Manipulation Of Host Signal Transduction Pathways And Cytoskeleton Functions By Invasive Bacterium Listeria Monocytogenes And Chlamydia Trachomatis

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MANIPULATION OF HOST SIGNAL TRANSDUCTION PATHWAYS AND CYTOSKELETON FUNCTIONS BY INVASIVE BACTERIUM LISTERIA MONOCYTOGENES AND CHLAMYDIA TRACHOMATIS

by

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ABSTRACT

Infectious disease remains one of the leading causes of morbidity and mortality worldwide. Many bacteria that cause disease have the capacity to enter into eukaryotic cells such as epithelial cells and tissue macrophages. Gaining access into the intracellular environment is one of the most critical steps in their survival and/or in pathogenesis. The entry mechanisms employed by these organisms vary considerably, but most mechanisms involve sabotaging and manipulating host cell functions. Invasion of epithelial cells involves triggering host signal transduction mechanisms to induce cytoskeleton rearrangement, thereby facilitating bacterial uptake. My work focuses on understanding the molecular mechanisms employed by bacterial pathogen *Listeria monocytogenes* and *Chlamydia trachomatis* to gain access into the host cells in order to cause the disease.

In first part of my thesis I investigated the mechanism of *Listeria monocytogenes* entry. *Listeria,* a facultative intracellular organism, is responsible for causing meningitis, septicemia, gastroenteritis and abortions. Critical for *Listeria* virulence is its ability to get internalized, replicates and spread into adjacent host cells. One of the pathways of *Listeria* internalization into mammalian cells is promoted by binding of its surface protein Internalin B (InlB) to host receptor MET. Studies done in the past demonstrated a critical role of host type IA Phosphoinositide (PI) 3-kinase in controlling cytoskeleton rearrangement and entry of *Listeria* downstream of MET. An important unresolved question was how activation of PI3K results in cytoskeleton rearrangements that promote *Listeria* entry. In this work, we identified 9 host signaling molecules, that

includes Rab 5c, SWAP 70, GIT1, PDK1, mTor, ARAP2, ARNO, DAPP1 & PKC-ζ, acting downstream of type IA Phosphoinositide (PI) 3-kinase to regulate changes in host cytoskeleton to cause *Listeria* entry.

Second part of my thesis involved studying the functions of chlamydial effector protein Tarp in its invasion. Infection caused by *Chlamydia Trachomatis* is the most common sexually transmitted disease resulting in uro-genital diseases, LGV, ectopic pregnancy and infertility. It is also responsible for causing trachoma, the leading cause of preventable blindness in third world countries. Being an obligate intracellular pathogen, gaining access into intracellular environment is the most critical step in lifecycle and pathogenesis of *Chlamydia*. Previous studies demonstrate the role of both chlamydial and host actin nucleators, Tarp and Arp2/3 complex respectively, in mediating *Chlamydial* entry into non-phagocytic cells. But the molecular details of these processes were not well understood. In this study, we demonstrate novel function of Tarp protein to form actin bundles by its ability to bind filamentous actin through newly identified FAB domains. And we also provide bio-chemical evidence that Tarp and Arp2/3 complex works in conjunction to cause changes in host cytoskeleton that effectively culminate into bacterial uptake by host cells.

Overall, this research was a significant step in enhancing our understanding, at a molecular level, to pathogenesis of infections caused by *Listeria monocytogenes* and *Chlamydia trachomatis*.

I dedicate this dissertation to my wonderful family. Particularly to my grandparents Sadruddin and Shirin Jiwani for their continuous support; My mother Rozma Jiwani for her faith and love; My sister Nasim, brother-in-law Anis, nephew's Shafaan and Arhaan for their inspiration; My beautiful wife, Nasrin, for her understanding and patience; And my brother-in-law Wasim for always making me smile during my toughest times. Finally, I dedicate this work to my late father Sherali Jiwani, who always believed in diligence, science, arts and the pursuit of academic excellence.

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AA	Amino acid
ABD	Actin binding domain
ADP	Adenosine di-phosphate
Arp2/3 complex	Actin related protein-2/3 complex
ATP	Adenosine tri-phosphate
CDC	Center for disease control and prevention
CFTR	Cystic fibrosis transmembrane receptor
CHO cells	Chinese hamster ovarian cell
DMEM	Dulbecco's modified eagles medium
EB	Elementary Body
eGFP	Enhanced green fluorescent protein
FAB	F-actin binding domain
F-actin	Filamentous actin
FGF	Fibroblast growth factor
G- actin	Globular actin
GAG	Glucose-amino-glycan
GAP	GTPase activating protein
GDI	GDP dissociation inhibitors
GDP	Guanosine di-phosphate
GEF	Guanine nucleotide exchange factors
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
GTP	Guanosine tri-phosphate
Hela cells	Henrietta Lacks cells
HGF	Hepatocyte growth factor
Hsp	Heat shock protein
HSPG	Heparan sulfate proteoglycan
IDO	Indolamine 2,3 deoxygenase
IFN-γ	Interferon gamma
IL	Interleukin
Inc	Inclusion membrane proteins
InlB	Internalin B
Jas	Jasplakinolide
LGV	Lymphogranuloma venerum
LLO	Listeriolysin O
LPS	Lipopolysaccharide
MOMP	Major outer membrane protein
MTOC	Microtubule organizing center
NPF	Nucleation promoting factor

NTC	Non template control
Omc	Outer membrane complex protein
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor receptor
PDI	Protein disulfide isomerase
PDK	Phosphoinositide dependent kinase
PhosD	Phosphorylation domain
PI 3-Kinase	Phosphatidylinositol 3-kinase
$PI(3,4)P_2$	Phosphatidylinositol 3, 4,-bisphosphate
$PI(3,4,5)P_2$	Phosphatidylinositol 3, 4,5-trisphosphate
PID	Pelvic inflammatory disease
PKC	Protein kinase C
PLcA	PhospholipaseC A
PLcB	PhospholipaseC B
PLCγ	PhospholipaseC gamma
Pmp	Polymorphic outer membrane protein
PRD	Proline rich domain
RB	Reticulate Body
RNAi	RNA interference
RQ	Relative quantitation
SEM	Scanning electron microscopy
SiRNA	Silencing RNA
T3SS	Type 3 secretion system
Tarp	Translocated actin recruiting protein
WASP	Wiscott-Aldrich syndrome protein
WAVE	WASP-family verproline-homology protein
WHO	World health organization

CHAPTER ONE: INTRODUCTION

The following chapter will introduce important concepts in the bacterial invasion of host cells. The broad focus of this thesis was to understand the subversion of host pathways by intracellular pathogens *Listeria monocytogenes* and *Chlamydia trachomatis*. This work was done to enhance our understanding of host-pathogen interaction with an ultimate goal to develop new preventive strategies to target these bacteria at the point of their entry.

1.1 Actin Cytoskeleton: A Key to Bacterial Invasion

Cytoskeleton dynamics plays a key role in many physiologic processes including cell migration and motility, chemotaxis, migration during embryogenesis endocytosis and phagocytosis [1-3]. It is no surprise that bacteria have evolved mechanisms to hijack cytoskeletal machinery for their own benefits. The main components of the cytoskeleton include microfilaments (7nm), intermediate filaments (10nm) and microtubules (25nm). The exploitation of microtubules has been reported only in case of a few organisms like entero-invasive *E. coli*, *C. jejuni, C. freundii* [4, 5]. In contrast, the subversion of actin filaments seems to be the common mechanism used by many pathogens. For example, entero-hemorrhagic *E. coli*, an extracellular pathogen modulate actin machinery to attach to host cells [6]. Intracellular bacteria like *Rickettsia, Chlamydia* and *Listeria* exploit the actin cytoskeleton for invasion as well as for intra and

intercellular movement [7, 8]. Below I will discuss the actin cytoskeletal machinery and how actin polymerization is regulated.

1.1.1 Actin and Actin Nucleation Machinery

1.1.1.1 Actin and Actin Turnover

First isolated in 1945 by Sczent Gyori, actin is a ~42 kDa ATP binding protein found in abundance in all eukaryotic cell types. It exists in two interchangeable forms, a monomeric G-actin and structurally twisted helical filaments (F-actin) [9, 10]. Actin filaments are formed by reversible polymerization of monomers by a process which involves ATP hydrolysis and release of inorganic phosphate. The two ends of actin filaments are biochemically distinct in that the addition of monomers is more favorable at one end [11, 12]. This fast growing end is called the barbed (+) end, whereas the opposite slow growing end is called as pointed (-) end. Actin dynamics is tightly regulated by monomer and filament binding proteins that control the free pool of monomers, regulate the polymerization process, organize the filaments into networks and also control filament depolymerization for recycling of monomers [13]. Profilin and cofilin are the two main actin binding proteins responsible for polarized growth of filaments [13, 14]. Profilin guides the ATP bound G-actin to the barbed end and favors the growth of filaments, whereas cofilin enhances dissociation of ADP bound G-actin from the pointed end to restrict the length of filaments [15, 16]. In unstimulated conditions the barbed ends are blocked by capping proteins to prevent unnecessary growth of filaments. Formation of new filaments depends on the presence of free

barbed ends that acts as template for addition of new monomers. Free barbed ends can be created by three distinct mechanisms; uncapping of barbed ends, severing of filaments or de novo nucleation [17, 18]. Nucleation is the rate limiting step in generation of new filamentous from actin monomers. It involves bridging of 3 monomers together to form a seed that favors addition of new monomers [19]. Spontaneous nucleation is kinetically unfavorable and requires the presence of free monomers above the certain critical concentration of actin [20, 21]. Thus the nucleation step is usually regulated by other cellular proteins called actin nucleators, which will be discussed below.

1.1.1.2 The Arp2/3 Complex

There are three different actin nucleating proteins identified so far in eukaryotic cells: the Arp2/3 complexes, formins and spire-like actin nucleators [22-24]. Each nucleator uses a distinct mechanism to nucleate actin. While formins and spire-like actin nucleators bind actin monomers and form linear filaments, the Arp2/3 complex bind monomers and preexisting filaments to promote actins branching [25-29].

The Arp2/3 complex is the most extensively studied actin nucleating protein because it plays such a central role in regulating actin nucleation. The Arp2/3 complex is a multimeric complex of 7 subunits including Arp2, Arp3, p16, p20, p21, p34 and p40 [24, 30]. The Arp2 and Arp3 subunits share structural similarity to actin. The other subunits provide structural conformation that allow binding to filamentous actin [25, 26]. The Arp2/3 complex by itself cannot nucleate actin and requires prior activation by

nucleation promoting factors (NPF) [31-33]. Activation of the Arp2/3 complex results in conformational change such that it binds to preexisting actin filaments via its ‘p’ subunits [34]. And Arp2 and Arp3 subunits mimic the barbed end of an actin filament which along with NPF promote actin nucleation [35]. This activity generates a new actin filament that forms a 70 degree branching pattern over a pre-existing filament [36].

1.1.1.3 Nucleation Promoting Factors

There are two known NPF family of proteins in eukaryotes that can directly activate the Arp2/3 complex which includes Wiskott- Aldrich syndrome protein (WASP) and Wasp- family verproline -homologus protein (WAVE) [37]. The WASP family includes two members (hematopoietic WASP and ubiquitously expressed N-WASP), whereas the WAVE family includes three members of which WAVE1 and WAVE3 are mainly neuronal and WAVE 2 is universally expressed. WASP and WAVE naturally exist in auto inhibitory conformations that do not favor interaction with the Arp2/3 complex [37]. Rho family GTPases interact with WASP and WAVE to induce conformational changes resulting in their activation [38, 39]. Both WASP and WAVE share certain common domains including the WCA domain, the Pleckstrin Homology domain (PH) and the proline rich domain (PRD) [37]. In an active state WASP/WAVE, via its WCA domain, simultaneously binds to G-actin and the Arp2/3 complex and promotes actin nucleation. The PH domain interacts with phosphatidylinositol (PI(3,4,5)P3 in WAVE and PI(4,5)P2 in WASP) which results in the recruitment of cytoplasmic WASP and WAVE complexes to plasma membrane [40, 41]. The PRD

domain binds to profilin that aids in supplying actin monomers [37]. Together, this results in the formation of actin filaments which alters the shape of the plasma membrane. WASP/Arp2/3 complex activity mainly results in fine extensions of plasma membrane called filopodia [42], whereas WAVE2/Arp2/3 complex activity is responsible for causing dramatic rearrangements of plasma membrane called lamellopodia [43, 44].

1.1.1.4 Rho Family GTPases

Rho family GTPases belong to a family of small GTP/GDP binding proteins that act as molecular switches in regulating appropriate cellular responses to extracellular stimuli [45]. Like other GTP binding proteins, Rho family GTPases harbor a GTP/GDP binding domain and also possess GTPase activity [45]. They alternate between active GTP bound and inactive GDP bound forms depending upon the upstream stimulus. When in an active conformation, these GTPases influence the cytoskeleton network by regulating the activity of nucleation promoting factors such as WASP and WAVE [45]. Cdc42 binds directly to the CRIB domain of N-WASP and Rac and, using several adaptor proteins, interacts indirectly with the PRD domain of WAVE-2 to facilitate an interaction with the Arp2/3 complex [37].

The activity of Cdc42/Rac is regulated by three groups of proteins: including GTPase activating proteins (GAPs), GDP dissociation inhibitors (GDIs), and Guanine nucleotide exchange factors (GEFs). GAP promotes the intrinsic activity of Rho GTPases to hydrolyze GTP, and GDI prevents GDP-GTP exchange by inhibiting the Rho GTPase activity [46, 47]. GEF facilitates the removal of GDP from Rho GTPases,

thereby promoting GTP binding due to GTP being present in the cytoplasm at a higher concentration than GDP [48]. Regulation of Rho GTPases is very complex as more than 100 regulatory proteins are present in eukaryotic cells, with each regulating one to several Rho GTPases. To add more complexity, different regulators can be stimulated by several upstream signaling proteins in response to many different extracellular stimuli. Elucidating these complex arrays of signaling events has been a major challenge in every field of molecular research including cellular microbiology.

1.1.2 Bacterial Entry: Zipper Vs Trigger Mechanism

The human body is composed of two types of cell: phagocytic and non-phagocytic cells. Phagocytic cells such as neutrophils, macrophages, dendritic cells and mast cells have the capacity to uptake large particles like bacteria. These cells play a key role in controlling bacterial infections by secretion of inflammatory mediators and antigen presentation [49-53]. A few intracellular bacteria like *Mycobacterium spp*, *Brucella spp, Listeria, spp,* and *Legionella spp* have evolved mechanisms to survive and replicate within phagocytes [54]. For most bacterial pathogens, the phagocytic environment is not a safe zone. Most pathogens prefer to invade non-phagocytic cells like epithelial cells, endothelial cells and fibroblasts. Unlike professional phagocytes, these cells lack the capacity to uptake large particles like bacteria. Thus, many intracellular organisms induce their own uptake in these non-phagocytic cell types by a process called “induced phagocytosis”. In 1995, Swanson and Baer , proposed the concept of “Zipper and Trigger mechanisms” of bacterial entry into non-phagocytic cells.

The main difference between the two mechanisms is that the former is stimulated from outside, whereas the latter is triggered from inside the host cells. The Zipper mechanism is widely used by gram positive and negative organisms, whereas the trigger mechanism is exclusively seen in entry of gram negative organisms.

1.1.2.1 The Zipper Mechanism

The zipper mechanism is a process that involves binding of bacterial surface proteins (adhesins) to host cell surface receptors. Each adhesin has the propensity to interact only with a specific type of host receptor, an interaction responsible for host specificity, tissue tropism and pathogenesis. Such interactions cause activation of host cell surface receptors resulting in initiation of downstream signaling pathways. These signaling pathways in turn activate actin nucleation machinery to cause changes that favor uptake of bacteria (**see figure 1**). In the zipper mechanism, the cytoskeletal changes do not involve major remodeling of the host cell surface; The plasma membrane remains in close association with the invading bacterium [55]. Many bacterial adhesins and their specific host receptors have been identified that promote uptake of an organism via the zipper mechanism. For example, *Yersinia enterocolitica* uses its surface protein invasin to bind and activate β-1 integrin whereas *Neisseria gonorrhea* uses OpaA to bind to heparan sulfate proteoglycan of the syndecan family to promote its own invasion [56-58]. *Listeria monocytogenes* uses InlA and InlB which bind E-cadherin and Met receptors present on the surface of many human cell types [59-61].

1.1.2.2 The Trigger Mechanism

The trigger mechanism is more complex and involves dramatic rearrangement of the host cell surface membrane in the form of filopodia and lamellopodia which promote passive uptake of organisms by a mechanism similar to macropinocytosis. Such process involves delivery of secreted bacterial effector proteins into the host cytoplasm using a needle-like Type 3 secretion system. These effector proteins modulate the cytoskeleton to favor bacterial uptake [55]. Effector proteins display a diverse range of functional capacity (**See figure 1**). For example, *Shigella*, which causes inflammatory diarrhea, delivers the effector protein IpaC which mimics the activity of the Rho GTPase cdc42 to mediate N-Wasp/Arp2/3 dependent actin polymerization [62]. RickA of *R. coronii* mimics a nucleation promoting factor (NPF) and directly activates Arp2/3 complex [63]. Recently, certain effector proteins like SipC of *Salmonella* and Tarp of *Chlamydia* were discovered to directly bind and nucleate actin to cause actin polymerization [64, 65].

1.2 Listeria monocytogenes

1.2.1 Background and Taxonomy

Listeria monocytogenes is a gram positive facultative anaerobic organism and is ubiquitously found in nature. Although the natural habitat of *Listeria* is believed to be decomposing vegetation, it is abundantly found in a variety of environmental sources including soil, sewage, silage, water and decaying dead animals [66]. It belongs to the genus *Listeria* which currently includes 8 other species: *L. ivanovii, L. seeligeri, L.*

innuocua, L. welshimeri, L. grayi, L. marthii, L. rocourtiae and *L. weihemstephanensis*. The genetic makeup of *Listeria* is well adapted to a saprophytic life style. It harbors multiple genes to facilitate the use of a variety of organic compounds as a source of carbon [67]. It expresses flagella which confers swimming motility for accessing nutrient sources [68]. Although *Listeria* does not form spores, it is very sturdy and survives a variety of environmental stresses including shifts in temperature, pH, osmolarity and oxidative stress [66].

While the majority of species in the genus *Listeria* are harmless saprophytes, *L. monocytogenes, L. ivanovii* and *L. seeligeri* are also potential pathogens. A Wide variety of warm blooded hosts are known to be the targets of *Listeria* including ruminants, sheep, goats, pigs, dogs, ferrets, raccoons, foxes, rats, gerbils, voles, hamsters, cats, horses, eagles and humans [69]. Once inside the infected host, *Listeria* can live either as a non-pathogenic extracellular bacterium or invade the tissues and become a deadly pathogen [69]. Interestingly, a comparative genomic analysis and evolutionary study has indicated that the loss of virulent genes from pathogenic species is responsible for evolution of non-pathogenic species. The Genus *Listeria* thus harbors the species of microorganisms that have evolved from a pathogenic to saprophytic life style.

1.2.2 Epidemiology, Infection and Pathogenesis

L. monocytogenes is mainly responsible for the vast majority of *Listeria* infections in humans, with only a few isolated reports of confirmed infections by *L. ivanovii* and *L. seeligeri.* Serovars 1/2a, 1/2b and 4b of *L. monocytogenes* cause more than 90% of all

human infections [70]. The Infectious disease caused by *L. monocytogenes* is known as listeriosis. It is one of the deadliest food-borne infections carrying a net mortality risk of approximately 30% [71]. It is responsible for causing nearly 2500 deaths per year in USA [72]. The transmission usually occurs via the oral route by consuming contaminated food. Infections can occur sporadically or can result in outbreaks affecting many people. The majority of the outbreaks are usually due to contamination of ready to eat (RTE) packaged foods. Milk and meat products such as salads, meat sandwiches, cheeses, etc. are the main sources of infection [73]. Outbreaks of listeriosis are evidently increasing over the last few decades because of the increase in industrialized farming and food processing. Contamination of processed food by *L. monocytogenes* has resulted in many food recalls in the past few years [73]. Recently, listeriosis has emerged as a disease of such a financial and public health concern that the FDA has approved a listeriocidal drug (a bacteriophage) as an food additive [74].

Host risk factors play a critical role in the development of disease following exposure to *L. monocytogenes*. The disease usually manifests in individuals with defects in T-cell mediated immunity.[71]. Patients who are at a higher risk include newborns, pregnant women, and the elderly. The disease usually manifests in adults with severe debilitating conditions like cancer, leukemia and lymphomas, Diabetes mellitus, chronic kidney or liver disease, as well as those undergoing immunosuppressive therapies. The current epidemic of HIV and cancers are also responsible for an upward trend observed in the incidence of listeriosis [71].

Pathogenesis of the disease caused by *L. monocytogenes* is a multi-step process. It involves : 1) the oral transmission and colonization of the gastro-intestinal tract; 2) invasion through the intestinal epithelium, 3) replication in the liver followed and 4) the systemic spread to other organs [71](**see figure 2**). The clinical manifestation of listeriosis depends on the organ system involved. The infection in early pregnancy can lead to placentitis, chorioamnionitis and abortion. In late pregnancy, the infection usually gets transmitted to the fetus and causes neonatal sepsis. In fact, it is one of the three leading causes of neonatal sepsis, along with group B streptococci and *E. coli*. Listeriosis usually manifests in adults as gastroenteritis and/or infections of the central nervous system leading to meningitis, encephalitis and meningo-encephalitis. Very rarely, it may also cause granulomatous hepatitis, granulomatous skin lesion and infections of other organs [75].

1.2.3 Intracellular Lifecycle of L. Monocytogenes

L. monocytogenes has the capacity to infect a wide variety of tissue specific host cells including macrophages, epithelial cells, endothelial cells, hepatocytes, fibroblasts, neurons and placental tissue [76-81]**.** Regardless of the cell type it parasitizes, the intracellular life cycle of *listeria* shares certain common characteristics (**see figure 3**).

1.2.3.1 Invasion

Invasion of the host cell is a critical steps in the pathogenesis of disease caused by *L. monocytogenes*. In order to enter the host cell, *Listeria* uses surface proteins such as Internalin A and Internalin B [76]. These proteins recognize the host cell surface

receptors and attach to them. Such interactions trigger the activation of host signal transduction pathways that cause changes to the host cytoskeleton that favor entry of *listeria*. This mechanism of entry is known as the zipper mechanism. It involves progressive wrapping of a bacterium by the host plasma membrane. The final product of this process is the engulfment by the host cell of a bacteria enclosed within a membrane bound vacuole [82].

1.2.3.2 Escape

Once inside the host cell, *Listeria* escape from the vacuole to enter the cytoplasm. Doing so is critical for *Listeria* to survive because overtime the vacuole will fuse with lysozomes which are rich in degradative enzymes and that would kill the bacterium [83-86]. In order to disrupt the vacuole, *Listeria* uses the enzymes Listeriolysin O (LLO) and Phospholipase C (PLcA and PLcB)[81, 86-89]. These enzymes insert into the vacuolar membrane, forming pores that provides pathways for *Listeria* to enter into the cytoplasm. LLO a is very potent enzyme and can potentially harm the host cells by disrupting the host cell membrane and other membrane bound organelles, though it is rapidly inactivated following its exposure to host cytoplasm. Thus, its activity is spatially and temporally restricted to endosomes containing *Listeria*. [90-94] **(see figure 3).**

1.2.3.3 Replication and Dissemination

Following its entry into a nutrient rich cytoplasmic environment, *Listeria* not only replicates, but also hijacks the actin cytoskeleton machinery to acquire intracellular

motility. This is essential because it helps *Listeria* to spread directly from cell to cell, thus avoiding the necessity of getting exposed to the hostile extracellular environment. To achieve such capabilities *Listeria* employs two proteins: Act A and Internalin C. Act A is a surface protein that *Listeria* expresses within the cytoplasmic environment. It harbors a domain that directly binds to monomeric actin [95-98]**.** Act A functions as a nucleation promoting factor and along with the host actin nucleator, the Arp2/3 complex, an “actin comet tail” that propels the bacterium, granting it motility[31] . Obviously, such activity suggests that Act A is localized to one pole of the bacterium. The polarization of Act A occurs during the active phase of intracellular replication [7, 89, 95, 99-101]. Another protein that favors intracellular dissemination is Internalin C (InlC). *Listeria* secretes InlC protein in the host cytoplasm. InlC acts on the intracellular junctions to perturb the adherent junctions, thereby enhancing the ability of motile bacteria to form a protrusion into an adjacent recipient cell [102]. These protrusions are called “listeriopods”. After listeriopods are completely taken up by the recipient cells, LLO and PLCB disrupts the membrane to release the *Listeria* back into the cytoplasm. [103, 104]. This allows a new replication cycle to begin. Such repetitions of replication and cell to cell dissemination are critical for pathogenesis of listeria infections and disruption at any stage may compromise pathogenicity [105].

1.2.4 Invasion into Mammalian Cells: The Zipper Mechanism

1.2.4.1 Attachment

Listeria can invade both phagocytic cells including neutrophils, tissue macrophages, and non-phagocytic cells including epithelial cells, endothelial cells, hepatocytes and trophoblasts [71, 106, 107]. The initial step in entry is the attachment of *Listeria* surface adhesins to the host cell surface receptors. *Listeria* express a variety of putative surface adhesins including Internalin A (InlA), Internalin B (InlB), InlJ, Ami, Vip, p60, LAP, FbpA and ActA. But only InlA and InlB were found to be critical for invasion. Other proteins have characteristic functions or expression patterns which suggest that they have evolved for the purpose other than invasion. Internalin A (InlA) and Internalin B (InlB) binds to the host surface receptors E-cadherin and MET tyrosine kinase receptors, respectively [60, 61]. The mode of entry depends on the type of receptor present on the specific host cell. In intestinal epithelium (which represent the initial entry point of *Listeria)*, internalization depends on InlA/E-Cadherin interaction [60, 108]. In most other tissues including hepatocytes (the predominant site of bacterial replication) the entry likely depends on the InlB/Met receptor interaction [107]. InlA/E-cadherin or InlB/MET interactions lead to the activation of signal transduction pathways that produce changes to the cytoskeleton that lead to engulfment of bacteria

1.2.4.2 Internalin Family and InlB

InlA and InlB are the only known virulence genes in *L. monocytogenes* that are involved in its entry into the eukaryotic host cell [106, 109]. They were first identified in a

genetic screen using transposon induced *listeria* mutants to test for their reduced invasiveness capabilities in Caco2 cell lines [76]. As *InlAB* gene locus was found to be critical for internalization of *listeria*, it was named Internalin. Multiple studies later confirmed the role of InlA and InlB in entry by employing several techniques such as genetic manipulations that rendered *L. monocytogenes* less invasive, confering invasiveness to non-invasive species or in-vitro assays using purified recombinant InlA and InlB proteins [76, 78, 110-114].

Four other internalin gene loci were subsequently identified in the *L. Monocytogenes* genome. These loci encode for seven Internalin homologs which includes InlC, InlC2, InlD, InlE, InlF, InlG and InlH (**See figure 4**). The studies aimed to identify the functions of these Internalins failed to demonstrate their role in entry [111, 115, 116]. However, InlC was later found to function at intracellular junctions and helps in cell to cell dissemination of *L. monocytogenes* [102]. The genes for InlC2, InlD, InlF, and InlG have also been identified in non-invasive species like *L. innocua,* suggesting that these are non-virulent genes. However the exact role of these internalins in *Listeria* biology and pathogenesis has not yet been determined [71, 76].

InlB structure: InlB is a 67 kDa protein which consists of a polypeptide having 630 amino acids. The N-terminal half of InlB harbors a signal peptide, the LRR domain and the IR region, whereas the C-terminal half harbors a GW module having three repeats of a GW domain (**see figure 4**) [117, 118].

The LRR domain is the most conserved domain of InlB. It consists of 7 leucine rich repeats of 22 amino acids having leucine placed at certain fixed positions (**See figure 3)** [119]**.** On X-Ray crystallography, each LRR repeat consists of a short β-strand followed by a helical region and a loop [120, 121]**.** Together this imparts a horseshoe shape to the LRR domain that has a concave and a convex surface [118]. The LRR domain interacts with the Met receptor through its concave surface [122, 123]**.** This domain is sufficient and necessary for the activation of the Met receptor and internalization of *Listeria***,** although the potency of LRR activity alone is less than that of full length InlB [61]**.** Thus the other domains of InlB also contribute in InlB mediated entry of *Listeria*. The IR region of InlB resembles the immunoglobin-like domain and participates in its interaction with the Met receptor [123, 124].

