less when rhEphA2 was pre-incubated with rhEphrin-A1 which shows that EphA2 directly interacts with EB and is specific. In addition, pre-incubation of *Ctr*-EB with recombinant EphA2 or using N-terminal specific EphA2 antibody prevents the binding of *Ctr* with cell surface EphA2. This further confirms that EphA2 acts as an essential and direct adherence receptor for *Ctr*.

Invasion assay studies performed using siRNA against EphA2 or antibodies directed against amino terminal-EphA2 or EphA2 tyrosine kinase inhibitor-dasatinib has affected *Ctr* invasion claiming to be also an important invasion receptor. Further in depth investigation of which *Ctr* factors, either the type three secreted effectors or the outer membrane protein complex, interacts and activates EphA2 to invade the cell has to be monitored. In addition, the signaling cascades formed during the infection downstream of EphA2 for invasion process should be identified via quantitative mass spectrometry analysis. This will unravel all the important early infection induced-interacting proteins of EphA2. *Ctr* attachment with host cell induces Rac activation [175] and Ephrin-A1 interaction with EphA2 induces Rac activation [176]. So *Ctr* by interacting with ligand binding domain of EphA2 may induce Rac activation during the early infection for the actin rearrangement and internalization process. Studies have to be performed to understand more on this which helps to disclose one way of *Chlamydia* invasion process.

Ctr-serovar D which cause genital and perinatal infections [177, 178], also enhanced EphA2 activation in infected HeLa cells. Similar to *Ctr*-L2, *Ctr*-serovar D also utilizes EphA2 for adherence and to invade the epithelial cells. EphA2 overexpression in majority of ovarian cancer patients predicts poor clinical outcome having shorter overall survival [120] and offer a hope of converting EphA2-targeted therapy into a clinical reality [179]. *Ctr* infections are known

to infect and damage human fallopian tube [180-183] and associates with ovarian cancer [184, 185]. The preliminary findings using EphA2 blocking antibody in Fimb cells suggests that targeting EphA2 and other Eph receptors or co-receptors could definitely act as a tool to control *Ctr*-induced ovarian cancer.

5.2. EphA2 internalizes during *Ctr* infection and associates with the inclusion

So far the studies revealed the activation of EphA2 upon *Ctr* infection which starts as early in min post infection whose total level increases from 4 h post infection. Thus, viable *Ctr* when binding with cell surface exposed EphA2 activates EphA2 probably needed for their internalization process and this was not seen with heat-inactivated *Ctr*. This shows the specificity of the *Ctr* factor binding with cell surface EphA2 and activates it. More interestingly, FACS analysis, plasma membrane isolation and TCA precipitation studies performed showed the increased surface EphA2 expression upon 30 min post infection got decreased after 3 h post infection which is an indication that EphA2 is internalized together with *Ctr*. Hence, EphA2 is necessary for *Ctr* invasion process in addition to the adherence process and the increased total EphA2 throughout *Ctr* infection is intracellular.

EphA2 has been identified as a receptor for hepatitis C virus [141] and Kaposi's sarcoma-associated herpes virus [142] binding and entry. This study illustrates the function of EphA2 as a receptor for a bacterial pathogen. Though EphA2 acts as a cellular receptor for virus, nothing was known about its role during mid and later phase viral infection. Whereas in *Ctr* infection, both activation and total levels seem to increase throughout infection and is MOI-dependent. Unlike Ephrin-A1-ligand induced degradation of EphA2, *Ctr* infection upregulates and activates EphA2 throughout the infection. Thus, this work demonstrates a tyrosine kinase receptor's function throughout a bacterial infection.

FACS analysis of mid-phase *Ctr* infection displayed similar EphA2 levels like that of uninfected cells, but showed increased EphA2 levels after permeabilisation stating that *Ctr* infection prevents the translocation of EphA2 to the surface and are kept intracellular for modulating the host signaling. Further, to prevent the re-infection and to protect the intracellular niche, *Ctr*-infected cells prevents EphA2 re-localization to the cell surface because entry of new bacteria can cause host cell nutrients deprivation. It is not surprising to see an increased intracellular EphA2 expression during *Ctr* infection because EphA2 and its interacting proteins controls several cellular processes all of which are essential to oncogenic/survival pathways. Recruitment of active EphA2 to the inclusion membrane during mid-phase of infection cycle indicates that EphA2-mediated signaling occurs at the cytosolic side of the inclusion membrane. EphA2 may initially remain associated with the endosomal membrane during bacterial entry. It has to be investigated in future regarding how the receptor is recruited to the inclusion at late infection time points and whether this requires secreted chlamydial ligands.