The B repeat is not well defined. The X-ray crystallography suggest that it does not interact with Met the receptor, but forms structures similar to the mucin binding protein or the ubiquitin-like proteins. The functional significance of such a structural conformation is yet to be determined [125]. Previous reports using truncated mutants of InlB suggests that the B domain, although not required, potentiates the effects of InlB to activate Met [126]. The reasons for such observations are yet to be determined.

The C terminal end of InlB harbors three GW domains [110]. Each GW domain consists of approximately 80 amino acids each starting with amino acids GW. InlB inserts into the bacterial cell surface via this GW domain [110]. This is a unique mechanism for protein-cell surface interaction in which the protein is partially buried in

the cell wall and uses lipotechoic acid as a ligand to interact with the cytoplasm [127]. However, such interactions are non-covalent and weak allowing dissociation of InlB from the bacterial cell surface. Thus in broth cultures, half of the InlB synthesized by *listeria* is found to be attached to the bacterial surface, whereas the other half is found in culture supernatant [127]**.**

1.2.4.3 Interaction of InlB with Host Cell Surface

1.2.4.3.1 Met RTK: A significant interaction

The Interaction of InlB with the Met is referred for bacterial entry tyrosine kinase family of receptors [61]. InlB binds with the extracellular domain of Met to promote invasion of *L. monocytogenes* into various cell lines [61, 112, 117, 120, 123, 126, 128-130]. Met is widely expressed in a variety of tissues including epithelial cells in the liver, kidney, gastrointestinal tract, vascular endothelial cells, the placenta and the central nervous system [131, 132]. It consists of a disulfide-linked heterodimer with an extracellular α-chain and a β-chain that has extracellular, trans-membrane and intracellular portions [133, 134]. The α-chain and the extracellular portion of the β-chain forms the ligand binding site. The intracellular portion of the β-chain has a cbl binding domain, the tyrosine kinase catalytic domain and a cytoplasmic tail, each harboring one to several tyrosine amino acids. The tyrosine at position 1003 specifically binds to cbl, an ubiquitin ligase, which promotes receptor degradation and recycling via endocytic machinery [135]. The tyrosine kinase catalytic domain has tyrosine residues at position 1234 and 1235 which get auto phosphorylated upon ligand binding and receptor dimerization resulting in activation of Met receptor [133]. The cytoplasmic tail harbors

two tyrosine residues at 1349 and 1356 which upon phosphorylation serve as a docking site for multiple SH2 domain containing cell signaling proteins including Gab1, Grb2, PLC-γ, p85 regulatory subunits of PI 3-Kinase and Src Kinase [133] . The only physiological ligand known to activate Met is hepatocyte growth factor (HGF) which is secreted by fibroblasts and stromal cells [133]. The binding of the HGF results in activation of Met receptor and phosphorylation of its tyrosine residues on the cytoplasmic tail. The cytoplasmic tail then recruits host cell signaling proteins to initiate a signal transduction cascade that ultimately results in cell survival, proliferation and/or motility [136]**.**

Although InlB shares no structural similarity with HGF, it acts as an agonist for Met [61, 120, 121]. Recently, the x-ray structure of the InlB-Met complex was determined which suggests that InlB and HGF do not share the same binding sites on Met. While the predominant site of the HGF-Met interaction is the Sema domain, Ig-3 and Ig-4 like domains of Met, the LRR domain of InlB primary binds to Met at its Ig-1 like domain and only a weak interaction between IR region of InlB with Sema domain of Met has been observed [122, 123, 137]**.** Regardless of these differences, InlB mimics functional activity of HGF including tubulogenesis, DNA synthesis and cell motility [61, 123]. However, the proliferative effects requires persistent stimulation of Met signaling, thus modulation of cell motility is the predominant effect observed during entry of *L. monocytogenes*.

1.2.4.3.2 gC1qr

Using an affinity chromatography approach, the gC1qR receptor was identified as one of the targets of InlB. gC1q receptors are predominantly expressed in WBC's, platelets and endothelial cells. The physiologic ligand for this receptor is C1q, a member of the classical complement cascade [138]. gC1qR binds to InlB at its GW module [139]. This interaction occurs only when InlB is in the soluble form, which is consistent with the finding that the GW domains are in association with the cell wall [117]. The data suggest that the InlB-gC1qr interaction is required for efficient entry of *L. monocytogene* into the host cell [139]**.** However, gC1qr receptors lack the intra cytoplasmic domain and are not known to directly initiate intracellular signaling events [138].

1.2.4.3.3 Heparan Sulfate Proteoglycans (HSPG)

Heparan sulfate proteoglycans (HSPG) are the negatively charged carbohydrate moieties that are abundantly found on eukaryotic cell surfaces. They function to maintain the interaction between cell surfaces and the components of the extracellular matrix [140]. Jonquires et al confirmed the direct interaction between GW modules of InlB with HSPG. This interaction, potentiates the binding and activation of Met by InlB and invasion of *L. monocytogenes* into host cells [119, 124, 128]. Similar to gC1qR, HSPG is not known to initiate intracellular signaling events.

How then, does an interaction of GW module with HSPG or gC1qR contribute in InlB mediated entry? GAG is essential for HGF signaling as it protects HGF from extracellular proteases, causes oligomerization of HGF and concentrates HGF at the site of Met receptors. Incubating *L. monocytogenes* with heparin (a soluble GAG)

results in dissociation and oligomerization of InlB [141]. Moreover, gC1qR binds only to a soluble form of InlB [117]. gC1qR also acts as a co-receptor for Met and helps with clustering of Met. Taken together this suggests that the interactions of GW domain with host cell surface dissociates InlB from bacterial cell membrane and presents it to Met receptor for its potent activation [107].

1.2.4.4 Intracellular events following InlB/Met Interaction

1.2.4.4.1 Activation of Pi 3-Kinase

Binding of InlB to the Met receptor causes activation of the catalytic kinase domain of Met resulting in its auto phosphorylation at critical tyrosine residues [61]. Adaptor proteins like Gab1, Shc, Grb2, cbl and CrkII are recruited to phosphorylated Met [130]. These adaptor proteins further recruit Type 1A PI 3-Kinase to Met resulting in its activation. Type IA PI 3-kinase has been identified as a critical signaling protein acting downstream of the MET/InlB interaction [61, 130, 142] and was shown to regulate F-actin rearrangement in InlB-mediated entry of *Listeria*(**See figure 5**) [143].

Type IA PI -3 kinase (PI3K) is a lipid kinase that regulates a variety of functions in eukaryotic cells, including mitogenic signaling, inhibition of apoptosis, control of intracellular trafficking and regulation of integrins and F-actin [144]. It mediates its action by converting phosphatidylinositol 3,4-bisphosphate ($PI(4,5)P_2$, or 'PIP2') to phosphatidylinositol 3,4,5 tris -phosphate ($PI(3,4,5)P_3$ or 'PIP3'). PIP3 acts as a second messenger that binds directly to several proteins, including various forms of the serine/threonine kinases PDK1 and Akt, and various regulators of small GTPases (Rac GEFs and ArfGAPs) [145]. By controlling PDK1, PI3K indirectly regulates the activity of

several protein kinases including protein kinase C (PKC) and its isoforms [146]. PI 3-kinase can also regulate many cell processes by direct interaction with other proteins to modulate their activity and/or compartmentalization [147-149]. Proteins that act downstream of PI 3-kinase that cause cytoskeleton changes and favor uptake of *L. monocytogenes* into the host cell are not yet known.

The Ras-Map kinase pathway is also activated following InlB /Met association. In fact, InlB is a more potent activator Ras-Map Kinase pathway than HGF. These differences are hypothesized to be due to different binding sites of HGF and InlB on Met.

1.2.4.4.2 Subversion of Host Actin Nucleation Machinery

Cytoskeleton rearrangements play a critical role in the entry of *Listeria*. Previous studies demonstrated that treatment of host cells with inhibitors of actin polymerization, such as cytochalasin D, resulted in a marked decrease in the entry of *L. monocytogenes* [77]. The role of the Arp2/3 complex, which generates branched actin filaments required for the formation of phagocytic cups, in entry of *L. monocytogenes* is well established. The activation of the Arp2/3 complex is mediated primarily via Rac/Wave and cdc42/ N-Wasp pathways [124, 150]**.** The contribution of Ena/Vasp and Abi proteins to preserve the newly formed actin filaments has also been demonstrated [151, 152]**.** The efficient uptake of *L. monocytogenes* also requires de-polymerization of the actin filaments. Such activities are mediated via the Cofilin/Lim kinase pathway [153]. AlThough, how these pathways are linked to Met activation following InlB stimulation is not yet known. Interestingly, the activation of PI 3-Kinase is found to be

essential for Rac1 activity following InlB stimulation [154]. PI 3-kinase can potentially regulate the activity of several guanine nucleotide exchange factors (GEF) needed for Rac1 and cdc2 activation. The GEFs which act downstream of PI 3-kinase to cause activation of Rac1 and cdc42 are not yet identified.

1.2.4.4.3 Subversion of the Clathrin Dependent Endocytic Machinery

Endocytosis is a process by which the cell mediates uptake of solutes, fluids and certain small molecules [155]. Endocytosis also plays a critical role in cell signaling as it allows recycling and degradation of several receptors, thus acting as a check point to prevent persistent cell stimulation. Consistently activated Met receptor also undergoes endocytosis mediated degradation following stimulation by HGF and purified InlB [156, 157]. Endocytic vesicles are typically small and allow internalization of only very small molecules [155]**.** Interestingly, some components of endocytic machinery, including clathrin, adaptor proteins Eps 15 and Grb2, ubiquitin ligase cbl and lipid rafts, have been shown to play essential roles in the entry of *L. monocytogenes*. Depletion of these molecules via RNA-interference abrogates *Listeria* entry [158, 159]. The mechanism by which these proteins help in the entry process is not yet known. One possibility is that clathrin forms large lattices to mechanically help the internalization process. Another possibility is that these molecules influence signal transduction events to regulate F-actin changes needed for *Listeria* entry. To support this notion, the role of lipid rafts in PI 3-Kinase dependent activation of Rac1 following InlB stimulation has been demonstrated [154]. The ability of clathrin to serve as a scaffolding protein in controlling the activity and/or membrane localization of many adaptor proteins and lipid kinases is

also known [160]. Cbl, an ubiquitin ligase, can directly bind and activate Pi 3-Kinase [161]. In turn, the activation of PI 3-Kinase can help the maturation of newly forming endosomes by recruiting essential molecules like Rab and SNX family of proteins [147]. However the association between the endocytic pathway and Pi 3-Kinase signaling in entry of *Listeria* is yet to be determined.

1.2.5 Hypothesis

PI-3 kinase mediated signaling events are critical for invasion of *L. monocytogenes.* PI-3 kinase can regulate the activity of many host cells proteins to cause cytoskeleton rearrangements. In order to understand the molecular mechanisms involved in invasion of *L. monocytogenes*, it is first essential to identify the host proteins that play a critical role in this process.

1.3 Chlamydia trachomatis

1.3.1 Background and Taxonomy

The order *Chlamydiales* includes organisms that possess unique biphasic developmental cycles, which alternate between an extracellular infectious form, 'the elementary body' (EB), and an intracellular replicative form, 'the reticulate body' (RB) [162]. The species in this order are broadly subcategorized into being environmental and pathogenic organisms. The ubiquitously found environmental organisms include the species in the families *Parachlamydiaceae*, *Waddliacea* and *Simkaniacae*. These organisms predominantly infect unicellular eukaryotes like amoebae [163, 164]. The pathogenic group includes species of the genera *Chlamydia and Chlamydophila* that

tend to infect complex eukaryotic organisms [163]. The genus Chlamydia includes three species, namely *C.trachomatis, C. muridarum and C. suis,* whereas the genus *Chlamydophila* include six species viz., *C. pneumonia, C. abortus, C. caviae, C. pecorum, C. psittaci* and *C. felis* [163]. In 2009, the Chlamydia basic research society meeting was held in Little rock, Arkansas, where the decision was made to merge the genus *Chlamydia* and *Chlamydophilia* into one family of *Chlamydia* (**see figure 6**). Within each species are many different serovars, which cause different diseases, elicit variable host immune responses, and show variations in their morphology. Serovars are distinguished based on variable host antibody response to the 'major outer membrane protein' (MOMP), the main surface antigen in pathogenic chlamydial species.

1.3.2 Epidemiology, Infection and Pathogenesis

Chlamydia can infect a wide variety of hosts including mice, hamsters, cattle, sheep, pigs, koalas, ferrets, pigeons, budgerigars, parakeets, horses and humans [165]. However, not all chlamydial species infect humans. Infections in humans are usually caused by *C. trachomatis, C. pneumoniae,* and *C. psittaci. C.trachomatis* serovars A, B, Ba and C are responsible for causing ocular disease 'trachoma,' whereas *C.trachomatis* serovars D-K cause urogenital infections resulting in cervicitis, urethritis, endometritis, salpingo-oophoritis, infertility and ectopic pregnancy. *C.trachomatis* serovars L1, L2 and L3 cause a more invasive disease called lymphogranuloma venereum, which mainly affects the lymph nodes. *C. pneumoniae* and *C. psittaci* usually infect the respiratory

tract resulting in a mild upper respiratory infection, but they can sometimes cause life threatening pneumonia [166].

Chlamydia trachomatis serovar A-C are responsible for causing trachoma, which is a leading cause of preventable blindness in developing and underdeveloped countries [167]. It accounts for 16% of the blinding population globally [168]. Poor hygiene, the unavailability of clean water and lack of health care resources are the main factors responsible for the high incidence and prevalence of trachoma [169]. The infection usually occurs in children within the age group of 0-4 years. Without treatment, the chronic irritation causes conjunctival and corneal scarring that leads to blindness[169]. 'SAFE,' a strategy whose components include *S*urgery, *A*ntibiotics, *F*acial cleanliness and *E*nvironmental improvement, is currently a major project funded by the WHO with an aim to eradicate trachoma by 2020[170].

Chlamydia trachomatis serovar D-F is the most common sexually transmitted disease in the United States [171, 172]. Each year it is responsible for over 1 million new cases in the U.S. and 92 million new infections globally. It is estimated that one out of every seventy-three individuals are infected by *Chlamydia* [173]. In 2007, a survey done by the Center for Disease Control and prevention (CDC) reported an infection rate of 13.2 % in females and 7.2 % in males between 16 and 24 years of age. The main risk factor for acquiring *C. trachomatis* infection is sexual promiscuity. It can be transmitted via vaginal, anal, or oral sexual contact. The majority of infections are asymptomatic and go unrecognized. Acute clinical symptoms include urethritis, proctitis and

epididymitis in males and cervicitis, urethritis and salpingitis in females. Subclinical or chronic infections lead to complications like pelvic inflammatory diseases, ectopic pregnancy and infertility[174]. Infection can also be transmitted from infected mother to newborn resulting in conjunctivitis and/or pneumonia [175].

Unlike *C. trachomatis* serovars A-K, which cause superficial mucosal infections, serovars L1- L3 infect deeper tissues, especially submucosa and the lymph nodes [176]. These serovars are responsible for the infectious disease known as Lymphogranuloma venereum (LGV). LGV is also a sexually transmitted disease which usually occurs in homosexuals and HIV positive patients. Clinical manifestation includes papular lesions followed by anal strictures and lymphedema [176]. Because of the rapidly changing sexual practices in general populations a number of recent outbreaks of LGV have been reported in Europe and the United States [177].

C. pneumoniae usually infects respiratory epithelium and causes acute respiratory tract infections including pneumonia, bronchitis, otitis media, pharyngitis and sinusitis [178, 179]. Human exposure to *C. pneumoniae* is very common and it is estimated that 80% of the world's population have been exposed to *C. pneumonia* at any given point [180]. However, acute infection usually occurs in children under 5 years of age [181, 182]. It is usually transmitted from person to person via respiratory droplets [182]. Recently, the role of *C. pneumonia* in chronic inflammatory conditions like atherosclerosis and destructive joint diseases has also been suggested [183].

C. psittaci is a zoonotic disease which usually occurs in birds like parrots, parakeets and canaries [184]. Humans are rare incidental hosts. It tends to occur in individuals having occupational exposure to birds like poultry farm workers and veterinarians [185-187]. The disease caused by *C. psittaci* is known as psittacosis, also referred to as parrot fever. It results in acute respiratory infection causing severe bronchitis and pneumonia [185]. Other symptoms include high fever, joint pain, conjunctivitis and epistaxis. In severe cases it carries a mortality risk of ~1%.

The majority of acute infections caused by chlamydia are cleared by host innate and adaptive immune responses without any major consequences. However, in susceptible individuals repeated or persistent infection can lead to chronic inflammation with subsequent scarring, fibrosis and anatomical defects [188].

Upon transmission into the humans, the first line of defense against *Chlamydia* is the innate immune response. Since chlamydia infects ocular, respiratory, or genital epithelium, the first line of defense is the mucosal barrier [189]. The mucosa not only serves as a physical barrier but also contains several antimicrobial proteins including lysozymes, defensins and complement proteins, which resist the establishment of a chlamydial infection [189, 190].

Epithelial cells respond to chlamydial infection by producing pro-inflammatory cytokines like IL-8, IL-6, GM-CSF and GRO-α [191]. These cytokines recruit and activate polymorphonuclear neutrophils, macrophages, peripheral blood monocytes, dendritic cells and natural killer cells [174]. These cells further potentiate the

inflammatory response by releasing IFN-γ and matrix metalloproteinase [174]. The high levels of IFN-γ activate Indoleamine 2,3 dioxygenase (IDO), which stops the production of tryptophan, an essential amino acid required for replication of EBs [174, 192]. IFN-γ also induces nitric oxide synthase in macrophages and epithelial cells leading to activation of nitric oxide based killing mechanisms [193, 194].

Adaptive immunity in the form of T and B cells also contribute to fight chlamydial infections. Antigen presenting cells such as macrophages and dendritic cells engulf chlamydia and infected epithelial cells and process chlamydial antigens via the MHC class II mediated pathway [195]. These antigens are recognized by CD4+ T-cells. CD4-TH1 cells are believed to play a significant role in clearing chlamydial infections by producing large amounts of inflammatory cytokines [192]. Infected epithelial cells also cause recruitment of CD8+ cytotoxic T-cells via MHC class I antigen presentation [174]. The role of B-cells in chlamydial infection is controversial [196, 197]. There are many reports confirming the production of chlamydia specific neutralizing antibodies by B-cells, which promotes opsonization and phagocytosis of EBs [196, 198, 199]. However, mice deficient in B-cells have been shown to fight chlamydial infections as efficiently as their normal counterparts [197]. Thus, it is believed that T-cell mediated responses are predominantly involved in chlamydial infection. However, such adaptive immunity is short lived and does not confer full protection to subsequent infections [200-202]. This is due to both rapid reduction in antibody titers as well as reduction in antigen specific T-cells [203, 204]. Interestingly, repeated exposures to chlamydia elicit more severe

inflammatory damage probably due to hyper immunization or delayed type hypersensitivity [198, 205, 206].

One mechanism by which chlamydia can respond to acute inflammatory host immune responses is by entering into a persistent state characterized by aberrant RBs. The persistent state is defined as the ability of the chlamydia to remain in a dormant and non-replicative form within the host for an extended period of time [207]. In tissue culture, EBs enter into the persistent state in response to penicillin treatment, IFN-γ, continuous infections or nutritional deficiency [208]. Aberrant RBs are characterized as enlarged forms of RBs, which produce less surface antigen such as MOMP, Outer membrane protein B (OmpB) and lipopolysaccharide (LPS) [207]. The DNA replication machinery is active in aberrant RBs and they continue to accumulate DNA. However, they are arrested in the growth cycle and do not divide or transform to EBs [209-211]. In the absence of adverse conditions, these aberrant RBs transition back and continue to propagate. The exact molecular details of such reversible transitions are not yet known. Such waxing and waning of the chlamydial developmental cycle is believed to be responsible for continuous residual infection causing chronic inflammatory damage to the host. There is ample clinical evidence to support this hypothesis. For example, chlamydial proteins, in absence of recoverable organism, can be detected at the site of infection long after the resolution of an acute episode of infection [212-214]. Children living in endemic areas have developed trachoma decades after moving to non-endemic zones probably due to the reactivation of an initial asymptomatic infection [215].

Persistent infection by *C. pneumoniae* is also believed to be a plausible etiology in development of asthma and atherosclerosis [216, 217].

Autoimmune inflammatory response to chlamydial antigens has been implicated in the pathogenesis of diseases caused by chlamydia. High expression of immunogenic Heat shock protein 60 (Hsp60) by aberrant RBs elicits strong antibody responses resulting in production of anti-Hsp antibodies by the host. Hsp from different organisms share great sequence and structural homology [218]. These antibodies can cross react with host Hsp to initiate autoantibody mediated destruction of host tissues [198]. Hsp proteins can also directly activate toll like receptors 2 (TLR2) and trigger production of inflammatory cytokines transforming growth factor-β (TGF-β) and fibroblastic growth factor (FGF) by many cell types including macrophages, T and B lymphocytes [219]. Together this can result in fibrosis and scarring causing anatomic defects [198].

Host susceptibility is also implicated in development of pathology following infection by *Chlamydia*. HLA-A31 and A6802 alleles have been strongly associated with development of pelvic inflammatory disease (PID) and trachoma respectively [220, 221]. Polymorphism in the promoter regions of inflammatory cytokines including TNF-α have also been associated with increased incidence of scarring following chlamydial infection [222].

1.3.3 Developmental Cycle of C. trachomatis

Species of *Chlamydia* utilize a unique developmental cycle in which bacteria transition from the infectious spore-like elementary body (EB) to the metabolically active

reticulate body (RB) within the protective confines of a membrane bound parasitophorous vacuole termed the inclusion [223]. The invasive EB is formed in the mid to late stages of the intracellular development cycle as the RBs differentiate back to EBs and are packed with metabolites and proteins designed to facilitate extracellular survival and reinfections. [224, 225]Additional infectious cycles arise from EBs that are released and disseminate from infected tissues (**see figure 7**) [226].

1.3.3.1 Attachment and Invasion

Being an obligate intracellular organism, invasion into eukaryotic host cells is the most critical step in the biology of chlamydia. The EB attaches to the mucosal epithelial cell membrane through a reversible electrostatic interaction [227]. Heparan sulfate proteoglycan (HSPG/GAG) mediates the initial reversible binding of EBs to the host cell surface, as preincubation of bacteria or host cell with heparan sulfate causes diminished entry of EBs [228-232]. Furthermore, treatment of *C. trachomatis* and *C. pneumoniae* EBs with monoclonal antibodies targeting heparan sulfate also reduces invasive capacity of EBs [233]. Similarly, chemically modified heparan sulfate or heparinase treatment of EBs or host cells prevented infection by *C. trachomatis* serovar E and L2 [231, 232]. Although the role of chlamydial GAG in attachment has been suggested in many studies, no chlamydial genes that can synthesize a structural analogue of GAG have yet been identified. It has also been proposed that chlamydia acquires GAG from the host golgi apparatus, which is a site of proteoglycan synthesis [234, 235].

The initial attachment is followed by temperature dependent irreversible binding of putative adhesins like MOMP, LPS, and Pmps to host cell surface receptors [236]. Many host cell surface receptors have been identified that favors irreversible interaction between chlamydia and host cell surface. This includes platelet derived growth factor receptor (PDGFR), fibroblastic growth factor receptor (FGFR), cystic fibrosis transmembrane conductance regulator (CFTR) [237-239]. Such interactions activate host signal transduction pathways and also favor contact dependent activation of the type III secretion machinery (T3SS) present on the surface of the EBs [240]. T3SS is a needle like apparatus that gram negative organisms use to deliver effector proteins into eukaryotic host cells[241] . Using the T3SS apparatus, EBs deliver several effector proteins into the host cells including CT456 (Tarp), CT166 and CT694 [242-244]. These events together cause dramatic rearrangement of the actin cytoskeleton favoring uptake of EBs into a membrane bound vacuole. Such effector mediated entry of microorganisms is known as a 'trigger mechanism.'

1.3.3.2 Transition from EBs To RBs

Immediately upon entry into the endocytic vesicle, EBs begin to differentiate into RBs. Such transition occurs within the first two hours post invasion and involves at least two defined mechanisms. The condensed nucleoid DNA of EBs begin to disperse to form free chromatin, which results in increased transcriptional activity. The transcriptional activity can be observed as early as 15 minutes following invasion [245].

Secondly, the infectious particles are rendered non-infectious and become metabolically active.

1.3.3.3 Intracellular Survival and Replication

Throughout its intracellular development, chlamydia remains confined within membrane bound vesicles called “the inclusion”. The naïve inclusion is initially resistant to degradation by endo-lysosomal fusion. Although the exact molecular details of non-fusogenic properties of early inclusions are elusive, the surface proteins of EBs are thought to be involved in this process. Another characteristic feature of early inclusion is the migration towards the peri-nuclear region that is close to the golgi apparatus. Inclusion migration requires dynein and other unknown phosphoproteins that drive migration toward the host microtubule organizing center (MTOC) [246, 247]. The transcriptionally active RBs decorate the inclusion membrane by expressing several inclusion membrane proteins. Such matured inclusions are not recognized by the host cells as a an endosome, but rather as an exocytic vesicle, thereby avoiding destruction via fusion with lysosomes.

Several genes for structurally and functionally distinct inclusion membrane proteins (Inc) have been identified in the chlamydial genome. Many of these Inc’s (IncD-IncG) are expressed by RBs during the early phase of the chlamydial developmental cycle [248, 249]. RBs use type III secretion apparatuses to secrete these Inc proteins within the inclusion. All Inc’s share a conserved hydrophobic domain of 40-60 amino acids, which allow these proteins to be inserted into the inclusion membrane [250].

While the functions of many Inc proteins are elusive, some of these Inc proteins are thought to mediate interaction of inclusion with host vesicles either directly or by recruiting host proteins involved in vesicular trafficking. IncA harbors a eukaryotic SNARE like domain that allows homotypic fusion of inclusions [227, 251]. Therefore, even if multiple EBs invade a cell, only one inclusion body per cell is eventually formed [249]. However, in the case of *C. pneumoniae,* the EBs do not coalesce and form multiple small inclusions. IncA can also directly mediate fusion of the inclusion with host vesicles. Some Inc proteins cause recruitment of Rab GTPases to the inclusion membrane resulting in its interaction with host vesicles. For example, *C. trachomatis* Inc CT229 has been shown to recruit Rab4, and *C. pneumoniae* Inc Cpn0585 recruits Rab1, Rab10 and Rab11 [252, 253]. Such interactions allow chlamydia to evade intracellular immune responses and also help in acquiring essential nutrients like sphingolipids, sterols and glycerophospholipids required for inclusion expansion [254-256].

Prevention of host cell cycle progression, apoptosis and inflammatory responses is critical for intracellular survival of developing inclusions. Chlamydia has developed several strategies to influence these host responses. Chlamydial infection into host cells causes cleavage and degradation of cyclin B1, which subsequently leads to cell cycle arrest [257]. IncG sequesters host protein 14-3-3β to prevent phosphorylation of pro-apoptotic BAD [258, 259]. NFKB signaling involved in pro-inflammatory cytokine

production is interrupted by effector proteins including CT441, ChlaDub1 and CP0236 [260, 261].

As the development of an inclusion progresses, RBs express another set of mid-cycle genes including transporter proteins and T3SS. This allows nutrient acquisition needed for the replication of RBs. As the RBs divide by binary fission, the inclusion body increases in size to accommodate newly dividing RBs. Some RBs lose contact with the inclusion membrane, possibly due to retraction of the T3SS apparatus, and revert back to the infectious EB [249, 262, 263]. This secondary differentiation from RB to EB involves prepackaging infectious EBs with proteins involved in invasion and nuclear condensation [209, 264, 265].

1.3.3.4 Egress

The intracellular developmental phase of chlamydia lasts for approximately 36 hours [266]. The extracellular release of newly formed EBs involves at least two distinct mechanisms. The first mechanism involves cysteine protease induced cell lysis, causing disruption of both the inclusion and host plasma membrane [226]. Thus, the naked EBs are directly released into the extracellular spaces. The second mechanism involves a cell lysis independent extrusion of the membrane bound inclusion. This mechanism involves N-wasp mediated actin polymerization and cytoskeleton rearrangements that ultimately push the entire inclusion out of the cell [226]. Once the contents of inclusion are released in the extracellular environment, the non-infectious RBs die, whereas the infectious EBs are ready to infect new host cells to repeat the cycle.

1.3.4 Invasion into Mammalian Cells: The Trigger Mechanism

1.3.4.1 Attachment

Following the initial reversible interaction between EBs and the epithelial cells, an irreversible binding step that is temperature dependent has been characterized[236]. The molecular details of such irreversible interactions are not yet understood. It is hypothesized that the irreversible interaction results from the insertion of a type III secretion apparatus or an as yet uncharacterized receptor-ligand interaction. Many putative surface adhesins and host receptors have been identified and will be discussed below. However, no single adhesin-receptor interaction is yet characterized, which is found to be essential for attachment or entry of all chlamydial species. It is believed that chlamydia uses multiple modes to interact with host cell surfaces for its efficient uptake. Genetic intractability of chlamydia, species specific differences in surface adhesins and the uses of several host cell lines has complicated the issues in characterizing the general mechanism used by chlamydial species to interact with the host cell surface.

Major outer membrane protein (MOMP)

MOMP is the most abundant protein found on the surface of both EBs and RBs [267]. It accounts for 50-60% of total outer membrane protein mass in EBs and almost 100% in RBs [267]. MOMP is conserved among all pathogenic chlamydial species. It consists of five constant domains that are identical among different chlamydial species, interspersed with four surface exposed variable domains [268]. The variable domains are linked to glucosamine containing sulfated polysaccharides [269]. Serovars of

chlamydia are distinguished based on the host antibody response to the variable domains of MOMP. Several observations suggest the role of MOMP in the attachment of EBs to the host cell surface. Treatment of host cells with MOMP antibodies can inhibit attachment and entry of *C. trachomatis* EBs[270]. The polysaccharide moiety of MOMP also seems to be important in host cell surface binding as N-glycanase treatment abrogates entry [269-271]. However, serovar specific differences were observed in the role MOMP as adhesin as trypsinization induced cleavage of variable domains of MOMP prevented attachment of *C. trachomatis* serovar B, but not of L2 [272, 273].

Outer membrane complex protein B (OmcB)

OmcB is a cysteine rich 60 kDa protein present on the surface of EBs. It has been thought to provide osmotic stability and structural rigidity to EBs. It is also predicted to function as adhesin and is thought to prevent fusion of the early inclusion with lysozomes [274, 275]. Heterologous expression of *C. pneumoniae* OmcB in yeasts resulted in binding of yeasts to epithelial cells [276]. OmcB possesses a 20 amino acid domain that binds to heparin [277]. Addition of heparin prevented binding of recombinant purified LGV OmcB to epithelial cells. Also, attachment of LGV EBs was abrogated in presence of excess recombinant OmcB protein. But, OmcB was not found to be important for attachment of *C. trachomatis* serovar E [278].

Polymorphic membrane protein (Pmp's)

Pmp's are a family of surface proteins that includes 9, 17 and 20 members in *C. trachomatis, C.psittaci* and *C. pneumoniae* respectively [279-281]. The pmp genes are

clustered in the chlamydial genome and show a great degree of variability in their structure [282]. The presence of several conserved pmp genes among different chlamydial species suggests its important role in chlamydial biology. Heterologous expression in yeast of many members of the Pmp family resulted in the attachment of yeast with epithelial cells suggesting the possible role of these Pmps as adhesin [283]. Crane et al recently demonstrated that antibodies targeting PmpD neutralizes chlamydial infection in vitro. Moreover, antibody targeting other surface membrane proteins like MOMP can block the neutralizing effects of anti-PmpD antibody, suggesting role of Pmp in decoy like immune evasion [284]. Recent evidences also suggest the role of some Pmps as auto transporters that help in early transition of EBs to RBs [285].

Lipopolysaccharide (LPS) and Heat shock protein 70 (Hsp70)

LPS is present in the cell wall of all gram negative bacteria. The chlamydial LPS differs from the other gram negative LPS in that it does not elicit strong endotoxic response in the host [286, 287]. Immunoprecipitation studies identified Cystic Fibrosis Transmembrane Receptor (CFTR) receptor as a probable interacting partner of LPS [239]. Hsp70 is another surface exposed protein present on the surface of EBs. Hsp70 is believed to mediate attachment of EBs with endometrial cells as heterologous expression of Hsp70 in *E. coli* favored attachment to endometrial cells [288].

The host receptors

Several putative host cell surface receptors have been proposed to favor attachment of EBs in a species/serovar specific manner. Platelet derived growth factor receptors (PGFR-β) have been shown to play a critical role in attachment of *C. trachomatis* serovar L2 to Hela and S2 cell lines [237]. Later studies implicated the role of PGFR-β in attachment of *C. pneumoniae* [289]. In vivo experiments using cystic fibrosis transmembrane receptor (CFTR) knockout mice suggest the role of CFTR in EB uptake. Chlamydial LPS has been shown to co-precipitate with CFTR receptors. Treatment of Hela cells with mAb targeting CFTR resulted in diminished entry of *C. trachomatis* EBs [239]. *C. pneumoniae* MOMP has been suggested to interact with Insulin-like growth factor receptors-2 (IGFR-2) as treatment with Mannose-6-phosphate abrogates attachment of EBs [290]. Protein disulfide isomerase (PDI) knock out Chinese hamster ovarian (CHO) cell lines show diminished attachment of *C. trachomatis* EBs, which can be reversed by ectopic expression of PDI suggesting the possible role of PDI in chlamydial entry. Recently, the requirement of PDI in the attachment of other chlamydial species has also been confirmed. The enzymatic activity of PDI is not necessary for EB attachment and it is believed that PDI acts as a co-receptor to favor attachment of EB's to yet unidentified host cell surface receptors [291].

1.3.4.2 Entry

Chlamydia is capable of inducing its own uptake so efficiently that the process is termed as 'parasite specific phagocytosis.' This process requires the formation of an

actin pedestal and hypertrophic microvilli on the host cell surface [292]. Attachment of EBs to the host cell surface causes recruitment of actin and treatment of host cells with cytochalasin D, an inhibitor of actin polymerization, abrogates chlamydia entry [292-295]. Actin polymerization during chlamydial entry involves active participation of both the host cytoskeleton machinery and chlamydial effector protein Tarp, which will be discussed below.

1.3.4.2.1 Host Factors

The Arp2/3 complex is recruited at the site of chlamydial attachment and disruption of its activity abrogates chlamydial entry, suggesting the important role of the Arp2/3 complex in chlamydial invasion [226, 296, 297]. Chlamydial species differentially regulate the activation of Arp2/3 complex. *C. trachomatis* requires Rac, whereas *C. caviae* requires both Rac and cdc42 for efficient uptake into host cells [298, 299]. Activation of Rac causes Abi and Wave2 dependent activation of the Arp2/3 complex [296]. Ezrin, a member of the ezrin-radixin-moesin (ERM) family, which links the actin cytoskeleton to the plasma membrane, has also been identified as a critical player in *C. trachomatis* entry [300]. Ezrin gets phosphorylated by yet unidentified kinases following EB-host interaction[300]. Phosphorylated ezrin then associates with actin near the tips of microvilli. Furthermore RNA interference of ezrin inhibited chlamydia entry [301]. Signaling events that occur downstream of EB-host interactions to activate host cytoskeleton machinery are not completely understood. Previously, PDGF-β receptor dependent activation of cortactin, Wave2, and Vav2 has been suggested to have a role in *C. trachomatis* entry. However, blocking of PDGF receptors only partially inhibited

entry [237]. FGFR receptor is recently identified to favor attachment of EBs [238]. However, its role in activation of the Arp2/3 complex to cause chlamydial entry is not yet determined. It is hypothesized that multiple pathways converge at multiple levels to activate the Arp2/3 complex and induce the efficient uptake of EBs. Some pathogenic microorganisms have been shown to harbor proteins that can directly activate Arp2/3 complex [6]. However, no such chlamydial effectors have been definitively identified with these capabilities.

1.3.4.2.2 Chlamydial Effector Proteins and The Tarp

One of the well characterized modes of chlamydial entry is via translocation of its effector proteins. At least three effector proteins have been identified so far that are believed to play an essential role in chlamydial entry including CT456 (Tarp), CT166 and CT684 [64, 242, 244]. These effectors exhibit different functional capabilities to regulate cytoskeleton rearrangement [64, 242, 244]. The Tarp has been demonstrated to increase the rate of actin polymerization in vitor [64]. CT166 has been shown to inhibit the activity of host Rac GTPases [244]. CT694 associates with host protein Desmoyokin (AHNAK) to modulate the stress fibers which could also be important in invasion [242].

The Tarp

Identification: Several phosphorylated proteins of varying molecular weights accumulate within the host cytoplasm in response to *C. trachomatis* infection [302, 303]. Initially these proteins were thought to be of host origin. Later, mass spectrometric analysis revealed one such protein to be of bacterial origin [243]. Because this protein

was found to be associated with the recruitment of actin, it was named translocated actin recruiting protein, “Tarp”. Tarp was observed to be translocated into the host cells using a heterologous expression system in *Y. pseudotuberculosis* [243]. Genomic analysis later revealed the presence of Tarp gene in all pathogenic species of chlamydia [243, 280, 304, 305]. However, Tarp orthologs from different chlamydial species differ in their sizes and share lower levels of sequence homology relative to other chlamydial Tarp genes [306]. At the amino acid level, the Tarp from *C. trachomatis serovar D, C. muridarum, C. caviae* and *C. pneumoniae* share 94.3, 59, 43 and 46 percent sequence homology to *C. trachomatis L2* Tarp, respectively [306]. In spite of these differences some important functional domains are found to be conserved among different Tarp orthologs identified so far (**see figure 8**) [307].

Structure: *C. trachomatis* serovar L2 Tarp (CT456/Tarp) consists of a single polypeptide of 1005 amino acids with a molecular weight of ~105kDa. The N-terminal 200 amino acids of Tarp possess a signal sequence that is recognized by chlamydial chaperone protein Slc-1(CT043), which shuttles the Tarp protein into a Type III secretion apparatus for effective delivery [308]. Tarp harbors three known functional domains including the N-terminal phosphorylation domain (PhosD), the C-terminal proline rich oligomerization domain (PRD) and the Wiskott-Aldrich syndrome protein (WASP)- homology-2 (WH2) like actin binding domain (ABD) [64, 243, 306]. While the phosphorylation domain is present only in Tarp that belongs to serovars in the *C.*

trachomatis group, the proline rich domain and actin binding domain are conserved among all Tarp orthologs (**see figure 8**) [306, 307].

The phosphorylation domain (PhosD) consists of tandem repeats of ~50 amino acids that are rich in tyrosine [306]. The PhosD in LGV strains of *C. trachomatis* harbor 6 Tyrosine rich repeats, whereas trachoma strains harbor only 3 such repeats (**See figure 8**). Four to five tyrosine amino acid residues are found within each repeat. The 2^{nd} and 3^{rd} tyrosine residues within the 1^{st} repeat and probably the corresponding tyrosine residues in each subsequent repeat are the target of host cell kinases [309]. A variety of host cells tyrosine kinases are identified to phosphorylate Tarp including Src, Fyn, Abl, Syk and Yes [309, 310].

The proline rich domain (PRD) consists of a polypeptide of ~25 amino acids that are rich in proline amino acids. The presence of several proline amino acid residues imparts hydrophobicity to Tarp. In eukaryotic cell lines, ectopically expressed Tarp tends to form aggregates in intra-cytoplasmic aqueous environment [243]. The ability of Tarp to form aggregates is essential for actin nucleation activity of certain Tarp orthologs that harbor only one ABD [307].

The actin binding domain (ABD) consists of a polypeptide of 10-12 amino acids that forms an α-helical structure like many other known actin binding domains [311]. Tarp can directly bind to monomeric (G-actin) and filamentous (F-actin) forms of actin via its ABD [64]. The number of actin binding domains varies among different Tarp orthologs. *C. trachomatis* serovar LGV and *C. pneumoniae* Tarp harbor one ABD, *C.*

trachomatis serovar D and *C. caviae* harbor three ABDs, whereas *C. trachomatis* serovar A and *C. muridarum* harbor four ABDs [307] (**See figure 8**).

Functions of Tarp in entry of *Chlamydia*: EBs are packaged with pre-synthesized Tarp. Tarp is not found to be surface exposed as antibodies targeting Tarp do not recognize EBs [243]. Interactions of EB with the host cell surface results in contact dependent activation of the T3SS apparatus and translocation of Tarp into the host cell cytoplasm [243]. Within minutes following its translocation into the host cell cytoplasm, *C. trachomatis* Tarp is tyrosine phosphorylated at its phosphorylation domain, although the significance of Tarp phosphorylation is not yet known. Tarp is hypothesized to participate in the observed cytoskeletal rearrangements required for chlamydia entry and remains associated to the inclusion membrane following invasion [243].

The first evidence suggesting a role for Tarp in entry of *Chlamydia* was the observation that ectopically expressed GFP-Tarp fusion co-localized with actin [243]. Jewett et al later demonstrated the functional ability of Tarp to bind and nucleate actin using an in vitro actin binding and polymerization assay [64]. The actin nucleating ability of Tarp is attributed to the presence of ABD and PRD domains. Tarp, via its actin binding domain, binds monomeric G-actin, while the PRD domain helps different Tarp molecules to form aggregates. Together this results in interaction of three monomers of actin, which is the rate limiting step to generate filamentous actin (F-actin). Some Tarp orthologs harbor 3 or 4 actin binding domains and can nucleate actin independent of

their ability to form aggregates. Jewett et al demonstrated in vivo functional significance of Tarp ABD by microinjecting the Hela cells with antibodies specifically targeting ABD which abrogated chlamydial entry [307].

An indirect role for Tarp in modulating host cytoskeleton machinery has also been suggested [296, 312]. There is evidence linking the phosphorylation domain of Tarp to the activation of Arp2/3 complex [312]. Biochemical assays using fusion of a single tyrosine rich repeats of Tarp with the N-terminal domain of CD4 receptors suggests phosphotyrosine dependent recruitment of Sos1/Abi/Eps8 or Vav2/PIP3 [296]. Formation of such a complex is believed to cause Rac dependent activation of Arp2/3 complex [299]. Interestingly, inhibition of Tarp phosphorylation using general kinase inhibitors does not prevent chlamydial entry, although it does prevent inclusion development [309]. In light of these controversial evidences, two models for entry of chlamydia have been proposed. One model proposes an independent role of Tarp in chlamydial entry, whereas the other model also links the phosphorylation domain of Tarp to Arp2/3 activation (**see figure 9**).

Tarp may harbor additional domains: Unlike other bacterial pathogens, up until recently chlamydial species were intractable to genetic manipulation. Most of our knowledge about the functions of chlamydial virulence factors come from scientific approaches that employed in vitro analysis. Large proteins like Tarp posed additional challenges as it was impossible to obtain recombinant full length proteins of optimum quality required for biochemical in vitro assays. Despite these challenges, several

scientific groups were successful in characterizing at least some functions of Tarp by employing its truncated mutants. However, such studies were very focused and directed towards one particular aspect of Tarp's function in chlamydial biology. In depth understanding of Tarp requires revisiting the known functions of individual domains in full length Tarp using in vitro and possibly in vivo studies.

Tarp possesses certain characteristic features that tempt one to speculate additional functions of Tarp in chlamydia entry. The actin binding domain of Tarp has been shown to bind both G-actin and F-actin. While the ability to bind G-actin is essential for Tarp's nucleating activity, the significance of Tarp's interaction with F-actin is yet unknown. Many proteins that bind and/or nucleate G-actin also have capacity to bundle, severe, cap or branch F-actin [311, 313, 314]. For example, eukaryotic Arp2/3 complex functions as both actin nucleator and branching protein [311]. Another eukaryotic protein, Villin, and a salmonella effector protein, Sip C, harbors an actin binding domain and an oligomerization domain and has been shown to bind and bundle F-actin [8, 314]. Plastin (Fimbrin), which harbors two actin binding domains, functions to bundle F-actin in microvilli [313]. Tarp orthologs also harbor one to several actin binding domains and a proline rich oligomerization domain. Moreover, the secondary structure of Tarp reveals additional α-helical domains similar to many other actin binding domains but has failed to bind G-actin in previous studies [64]. It is possible that these uncharacterized α-helices of Tarp specifically recognize F-actin to regulate cytoskeleton networks in addition to its nucleating capabilities.

1.3.5 Hypothesis

We hypothesize that cytoskeleton reorganization required for entry of chlamydia is the product of co-operative activity of Tarp and Arp2/3 complex. Tarp nucleates monomeric actin to make the linear filamentous actin, which then serves as a substrate for Arp2/3 complex activity to generate a branching pattern of actin filaments usually observed in the microvillus tip. We also hypothesize that Tarp plays an additional role in modulating the filamentous actin network, which is independent of its actin nucleation ability. A complete understanding of Tarp mediated cytoskeletal changes is required to reveal the mechanisms by which Chlamydia invades the host tissue.

1.4 Figures and Tables

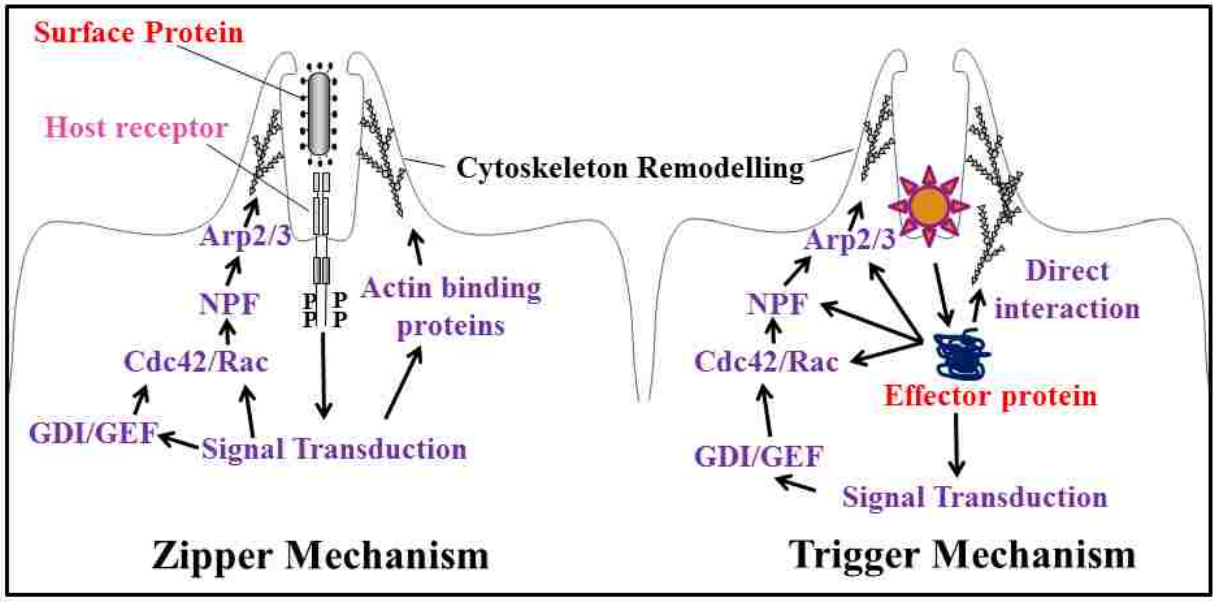

Figure 1 Schematic representation of the mechanisms of induced phagocytosis by bacterial pathogens.

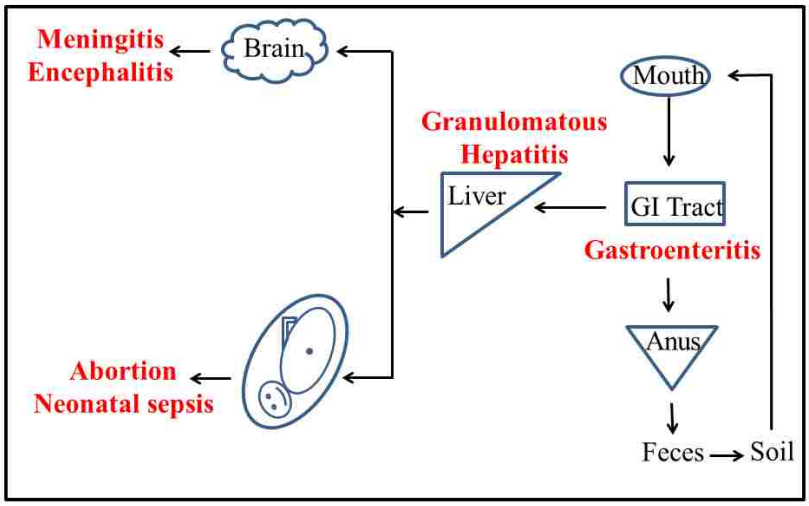

Figure 2 Schematic representation of pathophysiology and clinical manifestations of Listeriosis.

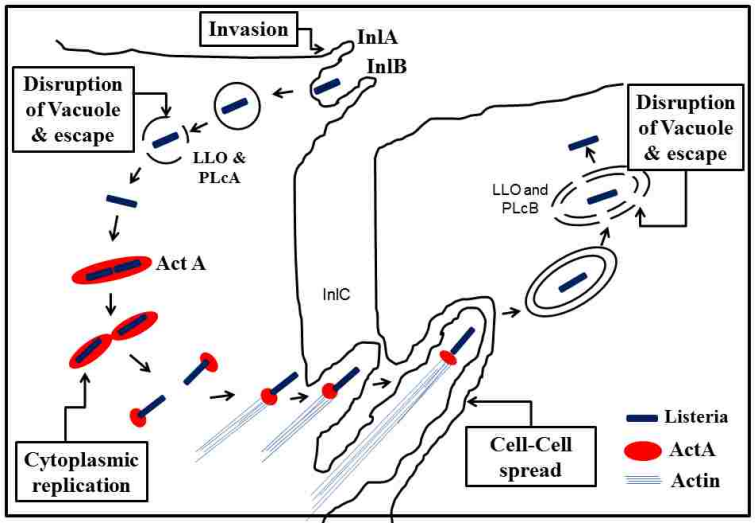

Figure 3 Diagrammatic representation of intracellular life cycle of L. monocytogenes.

Key virulent proteins and stages involved are depicted as follows, InlA and Inl B required for invasion, Listeriolysin O and Phospholipases (A and B) for escape from vacuole, ActA for actin based intracellular motility and InlC for cell-cell dissemination.

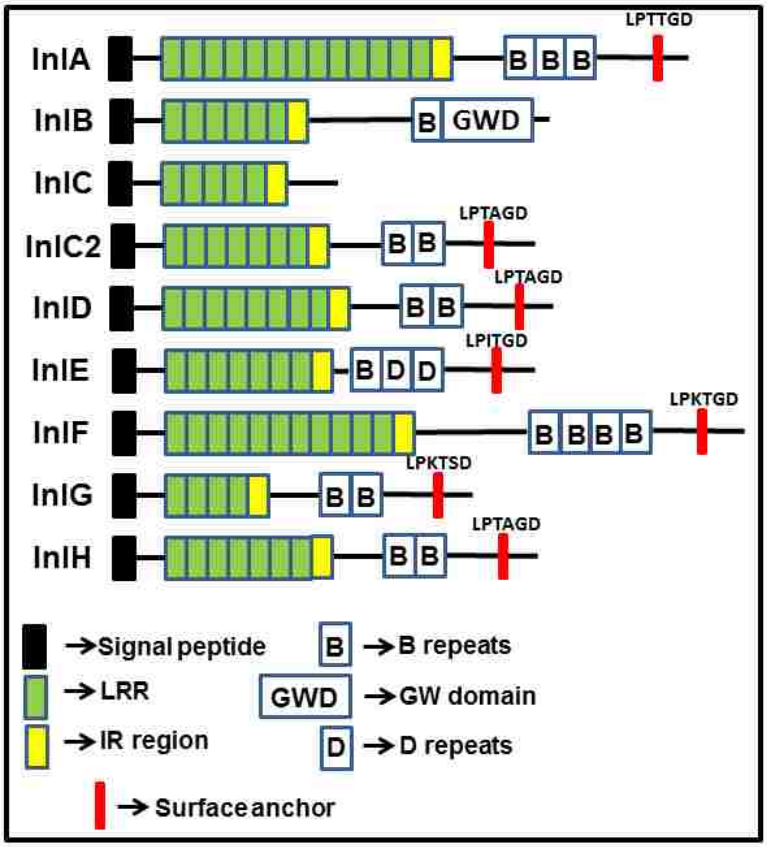

Figure 4 Schematic representation of members of Internalin family expressed in L. monocytogenes.

Important domains are represented in different colors. Signal peptide (Black box), Luciene rich repeat/LRR (green box), Inter-repeat/IR region (Yellow box), B domain (B), D domain (D) and surface anchors LPXXGD motif (Red box) or GW Domain (GWD).

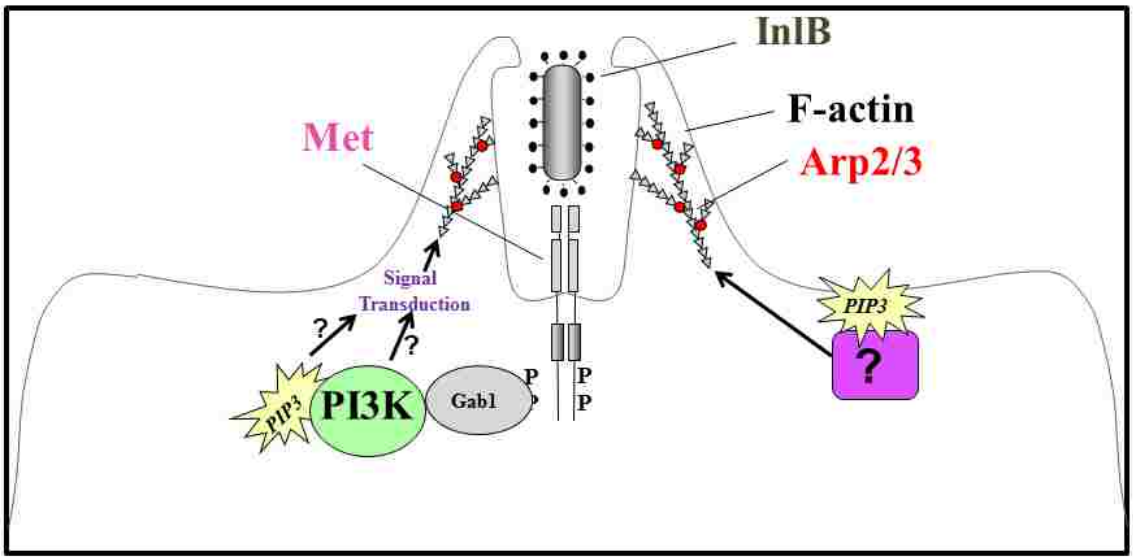

Figure 5 Model for InlB mediated entry of L. monocytogenes.
Met receptor is heterodimer consisting of extracellular alpha chain and a beta chain with extracellular, trans-membrane and intra-cytoplamic domain. Binding of InlB with Met receptor leads to dimerization and activation of Met receptors. Met gets auto phosphorylated at 1349 and 1356 tyrosine residues on the intra cytoplasmic domain. This results in recruitment of adaptor protein like Gab1. Gab1 causes recruitment of PI 3-kinase resulting in its activation by Met and generation of lipid second messenger PIP3. PI 3- kinase activity results in activation of Arp2/3 complex and F-actin recruitment by some unknown mechanism.

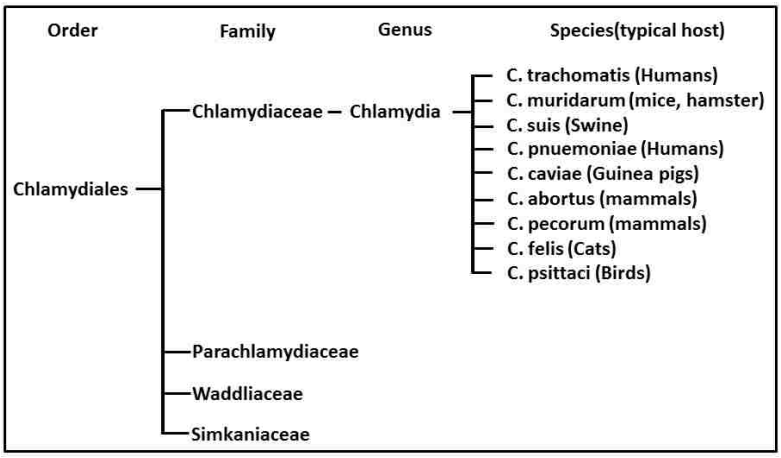

Figure 6 Chlamydiae taxonomy
Based on recommendations from Chlamydia Basic Research Society meeting held in 2009, Little Rocks, Arkansas, USA

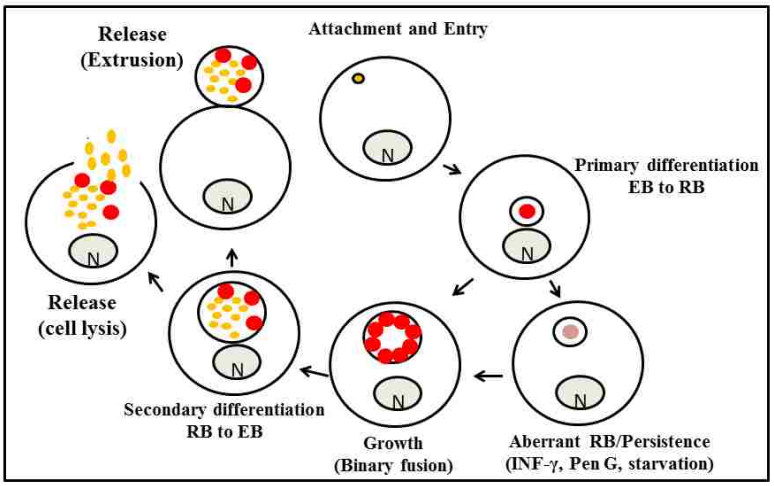

Figure 7 Diagrammatic representation of developmental cycle of C. trachomatis. Infectious elementary bodies (EB) enter host cells and remain confined in a membrane bound vacuole called inclusion. EB differentiate into metabolically active reticulate bodies (RB) which undergoes several rounds of replication. Following secondary differentiation RB reverts back to infectious EB. EBs gets released from the infected cells to start new infectious cycle.