5.3. Association of EphA2 with p85-PI3K is crucial for successful *Ctr* infection

Infection with *Ctr* activates both MEK/ERK and PI3K/Akt pathway to resist apoptosis during their infection. The factor upstream of PI3K activation was not known so far during *Chlamydia* infection. Several studies in past years have determined the association of activated EphA2 with p85-PI3K. This work revealed that EphA2 is an upstream factor of activated PI3K during *Ctr* infection. PI3K/Akt activation controls cellular phagocytic responses through multiple downstream targets, such as Ras and Rac, which are linked to actin polymerization and cytoskeletal dynamics [186, 187]. *Chlamydia* induces Rac activation for entry process [175]. Other gram-negative bacteria like *Pseudomonas aeruginosa* [188] *and Salmonella typhimurium* [189] also activates PI3K to gain the cellular entry. The flagellar motility of *P. aeruginosa* is linked to Akt activation in phagocytic cells and this activity is required for phagocytosis of

bacteria [188]. Activation of PI3K during chlamydial infection is, however, not completely dependent on activated EphA2 during chlamydial infection which indicates a possible role of other PI3K-inducing pathways. Recently, it was shown that EGFR is activated by *Ctr* infection and that *Ctr*-induced activation of Akt is EGFR-dependent [190]. Hence, apart from EphA2 and EGFR, there could be other tyrosine kinase receptors activated by *Ctr* that may also mediate Akt activation at different stages of their development.

Although PI3K activation upon chlamydial infection was well known to promote cell survival, the pathogen recognition receptor-mediated PI3K signaling may contribute to host innate immune defenses. However, *Chlamydia* effectively overcomes host immune response probably by their secreting enzymes like chlamydial protease-like activity factor (CPAF) and ChlaDub1. CPAF, a protease, degrades the host DNA allowing the bacteria to evade the host immune system by delaying the activation of gene responsible for the production of its major histocompatibility complex [191]. In addition, another chlamydial enzyme named ChlaDub1 inhibits the NF-ƙB activation which play a crucial role in inducing the host inflammatory stimuli [192]. Further, it was known that constitutive activation of the PI3K contribute to reduce TLR4-mediated TNF-α release [193, 194]. EphA2 modifies the immune response by altering T cell recruitment and cytokine production during *M. tuberculosis* infection [148]. Thus, *Chlamydia* may efficiently avoid host cell immune response by the prolonged activation of EphA2/PI3K signal thereby enhancing the survival.

5.4. *Ctr*-infected cells downregulates Ephrin-A1 to prevent the inhibition of host signaling pathway crucial for infection

This profile of EphA2 upregulation induced by chlamydial infection is strikingly different from that observed after Ephrin-A1 stimulation. EphA2 is rapidly endocytosed upon Ephrin-A1 ligation and degraded in lysosomes [195]. This so-called ligand-dependent signaling involves

phosphorylation of EphA2 at Tyr594 [167] and interferes with the activation of the PI3K and MEK/ERK pathway [105]. The effect of *Ctr* infection on EphA2 is in line with ligand-independent signaling that causes EphA2 activation, supports PI3K/Akt activation and prevents EphA2 degradation.