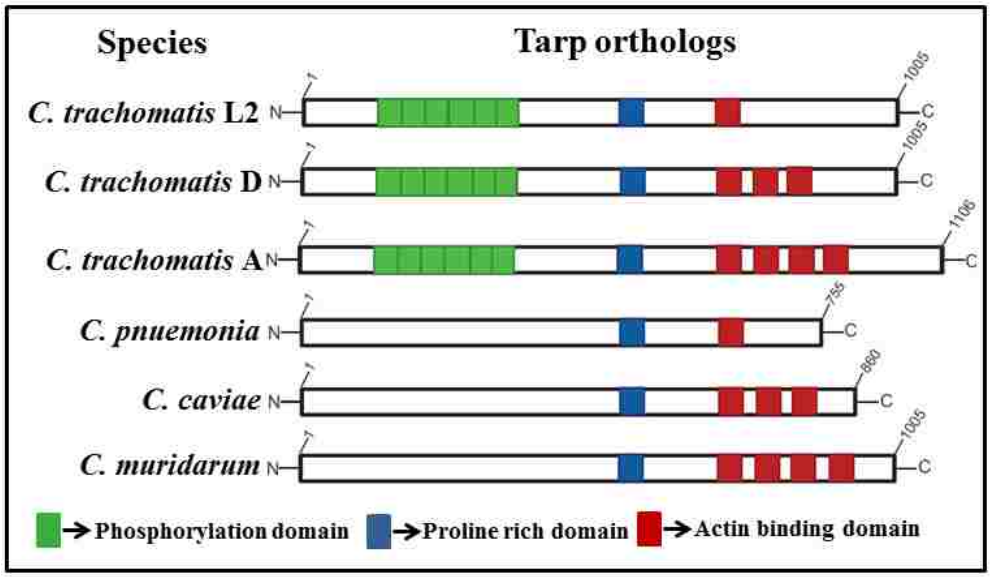

Figure 8 Schematic representation of Tarp proteins from C. trachomatis serovar L2, D and A, C. pnuemoniae, C. caviae and C. muridarum.

Known domains of Tarp are represented by different colors as follows, N-terminal phosphorylation domain (green box), Proline rich oligomerization domain (blue box) and C-terminal Actin binding domains (red box). Number represents the amino acid present in Tarp polypeptide. Note that phosphorylation domain is present only in Tarp from trachoma species. Variable number of actin binding domain is found in Tarp orthologs from different chlamydial species.

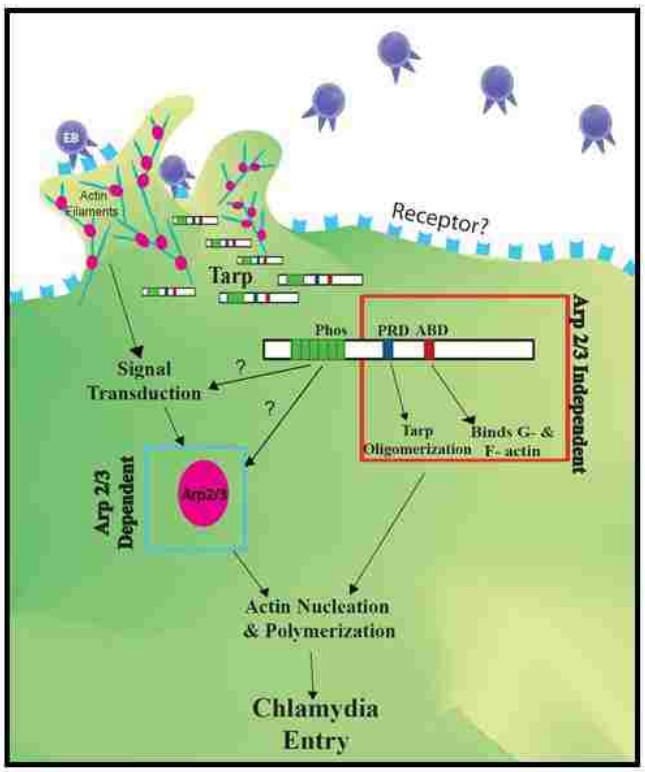

Figure 9 Model for Tarp mediated entry of C. trachomatis.
EB translocate Tarp into host cell cytoplasm. Tarp directly polymerizes actin via its actin binding domain (red box) and proline rich oligomerization domain (blue box). Host cell kinase phosphorylates Tarp at its N-terminal phosphorylation domain (green box). Phosphorylated Tarp causes activation of Arp2/3 complex (pink circle) which further mediates actin polymerization. Together, this results in uptake of EB into host cells. Arp2/3 complex activation is also proposed to be mediated via signaling events occurring downstream to interaction of EB with unknown host cell surface receptors.

CHAPTER TWO: IDENTIFICATION OF COMPONENTS OF THE HOST TYPE 1A PI 3-KINASE PATHWAY THAT PROMOTES INTERNALIZATION OF LISTERIA MONOCYTOGENES

2.1 Introduction

Listeria monocytogenes is a food-borne bacterial pathogen capable of causing severe infections culminating in meningitis or abortion [61, 315]. *Listeria* induces its own internalization ('entry') into host cells that are normally non-phagocytic. Entry of *Listeria* into enterocytes and hepatocytes plays an important role in virulence, by allowing bacteria to traverse the intestinal barrier and to colonize the liver [129]. Another potential role for *Listeria* internalization is infection of the placenta although this idea is controversial [316].

One of the major pathways of *Listeria* entry into host epithelial cells is mediated by interaction of the bacterial surface protein InlB with its host receptor, the Met receptor tyrosine kinase [61, 107]. InlB binds directly to the extracellular domain of Met, resulting in activation (tyrosine phosphorylation) of the receptor. Once activated, Met promotes signal transduction events that remodel the host cell surface, leading to bacterial engulfment [142, 143, 152, 153, 158]. Host surface remodeling is driven, at least in part, by localized polymerization of actin. One of the human signaling proteins that acts downstream of Met to stimulate F-actin assembly and internalization of *Listeria* is type IA phosphoinositide (PI) 3-kinase. Infection with *Listeria* induces localized activation of PI 3-kinase [142, 143]. Genetic or pharmacological inhibition of type IA PI 3-kinase results in a reduction in InlB-mediated actin polymerization and bacterial entry [130,

142, 143]. The molecular mechanism by which PI 3-kinase promotes internalization of *Listeria* is not known.

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Type IA PI 3-kinase is a heterodimeric enzyme comprised of a 110 kDa catalytic subunit and a 85 kDa regulatory subunit [317]. This PI 3-kinase controls a variety of processes in mammalian cells, including cell growth, survival, and motility. Type IA PI 3-kinase promotes its biological effects through at least two mechanisms. The best understood mechanism involves lipid kinase activity. PI 3-kinase produces phosphatidylinositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$], a lipid second messenger that

binds a plethora of downstream 'target' proteins [317, 318]. PI(3,4,5)P_3 recruits these target proteins to the plasma membrane, where they exert their biological activities. PI(3,4,5)P_3 is converted by phosphatases to phosphatidlinositol 3,4-bis phosphate [PI(3,4)P_2], another lipid with signaling activity [317, 318]. Apart from producing lipid second messengers, type IA PI 3-kinase can also regulate signal transduction through protein-protein interactions [147-149, 319].

In order to understand how type IA PI 3-kinase promotes internalization of *Listeria*, it is critical to identify human proteins that act upstream and downstream of this kinase to control pathogen uptake. In this work, we describe an RNA interference (RNAi) –based genetic screen to identify components of the type IA PI 3-kinase signaling pathway involved in *Listeria* uptake. The 64 host genes targeted in this screen encode proteins that bind PI(3,4,5)P_3 and/or PI(3,4)P_2, proteins that interact with catalytic or regulatory subunits of PI 3-kinase, and proteins that are indirectly regulated by PI 3-kinase. Our findings indicate that at least nine human genes known to participate in type IA PI 3-kinase signaling are involved in entry of *Listeria*. This work is an important first step in dissecting the molecular mechanism by which type IA PI 3-kinase mediates bacterial internalization.

2.2 Material and Methods

2.2.1 Bacterial Strains, Mammalian Cell Lines and Media

The *Listeria monocytogenes* strain BUG 947 was used for these studies. BUG947 contains an in-frame deletion in the *inlA* gene, and has normal expression of

InlB [129]. Consequently, BUG 947 is incapable of infecting HeLa or other host cells through interaction of the *Listeria* surface protein InlA with its host receptor E-cadherin [61, 320]. Instead, this bacterial strain enters into host cells in an InlB-dependent manner [61, 142]. The *Listeria* strain was grown in brain-heart-infusion (BHI, Difco) broth and prepared for infection as described [130].

The human epithelial cell line HeLa (ATTC CCL-2) was grown in Dulbecco's Modified Eagle's medium (DMEM) with 4.5 g of glucose per liter and 2 mM glutamine (11995-065; Invitrogen), supplemented with 10% fetal bovine serum (FBS). Cell growth, cell stimulation, and bacterial infections were performed at 37˚C in 5% CO2.

2.2.2 SiRNA

Sequences of siRNAs used to target human genes comprising the host type IA PI 3-kinase pathway are listed in Table2.2. These siRNAs were designed by and purchased from Ambion. As negative controls, two 'non-targeting' siRNAs were used. These control siRNAs (non-targeting control 1; Dharmacon cat. no. D-001210-01 or non-targeting control 2; Sigma-Aldrich cat. No. SIC001) contain two or more mismatches with all sequences in the human genome. Another control siRNA was directed against the nuclear gene lamin A/C (5'-CUGAGAGCCGCAGCAGCUUtt-3').

2.2.3 Antibodies, Inhibitors, and other Reagents

Polyclonal antibodies used were anti-mTor (2972; Cell Signaling), anti-phospholipase C-γ1 (sc-81; Santa Cruz Biotechnology), anti-Rab5c (HPA003426; Sigma-Aldrich), and anti-SWAP70 (sc-81991; Santa Cruz Biotechnology). The

monoclonal antibodies used were anti-GIT1 (611396; BD Biosciences), anti-PDK1 (611070; BD Biosciences), anti-PSCD2 (ARNO) (clone 6H5; WH0009266M2; Sigma-Aldrich), and anti-tubulin (T5168; Sigma-Aldrich). Secondary antibody horseradish peroxidase (HRPO) conjugates were from Jackson Immunolabs.

2.2.4 Transfection of Hela Cells with SiRNA

1.5×10^4 HeLa cells were seeded in wells of 24 well plates and grown for approximately 24 h. Transfection with siRNA and the lipid reagent LF2000 (Invitrogen) was as described [143]. In experiments in Figures 2.2-2.4, HeLa cells were transfected with pools of three different siRNA molecules for each target gene, except in the cases of ILK or HRAS. Targeting of ILK or HRAS involved transfection with a pool of two siRNAs or a single siRNA, respectively. The two or three siRNAs used in each pool are numbered as 1, 2, and 3 in Table 2.2. In the case of each siRNA pool (or of the HRAS single siRNA), the final total concentration of siRNA was 100 nM. Control conditions for the experiments in Figures 2-4 involved mock transfection in the absence of siRNA, transfection with 100 nM of either of two 'non-targeting control' (NTC) siRNAs, and transfection with a siRNA directed against the lamin A/C gene. For experiments involving single siRNA molecules (Figure 2.5), siRNAs were used at a final concentration of 100 nM. Control experiments employing a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [321] indicated that control siRNAs or siRNA pools targeting each of the 64 target genes shown in Tables 2.1 and 2.2 did not affect viability of HeLa cells 48 h after transfection.

2.2.5 Real Time PCR Analysis

HeLa cells in 24 well plates were used for analysis of gene expression about 48 h after transfection with siRNA. Cells were washed twice in PBS, and a TaqMan Gene Expression Cells to Ct kit (Applied Biosystems) was used to prepare cell lysates and cDNA. DNAse was included when making lysates in order to eliminate genomic DNA. In each experiment, a single sample of cells was used for each condition involving no siRNA, control siRNA, or siRNAs targeting a particular human gene. Real Time PCR was performed in triplicate on each cDNA sample using ABI7500 or ABI7900 instruments (Applied Biosystems). TaqMan gene expression assays (probes) used for each of the 64 target genes comprising the human type IA PI 3-kinase pathway are listed in Table 2.2. Each of these probes spans exon-exon junctions, and should not detect genomic DNA. The GAPDH gene (gene expression assay Hs99999905_m1; Applied Biosystems) was used as an endogenous control. Threshold cycle (Ct) values for the 64 target genes ranged from 23-32 in the various experiments. Ct values for the GAPDH endogenous controls were typically between 18-19. Data was analyzed by the comparative Ct method [322], normalizing Ct values for target gene expression to those for GAPDH. Relative quantity (RQ) values were calculated by the formula $RQ = 2^{-\Delta\Delta Ct}$. To obtain relative expression values in Figures 2.2-2.4, RQ values in a given experiment were normalized to the value in cells treated with non-targeting control siRNA 1 (NTC1). Relative expression values in Figure 2.5 were obtained by normalization to RQ values in cells mock transfected in the absence of siRNA (‘no

siRNA' condition). The data in Figures 2.2-2.5 are mean +/- SEM values from 3-7 independent experiments, depending on the gene and siRNA condition.

In the case of 10 out of the 64 human genes analyzed, expression could not be reliably detected using real time PCR and the available probe (Tables 2.2 and 2.3). In these situations, the Ct values obtained for the target genes ranged from 36 to undetectable. Control experiments with lysates that had not been subjected to reverse transcription yielded Ct values between 36 to undetectable for the various 64 genes. Based on these control experiments, any gene producing Ct values of 36 or greater, using reverse transcribed lysates, was considered to be expressed at levels too low for detection using the available probe. These genes were either not expressed in HeLa cells, expressed at levels below the limit of detection of real time PCR, or possibly incapable of being detected because of a flaw in probe design.

2.2.6 Bacterial Entry Assays

HeLa cells were used for bacterial infections approximately 48 hr after transfection with siRNA. Gentamicin protection assays to measure entry of *Listeria* (Figs. 11B, 12B, 13B, 14B) were performed by infecting for one h in the absence of gentamicin, and then incubating in DMEM with 20 µg/ml gentamicin for two h as described [61, 143]. Entry experiments involving HeLa cells transfected with siRNA pools (Figures 11-14) or single siRNAs (Figure 15) were performed 3-7 times, depending on the target gene. Entry efficiencies were first expressed as the percentage of the bacterial inoculum that survived gentamicin treatment. To obtain relative entry

values in Figures 11-13, absolute percent entry values in a given experiment were normalized to the value in cells treated with non-targeting control siRNA 1 (NTC1). Relative entry values in Figure 14 were obtained by normalization to percent entry values in cells mock transfected in the absence of siRNA ('no siRNA' condition).

2.2.7 Western Blotting and Immunoprecipitation

Approximately 48 h after transfection with siRNA, HeLa cells were solubilized in RipA buffer (1% Triton X-100, 0.25% sodium deoxycholate, 0.05% SDS, 50 mM Tris-HCl [pH 7.5], 2 mM EDTA, 150 mM NaCl, 1 mM PMSF, and 10 mg/L each of aprotinin and leupeptin). Western blots and detection using Enhanced Chemiluminescence (ECL) or ECL Plus reagents (GE Health Care) were performed as described [61, 130].

2.2.8 Statistical Analysis

Statistical analysis was performed using Prism (version 5.0a; GraphPad Software). When comparing data from three or more conditions, ANOVA was performed. The Tukey-Kramer test was used as a post-test. A *P* value of 0.05 or lower was considered as significant.

2.3 Results

2.3.1 Construction of a SiRNA Library Targeting Components of the Type IA PI 3-Kinase Pathway

In order to better understand the mechanism of InlB-mediated internalization of *Listeria*, we used RNAi to target human genes encoding proteins that participate in type IA PI 3-kinase signaling. A literature search was performed to compile a list of 64 host

genes whose products are involved in signal transduction mediated by type IA PI 3–kinase (**Table 1**). This list was then used to construct a short interfering RNA (siRNA) library, which was screened for effects on host target gene expression and *Listeria* entry. The human epithelial cell line HeLa was used for these studies, since this cell line is readily transfected, and has been extensively used to study InlB-mediated entry [143, 152, 158, 323]. When assembling the list of genes to be targeted by RNAi, those encoding neuronal or lymphocyte-specific proteins unlikely to be expressed in HeLa cells were excluded.

The human genes in the siRNA library were grouped in three categories, depending on the relationship of their protein products to type IA PI 3-kinase and its products $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ (**Table 1 and Figure 10**). A brief description of the categories follows. Category I contained 15 genes encoding proteins that are known to physically interact with catalytic and/or regulatory subunits of type IA PI 3-kinase. Some of these proteins, namely Ras GTPases, PI 3-kinase Interacting Protein 1, and PTK2/FAK control PI 3-kinase catalytic activity [324-326]. These proteins therefore act upstream of PI 3-kinase. Other proteins, for example Rab4 and Rab5 GTPases, have biochemical activities that are regulated by type IA PI 3-kinase [147]. These proteins might act downstream of the lipid kinase. For the remaining category I genes, insufficient information was available to propose where their protein products might act with regard to PI 3-kinase. Of these proteins, APPL and Cbl function at least partly as adaptor proteins [327-329] and their roles might lie in connecting the PI 3-kinase

pathway to other signaling pathways. Category II was comprised of 33 genes encoding proteins known to bind directly to $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$. For most of these proteins, phosphoinositide binding is mediated by one or more Pleckstrin Homology (PH), Phox Homology (PX), or DHR1 domains [330] (**Table 1**). A few of category II gene products (e.g. GIT1, GIT2, SWAP70) bind $PI(3,4,5)P_3$ through regions that are uncharacterized or that do not resemble other known protein-lipid interaction domains [331, 332]. Category III was composed of 16 genes whose products are indirectly regulated by type IA PI 3-kinase (**Table 1**). The scope of category III genes was not meant to be exhaustive. Instead, we focused on genes encoding substrates or effectors of some well-characterized direct targets of PI 3-kinase. Examples include isoforms of Protein Kinase C [333, 334], Protein Kinase N (substrates of the kinase PDK1) [315], or PAK kinases (substrates of PDK1) [335] and mTor (a kinase indirectly regulated by Akt proteins) [336].

2.3.2 RNAI Based Screen

siRNAs were designed that target the 64 genes encoding components of the type IA PI 3-kinase pathway (**Table 2**). For the vast majority of these genes (62 of 64), three different siRNAs were made and combined into pools to test for effects on host target gene expression and *Listeria* entry. In the case of the gene ILK, a pool of two siRNAs was used. The rationale for using a pooling approach was that screening multiple siRNAs together might increase the probability of effectively silencing target gene expression in situations in which not all of the individual siRNAs are potent. In the case

of one of the host genes (HRAS), only one siRNA was used. This latter siRNA effectively inhibited gene expression (see below).

siRNA pools (or a single siRNAs for HRAS) were transfected into HeLa cells. As controls, cells were mock transfected in the absence of siRNA or transfected with a control 'non-targeting' siRNA (referred to as 'NTC1') that has two or more mismatches with all known human mRNA transcripts. Approximately 48 hr post-transfection, gene expression was assessed by real time PCR (Materials and Methods). Of the 64 genes selected for analysis, 47 displayed a statistically significant reduction in gene expression by targeting siRNAs, compared to the control conditions (**Figs. 11A, 12A, and 13A**). For 10 of the 64 genes, expression could not be reliably detected by real time PCR using the available probes (**Table 3**). Effective antibodies against the product of one of these genes, PLC-γ1, was commercially available. Western blotting indicated depletion of PLC-γ1 protein (Fig. 12Aii). In the case of eight of the genes targeted, expression was detected by real time PCR, but siRNA pools targeting these genes did not reduce expression (**Table 3**). In summary, out of the 64 genes initially selected for silencing, 47 exhibited substantial RNAi-mediated reduction in expression at the mRNA or protein level. None of the siRNA conditions targeting these 47 genes affected cell growth or viability, as assessed by MTT assays [321] (data not shown).

The 47 genes whose expression was inhibited by siRNA were also examined for roles in *Listeria* entry. Internalization of *Listeria* into transfected HeLa cells was measured by gentamicin protection assays, in parallel with the gene expression studies

(**Figs. 11B, 12B, 13B**). Control conditions for the entry experiments included the absence of siRNA and NTC1 siRNA, used in the gene expression analysis. Additional controls were another non-targeting siRNA (NTC2) and a siRNA capable of silencing the nuclear protein lamin A/C. Compared to the controls, siRNAs against 21 of the type IA PI 3-kinase pathway genes resulted in statistically significant changes in entry of *Listeria* (**Figs. 11B, 12B, 13B**). For 19 of these genes, siRNAs reduced entry, suggesting a positive role in bacterial uptake. The extent of inhibition in entry ranged from ~ 70 to 95%, depending on the host gene targeted. As a reference, under the same cell growth and control transfection conditions, a bacterial mutant deleted for the *inlB* gene enters into HeLa cells at about a 90% reduced frequency compared to the isogenic *inlB*-positive strain [323]. In the case of two of the human genes targeted in the RNAi screen (KRAS2 and DOCK1), siRNAs augmented the efficiency of internalization, consistent with a negative role. The 21 host genes implicated in bacterial entry encode three proteins that bind to catalytic or regulatory subunits of PI 3-kinase (**Fig. 11**), twelve proteins that interact with $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ (**Fig. 12**), and six proteins that are indirectly regulated by PI 3-kinase (**Fig. 13**). Collectively, the gene expression and bacterial entry results in Figures 11-13 indicate that several members of the type IA PI 3-kinase pathway play important roles in InlB-mediated internalization of *Listeria*. In the Discussion section, we comment on known cellular functions of key host proteins that emerged from the RNAi screen. Also discussed are possible mechanisms by which some of the PI 3-kinase pathway proteins might promote entry of *Listeria.*

2.3.3 Addressing Possible Off-Target Effects of SiRNAs

Although RNAi is a powerful method of genetic analysis, a potential weakness of this approach is the occurrence of 'off-target' effects [337]. 'Off-target' refers to a situation in which an siRNA affects an mRNA apart from the desired target mRNA [337]. One common method of minimizing the possibility of off-target effects is to confirm that several different siRNA molecules recognizing distinct regions in a given mRNA cause the same biological phenotype[337]. We selected eight of the 21 human genes implicated in *Listeria* entry (**Figures 11-13**), and tested whether multiple siRNAs inhibiting target gene expression also impaired bacterial uptake. The genes selected were the category I gene RAB5C, category II genes PSCD1, DAPP1, GIT1, PDPK1, and SWAP70, and category III genes FRAP1 and PRKCZ. The multiple siRNAs used consisted of the three individual components of the siRNA pools employed in experiments in Figures 11-13. In some cases (e.g. PDPK1 and FRAP1), a fourth siRNA was also tested. Data are presented in Figure 14. Importantly, for each of the eight selected genes, three siRNAs of unique sequence inhibited both bacterial entry and gene expression at the mRNA level. For five out of these eight human genes, effective antibodies were commercially available. We used these antibodies to confirm inhibition of expression at the protein level (**Fig. 15**). Altogether, the findings in Figures 14 and 15 indicate that off-target effects for the eight selected host genes are unlikely. The data support the idea that these genes have bona-fide roles in *Listeria* internalization.

In previous work, we confirmed that multiple siRNAs targeting the human CENTD1 gene (encoding the protein ARAP2) impair gene expression at the mRNA and protein level, and also block *Listeria* entry [323]. Based on the present study and previous work, we conclude that our RNAi-based screen has identified at least nine human genes that are required for efficient InlB-mediated entry of *Listeria*.

2.4 Discussion

In this work, we describe an RNAi screen that led to the identification of at least nine human genes encoding proteins in the type IA PI 3-kinase pathway that play important roles in entry of *Listeria*. One of the genes identified from the screen, RAB5C, codes for a protein that interacts with regulatory and catalytic subunits of type IA PI 3-kinase [147-149]. Six of the host genes, CENTD1, PSCD2, DAPP1, GIT1, PDPK1, and SWAP70, encode proteins that bind directly to the PI 3-kinase lipid products $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$ [331, 332, 338-341]. Two of the genes identified from the screen, FRAP1 and PRKCZ, code for proteins that are indirectly regulated by PI 3-kinase [338, 342, 343]. In addition to the nine host genes described above, it seems likely that other members of the type IA PI 3-kinase signaling pathway are involved in InlB-dependent entry of *Listeria*. Results with siRNA pools implicated 21 different human genes in bacterial internalization (**Figs. 11-13**). Nine of these 21 genes were further examined for roles in *Listeria* entry by testing multiple individual siRNAs (**Fig. 14**). The results indicated that off-target effects were unlikely, and that the nine host genes therefore have important roles in bacterial uptake. Future work using single siRNAs will determine

which of the remaining 12 host genes have bona fide functions in *Listeria* entry.

In the case of 26 of the host genes targeted in our study, siRNA-mediated inhibition in expression failed to affect *Listeria* entry in a statistically significant fashion. These findings suggest that many of these 26 genes do not have important roles in bacterial internalization, at least not in the conditions employed in our work. It is worth noting that two of these 26 human genes, PLD2 and CBL, have been previously implicated in InlB-mediated uptake. In the case of PLD2, an earlier study used a cell line other than HeLa [344]. The apparent discrepancy between our data and this prior study is most likely due to cell line differences. Previous siRNA studies targeting CBL in HeLa cells suggested that this host gene was needed for efficient *Listeria* entry [158]. In our work, siRNAs directed against CBL reduced internalization of *Listeria* by approximately 70%, but the effect was not statistically significant.

As described above, our RNAi screen identified at least nine human genes that have important functions in InlB-mediated entry. How might these genes control internalization of *Listeria?* Below, we describe what is known about the cellular functions of the protein products of the genes. Molecular mechanisms by which these proteins might regulate bacterial entry are discussed below.

***Rab5c*.** Rab5c is one of three Rab5 GTPases that control clathrin-dependent endocytosis and early endosome fusion [345]. Results from the RNAi-based screen indicated a role for Rab5c, but not Rab5a or Rab5b proteins, in *Listeria* entry (**Figs. 11, 14, 15**). Activated Rab5 proteins bind to the p110 catalytic and p85 regulatory subunits

of type IA PI 3-kinase [147, 149, 346]. Interaction of Rab5 proteins with p85 results in enhanced GTP hydrolysis on Rab5 [346]. These findings suggest that PI 3-kinase might control Rab5-mediated endocytosis and/or subsequent vesicular trafficking. Importantly, InlB-dependent entry of *Listeria* requires clathrin and several other human proteins that regulate clathrin-mediated endocytosis [158]. It is possible that Rab5c works together with the endocytic machinery previously reported to control *Listeria* uptake. How might Rab5-dependent endocytosis promote bacterial entry? Recent results indicate an important role for Rab5 in signal transduction and actin polymerization mediated by the Met receptor [347]. Activation of Met by its mammalian ligand Hepatocyte Growth Factor (HGF) leads to the Rab5-dependent stimulation of the GTPase Rac1 and subsequent actin polymerization. Rab5 promotes internalization of Rac1 into endosomes, where Rac1 is then activated. Activated, endosomal Rac1 is then delivered to specific sites on the plasma membrane, resulting in cortical actin polymerization. Importantly, Rac1 is needed for InlB-mediated internalization of *Listeria* [153]. The recent findings with Rab5 and Met raise the possibility that one role of Rab5c in *Listeria* entry might be to facilitate the localized delivery of Rac1 to promote subsequent cytoskeletal remodeling.