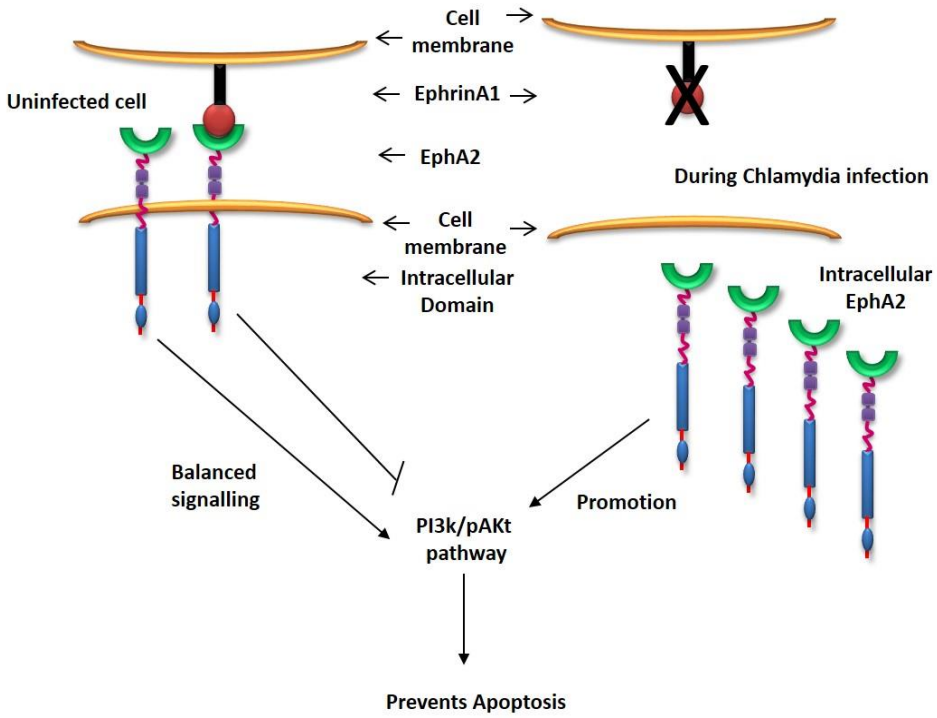

Figure 5.1. EphA2 signaling in uninfected cell Vs *Ctr*-infected cell: **Under normal conditions (uninfected cells), EphA2 and Ephrin-A1 are properly expressed and engaged with each other on the cell surface which leads to balanced PI3K signaling which contributes to the maintenance of epithelial homeostasis. *Ctr* infection leads to down regulation of Ephrin-A1 and upregulation of EphA2. Activated EphA2 interacts and activates PI3K necessary for the infected cell survival.**

It was previously identified that EphA2 is a direct transcriptional target of the RAS-RAF-MAPK pathway [138]. Thus, infection induced ERK-mediates up-regulation of EphA2. This work shows that *Ctr*-mediated ERK activation, apart from transcriptionally regulating the expression of EphA2, also regulates the activation of EphA2. Hence, it is possible that ERK activation induces expression of any host protein which acts as a ligand in keeping EphA2 active during chlamydial infection. In addition to that, ERK activation is necessary for the *Ctr* infection and inhibiting ERK

activation by UO126 affects *Ctr* infection [65, 70]. In that case, some unknown chlamydial ligand might keep EphA2 active.

The activation of the MAPK pathway inhibits levels of Ephrin-A1 [138]. Ephrin-A1 levels are reduced in *Ctr*-infected cells which show how *Ctr* can impede and modulate the host signaling pathways for their benefit (Figures 4.25 and 4.26). Thus, the reduced levels of Ephrin-A1 could be mediated by ERK activation during *Ctr* infection. The results are similar to glioblastoma cells and breast cancer cells where high levels of Ephrin-A1 exhibit low EphA2 and the condition is reversed by siRNA mediated knockdown of Ephrin-A1 [138]. In contrast, EphA2 overexpression exhibit low Ephrin-A1 [138, 139]. These findings propose a feedback loop mechanism also in *Ctr* infection which increases EphA2 expression and activation that leads to the suppression of its ligand (Figure 5.1). Thus, downregulation of Ephrin-A1 during *Ctr* infection is to prevent EphA2 interaction with EphrinA1, thereby preventing the EphrinA1-EphA2 mediated inhibition of PI3K and MAPK pathway crucial for *Ctr* infection.

5.5. *Ctr* infection exploits intracellular EphA2 kinase function for enhancing the infection and inhibiting apoptosis

Ctr mid-phase infection mediated-activation and -upregulation of EphA2 also associates with PI3K showing that *Ctr* uses EphA2 to control the downstream signaling throughout its infection. This association is definitely mediated by *Ctr* infection as knockdown or overexpression of full length EphA2 or mutant EphA2 lacking cytoplasmic domain influenced PI3K activation and further *Ctr* infection which collectively reveals an important role for EphA2 in productive *Ctr* infection. One could argue that the observed bigger inclusion in EphA2 overexpressing cells is due to the increased invasion rate of *Ctr*. But MOI-1-2 was used for all mid phase infection to ensure similar initial infection irrespective of the EphA2 status. Therefore the observed bigger inclusion in EphA2 overexpressing cells must be due to the EphA2–mediated downstream

signal which fastens the growth of *Ctr*. On the other hand, the smaller inclusion in EphA2ΔIC overexpressing cells could be missing of the EphA2 cytoplasmic domain mediated-downstream signals. Thus, the downstream signaling in overexpressed EphA2 fastens the *Ctr* infection and mutant EphA2 signaling slowed down *Ctr* infection.