Regulators of Arf GTPases (ARAP2, GIT1, ARNO). Results from the RNAi-based screen indicated that at least six host genes encoding proteins that bind $PI(3,4,5)P_3$ have important functions in internalization of *Listeria*. Three of these host proteins, ARAP2 (encoded by CENTD1), GIT1, and ARNO (encoded by PSCD2), are

regulators of small GTPases of the Arf family [348]. ARAP2 and GIT1 are GTPase Activating Proteins (GAPs) capable of antagonizing various Arf GTPases [348]. Binding of PI(3,4,5)P_3 to ARAP2 or GIT1 enhances the GAP activities of these proteins [332, 341]. These findings indicate that type IA PI 3-kinase can inhibit Arf GTPases through ARAP2 and GIT1. In previous work, we found that ARAP2 promotes entry of *Listeria*, in part, by restraining the activity of Arf6 [323]. The mechanism by which ARAP2-mediated inhibition of Arf6 facilitates bacterial uptake is not known. The observation that uncontrolled activation of Arf6 leads to sequestration of cholesterol and PI(4,5)P_2 in internal membranes [349, 350] prompted an hypothesis that ARAP2 might maintain the normal plasma membrane localization of lipids critical for *Listeria* uptake [323]. Like ARAP2, it is possible that GIT1 promotes internalization of *Listeria* by antagonizing Arf6. This idea would imply that neither ARAP2 nor GIT1 alone is sufficient to fully inactivate Arf6. Alternatively, GIT1 could act through regulation of other Arf GTPases, such as Arf1, Arf2, Arf3, or Arf5 [332, 351]. A third way that GIT1 could mediate *Listeria* uptake is by contributing to the activation of Rac1 and/or Cdc42 GTPases. Both Rac and Cdc42 are needed for InlB-mediated entry [152, 153]. GIT1 promotes the activation of these two GTPases by binding to the guanine nucleotide exchange factor (GEF) α-PIX [351, 352]. ARNO, the third Arf regulator identified in our RNAi screen, is a GEF that activates Arf1 or Arf6 GTPases [348]. PI(3,4,5)P_3 binds to a pleckstrin homology (PH) domain in ARNO, thereby recruiting this GEF to the plasma membrane [340, 348]. In previous work, we found that siRNA-mediated depletion of Arf6 did not impair *Listeria*

entry [323]. This finding indicates that ARNO does not mediate bacterial internalization through Arf6. It is possible that ARNO acts on Arf1 to stimulate *Listeria* uptake. Experiments involving RNAi-mediated depletion of Arf1 indicate an important role for this GTPase in InlB-mediated bacterial entry (P. Le and K. Ireton, unpublished data). Arf1 could control entry by promoting $PI(4,5)P_2$ synthesis through PI 4-phosphate 5-kinase [348]. Alternatively or additionally, ARNO might stimulate *Listeria* uptake by promoting recycling of integrin receptors to the plasma membrane [353]. Integrins contribute to signaling downstream of the Met receptor [354], and are also needed for efficient InlB-mediated entry of *Listeria* [355].

SWAP70*.* Another $PI(3,4,5)P_3$ –interacting protein that is required for *Listeria* entry is SWAP70, a known direct activator of Rac1 GTPase [331]. As previously mentioned, Rac1 promotes InlB-mediated entry by eliciting actin cytoskeletal changes through the Arp2/3 complex [152, 153]. SWAP70 is a Rac1 GEF whose activity is stimulated by $PI(3,4,5)P_3$ [331]. Our findings suggest that Rac1 activation downstream of the Met receptor during *Listeria* entry might be promoted by SWAP70. Apart from SWAP70, our RNAi-based screen targeted several other $PI(3,4,5)P_3$ –regulated GEFs for Rac1. The results indicated that the GEFs Dock180, SOS1, and Vav2 are dispensable for internalization of *Listeria* (**Fig. 12**).

DAPP1. The DAPP1 gene is critical for *Listeria* entry and encodes a $PI(3,4,5)P_3$ –binding adaptor protein. DAPP1 protein contains a PH domain that interacts with $PI(3,4,5)P_3$, an SH2 domain, and a tyrosine residue capable of being phosphorylated

[356, 357]. Known functions of DAPP1 include regulation of phospholipase C-γ1 and activation of Rac1 GTPase [356, 358]. The lack of a recognizable GEF domain in DAPP1 indicates that the effect on Rac1 activity is likely indirect. Our RNAi data suggested an important role for phospholipase C-γ1 in InlB-mediated entry (**Fig. 12**). Possible ways that DAPP1 could promote *Listeria* uptake include activation of PLC-γ1 and/or Rac1.

PDK1 and PKC-ζ. Results from the RNAi screen indicated important functions for the serine/threonine kinase PDK1 (encoded by the PDPK1 gene) in *Listeria* entry. PDK1 is a 'master kinase' that phosphorylates the activation loop of more than 20 serine/threonine kinases of the AGC family [334, 338]. PDK1-mediated phosphorylation is critical for the activity of these AGC kinases. Our siRNA library targeted 13 genes encoding AGC kinases that are PDK1 substrates (**Table 1**). Eight of these 13 genes were expressed in HeLa cells and effectively silenced by siRNA (**Fig. 13; Table 2**). These eight genes were Akt1, Akt2, PRKCD (encoding PKC-δ), PRKCG (encoding PKC-γ), PRKCZ (encoding PKC-ζ) PKN1, PKN2, and SGK1. Results with siRNA pools suggested that PRKCD, PRKCZ, PKN1, and PKN2 might have important functions in *Listeria* internalization. Experiments with single siRNAs confirmed that PRKCZ plays a crucial role in InlB-mediated entry. Importantly, the product of PRKCZ, PKC-ζ is known to regulate the actin cytoskeleton [356, 359, 360]. Potential substrates of PKC-ζ include proteins that cross link F-actin to the plasma membrane (MARCKs and ERM proteins), actin binding proteins of the coronin family, the actin capping protein adducin, the actin

bundling protein fascin, and the anti-capping protein VASP [333, 361]. PKC-ζ could mediate entry of *Listeria*, at least in part, by stimulating actin remodeling through phosphorylation of one or more of these substrates. Another important activity of PKC-ζ is in promoting exocytosis- the fusion of intracellular vesicles with the plasma membrane [360, 362]. Specifically, PKC-ζ phosphorylates VAMP2, a vesicular protein that mediates vesicle docking to the plasma membrane [363]. Thus far, a role for exocytosis in entry of *Listeria* has not been described. During Fc-γ receptor-mediated phagocytosis in macrophages, exocytic delivery of vesicles to the phagosome replenishes membrane that would be otherwise lost due to particle internalization [364]. It is possible that exocytosis occurs during *Listeria* uptake and serves a similar function. Another potential function for exocytosis could be to provide membrane needed for extension of pseudopods around adherent bacteria.

mTor. The RNAi screen revealed a critical role for the host protein mTor in InlB-mediated entry of *Listeria*. mTor (encoded by the FRAP1 gene) is a serine/threonine kinase that functions downstream of type IA PI 3-kinase to regulate several biological processes, including translation, ribosome biogenesis, autophagy, and cytoskeletal organization [336, 343]. mTor is present in two different multi-protein complexes termed mTORC1 and mTORC2. mTORC1 controls protein synthesis, cell growth, and autophagy. In contrast, mTORC2 regulates the actin cytoskeleton. Many of the cellular functions of mTORC1 are inhibited by the drug rapamycin [343], whereas mTORC2 is thought to be insensitive to this compound. Interestingly, treatment of HeLa cells with

rapamycin fails to impair InlB-mediated internalization of *Listeria* (data not shown), suggesting that the mTor complex involved in bacterial uptake is likely mTORC2, and not mTORC1. mTORC2 is known to control the actin cytoskeleton through phosphorylation of Protein Kinase C (PKC)-α or activation of Rac1 GTPase [336]. Future work will determine whether mTor promotes internalization of *Listeria* through PKC-α, Rac1, or via a previously unrecognized pathway.

The RNAi-based screen described in this work represents a key first step towards understanding how type IA PI 3-kinase promotes InlB-mediated entry of *Listeria*. Future studies will examine the molecular mechanisms by which the proteins encoded by the various host genes identified in the screen control *Listeria* uptake. Such work will contribute to a better understanding of how bacterial activation of the Met receptor elicits actin cytoskeletal changes that drive *Listeria* internalization. Future studies on human proteins identified from the screen also have the potential to uncover novel host cell events needed for bacterial entry, such as localized exocytosis.

Importantly, human type IA PI 3-kinase plays a critical role in internalization of several microbial pathogens apart from *Listeria*. Such microbes include bacterial pathogens that cause anthrax (*Bacillus anthracis*) [365], respiratory infections (*Pseudomonas aeruginosa* and *Chlamydia pneumoniae*) [366], and food-borne disease (*Campylobacter jejuni* and *Yersinia enterolitica*) [367, 368]. Host type IA PI 3-kinase also promotes entry of Ebola virus [369], and parasites causing Chagas' disease (*Trypanosoma cruzi*) [370] or toxoplasmosis (*Toxoplasma gondii*) [371]. To the best of

our knowledge, our work is the first systematic study to identify components of the host type IA PI-3 kinase pathway involved in infection by a microbial pathogen. Human proteins identified as critical for *Listeria* entry may also be viable candidates for host factors mediating infection by these other important pathogens.

2.5 Figures and Tables

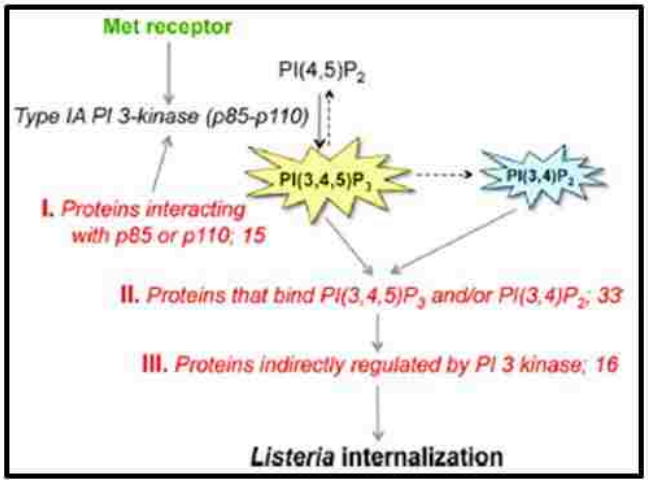

Figure 10 Human type 1A PI 3-kinase pathway components targeted in the RNAi based screen.

During infection with *Listeria*, host type IA PI 3-kinase is activated downstream of the Met receptor and plays a critical role in bacterial internalization [130, 142, 305]. Type IA PI 3-kinase uses $PI(4,5)P_2$ as a substrate and produces the lipid second messenger product $PI(3,4,5)P_3$ (9, 28). $PI(3,4)P_2$ is another second messenger that is generated from $PI(3,4,5)P_3$ by phosphatases [318]. The RNAi-based screen performed in this study targeted three categories of host genes encoding proteins that act on the type IA PI 3-kinase signaling pathway. Category I genes encode proteins that interact with the 85 kDa regulatory and/or 110 kDa catalytic subunits of PI 3-kinase. Category II genes code for proteins that bind to the PI 3-kinase lipid products $PI(3,4,5)P_3$ and/or $PI(3,4)P_2$. Category III genes encode products that are indirectly controlled by type IA PI 3-kinase.

Table 1 Genes targeted in SiRNA library

Gene category and name	Gene Bank accession no.	Protein Name	Biochemical activity	Biological function
Category I. Genes encoding proteins that bind subunits of Type 1A PI 3-kinase				
Rab4A	NM 004578	Rab4a	Binds p85 regulatory subunit of PI 3- Kinase	Receptor recycling, exocytosis
Rab4B	NM 016154	Rab4b	Binds p85 regulatory subunit of PI 3- Kinase	Receptor recycling, exocytosis
Rab5A	NM 004162	Rab5a	Binds p85 and p110 subunit of PI 3- Kinase	Endocytosis (formation and fusion of clathrin coated vesicle)
Rab5B	NM 002868	Rab5b	Binds p85 and p110 subunit of PI 3- Kinase	Endocytosis (formation and fusion of clathrin coated vesicle)
Rab5C	NM 201434	Rab5c	Binds p85 and p110 subunit of PI 3- Kinase	Endocytosis (formation and fusion of clathrin coated vesicle)
HRAS	NM 004985	H-Ras	Binds and activates p110 catalytic subunit of PI 3-Kinase	Actin cytoskeleton, cell growth
KRAS2	NM 004985	Ki-Ras2	Binds and activates p110 catalytic subunit of PI 3-Kinase	Actin cytoskeleton, cell growth
MRAS	NM 012219	M-Ras/ R-Ras3	Binds and activates p110 catalytic subunit of PI 3-Kinase	Actin cytoskeleton
NRAS	NM 002524	N-Ras	Binds and activates p110 catalytic subunit of PI 3-Kinase	Actin cytoskeleton, cell growth
RRAS2	NM 012250	R-Ras2/ TC21	Binds and activates p110 catalytic subunit of PI 3-Kinase	Actin cytoskeleton
APPL	NM 012096	APPL	Adaptor protein; binds p110	Enhancer of Akt1 activation
CBL	NM 005188	Cbl	E3 ubiquitin ligase; Binds p85 regulatory subunit of PI 3- Kinase	Ubiquitination, receptor trafficking, actin cytoskeleton
CTNNB 1	NM 001904	β-catenin	Component of adherens junction; Binds p85 regulatory subunit of PI 3- Kinase	Cell-cell junctions, transcriptional regulation
PIK3IP 1	NM 052880	PI 3-Kinase-interacting protein 1	Transmembrane protein containing kringle motifs; binds and inhibits p110 catalytic subunit of PI 3-kinase	Apoptosis
PTK2	NM 153831	FAK/PTK2	Cytosolic tyrosine kinase; binds p85 regulatory subunit of PI 3-kinase, stimulate PI 3-kinase activity	Cell adhesion and migration

References: [147, 324-329, 372]

Gene category and name	Gene Bank accession no.	Protein Name	Biochemical activity	Biological function
Category II. Genes encoding Proteins that binds PI(3,4,5)P3 and/or PI(3,4)P2				
AKT1	NM 005163	Akt1/ protein kinase B1	Ser/thr kinase; substrate include BAD, forkhead transcription factors, GSK-, ERM proteins; PH domain binds PI(3,4,5)P3 and/or PI(3,4)P2	Cell survival, growth, motility
AKT2	NM 001626	Akt2/ protein kinase B1	Ser/thr kinase; substrate include BAD, forkhead transcription factors, GSK-, ERM proteins; PH domain binds PI(3,4,5)P3 and/or PI(3,4)P2	Cell survival, growth, motility, insulin induced glucose uptake, tumorigenesis
ILK	NM 004517	Integrin-linked kinase	Ser/thr kinase; substrate include MLC-20; PH domain binds PI(3,4,5)P3	Integrin-mediated cell adhesion and survival
PDPK1	NM 002613	PDK1	Ser/thr kinase; substrate include Akt, PKC, PAK1, PAK2, p70S6 kinase and SGK1, PH domain binds PI(3,4,5)P3 and/or PI(3,4)P2	Cell survival, growth, motility, insulin signaling, T-cell differentiation
ARAP3	NM 022481	ARAP3/ centauri n-3	GAP for Arf6 and RhoA; PH domain binds PI(3,4,5)P3	Actin cytoskeleton, cell motility
CENTA1	NM 006869	Centauri n-1	GAP for Arf6 and RhoA; PH domain binds PI(3,4,5)P3	Actin cytoskeleton, cell motility
CENTD1	NM 139182	ARAP2/ centauri n-1	GAP for Arf6, PI(3,4,5)P3 stimulates GAP activity by binding PH domain	Actin cytoskeleton, Focal adhesions
CENTD2	NM 015242	ARAP1/ centauri n-2	GAP for Arf1, Arf5, RhoA and Cdc42; PH domain binds PI(3,4,5)P3	Golgi structure, actin cytoskeleton
GIT1	NM 014030	GIT1	GAP for Arf1, Arf2, Arf3, Arf5 and Arf6; PI(3,4,5)P3 stimulates GAP activity	Actin cytoskeleton, focal adhesions, membrane trafficking
GIT2	NM 057169	GIT2	GAP for Arf1, Arf2, Arf3, Arf5 and Arf6; PI(3,4,5)P3 stimulates GAP activity	Actin cytoskeleton, focal adhesions, membrane trafficking

Reference:[332, 338, 340, 341, 351, 373-377]

Gene category and Name	Gene Bank accession no.	Protein Name	Biochemical activity	Biological function
PSCD1	NM 004762	Cytohesin-1	GEF for Arf1 and Arf3; PH domain binds PI(3,4,5)P3	Integrin-mediated cell adhesion
PSCD2	NM 017457	ARNO/cytohesin-2	GEF for Arf1 and Arf6; PH domain binds PI(3,4,5)P3	Actin cytoskeleton, membrane trafficking, integrin recyclin
PSCD3	NM 004227	Grp1/cytohesin-3	GEF for Arf1, Arf3 and Arf6; PH domain binds PI(3,4,5)P3	Membrane trafficking, Golgi function, cell adhesion
ARHGAP9	NM 032496	Rho-type GTPase-activating protein 9	GAP for Cdc42 and Rac1; PH domain binds PI(3,4,5)P3	Cell adhesion
ARHGEF6	NM 004840	-PIX/Rac/Cdc42 GEF/Cool-2	GEF for Rac and Cdc-42	Cell adhesion
DAPP1	NM 014395	DAPP1/Bam32	Adaptor protein, enhance Rac activity; PH domain binds PI(3,4,5)P3 and/or PI(3,4)P2	Actin cytoskeleton
DEPDC2	NM 024870	P-REX2/DEPDC2	GEF for Rac	Actin cytoskeleton
DOCK1	NM 001380	Dock180	GEF for Rac, DHR1 domain binds PI(3,4,5)P3	Actin cytoskeleton, cell motility, phagocytosis
SOS1	NM 005633	Sos1	GEF for Rac and Ras	Actin cytoskeleton, cell growth
SWAP70	NM 015055	SWAP70	GEF for Rac	Actin cytoskeleton
TIAM1	NM 003253	Tiam1	GEF for Rac, PH domain binds PI(3,4,5)P3	Actin cytoskeleton, cell motility
VAV2	NM 003371	Vav2	GEF for Rac, PH domain binds PI(3,4,5)P3	Actin cytoskeleton
VAV3	NM 006113	Vav3	GEF for Rac, PH domain binds PI(3,4,5)P3	Actin cytoskeleton

References: [331, 348, 357, 378-386]

Gene category and Name	Gene Bank accession no.	Protein Name	Biochemical activity	Biological function
RASA2	NM 006506	Gap1m	GAP for Ras, PH domain binds PI(3,4,5)P3	Cell growth inhibitor
RASA3	NM 007368	Gap1/ P4BP	GAP for Ras and Rap, PH domain binds PI(3,4,5)P3	Cell growth inhibitor
PLCG1	NM 002660	PLC-1	Converts PI(4,5)P2 to IP3; PH domain binds PI(3,4,5)P3	Actin cytoskeleton, cell growth, phagocytosis
PLD1	NM 002662	Phospho lipase D1	Converts phosphatidylcholine to phosphatidic acid, activated by PI(4,5)P2 or PI(3,4,5)P3; PH domain binds PI(3,4)P2	Actin cytoskeleton, membrane trafficking
PLD2	NM 002663	Phospho lipase D2	Converts phosphatidylcholine to phosphatidic acid, activated by PI(4,5)P2 or PI(3,4,5)P3; PH domain binds PI(3, 4)P2	Actin cytoskeleton, membrane trafficking
PLEKHA1	NM 001974	PLEKHA 1/ TAPP1	Adaptor protein; PH domain binds PI(3, 4)P2	Actin cytoskeleton, inhibition of insulin and PI 3-kinase pathway
BMX	NM 203281	BMX/ Etk	Tec Family tyrosine kinase; PH domain binds PI(3,4,5)P3	Cell growth, motility, apoptosis
MYOX	NM 012334	MyosinX	Actin dependent motor protein; PH domain binds PI(3,4,5)P3	Contractility, phagocytosis
SNX1	NM 003099	Sortin nexin-1	PX domain binds PI(3,4,5)P3	Endocytosis, receptor trafficking
SNX5	NM 152227	Sortin nexin-5	PX domain binds PI(3,4)P2	Endocytosis, receptor recycling from endosomes to Golgi

References: [339, 387-394]

Gene category and name	Gene Bank accession no.	Protein Name	Biochemical activity	Biological function
Category III. Genes encoding proteins indirectly regulated by Type 1A PI 3-Kinase				
FRAP1	NM 004958	mTor/FRAP1	Ser/Thr Kinase; regulated by Akt; substrates include Akt, PKC isoforms, p70S6 kinase, SGK1	Regulation of protein synthesis, cell migration
GSK3A	NM 019884	Glycogen synthase Kinase-3	Regulates glycogen synthase; substrate of Akt proteins	Cell survival, tumorigenesis, regulation of gene expression
PAK1	NM 002576	PAK1	Ser/Thr Kinase; phosphorylated by PDK1	Actin cytoskeleton, cell migration
PAK2	NM 002577	PAK2	Ser/Thr Kinase; phosphorylated by PDK1	Actin cytoskeleton, cell migration
PKN1	NM 213560	Protein Kinase N1	Ser/Thr Kinase; phosphorylated by PDK1; substrate include α-actinin, adducin and cortactin	Actin cytoskeleton, cell migration, cell adhesion
PKN2	NM 006256	Protein Kinase N2	Ser/Thr Kinase; phosphorylated by PDK1; substrate include α-actinin, adducin and cortactin	Actin cytoskeleton, cell migration, cell adhesion
PRKCD	NM 006254	PKC-delta	Ser/Thr Kinase; phosphorylated by PDK1and mTor; substrate include MARCKS, adducin and ERM proteins	Actin cytoskeleton, cell migration
PRKCE	NM 005400	PKC-ε	Ser/Thr Kinase; phosphorylated by PDK1and mTor; substrate include MARCKS, adducin and ERM proteins	Actin cytoskeleton, cell migration
PRKCG	NM 002739	PKC-γ	Ser/Thr Kinase; phosphorylated by PDK1and mTor; substrate include MARCKS, adducin and ERM proteins	Actin cytoskeleton, cell migration
PRKCI	NM 002740	PKC-I	Ser/Thr Kinase; phosphorylated by PDK1and mTor; substrate include MARCKS, adducin and ERM proteins	Actin cytoskeleton, cell migration

References: [315, 333-336, 343, 395]

Gene category and name	Gene Bank accession no.	Protein Name	Biochemical activity	Biological function
PRKCQ	NM 006257	PRK-Q	Ser/Thr Kinase; phosphorylated by PDK1and mTor; substrate include MARCKS, adducin and ERM proteins	Actin cytoskeleton, cell migration
PRKCZ	NM 002744	PKC-Z	Ser/Thr Kinase; phosphorylated by PDK1and mTor; substrate include MARCKS, adducin and ERM proteins	Actin cytoskeleton, cell polarity
RPS6KA1	NM 002953	P70S6 Kinase 1/ RSK1	Substrate of PDK1; phosphorylates several transcription factors including SRF and c-Fos	Regulation of translation, cell growth
RPS6KA3	Nm 004586	P70S6 Kinase 2/ RSK2	Substrate of PDK1; phosphorylates several transcription factors including SRF and c-Fos	Regulation of translation, cell growth
SGK1	NM 005627	Serum/glucocorticoid regulated kinase 1	Ser/Thr kinase; phosphorylated by PDK1 and mTor; substrates include NEDD4L and FOXO3A	Cell survival, ion transport

References: [343, 396]

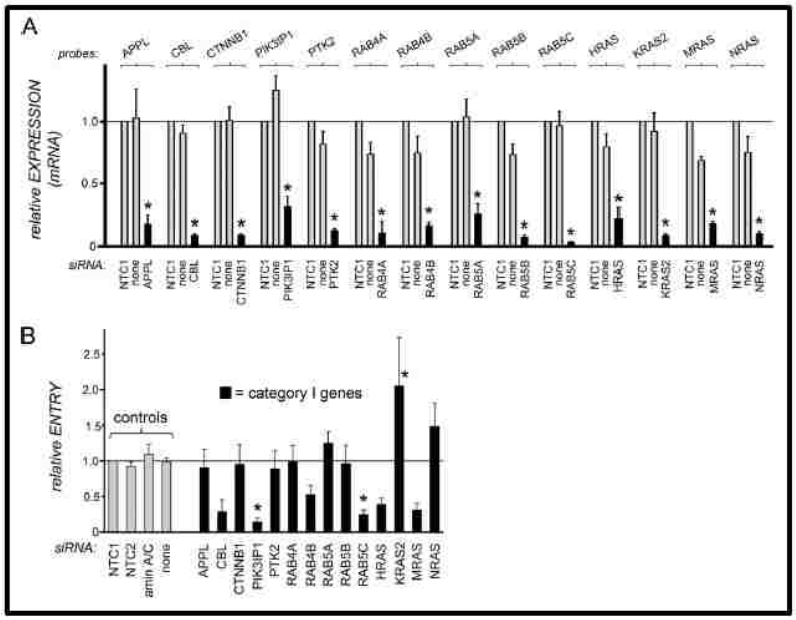

Figure 11 Effect of SiRNA targeting category 1 host genes on gene expression and InlB mediated entry of Listeria.

(A). Inhibition of host gene expression by siRNA pools. HeLa cells were transfected with siRNA pools targeting the indicated host category I genes. As controls, cells were transfected with a non-targeting control siRNA (NTC1) or mock transfected in the absence of siRNA (none). Approximately 48 hr after transfection, cells lysates were prepared. Gene expression was analyzed by real time PCR. Relative gene expression values were determined as described in the Materials and Methods. Data are mean +/- SEM of 3-5 experiments, depending on the siRNA condition. Statistical analysis by ANOVA indicated $P < 0.0001$. *, $P < 0.05$ relative to the NTC1 or no siRNA controls (Tukey-Kramer post-test). (B). Impact of siRNA pools on internalization of *Listeria* into host cells. HeLa cells were transfected with siRNA pools directed against the indicated host category I gene. As controls, cells were transfected with either of two non-targeting control siRNAs (NTC1, NTC2), a siRNA directed against lamin A/C, or mock transfected in the absence of siRNA (none). About 48 hr post-transfection, bacterial entry was assessed using gentamicin protection assays. Relative entry values were obtained by normalizing to entry in cells treated with the NTC1 control, as described in the Materials and Methods. Data are mean +/-SEM values. Results for siRNAs targeting category I genes are from 3-7 experiments, depending on the siRNA condition. Data with the no siRNA (none), NTC2, or lamin A/C siRNA control conditions are from 46, 13, or 13 experiments, respectively. Statistical analysis by ANOVA indicated $P < 0.0001$. *, $P < 0.05$ relative to the NTC1, NTC2, lamin A/C, or no siRNA controls (Tukey-Kramer post-test)**.**

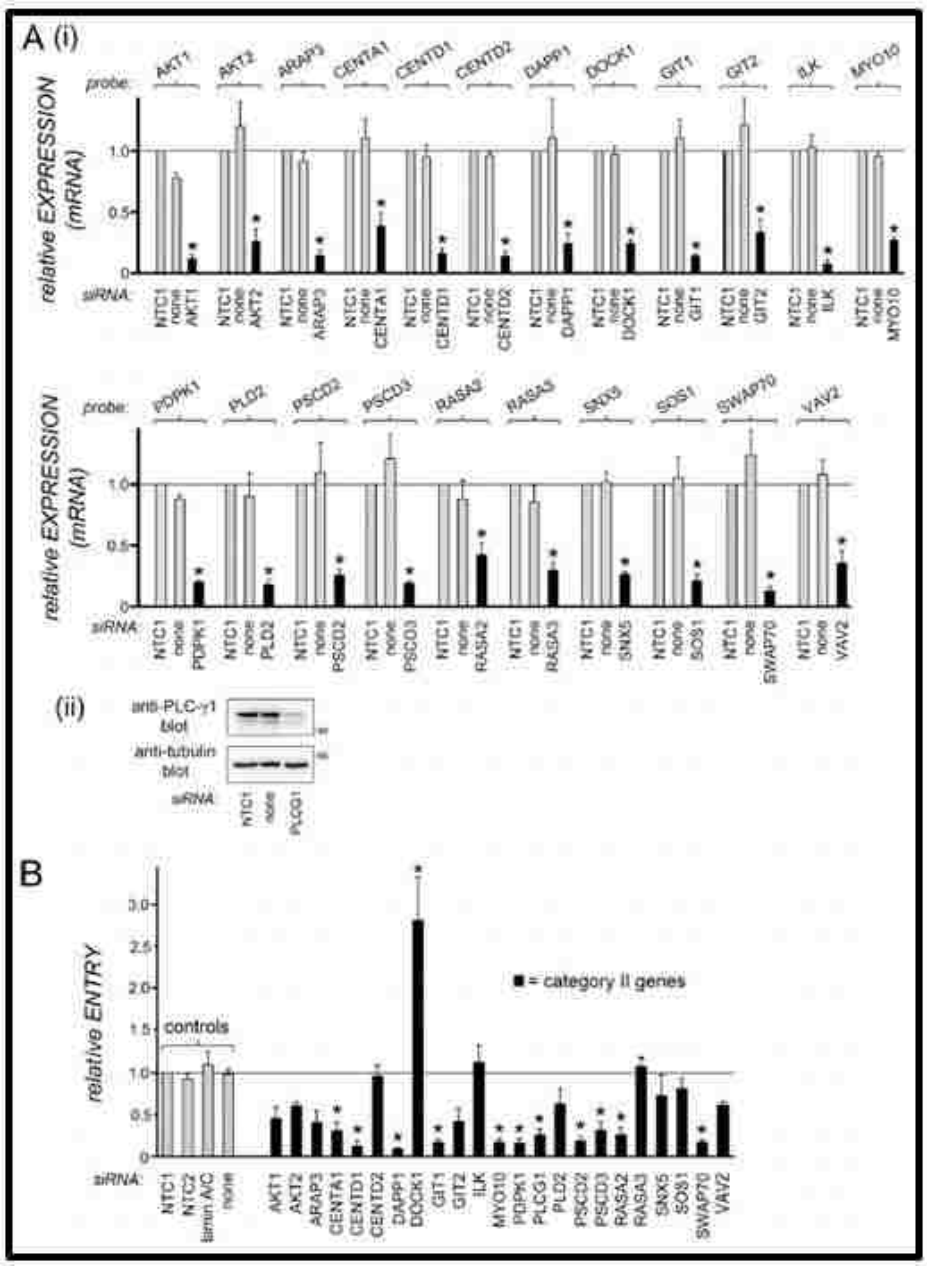

Figure 12 Effect of SiRNA s directed against category II host genes on gene expression and Listeria internalization.