Both chemical inhibition of EphA2 kinase activity and of PI3K [70, 89] prevented *Ctr* infection if the inhibitor was added either prior to or simultaneously with infection. These effects were milder if the inhibitors were applied after the EB to RB transition. Therefore the early activation of the EphA2-PI3K pathway is important for chlamydial development. During this phase of infection, EphA2 levels remain largely unchanged indicating that post-translational modification by phosphorylation is the main mechanism of activation. During the middle and late phase of infection, the PI3K pathway appears to be less important since *Ctr* is able to develop irrespective of whether PI3K is active or not [70]. The dose-dependent downregulation of mid phase-chlamydial infection by dasatinib, which can inhibit the activity of EphA2, Src, BCR-ABL, KIT and PDGFR [164, 165], did not correlate well with the downregulation of the PI3K pathway. However, a kinase inactive EphA2 mutant also affected PI3K activation and *Ctr* infection, demonstrating a role of the EphA2 kinase activity in chlamydial infection. Clearly, the effect of the EphA2ΔIC was stronger suggesting, that the cytoplasmic domain of EphA2 in addition to its kinase activity has an important function.

Ctr can replicate inside the host cell by preventing apoptosis. They are known to prevent apoptosis induced by various stimuli like staurosporine, etoposide, TNF-α, FAS antibody and granzyme B/perforin [53, 70, 196]. There are several accumulating evidences for EphA2 being an anti-apoptotic gene [154, 170]. Since the previous results showed changes in the activation of the survival signal (PI3K/Akt) upon EphA2 knockdown or overexpression, Akt activation in EphA2-siRNA transfected cells during mid to late phase *Ctr* infection was tested. Akt activation

was reduced in EphA2 knockdown cells during mid *Ctr* infection (16 h to 24 h) but not in later *Ctr* infection (48 h). Therefore, testing 16 h EphA2 knockdown *Ctr*-infected cells had underwent apoptosis induced by TNF-α demonstrating that EphA2 is required to maintain apoptosis resistance during *Ctr* infection. Similarly, *Ctr*-serovar D could also prevent cell death induced by TNF-α by EphA2 signaling. Thus, at least two different serovars of *Ctr* appear to be capable of amplifying EphA2 signaling crucial for infection.

This function very likely depends on the role of RTKs in activation of the PI3K pathway which plays a role in at least two anti-apoptotic signaling pathways mediated by *Ctr* infection. The first pathway involves the inactivation of the pro-apoptotic BH3-only protein Bad [89]. The phosphorylated Bad gets dissociates from Bcl-2/Bcl-xL complex and the released Bcl-2/Bcl-xL inhibits Bax/Bak triggered intrinsic apoptosis. The second pathway involves the stabilization of the anti-apoptotic Bcl-2 family member Mcl-1 by Akt pathway [70], as interfering with Mcl-1 levels sensitized *Ctr*-infected cells to apoptosis.

It is very likely that maintaining apoptosis resistance supports the integrity of the host cell during chlamydial replication and thus supports the infection, although direct experimental proof for this hypothesis is missing. The crucial role of EphA2 for chlamydial replication and its long-lasting upregulation during the course of infection may have an important consequence. Taken together, the findings provide the first insight in the pathogenesis of *Ctr* where a host cell surface receptor is exploited for invasion and to facilitate the infection by exploiting the receptor-mediated intracellular signaling cascades.

5.6. Model: EphA2 signaling during *C. trachomatis* infection

Chlamydial-EB interacts with EphA2 (may be also together with other co-receptors) expressed on the cell surface that facilitates the entry of EB inside the cell (probably by the known chlamydial effector proteins secreted though T3SS like TARP protein). This binding of *Ctr*-EB

with EphA2 recruits and activates the PI3K pathway via C-terminal SH2 domain of the p85 subunit of PI3K [197]. The p85 subunit of PI3K binding to the intracellular domain of EphA2 leads to conformational changes in the catalytic domain of PI3K (p110) and this leads to consequent kinase activation. The recruitment of EphA2 interacting with PI3K will help *Chlamydia* to use-up the downstream activated Akt pathway to inhibit apoptosis. *Ctr* infection-induced activation of MEK/ERK pathway enhances the transcription rate of EphA2. The ligation of EphA2 with Ephrin-A1 antagonizes other growth factor and receptors mediated activation of MAPK signaling [198, 199]. Therefore by inhibiting the expression of Ephrin-A1, *Chlamydia* can effectively block the Ephrin-A1-EphA2 signaling mediated inhibition of PI3K/MAPK signaling pathway. EphA2 which interacts with PI3K are recruited to the chlamydial inclusion through unknown signaling caused by chlamydial secreted effectors. By recruiting EphA2 together with PI3K, chlamydial controls and benefits the downstream Akt signaling pathway (Figure 5.2).

Apart from the PI3K, there are several other downstream targets of EphA2 like Ras-GTPase activating protein (RasGAP), Src, Abl family of non-receptor tryosine kinases and Rac1. During chlamydial infection, the Src kinase FYN has been shown to be essential for sphingomyelin trafficking to the *Ctr* inclusion [50]. Moreover, Rac1 activation is involved in chlamydial invasion into the host cells [200]. In addition, EphA2 is required for the association of Rab5 with internalized KSHV and productive trafficking of KSHV [109]. Further, Eph-EphA2 complexes are shown to be involved in various forms of endocytosis and cellular trafficking pathways [95]. Thus, the central role of EphA2 in cytoskeletal regulation and intracellular trafficking may be exploited by *Ctr* for efficient cell entry, replication and development. Taken together, EphA2 acts as a critical invasion receptor as well as an intracellular master regulator receptor essential for *Ctr.*