(A). Inhibition of host gene expression. HeLa cells were transfected with siRNA pools targeting the indicated host category II genes. Control transfection conditions and analysis of gene expression were as described in the legend for Fig. 2. Data are mean +/- SEM of 3-5 experiments, depending on the siRNA condition. Statistical analysis by

ANOVA indicated $P < 0.0001$. *, $P < 0.05$ relative to the NTC1 or no siRNA controls (Tukey-Kramer post-test). (B). Effect of siRNA pools on internalization of *Listeria*. HeLa cells were transfected with siRNA pools directed against the indicated host category II gene. Control transfection conditions and measurement of bacterial entry was as described in the legend for Fig. 2. Data involving siRNAs targeting category II genes are mean +/-SEM of 3-5 experiments. Data with the no siRNA (none), NTC2, or lamin A/C siRNA control conditions are from 46, 13, or 13 experiments, respectively. Statistical analysis by ANOVA indicated $P < 0.0001$. *, $P < 0.05$ relative to the NTC1, NTC2, lamin A/C, or no siRNA controls (Tukey-Kramer post-test).

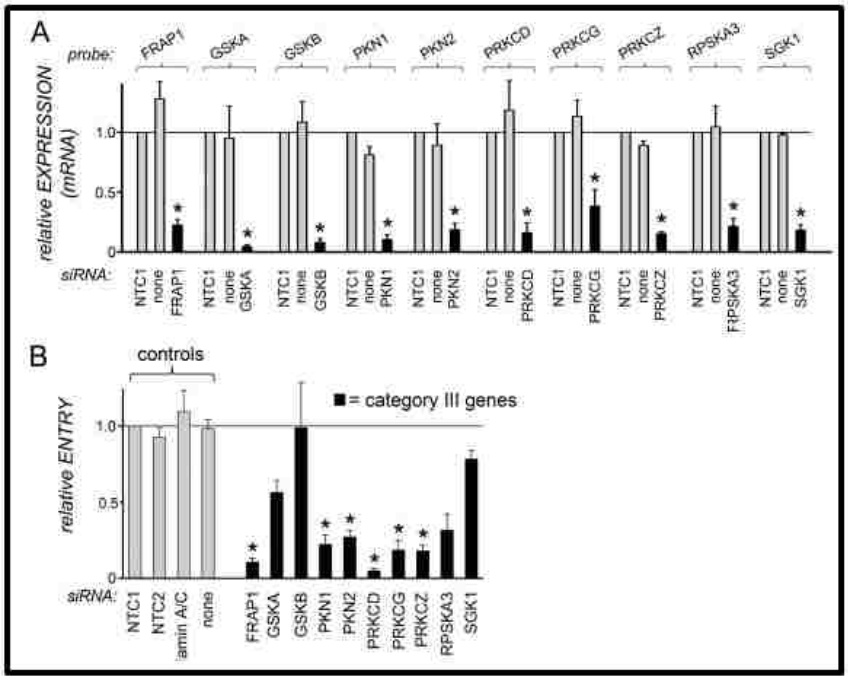

Figure 13 Effect of SiRNA targeting category III host genes on gene expression and Listeria internalization.

(A). Inhibition of host gene expression. HeLa cells were transfected with siRNA pools targeting the indicated host category III genes. Control transfection conditions were as described in the legend for Fig. 2. Gene expression was analyzed by real time PCR for all but one of the genes (i). In the case of PLCG1 (encoding PLC-□1 protein), gene expression was assessed by Western blotting (ii), since the probe did not detect expression (Materials and Methods). For experiments involving siRNAs targeting category III genes, data are mean +/- SEM values of 3-5 experiments, depending on the siRNA condition. Statistical analysis by ANOVA indicated $P < 0.0001$. *, $P < 0.05$ relative to the NTC1 or no siRNA controls (Tukey-Kramer post-test). (B). Impact of siRNA pools on entry of *Listeria*. HeLa cells were transfected with siRNA pools directed against the indicated host category III genes. Control transfection conditions and measurement of bacterial entry were as described in the legend for Fig. 2. Data with siRNAs targeting category III genes are mean +/- SEM of 3-7 experiments. Data with the no siRNA (none), NTC2, or lamin A/C siRNA control conditions are from 46, 13, or 13 experiments, respectively. Statistical analysis by ANOVA indicated $P < 0.0001$. *, $P < 0.05$ relative to the NTC1, NTC2, lamin A/C, or no siRNA controls (Tukey-Kramer post-test).

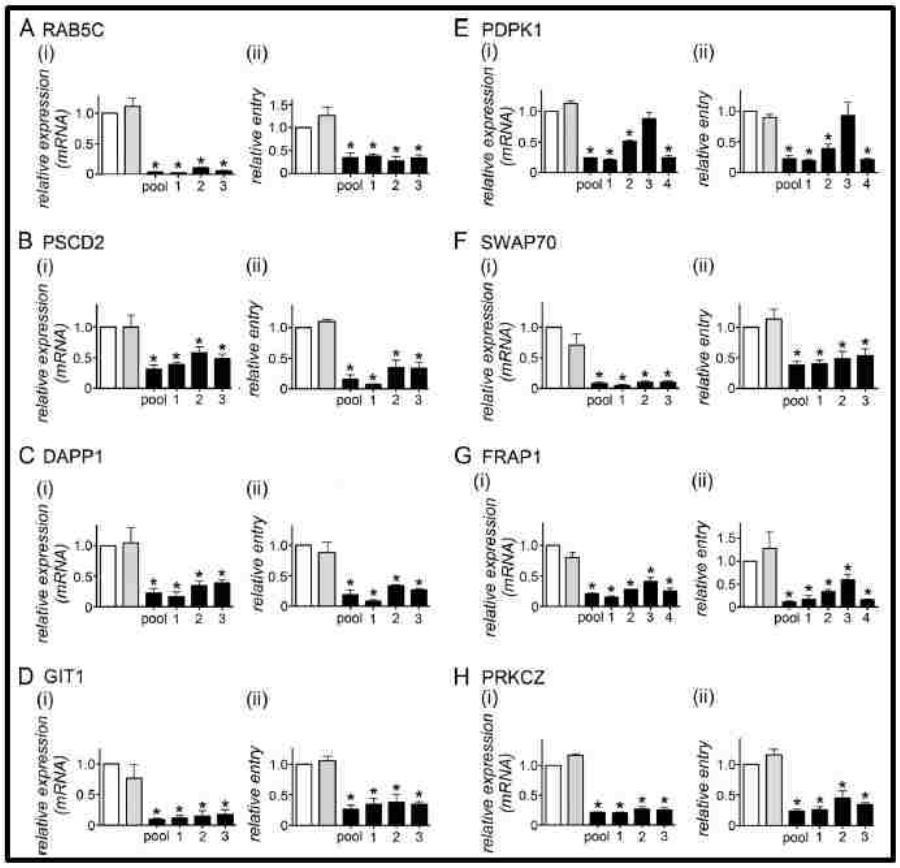

Figure 14 Multiple SiRNA inhibiting target gene expression impairs internalization of Listeria.

HeLa cells were transfected with siRNA pools or individual siRNAs targeting the human gene indicated in A-H. Four individual siRNAs were tested for PDPK1 and FRAP1, whereas three siRNAs were tested for all other genes. As controls, cells were mock transfected in the absence of siRNA (no siRNA) or transfected with control non-targeting siRNA 1 (NTC1). (i). Effect of siRNAs on host gene expression. Gene expression was analyzed by real time PCR. Data are mean +/- SEM of 3-4 experiments, depending on the siRNA condition. ANOVA indicated $P <$ 0.0001 for Rab5c (A), PDPK1 (E), SWAP70 (F), FRAP1 (G), and PRKCZ (H), $P = 0.0001$ for GIT1 (D), $P = 0.0003$ for DAPP1 (C), and $P = 0.0012$ for PSCD2. *, $P < 0.05$ relative to the no siRNA or NTC1 controls (Tukey-Kramer post-test). (ii). Effect of siRNA pools on internalization of *Listeria*. Data are mean +/- SEM of 3-7 experiments, depending on the siRNA condition. Statistical analysis by ANOVA indicated $P < 0.0001$ for all data in A-H. *, $P < 0.05$ relative to the no siRNA or NTC1 controls (Tukey-Kramer post-test)

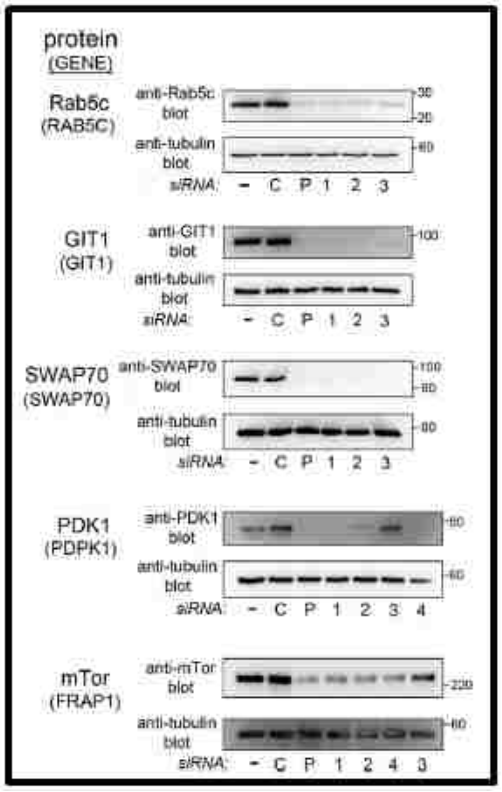

Figure 15 Confirmation that SiRNA inhibits expression at protein level.

Human proteins evaluated for expression are indicated on the left. Gene names are given in parentheses. HeLa cells were transfected with siRNA pools (P) or individual siRNAs directed against the indicated human gene. As controls, cells were either mock transfected in the absence of siRNA (-) or transfected with non-targeting control 1 siRNA (C). Approximately 48 hr after transfection, cells were solubilized in RipA buffer. The indicated target protein was detected by Western blotting as described in the Materials and Methods. In order to confirm equivalent loading, membranes were then stripped and probed a second time with anti-tubulin antibodies.

2.6 Supplemental Information

Table 2 siRNA and probe for measuring gene expression

Gene[a]	GenBank accession no.[b]	siRNAs used[c]	Probe used for Real time PCR[d]
APPL	NM_012096	(1) GGUGAUGAUGAAGUUAUGAtt (2) GCUAUGCAUCGGAUUUAUGtt (3) CGAUAUUGUUUUCAGAUCAtt	Hs00179382_m1
AKT1	NM_005163	(1) GGAGCAAGGUUUAAAUUUGtt (2) GGCUCCCCUCAACAACUUCtt (3) GGGCACUUUCGGCAAGGUGtt	Hs00178289_m1
AKT2	NM_001626	(1) GGUACUUCGAUGAUGAAUUtt (2) GGACCUUCCACGUGGAUUCtt (3) GGAUGAAGUCGCUCACACAtt	Hs00609846_m1
ARAP3	NM_022481	(1) GGGAAACUAUGUCUUCCAGtt (2) CCAGUGAUGACCUCAUUUCtt (3) GCCCAUUUAUCUAUACCAUtt	Hs00223903_m1
ARHGAP9	NM_032496	(1) GGAACAAUGAUGUCCUGCAtt (2) GGAAGACUUGCCGUCAGAAtt (3) GGAUAUCCCUUUAAAUCUCtt	Hs00261256_m1
ARHGEF6	NM_004840	(1) GGAUUUGAAACUGCUCCACtt (2) GCAAUCGUAGCAUUCAAAAtt (3) GCCGUCAAAGGAUUUGAAAtt	Hs00374462_m1
BMX	NM_203281	(1) CCUGCUGGUCAAGUACCAUtt (2) GGGAAGACUUCCCUGACUGtt (3) GGAAACAAAAGAUUCCCUUtt	Hs00176455_m1
CBL	NM_005188	(1) CGGUGGACAAGAAGAUGGUtt (2) GGAGGGAAAAGAAAGAAUGtt (3) GCUCGGCUCCAGAAAUUCAtt	Hs00231981_m1
CENTA1	NM_006869	(1) GGUUUGAGUCCAAAGUACCtt (2) GGCUCAUGUACUUCAAAGAtt (3) CCUCCACGACUAUUUAUUGtt	Hs00199317_m1
CENTD1	NM_139182	(1) GGGUGUAUUAAGUCAAGAGtt (2) GGCUGGUUUGCUAUGGACAtt (3) GGAUGCAAGAAGCUUUAAAtt	Hs00367391_m1
CENTD2	NM_015242	(1) GGUAGUGAAAAUGAAAUGCtt (2) CCUUCAUCGACAUGAGCGUtt (3) GGAAGGUGUGGACAGAAACtt	Hs00362929_m1
CTNNB1	NM_001904	(1) GGCAAUCCUGAGGAAGAGGtt (2) GGUGGUGGUUAAUAAGGCUtt (3) GGCUACUGUUGGAUUGAUUtt	Hs00170025_m1
DAPP1	NM_014395	(1) GCGAGACAGGCACUCUGAUtt (2) GCUGUACAAUUCGAUUAUUtt (3) CCAUUCUGAUAGAAAGUGCtt	Hs00183937_m1

Gene[a]	GenBank accession no.[b]	siRNAs used[c]	Probe used for Real time PCR[d]
DEPDC2	NM_024870	(1) GGGUGAGAAACUUUAUAAAtt (2) GGCAUCUACAGAUGGACAUtt (3) GGUGAUGGAAGUGGAGAAUtt	Hs00228571_m1
DOCK1	NM_001380	(1) CGUUACGAAAAAAGUCUAAtt (2) CGAUUAGAGCAUGUGAUUUtt (3) GGACAAGAAGAAGGAAAAAtt	Hs00169192_m1
FRAP1	NM_004958	(1) GGAAAUGGGUUGAUGAACUtt (2) GGAGUCUACUCGCUUCUAUtt (3) GGAGCUCCAGCACUAUGUCtt (4) GCUCGUAGUUGGGAUAACAtt	Hs00234508_m1
GIT1	NM_014030	(1) GGUGGAUCGAAGAGAAAAUtt (2) GGCUGGUUGAGUGCCAAUAtt (3) GGCCUUUUCCAUGUAUGAAtt	Hs00204031_m1
GIT2	NM_057169	(1) GGAAACCAGAUCACAAAAAtt (2) GCUCAUACUGAAAACCAUCtt (3) GGAUAAAGUACAUCCCAAUtt	Hs00207498_m1
GSK3A	NM_019884	(1) GGCUGAGAUACUUUUUCUAtt (2) GGCCAAGUUGACCAUCCCUtt (3) GGGAACUAGUCGCCAUCAAtt	Hs00219856_m1
GSK3B	NM_002093	(1) GGACAUUUCACCUCAGGAGtt (2) GGAGAACCCAAUGUUUCGUtt (3) GGACAAGAGAUUUAAGAAUtt	Hs00275656_m1
HRAS	NM_004985	(1) GUGCCUGUUGGACAUCCUGtt	Hs00610483_m1
ILK	NM_004517	(1) CUCAAUAGCCGUAGUGUAAtt (2) CUCAAUAGCCGUAGUGUAAtt	Hs00177914_m1
KRAS2	NM_004985	(1) GGAUUCCUACAGGAAGCAAtt (2) GGGCUUUCUUUGUGUAUUUtt (3) GGACAUCACUUACUAUCCAtt	Hs00270666_m1
MRAS	NM_012219	(1) CGUCAAAGUAACCUAAUGUtt (2) GCCUUCCAUGACCUCGUUAtt (3) CCAAACACAAUAUUCCGUAtt	Hs00171926_m1
MYOX	NM_012334	(1) GCCGAUUUGACUUUAUCUAtt (2) GGAGAAGACAUCCUGUGUUtt (3) GGUAUUCACUUACAAGCAGtt	Hs00202485_m1
NRAS	NM_002524	(1) GCAGUGAUGAUGGGACUCAtt (2) GGCUUCCUCUGUGUAUUUGtt (3) GGAUUCUUACAGAAAACAAtt	Hs00180035_m1

Gene[a]	GenBank accession no.[b]	siRNAs used[c]	Probe used for Real time PCR[c]
PAK1	NM_002576	(1) GGAGAAGAAAAAGAAGGACtt (2) GGACCGAUUUUACCGAUCCtt (3) GGGUGGUUUAUGAUUAAGGtt	Hs00176815_m1
PAK2	NM_002577	(1) GGUAGUAGCUAAAAUUAGAtt (2) GGCAAAGACCCUUUGUCAGtt (3) CGAGUAAUUGUGAAGCAUAtt	Hs00605586_m1
PDPK1	NM_002613	(1) GGCUCUUUUUCCACGGUUGtt (2) CGAGAGUGGCUCUUUUUAGtt (3) GCCAAUUUCCUUGUUUUGGtt (4) GGGUUUAUUUGCAAGACGAtt	Hs00176884_m1
PIK3IP1	NM_052880	(1) CCUGGAAACUAAGCUCAGAtt (2) GGCUAACCUGGAAACUAAGtt (3) GGAGAAAAAGGACCUGGGAtt	Hs00364627_m1
PKN1	NM_213560	(1) GGCCCAGGAGAAAUUGACAtt (2) GCCAUCGGCAUCAUGAGAAtt (3) GGACAGUAAGACCAAGAUUtt	Hs00177028_m1
PKN2	NM_006256	(1) GGAUCGAAUUAAGAGAGAAtt (2) GGAGCUGAAAAUCUGAGGAtt (3) GGAUUCAGAGACUGUUUUUtt	Hs00178944_m1
PLCG1	NM_002660	(1) GGAUGAAGUGAACAUGUGGtt (2) CCCUUACCACCAAGAUCAAtt (3) CCCUUACCACCAAGAUCAAtt	Hs00234046_m1
PLD1	NM_002662	(1) GGAUUUAAGGAGCCUAAUAtt (2) GGGCAUAGAAGGUAUGAUAtt (3) GGCAAAUGAAGAGAUUUUUtt	Hs00160118_m1
PLD2	NM_002663	(1) GGCCCUACUACCCAUUGUUtt (2) GGUGCCAUCUCAUUUGUUCtt (3) CCCGUUUCUGGCCAUCUAUtt	Hs00160163_m1
PLEKHA1	NM_001001974	(1) GCAAGUGUCUUACAGAACUtt (2) GCGACAUAAUGAUGAGGGAtt (3) GCCAUUAAGCUUACCUACAtt	Hs00608662_m1
PRKCD	NM_006254	(1) GGCUACAAAUGCAGGCAAUtt (2) GGCCAAAAUCCACUACAUCtt (3) GUUGAUGUCUGUUCAGUAUtt	Hs00178814_m1
PRKCE	NM_005400	(1) GCCUUGUCAUUUGACAACCtt (2) GGAAAUAAAAGAACUUGAGtt (3) GGGAUACCAGUGUCAAGUCtt	Hs00178455_m1

Gene[a]	GenBank accession no.[b]	siRNAs used[c]	Probe used for Real time PCR[d]
PRKCG	NM_002739	(1) CGAUGCCACGAAUUUGUGAtt (2) GCUUUUGUUCUAGACUUCCtt (3) GGCCCGUAACCUAAUUCCUtt	Hs00177010_m1
PRKCI	NM_002740	(1) GGAAGGAGACCCGUGUACAtt (2) GGUUCGAGACAUGUGUUCUtt (3) GGAGACCCGUGUACAGUAUtt	Hs00702254_s1
PRKCQ	NM_006257	(1) GGGCGAGGCUGUUAACCCUtt (2) GGGAAGAGUCAUGCAGAUCtt (3) GGAUUUUAUCUUGCACAAAtt	Hs00234697_m1
PRKCZ	NM_002744	(1) GGCUUUGAGUAUAUCAACCtt (2) GGAAGCAUAUGGAUUCUGUtt (3) GGACCUUAAGCCAGUUAUCtt	Hs00177051_m1
PSCD1	NM_004762	(1) CCCUGCACCAUAAAUACAUtt (2) GCCACAUACUAUAAAGAUCtt (3) GGAUGAGAUAGCAGAAGUAtt	Hs00245092_m1
PSCD2	NM_017457	(1) GGAAGAAGUUCAACAUGGAtt (2) GUUCUUGGUGGAGAAUGAAtt (3) ACCACAUGGUGUACCGGAUtt	Hs00244669_m1
PSCD3	NM_004227	(1) GGAGAGCAAAACGACUCAGtt (2) GCUAUAAAGUUAACUCCUGtt (3) GCAAAACGACUCAGAGGAAtt	Hs00188456_m1
PTK2	NM_153831	(1) GGAGUGGAAAUAUGAAUUGtt (2) GGCAUGGAGAUGCUACUGAtt (3) GGAGGUUCACUGGCUUCACtt	Hs00178587_m1
RAB4A	NM_004578	(1) CCUACAAUGCGCUUACUAAtt (2) GGUCCGUGACGAGAAGUUAtt (3) CGAUUCAGGUCCGUGACGAtt	Hs00190157_m1
RAB4B	NM_016154	(1) GGAACUGGCAAAUCAUGUCtt (2) GGACUCCAACCACACAAUCtt (3) GGAGAAUGAGCUGAUGUUCtt	Hs00535053_m1
RAB5A	NM_004162	(1) GCCUAGUGCUUCGUUUUGUtt (2) GGCAAGCAAGUCCUAACAUtt (3) GCAAGUCCUAACAUUGUAAtt	Hs00702360_s1
RAB5B	NM_002868	(1) GGAGAGGUUAUAGAUAAAAtt (2) GCCUGGUAUUACGUUUUGUtt (3) GGGCAGUUCCAUGAGUACCtt	Hs00161184_m1
RAB5C	NM_004583	(1) CCAAUCGCACUUAACGACUtt (2) CCAACACAGAUACAUUUGCtt (3) GGGACAGUUUCACGAGUACtt	Hs00426044_m1

Gene[a]	GenBank accession no.[b]	siRNAs used[c]	Probe used for Real time PCR[d]
RASA2	NM_006506	(1) CCAGGAAGAAGUUUAUCGUtt (2) CGUAUUAAGAACUGAUUCCtt (3) GCCUGUUUGACUCCAAUUCAtt	Hs00183550_m1
RASA3	NM_007368	(1) CGUGGACAAGCUCGAAAUCtt (2) CGUGUCGUCAUUUGAUUCUtt (3) CGUGUCGUCAUUUGAUUCUtt	Hs00183698_m1
RPS6KA1	NM_002953	(1) GGGACAGGGUCUUUUUUCAtt (2) GGCUGGCUCUGAGAAGGCUtt (3) GGAGAUCUCCAUCACGCACtt	Hs00177257_m1
RPS6KA3	NM_004586	(1) GGAGGAGAUUAACCCACAAtt (2) GGACAUGAAAAGGCAGAUCtt (3) GGUUUUAAAAAGACAAGGCtt	Hs00177936_m1
RRAS2	NM_012250	(1) CGGAUUAUGAUCCAACCAUtt (2) GCUUAAGGUAACAUACAUGtt (3) GCUACAGGUUGUGAAAUAAtt	Hs00273367_m1
SGK1	NM_005627	(1) GGAGCCUGAGCUUAUGAAUtt (2) GGUUCUUCUAGCAAGACACtt (3) GGAUGGGUCUGAACGACUUtt	Hs00178612_m1
SNX1	NM_003099	(1) GGAAGAUUCUUCUUCUGCAtt (2) CCACGUGAUCAAGUACCUUtt (3) GGAUUUCAACAGUGGUCCGtt	Hs00162052_m1
SNX5	NM_152227	(1) GGCAACAUGAAGACUUUGUtt (2) CCAGAUCUCAUAAAAAUGUtt (3) GGUUGCUGAGCUAUUUGAAtt	Hs00429583_m1
SOS1	NM_005633	(1) GGAGGUCCUAGGUUAUAAAtt (2) GGUUUCUGUUUACAUAGUAtt (3) GGUUUCUGUUUACAUAGUAtt	Hs00362308_m1
SWAP70	NM_015055	(1) CGUCAGUUAUGUAGAUACUtt (2) GGACAAGUAUCCAUUAAUUtt (3) CAACAUAAUUUCUUACUAUtt	Hs00929832_m1
TIAM1	NM_003253	(1) GGAUGCACGCUAUUUUUCUtt (2) GGGAAUAUUUGAUGACAUUtt (3) CCUGUGUCUUACACUGACUtt	Hs00180075_m1
VAV2	NM_003371	(1) GGAUGGAAAAUUUAAGCUGtt (2) CGAUAAAUUUGGAUUAAGGtt (3) GGAAAAUCAGCGAAUUUCAtt	Hs00610104_m1
VAV3	NM_006113	(1) GGACAAAAUGGGUUAGAAUtt (2) GGACUUGGCACAAUAUGUGtt (3) CGACUUUCUCGAACACCUAtt	Hs00198125_m1

Table 3 Human genes whose expression was not detected by real time PCR or failed to be inhibited by siRNA

Gene[a]	GenBank accession no.[b]	Expression[c]	Inhibition by siRNA pool[d]
ARHGAP9	NM_032496	Detected	No
ARHGEF6	NM_004840	Not detected	
BMX	NM_203281	Not detected	
DEPDC2	NM_024870	Not detected	
PAK1	NM_002576	Not detected	
PAK2	NM_002577	Not detected	
PLD1	NM_002662	Not detected	
PLEKHA1	NM_001001974	Not detected	
PLCG1	NM_005188	Not detected[e]	
PRKCE	NM_005400	Detected	No
PRKCL	NM_002740	Detected	No
PRKCQ	NM_006257	Not detected	
PSCD1	NM_004762	Detected	No
RPSK6A1	NM_002953	Detected	No
RRAS2	NM_012250	Detected	No
SNX1	NM_003099	Detected	No
TIAM1	NM_003253	Not detected	
VAV2	NM_003371	Detected	No

[a] Genes are ordered alphabetically.

[b] GenBank database 2011 (http://www.ncbi.nlm.nih.gov/)

[c] Expression was measured by real time PCR. Expression was considered to be undetectable if the average Ct value was 36 or higher. The cutoff Ct value of 36 was selected based on control experiments using lysates lacking cDNA (Materials and Methods). Ct values for the remaining genes 53 genes whose expression was detected by real time PCR ranged from 23-32.

[d] Gene expression was considered to be inhibited if the siRNA pool directed against the target gene caused a two fold or greater decrease in mRNA levels, compared to the no siRNA control.

[e] Gene expression was not detected by real time PCR using the probe listed in Table S1. However, expression of the corresponding protein was detected by Western blotting (Fig. 3A ii).

CHAPTER THREE: CHLAMYDIA TRACHOMATIS TARP COOPERATES WITH THE ARP2/3 COMPLEX TO INCREASE THE RATE OF ACTIN POLYMERIZATION

3.1 Introduction

C. trachomatis, a gram negative obligate intracellular pathogen, is responsible for the most common sexually transmitted disease in the United States. It also causes trachoma, which is the most common form of preventable blindness in underdeveloped countries [397]. Chlamydia exhibits a unique developmental cycle that alternates between the extracellular infectious and metabolically inactive form, the elementary body (EB), and the intracellular non-infectious replicative form, the reticulate body (RB) [162]. Being an obligate intracellular organism, gaining access into the host cell is the most critical step in survival of *C. trachomatis*. Upon internalization, the EB transforms into a RB, which then undergoes several rounds of replication before reverting back to infectious EB [162].

Chlamydia induces its uptake so efficiently that the process has been distinguished as “parasite- specified phagocytosis.” The uptake of an EB involves its attachment to the host cell surface followed by its entry [162, 297]. The initial step in the attachment is the reversible electrostatic interaction of the EB with the host cell surface. There is much evidence to suggest the role of heparan sulfate proteoglycans in such an interaction in many, if not all, strains of *C. trachomatis* [228, 278]. Studies employing defective mutants of CHO cell lines suggest a second irreversible step of attachment occurring before entry [236]. Despite considerable efforts, neither the chlamydial ligands

nor the host cell surface receptors critical for such irreversible interactions have yet to be identified. Although several putative host receptors have been suggested, including PDGF-β R and FGFR, blocking of these receptors only partially inhibited entry [237, 238]. This suggests that irreversible attachment involves multiple interactions of the EB with the host cell surface. While the molecular mechanism of attachment remains undefined, the post-attachment events of chlamydial invasion have begun to be characterized.

Like many other intracellular bacteria, the entry of chlamydia is also driven by the rearrangement of the host actin cytoskeleton [292]. The attachment of an EB to the host cell surface results in the recruitment of actin, and the treatment of host cells with inhibitors of actin polymerization, such as cytochalasin D, inhibits chlamydial entry [292]. Efficient entry of chlamydia requires active participation of both the host and chlamydial proteins. Activation of the Arp2/3 complex, a host actin nucleator, is a critical event in regulating actin dynamics during chlamydial entry as the disruption of Arp2/3 complex activation abrogates entry [296, 299]. Upon entry of *C. Trachomatis*, Arp2/3 complex is regulated via activation of Rac GTPases, which in turn are regulated via host signaling [296, 299]. Because the surface receptor essential for chlamydial entry is not known, the signaling events that result in activation of Rac GTPases have remained elusive for the most part. It is believed that multiple mechanisms are involved in activation of Arp2/3 complex. Some bacterial effector proteins are also known to regulate Arp2/3 complex either by direct binding and activation of the Arp2/3 complex or by manipulating

the activity of Rho family GTPases [63, 95]. No chlamydial effectors are yet identified that can directly interact with Arp2/3 complex. However, controversial evidences do exist regarding the indirect regulation of Arp2/3 by a chlamydial effector protein Tarp [296, 306].

Chlamydia also mediates its entry by a second mechanism involving its type III effector protein called Tarp [64, 243, 307]. Tarp, which stands for translocated actin recruiting protein, is conserved among all pathogenic species of chlamydia [306]. EBs deliver pre-synthesized Tarp into the host cytoplasm following its initial attachment [243]. In vitro functional analysis of Tarp demonstrated its ability to polymerize actin [64]. Such capacity is attributed to the presence of a C-terminal actin binding domain (ABD) and a proline rich oligomerization domain (PRD), which clusters multiple actin monomers to nucleate new actin filaments. Chlamydial EBs showed reduced invasive capacity in host cells that were microinjected with neutralizing antibodies that target ABD [307]. This implicates the bona fide role of Tarp in chlamydial entry.

Tarp harbors an additional N-terminal phosphorylation domain, which consists of a variable number of tyrosine rich repeats of ~50 amino acids, with LGV serovars harboring 6 and the trachoma group harboring 3 such repeats [306]. Tarp becomes phosphorylated at several tyrosine residues by Src, Abl and Syk kinases immediately following its translocation into the host cell [237, 309]. The phosphorylated Tarp remains in close association with the developing inclusion [243]. However, the significance of such associations is not completely understood. Recently, the interaction of

phosphorylated Tarp with SH2 domain containing adaptor protein SHC1 and PI-3 kinase became evident [312, 398]. The association of Tarp with SHC1 has been linked with survival of the infected host, which is essential for inclusion development [398]. As far as the role of the phosphorylation domain in entry is concerned, the effect of such phosphorylation on Tarps ability to nucleate actin is not yet known. Previous studies employed an unphosphorylated form of recombinant Tarp to investigate its role in actin dynamics [64, 307]. Since Tarp naturally exists in phosphorylated form within the host cytoplasm, it is essential to test if phosphorylation enhances or hampers Tarps ability to nucleate actin. While the possibility of Tarp or phosphorylated Tarp interacting directly with Arp2/3 complex to cause its activation has never been tested, controversial evidence does exist linking the phosphorylated Tarp with Arp2/3 complex activation. Lane et al suggested participation of the phosphorylation domain in activation of Rac GTPases via recruitment of GEF complexes including Sos/Abi/Aps8 and Vav2 [312]. Interestingly, another study suggested that Tarp phosphorylation is not necessary for the invasion of EBs as the prevention of Tarp phosphorylation had no effect on entry [306, 309]. Moreover, the phosphorylation domain is not conserved in other chlamydial species other than *C. Trachomatis*, even though invasion is critical for the survival of all species [306]. This implies that either the phosphorylation domain is not essential in entry of *C. trachomatis,* or that other species have evolved other mechanisms of initiating signaling events. Regardless of our lack in knowledge about exact signaling events, Tarp and Arp2/3 complex are both required for efficient entry of chlamydia.

Whether these two nucleators play an independent role in entry or if they cooperate in regulating the actin dynamics is not yet known.

In this study, we first reconfirmed the role of Arp2/3 complex in chlamydial entry using two newly identified Arp2/3 inhibitors, CK-666 and CK-869. We also provide the first bio-chemical evidence that Tarp and Arp2/3 complex cooperate to form a branched network of actin filaments, which might be necessary for entry of chlamydia, and that phosphorylation of Tarp has no effect on its cooperative activity with Arp2/3 complex. And importantly, we provide evidence that Tarps ability to recruit actin is independent of its phosphorylation domain.

3.2 Material and Methods

3.2.1 Invasion Assay

Chlamydia trachomatis invasion of HeLa cells was determined as previously described using fluorescently labeled elementary bodies (EBs) [296]. Briefly, to determine the percent of internalized *C. trachomatis* elementary bodies in drug treated cells, HeLa 229 (ATCC) host cells were pretreated for 1.5 hours with the recently characterized Arp2/3 chemical inhibitors CK-869 and CK-666 (Calbiochem) and infected with CellTracker^{TM} CMTPX (Invitrogen) labeled *C. trachomatis* L2 (MOI ~30) [399]. Two inactive inhibitor controls (CK-312 and CK-689) and DMSO alone served as negative controls. All compounds were used at a media concentration of 200□M. Infected host cells were fixed 30 minutes post infection with 4% paraformaldehyde for 15 minutes, washed in PBS and blocked with 10% fetal bovine serum. The cells were

not permeabilized. Extracellular EBs were labeled for 1 hour with antibodies specific for chlamydial momp (L2). After four washes in PBS, secondary antibody conjugated to Alexa 488 (green) was added for 1 hour. Coverslips were rinsed and mounted in ProLong Gold antifade reagent (Invitrogen). Cells were examined with a Zeiss Axio Observer A1 microscope equipped with phase contrast and epifluorescence optics. Images were obtained using an AxioCam MRm camera controlled by AxioVision 4.8.2 and further processed using Adobe Photoshop CS2. The percentage of internalized bacteria was taken as the total EBs (red)−extracellular EBs (green)/total EBs (red)×100. Protein samples collected from drug treated host cells identical to those described above were infected with *C. trachomatis* L2 at an MOI of 1000 and were examined by Western blot analysis.

3.2.2. SDS-PAGE and Western Blotting

Proteins were separated on SDS-10% polyacrylamide gels and stained with coomassie R-250 (Pierce) or transferred to 0.45□m pure nitrocellulose transfer and immobilization membrane (Schleicher & Schuell, Keene, NH). Immunoblotting employed peroxidase conjugated secondary antibodies (Chemicon International, Temecula, CA) and Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). The anti-phosphotyrosine 4G10 monoclonal antibody was purchased from Upstate (Millipore). Polyclonal rabbit antibodies directed towards *C. trachomatis* L2 LGV 434 Tarp was developed at Rocky Mountain Laboratories as previously described [243]

3.3.3 Cloning, Protein Expression and Purification

An in frame glutathione-S-transferase (GST) and polyhistidine *C. trachomatis* L2 LGV 434 Tarp fusion protein was generated by PCR amplifying the corresponding coding regions from *C. trachomatis* genomic DNA (QIAGEN genomic purification kit, Valencia CA) using custom synthesized oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) engineered with SalI, SacI or NotI linkers. PCR products were purified (QIAGEN), digested with restriction enzymes (New England Biolabs, Beverly, MA) and subcloned into linearized pGEX-6P-1 to generate translational fusions with GST at the N-terminus and polyhistidine at the C-terminus. The PCR fragments described above harboring *tarP* were also cloned into pEGFP-C3 (BD Biosciences Clontech) to allow for the ectopic expression of eGFP-Tarp in HeLa cells.

pGEX-6P-1 plasmids were transformed into BL21 strain of *E. coli* (Novagen, Madison WI). Protein expression and purification were performed according to the procedures outlined for Ni Sepharose 6 Fast Flow and Glutathione sepharose 4B in the Bulk GST Purification Module (GE health sciences, Piscataway, NY).

3.3.4 Actin Polymerization Assay

The rate of actin polymerization in the presence of Tarp and the Arp2/3 complex was monitored according to the methods outlined in the Actin Polymerization Biochem Kit BK003 (Cytoskeleton, Denver CO). Briefly, monomeric pyrene labeled actin was prepared by diluting 500 ng of lyophilized pyrene actin into 5 mls of 5 mM Tris (pH 8.0), 0.2 mM $CaCl_2$, and 0.2 mM ATP (G-buffer) and incubating for 1 hour at room

temperature followed by an additional hour of incubation at 4^{o}C. Monomeric pyrene actin was obtained by collecting the supernatant following a 2 hour, 100,000 rcf, 4^{o}C spin in a Beckman Optima TLX Ultracentrifuge using a TLA 100.3 rotor (Beckman Coulter Inc., Fullerton, CA). Approximately 40 µg of pyrene labeled actin was gently mixed with 2-5 ng of test proteins in a volume of 500 µl for 5 minutes prior to the addition of 1/20th volume of polymerization buffer (500 mM KCl, 20mM $MgCl_2$, and 10mM ATP). The reaction was monitored over one hour with an LS 55 Luminescence spectrophotometer equipped with a biokinetics accessory and directed by FL WinLab software version 4.0 (Perkin Elmer, Beaconsfield, BUCKS, UK) with 2.5 nm bandwidth at 365 nm excitation wavelength and 2.5 nm bandwidth at 407 nm emission wavelength.

3.3.5 Scanning Electron Microscopy

Actin filaments were added to an SEM type 3 mount and sputter coated with 8-10nm chromium. Coated filaments were examined in a Hitachi S5200 scanning electron microscope at 30 kV accelerating voltage.

3.3.6 Transfection of Hela Cells and Indirect Immunofluorescence Microscopy

HeLa cells (2 X 10^{5}) were seeded in 6 well plates with coverslips and grown for 24 hours in DMEM containing 10% FBS. Cells were then transfected with transfection mixture containing 8 µl of Fugene HD (promega) and 2.5 mg of respective plasmid. Following 24 hours, cells were fixed by adding 4 % paraformaldehyde and incubating at 4^{0} C for 15 minutes. Cells were then treated with ice cold 0.4 % Triton-X for 10 minutes, followed by blocking with 5% BSA for 45 minutes. To visualize tyrosine phosphorylated

protein, cells were first incubated with anti-phosphotyrosine primary antibody (upstate) at 1:1000 dilutions in 0.5 % BSA at RT for 45 minutes followed by incubation with anti-mouse secondary antibody conjugated to Alexa 350 (invitrogen). To simultaneously visualize actin, phalloidin conjugated to Alexa 568 (invitrogen) was added to the above mixture containing secondary antibodies. To stain for Arp2/3 complex, cells were first incubated with anti-Arp3 primary antibody (upstate) at 1:100 dilutions in 0.5 % BSA at RT for 45 minutes followed by incubation with anti- rabbit secondary antibody conjugated to Alexa 594 (invitrogen). Coverslips were rinsed and mounted in Prolong Gold antifade reagent (invitrogen). Cells were examined with a Zeiss Axio Observer A1 microscope equipped with phase contrast and epifluorescence optics. Images were obtained using an AxioCam MRm camera controlled by AxioVision 4.8.2 and further processed using Adobe Photoshop CS2.

3.3 Results

3.3.1 Arp2/3 Complex is Essential for Entry of C. Trachomatis

The role of Arp2/3 complex in chlamydial entry was previously confirmed using RNA interference and dominant-negative overexpression techniques. In order to test the effects of the new pharmacological inhibitors of Arp2/3 complex, CK-666 and CK-869, on entry of *C. trachomatis*, we did an invasion assay using HeLa cell lines that were pre-incubated with either media alone or media containing DMSO, Arp2/3 inhibitors (CK-666 and CK-869), control inhibitors (CK-312 and CK-689), or Jasplakinolide (Jaz), an actin filament stabilizer. Entry of *C. trachomatis* was inhibited in HeLa cells that were

pre-incubated with Arp2/3 complex inhibitors (CK-666 and CK-869) as compared to HeLa cells that were either untreated or treated with DMSO or control inhibitors (CK-312 and CK-689). These differences are statistically significant. Moreover, treatment of HeLa cells with Jasplakinolide (Jaz), a compound that stabilizes filamentous actin, also resulted in decreased entry (**Fig 16A**). To test whether pharmacological inhibition of Arp2/3 complex altered the delivery of Tarp into the host cytoplasm, we infected pharmacologically pretreated HeLa cells (as mentioned above for invasion) with EBs for 30 minutes and prepared the samples for western blot analysis. The samples were run on SDS-PAGE gel and a western blot analysis with phosphotyrosine Ab was done to detect translocated Tarp. The results indicate that the delivery of Tarp is not affected by treating the HeLa cells with either Arp2/3 complex inhibitors or Jaz (**Fig 16B**). Overall, the above result confirms the ability of CK-666, CK-869 and Jaz to inhibit entry of *C. trachomatis*, and also that such observed effects were not due to inhibition of effector delivery.

3.3.2 Tarp Cooperates, but does not directly activate Arp2/3 Complex to Polymerize Actin

Nucleating activity of Tarp differs from Arp2/3 complex in that Tarp generates linear actin filaments, whereas Arp2/3 uses linear filaments to form the branching pattern of filamentous actin [64, 400]. To test if these two nucleators cooperate to alter the kinetics of F-actin, an in vitro actin polymerization assay was done using fluorescently labeled actin. Mixtures containing actin alone or actin in combination with

Tarp, activated Arp2/3(Arp2/3 + VCA), or both were allowed to polymerize following the addition of polymerization buffer. The increase in fluorescence intensity was measured over time and graphed as a readout of the rate of actin polymerization (**Fig 17B**). The sample containing both nucleators showed an increased rate of actin polymerization as compared to other samples containing either a single nucleator or actin alone. Moreover, the lag phase that represents initial actin nucleation was not observed in samples containing Tarp. This suggests that Tarp and Arp2/3 complex cooperate to promote actin polymerization. To test whether phosphorylation alters the ability of Tarp to cooperate with Arp2/3 complex, Tarp was phosphorylated using commercially purified Fyn kinase. Phosphorylation of Tarp was confirmed by western blot analysis using phosphotyrosine antibody (**Fig.17C**) and actin polymerization kinetics of phosphorylated Tarp were assessed in combination with activated Arp2/3 complex. Phosphorylated Tarp retained the ability to cooperate with activated Arp2/3 complex (**Fig. 17D**). To test whether Tarp or phosphorylated Tarp can directly activate Arp2/3 complex, an actin polymerization assay was performed using Tarp or phosphorylated Tarp in presence of inactive Arp2/3 complex (Arp2/3-VCA). No alteration in the kinetics of actin polymerization was observed in samples containing both Tarp (phosphorylated or not) and inactive Arp2/3 complex as compared to Tarp or phosphorylated Tarp alone (**Fig. 17B and 17D**). Finally, to visualize the patterns of actin filaments generated by actin alone or in presence of either single or double nucleators, an actin polymerization assay was run for 10 minutes and actin filaments were then visualized using scanning electron

microscopy. Actin filaments in samples containing Tarp and activated Arp2/3 showed more branching patterns as compared to samples containing either actin alone or single nucleators (**Fig. 17E**). Overall, this data suggests that Tarp and Arp2/3 complex cooperates to promote actin polymerization, but Tarp does not directly activate Arp2/3 complex.

3.3.3 Ectopically expressed eGFP-Tarp Co-localize with F-Actin, but not with Arp2/3 Complex

Tarp co-localization with actin has been studied in the past [243]. Full length and C-terminal domain of Tarp were found to co-localize with actin aggregates [243]. Later, the ABD in the C-terminal domain of Tarp was identified as the site responsible for binding actin and causing actin aggregation [64]. Phosphorylated Tarp peptide was previously demonstrated to interact with recruitment of SOS and Vav, a guanine nucleotide exchange factor (GEF) responsible for Rac GTPase activation [312]. To examine whether phosphorylated Tarp can recruit Arp2/3 complex to alter actin dynamics in vivo, we constructed mammalian expression plasmids with EGFP-fusions of full length Tarp, the N-terminal mutant harboring the phosD and PRD domains ($Tarp^{1-748}$), and the C-terminal mutant harboring the PRD and ABD domains ($Tarp^{625-1005}$)(**Fig 18A**). The mutants were specifically engineered to study the individual contribution of the actin binding domain and the phosphorylation domain of Tarp in regulating actin dynamics. It was previously demonstrated that expression of the phosphorylation domain without PRD does not form aggregates and appears to co-localize in the

nucleus [306]. Thus, the mutants were designed to retain the PRD domain responsible for Tarp aggregation. Following 24 hour transfection in HeLa cells, we fixed and stained cells to detect Arp2/3 complex, actin, and also to study the phosphorylation pattern of Tarp protein. The full length Tarp formed EGFP aggregates, was phosphorylated, and co-localized with actin (**Fig 18B and 18C**). The mutant with the C-terminal half, harboring PRD and ABD, formed EGFP aggregates that were not phosphorylated but co-localized with actin (**Fig 18B and 18C**). Interestingly, the mutant with the N-terminal half of Tarp, harboring the phosphorylation domain and PRD, formed intracytoplasmic EGFP aggregates that appeared to be phosphorylated but failed to co-localize with actin (**Fig 18B and 18C**). Furthermore, neither the full length nor the mutant Tarp constructs were able to recruit Arp2/3 complex (**Fig. 18C**).

3.4 Discussion

Invasion of bacteria is a complex and highly regulated process that requires coordinated rearrangement of host actin cytoskeleton. In chlamydial entry, such cytoskeletal rearrangement requires the activity of the chlamydial and the host actin nucleator, Tarp and Arp2/3 complex respectively [307, 312]. Previous studies employed RNA interference and dominant negative overexpression of Arp2/3 activators to confirm the role of Arp2/3 complex in chlamydial entry. Recently, specific inhibitors of Arp2/3 complex CD-666 and CD-869 have been described [399]. Since pharmacological inhibition is a relatively simple, cheap, and fast technique to use as compared to gene manipulation, we decided to test the effects of these inhibitors on entry of chlamydia.

CD-666 and CD-869 mediated inhibition of Arp2/3 complex abrogated chlamydial entry as compared to their control counter parts in a statistically significant manner. This data further confirms the role of the Arp2/3 complex in the entry of chlamydia and that these inhibitors can be used as a scientific tool to elucidate chlamydial entry mechanisms in future studies. Interestingly, Jasplakinolide, a compound that stabilizes filamentous actin and prevents depolymerization, also inhibited chlamydial entry [401]. This suggests that chlamydial entry is a product of the coordinated rearrangement of the actin cytoskeleton requiring spatially and temporally restricted patterns of actin polymerization and depolymerization. Consistently, a chlamydial effector protein CT166 was recently identified and implicated in inhibition of Rac GTPases [244].

Because Tarp and Arp2/3 are both essential for chlamydial entry, it was previously hypothesized that these two nucleators may cooperate to cause efficient chlamydial uptake. However, the molecular mechanisms of such cooperation between Tarp and Arp2/3 complex were not known. One possibility was that the Arp2/3 complex activity might be essential in priming the host cells for delivery of effector proteins by chlamydia, a mechanism which is recently identified to be essential for effector delivery by *E. coli* [402]. However, *Chlamydia* retained the ability to deliver Tarp in host cells pharmacologically inactivated for the Arp2/3 complex (**Fig 16B**). This suggests that Arp2/3 complex activation plays a critical role in entry mechanism following delivery of effector protein Tarp.

Previous evidence implicates the distinct patterns of actin filaments generated by Tarp and Arp2/3 complex activity. Tarp binds and nucleates actin monomers to form linear actin filaments, whereas Arp2/3 complex interacts with pre-existing linear filaments and branch it at 70^0 angles [64, 403]. Thus, it is possible that Tarp activity provides linear actin filaments as a substrate for Arp2/3 complex activity, which together results in the rapid rearrangement of the actin necessary for efficient chlamydial uptake. Indeed, in an in vitro actin polymerization assay when Tarp and activated Arp2/3 complex were added together, the rate of actin polymerization was increased as compared to samples containing solely these individual nucleators. Scanning electron microscopy further confirmed these findings as extensive branching patterns were observed when Tarp and Arp2/3 were added together. One of the ways in which the functions of many proteins are altered is via the addition or removal of the phosphate moiety. Since Tarp is phosphorylated inside the host cytoplasm, we were interested in studying the effects of phosphorylation on the ability of Tarp to cooperate with Arp2/3 complex. Tarp proteins phosphorylated with Fyn kinase retained the ability to cooperate with Arp2/3 complex. Together, our data provides the first biochemical evidence indicating that Tarp and Arp2/3 complex cooperate to increase the rate of actin polymerization.

While Tarp is inherently active, the Arp2/3 complex is highly regulated and requires activation by nucleation promoting factors (NPF) like WASP and WAVE [404]. Many eukaryotic signaling cascades that are initiated following receptor-ligand

interaction converge to regulate the interaction between NPF and Arp2/3 complex. Interestingly, some bacterial effector proteins, like Act A in *L. monocytogenes* and RickA in *R.coronii,* mimic the activity of NPF to cause direct activation of Arp2/3 complex, whereas other bacterial effectors in *E.coli*, Salmonella and Shigella promotes activation of Arp2/3 complex by influencing upstream host signaling proteins like Rho family GTPases [65, 405-408]. Whether or not Tarp exhibits NPF activity has never been tested. Our actin polymerization assay demonstrated that addition of Tarp (either phosphorylated or not) and Arp2/3complex in the absence of known NPF produces no change in actin polymerization kinetics as compared to Tarp alone. This data clearly indicates that Tarp alone is not efficient to activate Arp 2/3 complex. Previous evidences also implicate the role of phosphorylated Tarp in the recruitment of Sos1 and Vav2, two guanine nucleotide exchange factors, which in turn participate in WAVE2-Arp2/3 complex recruitment and activation. To examine whether Tarp, via its phosphorylation domain, indirectly regulates Arp2/3 activation to alter actin dynamics in vivo, we ectopically expressed an EGFP fusion of Tarp harboring the phosphorylation and proline rich domains, but missing the actin binding domain, in HeLa cells. Our data demonstrates that although EGFP fusion proteins were phosphorylated, they failed to co-localize with Arp2/3 complex or actin. Such discrepancy with the previous observations could be due to different scientific techniques applied to study function of Tarp. Previous observations were based on the ectopic expression of a single tyrosine rich repeat of the phosphorylation domain in Tarp, whereas we had engineered Tarp

constructs so that they retain the entire phosphorylation domain as it is found in the full length Tarp protein. Moreover, we also did not observe the recruitment of Arp2/3 complex in HeLa cells that expressed full length or the C-terminal domain of Tarp protein, while these constructs retained the capacity to recruit actin. Together this data suggests that Tarps ability to alter actin dynamics is solely a function of the C-terminal domain of Tarp and that phosphorylated Tarp is not responsible for Arp2/3 activation or actin remodeling.

Collectively, we have demonstrated that Arp2/3 complex inhibitors CK-666 and CK-869 can be used as pharmacological tools in studying the mechanism of chlamydial entry. Importantly, we demonstrated for the first time that Tarp and Arp2/3 complex cooperate to increase the rate of actin polymerization. Furthermore, Tarp plays no role in the activation of Arp2/3 complex. While the signaling event responsible for activation of Arp2/3 complex is still a mystery, recent evidences implicate the role of fibroblast growth factor receptors (FGFR) in chlamydial entry [238]. FGFR mediated signaling can activate Arp2/3 complex via Ras GTPase activating like protein 1 (IQGAP-1) in other systems [409]. The role of IQGAP-1 in chlamydial entry is not yet studied. Whether Arp2/3 complex activation is the result of FGFR mediated signaling and/or some other host signaling event(s) remains to be determined.

3.5 Figures

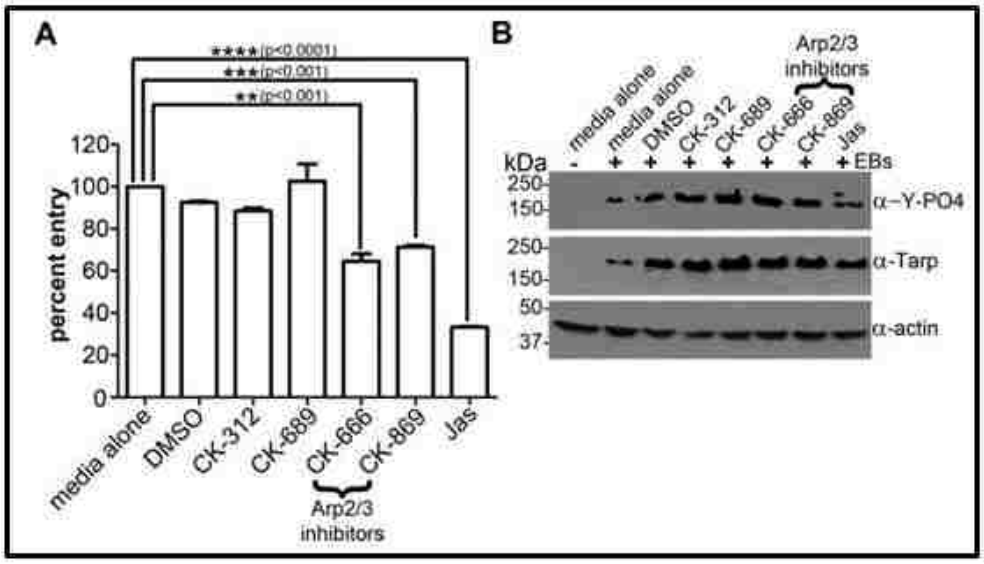

Figure 16 Arp2/3 complex is required for entry of Chlamydia trachomatis.
Graphical representation of *C. trachomatis* EB invasion assay in HeLa cells pharmacologically treated with Arp2/3 complex inhibitors CK-666 and CK-869. HeLa cells were treated for one and a half hours at 37^0C with 200 uM concentration of either Arp2/3 complex inhibitors (CK-666 and CK-869), control inhibitor (CK-312 and CK-689) or Jasplakinolide (Jaz, an actin stabilizer). Invasion assay was performed using CMPTX labeled florescent EBs (Red). After allowing 30 minutes of invasion, extracellular EBs were counterstained with antibodies targeting MOMP (L2) and anti-mouse antibody conjugated to Alexa 488 (Green). EB entry was determined as percent invasion. Media alone or DMSO served as an additional negative control. Graph represents cumulative result from three independent experiments (B) Delivery of Tarp into host cells is not affected by Arp2/3 complex inhibitor. Protein samples collected from drug treated host cells identical to those described above were infected with *C. trachomatis* L2 at an MOI of 1000 and were examined by Western blot analysis. Phosphotyrosine antibody was used to detect intracytoplasmic phosphorylated Tarp. Tarp antibody was used to detect total Tarp protein and Actin blot served as loading control.