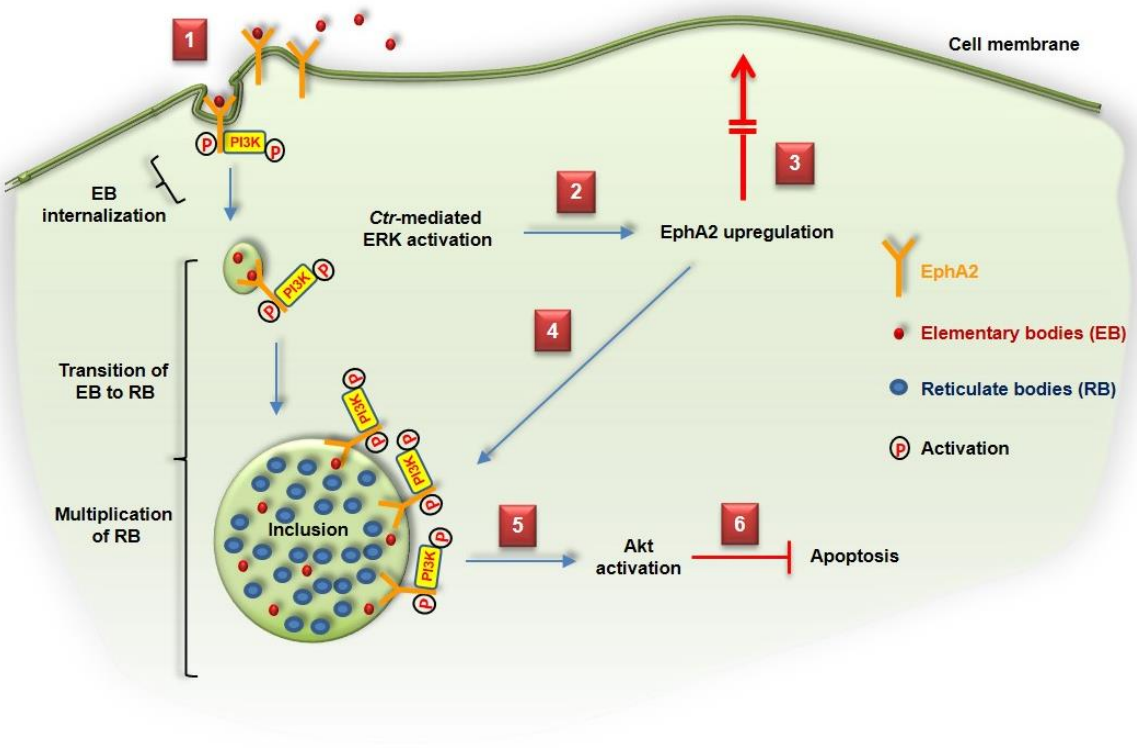

Figure 5.2. Schematic representation of EphA2 signaling during *C. trachomatis* infection: (1) *Ctr*-EB binds EphA2 and enters the cell with the activated receptor which interacts with and activates PI3K. (2) *Ctr*-mediated ERK activation induces EphA2 upregulation and activation. (3) Upregulated EphA2 is prevented from transporting to the cell surface. (4) Intracellular EphA2 is associated with the *Ctr* inclusion which interacts with p85-PI3K. (5, 6) EphA2-mediated downstream signaling cascade (pAkt) prevents apoptosis and enhance the infection.

6. OUTLOOK

Successful infection by intracellular bacterial pathogens needs cellular adherence and invasion followed by intracellular multiplication and development by procuring nutrients through manipulation of host cell signaling pathways. This mechanism of bacterial attachment and successive ingress into the cells remains unclear. Anti-bacterial strategies could be developed once the host factors that are exploited by bacteria for the adherence and invasion process were identified and blocked. The present study was an attempt to investigate and understand the molecular mechanisms of a tyrosine kinase cell surface receptor, EphA2 that governs *Ctr* infection. The importance of EphA2, besides being an entry receptor, as an intracellular signaling receptor for *Ctr* survival was identified.

The role played by EphA2 in other serovars like serovar A-C which causes infection in the eye and in other *Chlamydia* species like *C. pneumoniae,* a major cause of pneumonia have to be investigated. Human Herpes viruses and *Chlamydia* are both detected together in different human disorder like chronic fatigue syndrome [201]. In the preliminary studies, *Ctr* co-infection with HHV-6 showed decrease in EphA2 regulation compared to only *Ctr* infection which further illustrates the specific importance of EphA2 in *Ctr* infection. More studies have to be performed on this direction to understand the precise role of EphA2 during *Ctr and* HHV-6 co-infection.

EphA2 was previously identified as GP96 client protein which is an essential mediator of EphA2 stability and function [202]. Our group identified that GP96 plays a crucial role for epithelial cell infection with *Ctr* (Karunakaran et al., submitted). Therefore, GP96-dependent targeting of EphA2 may represent an important therapeutic strategy to impair EphA2 signaling essential for early *Ctr* infection. It is well known, that 50% to 70% of the patients infected with sexually transmitted *Ctr* are also positive for the *Neisseria gonorrhoeae*, a bacterium that causes gonorrhea in more than 100 million patients every year [203]. The reason for this high co-

infection incidence is unknown. Interestingly, our group (Karunakaran et al., in preparation) found that *Chlamydia*-infected cells with down regulated GP96 are highly susceptible for N*. gonorrhoeae* infection. Preventing GP96 depletion and shedding prevents gonococcal co-infection, but increases chlamydial re-infection and potential competition for host cell nutrients. Despite the observation that EphA2 expression is increased upon *N. gonorrhoeae* infection [140], the role played by EphA2 in the pathogenesis of *N. gonorrhoeae* has not been examined. Hence this needs further investigation.

The role of other Eph receptors and its downstream signaling whether or not regulated during *Ctr* infection has to be explored. This study could be further expanded to mouse models to understand the role of EphA2 *invivo*. Targeting EphA2 and probably other Eph receptors could be a therapeutic base to control *Ctr* infection. Most importantly, the bacterial effector/s that interacts with EphA2 has to be investigated. Furthermore, the important question how EphA2 is recruited to the inclusion and whether they play any role in *Ctr* intracellular trafficking should be studied. Thus, I have developed a working model to guide for additional studies to understand more of the role played by EphA2 signaling during *Ctr* infection. Further studies are vital to interpret the downstream targets of EphA2 that favors *Ctr* infection. Identification of RTKs would be an important host factor regulating *Ctr* infection and thus targeting RTKs would be a promising therapeutic tool for controlling *Ctr* infection.

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8. CURRICULUM VITAE

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RESEARCH INTERESTS:

Host-Pathogen interactions, Identifying disease-specific molecular mechanisms.

EDUCATION BACKGROUND:

Doctoral Studies

Institute:	Biozentrum, Department of Microbiology,
Graduate School:	University of Würzburg, Würzburg, Germany.
Period:	1st March 2011 – 2015
Project Supervisor:	Prof. Dr. Thomas Rudel

Graduate Education

Degree:	Master's in Molecular Medicine (2008-2010)
Institution:	Charité - Universitätsmedizin Berlin, Germany

Under Graduate Education

Degree:	Bachelor of Technology in Biotechnology (2003-2007)
University:	Anna University, India

PhD PROJECT (2011-2015):

"EphA2 is an invasion and an intracellular signaling receptor for *Chlamydia trachomatis*"

Institute: Department of Microbiology, University of Würzburg, Germany

Supervisor: Prof. Dr. Thomas Rudel

Other projects during PhD (2011-2015)

- Studying the importance of multiprotein complex (Hexokinase-I, Bad, AKAP1, PKA) during *Ctr* infection (2012-2015)
- Do HHV-6 interacts with EphA2 and affects the *Ctr*-induced EphA2 activation during co-infection? (2014-2015)
- Development of chlamydial strains expressing recombinant IncA or IncG (2012-2013)
- *Chlamydia trachomatis* inclusion proteome mass spectrometry data (2011-2014)
- *Chlamydia* specific IncA-antibody production and purification (2011-2012)

MASTER'S THESIS:

"Expression and regulation of Interleukin-33 in *S. pneumoniae*-infected human cells"

Dept. of Internal Medicine/Infectious Diseases and Pneumology,

Charité Universitätsmedizin Berlin, March 2010 - September 2010.

Supervisor: Prof. Dr. Bastian Opitz.

PRESENTATIONS:

Scientific talks during PhD,

- 12th Deutscher Chlamydien Worshop, Berlin, 2014
- 8th International GSLS symposium (Scientific crosstalk), Würzburg, 2013
- 3rd Pathogen-Wirtsinteraktom und Signalkomplexe in bakteriellen Infektionen Glashutten, BMBF meeting, Frankfurt, 2013
- 11th Deutscher Chlamydien Worshop, Würzburg, 2013
- 2nd Pathogen-Wirtsinteraktom und Signalkomplexe in bakteriellen Infektionen, BMBF meeting, Hamburg, 2012
- 7th International GSLS symposium (Scientific crosstalk), Würzburg, 2012
- 1st Pathogen-Wirtsinteraktom und Signalkomplexe in bakteriellen Infektionen, BMBF meeting, Gottingen, 2011

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I hereby assure that the provided information above is true to the best of my knowledge.

Prema Subbarayal
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9. LIST OF PUBLICATIONS

1. EphrinA2 receptor (EphA2) is an invasion and intracellular signaling receptor for *Chlamydia trachomatis.* (Revision, PlosPathogens., 2015)

Prema Subbarayal, Karthika Karunakaran, Ann-Cathrin Winkler, Marion Rother, Erik Gonzalez, Thomas F. Meyer and Thomas Rudel

2. Gp96 shedding induced by *Chlamydia trachomatis* prevents chlamydial re-infection. (Submitted., 2015)

Karthika Karunakaran, Prema Subbarayal, Nadine Vollmuth and Thomas Rudel

3. *Chlamydia trachomatis* secreted deubiquitinase stabilises Mcl-1 to mediate apoptosis resistance. (Submitted., 2015)

Annette Huber, Swarna Oli, Bhupesh Prusty, Prema Subbarayal, Tanja Schirmeister and Thomas Rudel

4. Septins arrange F-actin containing fibers on the *Chlamydia trachomatis*-inclusion and are required for the normal release of the inclusion by extrusion. mBio., 2014

Larisa Volceanov, Katharina Herbt, Martin Biniossek, Oliver Schilling, Dirk Haller, Thilo Nölke, Prema Subbarayal, Thomas Rudel, Barbara Zieger and Georg Häcker.

5. Mutation of megalin leads to urinary loss of selenoprotein P and selenium deficiency in kidneys and brain. Biochemical Journal., 2010

Jazmin Chiu-Ugalde, Franziska Theilig, Thomas Behends, Julia Drebes, Carolin Sieland, Prema Subbarayal, Josef Kohle, Annette Hammes, Lutz Schomburg And Ulrich Schweizer