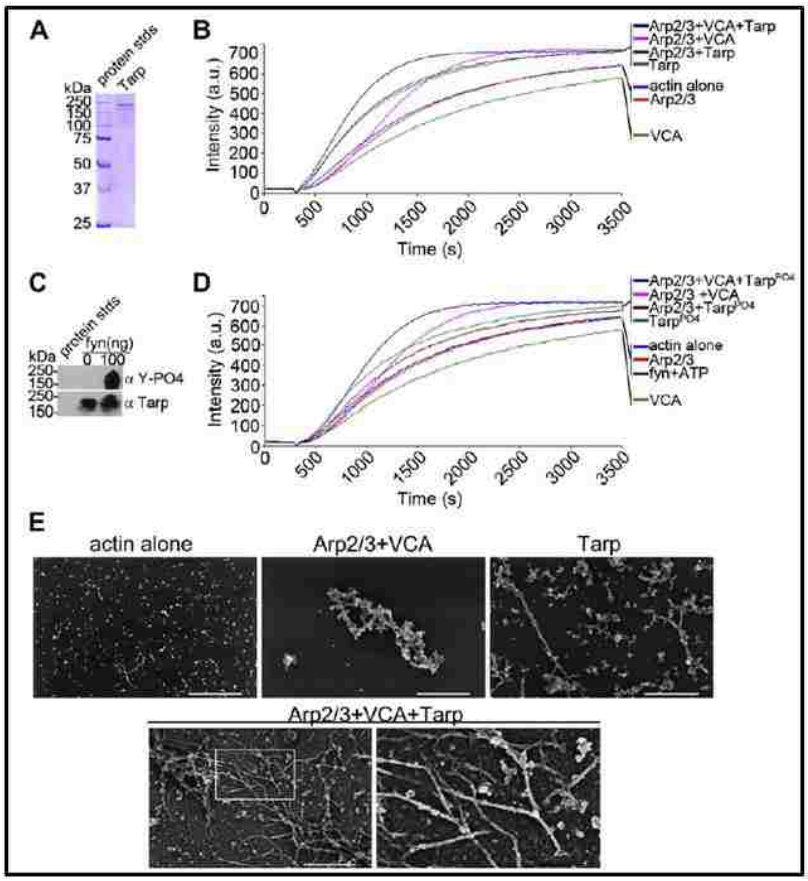

Figure 17 Tarp co-operates, but does not activate Arp2/3 complex to polymerize actin.

(A) Dual Tag (N-terminal GST and C-terminal poly histidine) recombinant full length Tarp protein (Tarp) was double purified using gluthathione sepharose and nickel beads and subsequently resolved on 10% SDS page gel to analyze for purity and quantity by staining with coomassie blue. The same quantity of protein represented in the figure was used for subsequent analysis. (B) Actin polymerization assay was performed by mixing 1 mM of fluorescently labeled pyerene conjugated actin with Tarp, activated Arp2/3 complex (Arp2/3 + VCA), Tarp and activated Arp2/3(Tarp+ Arp2/3+VCA), or Tarp and Arp2/3 (Tarp+ Arp2/3). The rate of actin polymerization was measured by the increase in fluorescence intensity (Intensity a. u.) over 1hr following addition of polymerization buffer at 300 sec. Actin alone or addition of GST, VCA, or Arp2/3 served as controls. (C) Tarp was phosphorylated by incubating Tarp with purified Fyn kinase and ATP at 37^0C for 5 mins. The phosphorylation of Tarp was confirmed by performing SDS-PAGE and western blot analysis using phosphotyrosine specific and total Tarp antibodies. (D) Actin polymerization assay was performed exactly as described in (B) except using phosphorylated Tarp ($Tarp^{PO4}$) instead of Tarp.

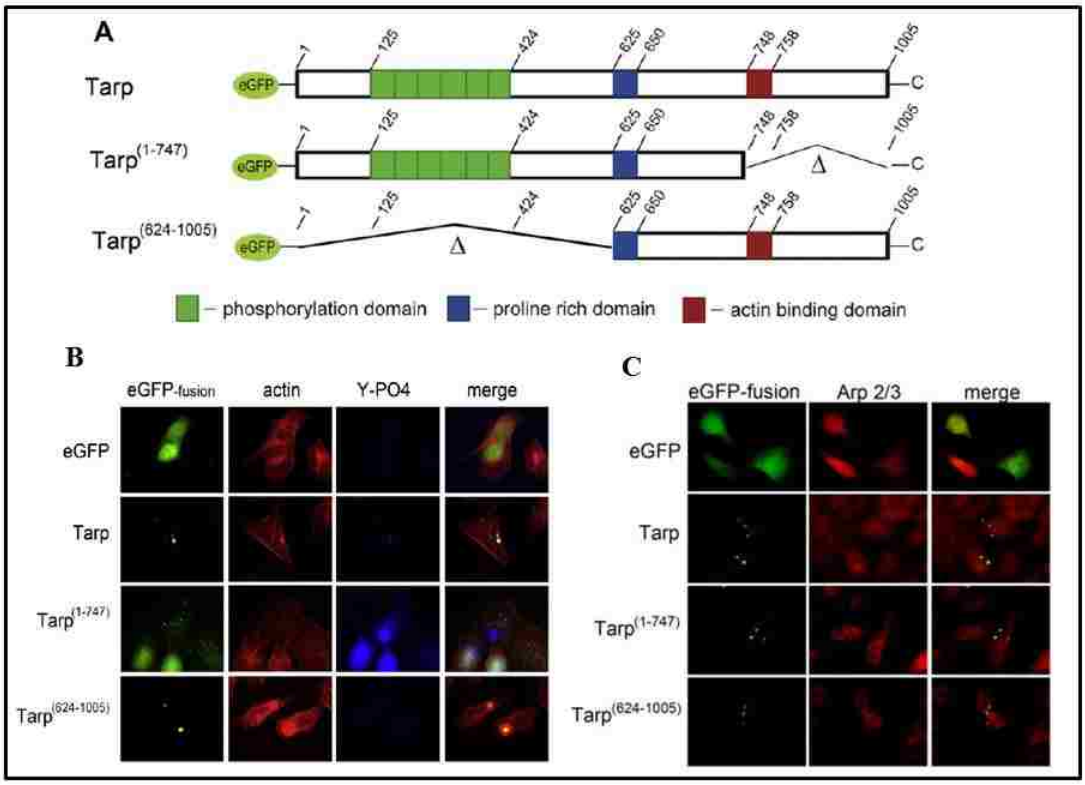

Figure 18 Ectopically expressed egfp-Tarp co-localizes with actin, but not with Arp2/3 complex.

(A) Schematics of EGFP-fusion of full length (Tarp), actin binding domain deletion mutant ($Tarp^{(1-747)}$) and phosphorylation domain deletion mutant ($Tarp^{(624-1005)}$) of Tarp protein used for immunofluorescence Assay. The domains of Tarp are color coded as follows: phosphorylation domain (green box), proline rich domain (blue box) and actin binding domain (red box). Δ represents amino acid deletion and number represents amino acid position in Tarp polypeptide. (B) HeLa cells were transfected with EGFP-fusions of full length Tarp, $Tarp^{(1-747)}$, and $Tarp^{(624-1005)}$ for 24 hours, followed by fixation and staining with Alexa-568 conjugated phalloidin (Red) and phosphotyrosine specific primary and corresponding Alexa-350 conjugated secondary antibodies (Blue). (C) Similar transfection as described in B was performed and samples were stained with primary Arp 3 and corresponding Alexa-568 conjugated secondary antibodies. Images were obtained using an AxioCam MRm camera controlled by AxioVision 4.8.2 and further processed using Adobe Photoshop CS2.

CHAPTER FOUR: *CHLAMYDIA TRACHOMATIS* TARP HARBORS DISTINCT G AND F ACTIN BINDING DOMAINS WHICH BUNDLE ACTIN FILAMENTS.

4.1 Introduction

The obligate intracellular bacterium, *Chlamydia trachomatis,* is the most frequently reported sexually transmitted bacterial disease in the United States with over one million cases reported to the Centers for Disease Control annually. Worldwide, ocular infection with *C. trachomatis* (trachoma) is the leading cause of preventable blindness and is the subject of a global initiative led by the World Health Organization to eradicate Trachoma by 2020[410].

Species of *Chlamydia* utilize a unique developmental cycle in which bacteria transition from the infectious spore-like elementary body (EB) to the metabolically active reticulate body (RB) within the protective confines of a membrane bound parasitophorous vacuole termed the inclusion [223]. The invasive EB is formed in the mid to late stages of the intracellular development cycle as the RBs differentiate back to EBs and are packed with metabolites and proteins designed to facilitate extracellular survival and reinfections. [224, 225]Additional infectious cycles arise from EBs that are released and disseminate from infected tissues [226].

C. trachomatis invasion is induced by cytoskeletal rearrangements initiated upon microbe contact with the host cell surface [292]. Alterations of the host cytoskeleton are required for bacterial uptake as drugs such as Cytochalasin D which disrupt the cytoskeleton prevent *C. trachomatis* infections [292]. A number of intracellular

microorganisms harbor proteins that directly alter actin dynamics which favor pathogen survival and propagation [411]. These virulence factors can drive the formation of actin filaments, and actin bundles or can lead to the disassembly of actin filaments. Cytoskeletal rearrangements initiated upon EB contact with the host cell surface may in part be triggered by the translocation of type III secreted effectors [242, 243]. One of the effector proteins called **t**ranslocated **a**ctin **r**ecruiting **p**rotein (Tarp) is able to increase the rate of actin filament formation by directly nucleating actin [64]. In addition, Tarp and the host cell Arp2/3 actin nucleating complex cooperate to increase the rate of actin filament formation and both host and bacterial derived actin nucleators are implicated in *C. trachomatis* invasion [296, 307, 412].

Tarp contains a C- terminal actin binding and oligomerization domain required for actin nucleation and a N-terminal phosphorylation domain implicated in host cell signaling via association with host derived proteins such as phosphoinositide 3-kinase (PI3K) and Src homology 2 (SH2) domain containing transforming protein 1 (SHC-1) [64, 306, 312, 398]. Phosphorylated Tarp peptides have also been shown to immunoprecipitate a complex of proteins containing Sos1, Vav2, two Rac guanine nucleotide exchange factors thought to participate in WAVE2 and Arp2/3 complex recruitment [312]. Co-localization studies of ectopically expressed eGFP-Tarp indicate that actin filament recruitment is restricted to the C-terminal half of the effector and is presumably associated with Tarp via the previously identified actin binding alpha helix required for actin nucleation *in vitro* [306, 412]. In this work we examined the effect of

domain specific mutations on actin filament co-localization with eGFP-Tarp. Herein we describe that *C. trachomatis* L2 Tarp harbors two distinct F-actin binding sites that allow the Tarp effector to bundle actin filaments. Furthermore, Tarp mediated actin bundling did not require actin nucleation as the ability to bundle actin filaments was observed in mutant Tarp proteins deficient in actin nucleation. These findings attribute a novel activity to the critical Tarp protein and shed molecular insight into the complex cytoskeletal rearrangements required for *C. trachomatis* entry into host cells.

4.2 Material and Methods

4.2.1 Cloning and Protein Expression

In-frame amino-terminal GST and carboxyl-terminal polyhistidine fusion proteins for full-length wild-type Tarp was generated by PCR by amplifying the corresponding coding regions from *Chlamydia trachomatis* servovar L2 LGV 434 genomic DNA (Qiagen genomic purification kit, Valencia, CA) as previously described using custom synthesized oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) engineered with SalI, SacI or NotI linkers. PCR products were purified (Qiagen), digested with restriction enzymes (New England BioLabs, Beverly, MA), and subcloned into linearized pGEX-6P-1 to generate translational fusions with GST and polyhistidine. Tarp domain deletion mutants: phosphorylation domain deletion (ΔphoD =Δ D125-Y424), proline rich domain deletion (ΔPRD = ΔS625-N650), actin binding domain deletion (ΔABD = Δ748-758), F-actin binding domain deletion 1 (ΔFAB1 = ΔL871-L882) and F-actin binding domain deletion 2 (ΔFAB2 = ΔN942-G967) were generated by

inverse PCR by amplifying the pGEX-6P-1 plasmids encoding the wild type Tarp fusion protein. Multiple domain deletions in a single *tarP* gene (for example: ΔABD, ΔFAB1 & ΔFAB2) were generated sequentially by inverse PCR or by ligating individual deletion mutants together. The Tarp mutants described above were also cloned into pEGFP-C3 (BD Biosciences Clontech) to allow for ectopic expression of eGFP-Tarp in HeLa cells. All pGEX-6P-1 plasmids were transformed into the BL21 strain of *Escherichia coli* (Novagen, Madison, WI). Protein expression and purification were performed according to the procedures outlined for the Ni Sepharose 6 Fast Flow and Glutathione Sepharose 4B in the Bulk GST Purification Module (GE health sciences, Piscataway, NY).

4.2.2 GST Fusion Pull-Down Experiment

HeLa 229 cells were suspended in 100mM KCl, 10mM HEPES (pH 7.7), 2mM $MgCl_2$ and 2mM ATP (buffer A) and disrupted by sonication delivered in four consecutive bursts of 20 second intervals on setting #4 (ultrasonic sonicator processor XL equipped with microtip: Misonix Incorporated, Farmingdale, NY). Insoluble material was removed by centrifugation (12,000 rcf, 25 min., 4^{o}C). Glutathione-sepharose beads were incubated with 10mg of GST fusion proteins or GST for 1 hour at 4^{o}C in PBS. (GE health sciences). GST-fusion protein coated beads were washed twice with PBS and once with buffer A prior to the addition of approximately 100mg of HeLa extract. Extracts and beads were incubated together for 2 hours at 4^{o}C, washed three times with fresh buffer A and bound proteins were eluted using sample buffer.

4.2.3 SDS-PAGE and Immunoblotting

Proteins were separated on SDS-10% polyacrylamide gels and transferred to 0.45□m pure nitrocellulose transfer and immobilization membrane (Schleicher & Schuell, Keene, NH). Immunoblotting employed peroxidase conjugated secondary antibodies (Chemicon International, Temecula, CA) and Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). The anti-actin C4 monoclonal antibody was purchased from Chemicon International. The anti-phosphotyrosine 4G10 monoclonal antibody was purchased from Upstate (Millipore). Polyclonal rabbit antibodies directed towards Chlamydia trachomatis L2 LGV 434 Tarp (CT456) was developed at Rocky Mountain Laboratories as previously described [243]. Peptide antibodies directed toward the Tarp actin binding domain and proline rich domain were generated and purified by Sigma Genosys (Spring, TX) as previously described [307].

4.2.4 Transfection of Hela Cells and Indirect Immunofluorescence Microscopy

Hela cells (2×10^5) were seeded in 6 well plates with coverslips and grown for 24 hours in DMEM containing 10% FBS. Cells were then transfected with transfection mixture containing 8 □l of Fugene HD (promega) and 2.5 mg of respective plasmid. Following 24 hours, cells were fixed by adding 4 % paraformaldehyde and incubating at 4^0 C for 15 minutes. Cells were then treated with ice cold 0.4 % Triton-X for 10 minutes, followed by blocking with 5% BSA for 45 minutes. To visualize tyrosine phosphorylated protein, cells were first incubated with anti-phospho tyrosine primary antibody (upstate) at 1:1000 dilutions in 0.5 % BSA at RT for 45 minutes followed by incubation with anti-

mouse secondary antibody conjugated to Alexa 350 (invitrogen). To simultaneously visualize actin, phalloidin conjugated to Alexa 568 (invitrogen) was added to the above mixture containing secondary antibodies. To stain for Arp2/3 complex, cells were first incubated with anti-Arp3 primary antibody (upstate) at 1:100 dilutions in 0.5 % BSA at RT for 45 minutes followed by incubation with anti- rabbit secondary antibody conjugated to Alexa 594 (invitrogen). Coverslips were rinsed and mounted in Prolong Gold antifade reagent (invitrogen). Cells were examined with a Zeiss Axio Observer A1 microscope equipped with phase contrast and epifluorescence optics. Images were obtained using an AxioCam MRm camera controlled by AxioVision 4.8.2 and further processed using Adobe Photoshop CS2.

4.2.5 F-Actin Binding and Bundling

Briefly, 5 µg of GST fusion proteins or control proteins (GST and a-actinin) were added to 40 µg of filamentous actin [F actin; generated by adding 1/10th volume of polymerization buffer to G actin and incubating at room temperature (RT) for 1 h] and allowed to incubate at RT for 30 min. F actin and bound proteins were separated by differential sedimentation at 100,000 ´ *g* for 2 h at RT in a Beckman Optima TLX Ultracentrifuge using a TLA 55 rotor (Beckman Coulter, Fullerton, CA). Proteins associated with the F actin pellets were compared to unbound proteins that remained in the supernatant by resolving proteins on 10% SDS-polyacrylamide gels followed by Coomassie staining.

4.2.6 G Actin Binding Assay

Monomeric actin was prepared by diluting 100 mg of lyophilized rabbit muscle actin (Cytoskeleton, Denver, CO) into 2 ml of 5 mM Tris (pH 8.0)/0.2 mM $CaCl_2$/0.2 mM ATP/0.1% Tween 20 (G buffer with tween) and incubating for 1 h at RT. Monomeric actin was obtained by collecting the supernatant after a 2-h 100,000 ´*g* 4°C spin in a Beckman Optima TLX Ultracentrifuge using a TLA 55 rotor (Beckman Coulter). Monomeric actin was incubated with »5 mg of GST fusion proteins immobilized to glutathione Sepharose beads (prepared as described above). After gentle mixing for 2 h at 4°C, beads were washed three times with fresh G buffer, and bound proteins were eluted using sample buffer.

4.2.7 Actin Polymerization Assay

Briefly, monomeric pyrene labeled actin was prepared by diluting 100 mg of lyophilized pyrene actin (Cytoskeleton) into 2 ml of 5 mM Tris (pH 8.0)/0.2 mM $CaCl_2$/0.2 mM ATP (G buffer) and incubating for 1 h at RT followed by 1 additional hour of incubation at 4°C. Monomeric pyrene actin was obtained by collecting the supernatant after a 2-h 100,000 ´*g* 4°C spin in a Beckman Optima TLX Ultracentrifuge using a TLA 55 rotor (Beckman Coulter). Approximately 20 mg of pyrene-labeled actin was gently mixed with 5 mg of GST fusion proteins in a volume of 500 ml for 10 min before the addition of 1/20th volume of polymerization buffer (500 mM KCl/20 mM $MgCl_2$/10 mM ATP). The reaction was monitored over 1 hr with an LS 50B Luminescence spectrophotometer directed by FL WinLab software version 4.0 (Perkin-

Elmer, Beaconsfield, Bucks, UK) with 2.5-nm bandwidth at 365-nm excitation wavelength and 2.5-nm bandwidth at 407-nm emission wavelength.

4.3 Results

4.3.1 Mutant Tarp Proteins Exhibit Unique Actin Binding and Polymerization Kinetics.

The actin nucleating activity of Tarp results from distinct actin binding and proline rich oligomerization domains *in vitro* [64] and potentially from a signaling cascade involving the recruitment and activation of Vav2, Sos1, Eps8, Abi1, Rac1 and WAVE2 *in vivo* [296, 299, 312]. *C. trachomatis* L2 Tarp is a large 1005 amino acid protein and studies to date have primarily focused on recombinant Tarp truncation mutants or Tarp peptides to identify the domains of the protein responsible for Tarp mediated actin polymerization [64, 243, 307]. To confirm that the previously identified domains were sufficient for actin binding and actin nucleation in the entire Tarp effector we have generated a series of GST and eGFP recombinant full length L2 Tarp deletion mutants that are missing the phosphorylation, the actin binding and/or the proline rich oligomerization domain to examine the contribution that each domain has on actin kinetics biochemically and in HeLa cells (**Fig. 19A**). Wild type Tarp and deletion mutant Tarp proteins with dual N-terminal GST and C-terminal poly histidine affinity tags were purified and employed in actin binding and actin polymerization assays (**Fig. 19B, 19C**). All full length Tarp mutants were able to associate with host cell actin in a GST–pulldown assay except for the ΔABD Tarp mutant harboring an 11 amino acid deletion

(aa 748-758) of the previously characterized actin binding domain (**Fig. 19B**) [64]. The purified Tarp proteins also demonstrated distinct actin polymerization kinetics as observed in *in vitro* pyrene actin polymerization assays (**Fig. 19C**). An increase in the rate of actin polymerization was observed in wild type Tarp and the Tarp effector harboring a deletion in the phosphorylation domain compared to actin alone controls which is in agreement with reports localizing the Tarp actin nucleating activity to the C-terminal half of the protein [64, 243, 307, 412]. Consistent with previous studies, removal of a short proline rich domain of 25 amino acids implicated in oligomerization activity was also required for actin nucleation (**Fig. 19C**) [64]. Surprisingly, when actin filament co-localization with eGFP-Tarps was examined in HeLa cells with alexa-conjugated phalloidin, Tarp mutants lacking the actin binding domain (Tarp ΔABD) retained the ability to co-localize with actin filaments (**Fig. 19D**). A Tarp eGFP fusion lacking the amino acids 748-1005 ($Tarp^{1-747}$) did not co-localize with phalloidin and served as a negative control (**Fig.19D**). eGFP-Tarp harboring mutations in both the phosphorylation and actin binding domain simultaneously also were able to co-localize with actin filaments (data not shown). These data suggest that Tarp may harbor an as yet uncharacterized F-actin binding domain(s) distinct from the previously characterized G-actin binding domain that is essential for actin nucleation.

4.3.2 C. Trachomatis L2 Tarp Harbors A Distinct F-Actin Binding Domain.

Tarp orthologs contain between one and four actin binding domains according to GST-pull down assays performed with HeLa extracts [307]. Previous reports have

indicated that *C. trachomatis* L2 Tarp harbors one actin binding domain which is consistent with the data presented in figure 19[64, 307]. Deletion of 11 amino acids contained within the L2 Tarp actin binding domain was sufficient to prevent actin binding in the GST-pull down assay compared to the wild type control. Interestingly a second putative actin binding domain which is similar in sequence to the characterized L2 Tarp actin binding domain was previously identified bioinformatically but was unable to associate with host cell actin in a GST-pull down assay [307]. Our contradictory findings suggested that Tarp may harbor protein domains which differentiate between monomeric (globular) actin found predominately in the HeLa generated protein lysates and filamentous actin detected by fluorescent phalloidin in the transfected host cells. In order to examine whether the second actin binding-like domain found in *C. trachomatis* L2 Tarp sequence was able to differentially associate with globular versus filamentous actin, GST-Tarp fusions to this domain was tested for the ability to associate with actin generated from HeLa lysates in a GST-pulldown and an F-actin co-sedimentation binding assay (**Fig. 20**). Similar to our previous findings, the 100 amino acid peptide harboring the original actin binding domain was able to associate with actin generated from a HeLa lysate, however, the additional putative sequence did not associate with actin generated from the same lysate (**Fig. 20A**) [64, 307]. Interestingly the two domains did co-sediment with filamentous actin indicating the second domain preferentially associates with filamentous actin while the original actin binding domain is able to associate with both monomeric and filamentous actin as previously described (**Fig.20B**)

[64]. Since the second actin binding site prefers F-actin we have called this site the F-actin binding site (FAB1) to differentiate it from the originally characterized actin binding domain (ABD).

4.3.3 Tarp Harbors Multiple F-Actin Binding Sites

Identification of an alternate F-actin binding site in Tarp was examined with a GST fusion to 115 amino acids of Tarp representing the region of the protein adjacent to the previously characterized actin binding domain. This domain contains a 13 amino acid peptide with similarity to the described ABD which is also predicted to form the alpha helix secondary structure required for actin binding. Further secondary structure predictions of the c-terminal domain of Tarp revealed an additional alpha helix located between amino acid 942-967 although this peptide does not share sequence similarity with the former two sites (data not shown). In order to determine if the ABD and two additional alpha helical domains were responsible for co-localization with filamentous actin in tissue culture cells, an eGFP fusion to Tarp harboring all three alpha helical deletions was tested for actin co-localization (**Fig. 21**). An eGFP-Tarp fusion harboring the single ABD and an eGFP-Tarp fusion harboring a double mutant ABD and the first putative F-actin binding site were also tested. As previously observed the ΔABD mutant was able to co-localize with actin filaments as was the double mutant harboring both the ΔABD and a deletion in the first alpha helix (ΔFAB1). Interestingly when all three sites were removed from Tarp, F-actin co-localization was not observed suggesting that all three sites may serve as F-actin binding domains (**Fig. 21C**). We have termed the last

alpha helix the “F-actin binding domain 2” (FAB2). To confirm the eGFP co-localization results, GST fusions of Tarp harboring deletions in ABD, FAB1 and FAB2 were used to biochemically examine co-sedimentation of actin filaments with recombinant proteins. Consistent with the eGFP results, Tarp proteins (with the GST removed) demonstrated a reduction in their ability to co-sediment with preformed actin filaments as each domain of the protein was removed. This could be observed as both a reduction in the quantity of Tarp mutants fractioned to the pellet, and an increase in the Tarp proteins retained in the supernatant (**Fig. 21B**). Tarp proteins lacking all three actin binding sites showed the least F-actin binding (**Fig. 21B**).

4.3.4 Tarp Bundles Actin Filaments

Tarp has previously been shown to function as an actin nucleator [64]. The actin nucleating activity was localized to a 200 amino acid region of the Tarp protein sequence that was found to contain a proline rich region responsible for protein oligomerization and a solitary actin binding domain [64]. This actin binding domain was able to associate with monomeric and filamentous actin [64]. In light of the identification of two additional F-actin binding domains we sought to examine whether Tarp was capable of bundling actin filaments. Actin bundles sediment at a faster rate compared to actin filaments and monomeric actin. Therefore, proteins capable of bundling actin filaments will appear in the pellet upon a low speed centrifugation. Interestingly, Tarp was capable of bundling actin filaments (**Fig. 20A**) and actin bundling was not dependent on Tarp mediated actin nucleation as the Tarp ΔPRD mutant which fails to

nucleate actin in vitro (**Fig. 19**) retained actin bundling activity (**Fig. 22B**). The Tarp triple mutant lacking the ABD, FAB1 and FAB2 alpha helices was unable to bundle actin filaments which is consistent with both the eGFP co-localization and F-actin co-sedimentation results (**Fig. 22A**).

4.4 Discussion

The Tarp effector is a multifunctional protein which primes the host cell for bacterial entry and residence. We now demonstrate that Tarp harbors distinct G-actin binding and nucleating as well as F-actin binding and bundling activity. All three domains are similar in that they mediate a direct link to the host cytoskeleton yet biochemically they are distinct sites that differentially associate with globular or filamentous actin.

EB attachment to the surface of an epithelial cell ultimately results in the formation of an actin rich pedestal at the site of contact and is associated with bacterium invasion [292]. Actin filament destabilizing drugs such as cytochalasin D inhibit the formation of these projections and subsequent uptake of *C. trachomatis* [292]. The arrangement of the actin filaments within the pedestal is unknown, but presumably the actin filaments form polarized actin bundles (actin filaments sharing the same orientation with relation to their barbed (+) and pointed ends (-)) similar to those characterized in microvilli and filopodia [413]. Actin bundling proteins such as fascin-1 co-localize with filopodia on the leading edge of the growth cone of developing nerve cells and are implicated in the formation of actin bundles [414]. Similarly, Tarp may

serve a role in the formation of actin bundles located directly beneath the invading microbe. *C. trachomatis* entry into host cells *in vitro* is temperature dependent and involves the recruitment of actin to the site of EB attachment [292]. Once internalized, the recruited actin quickly disseminates. The molecular details of actin disassembly are not well defined, and it is possible that actin depolymerization itself drives EB entry. Actin filament stabilizing drugs such as Jasplakinolide (Jas) inhibit EB entry [299, 412], although the effects of Jas on actin filaments *in vivo* is controversial as changes in cell morphology that are consistent with a reduction in filamentous actin are observed in some Jas treated cells[401].

Similar to Tarp, the *Salmonella enterica serovar* Typhimurium SipC effector is able to nucleate actin and bundle actin filaments [308, 415]. Recently, mutant bacteria lacking the C-terminal region of SipC responsible for F-actin binding and bundling were found to be less invasive compared to wild type *Salmonella* suggesting that the bundling activity of SipC plays a role in pathogen entry into HeLa cells [8]. Whether Tarp's ability to bundle actin filaments also contributes to pathogen entry is unknown but worthy of investigation as new molecular tools continue to be developed to examine the genetic requirements of *C. trachomatis* pathogenicity [416, 417].

Actin bundles are tightly controlled by a variety of actin binding proteins (ABPs) which drive specific cytoskeletal processes and result in actin assemblies of defined thickness, length and organization. The architecture of Tarp mediated actin bundles has not been investigated and it will be intriguing to compare them to bundles formed in the

presence of other bacterial effectors such as SipC. It is possible that evolutionally divergent bacterial species employing unique effector proteins can create similar actin bundles for the same purpose. Whether actin bundles actually contribute to microvilli morphogenesis is controversial as a recent study has demonstrated that mice deficient in three actin bundling proteins, villin, espin and plastin-1still developed microvilli albeit with slightly altered actin architecture.

Following translocation into host cytoplasm, Tarp gets phosphorylated at its n-terminal phosphorylation domain by variety of host cell kinases including Src, Abl and Syk[306, 309]. In vitro analysis has revealed the ability of phosphorylated Tarp to interact with SH2 domain containing proteins including PI3-Kinase and SHC1 adaptor protein [398]. However, if phosphorylation or protein interaction has any effect on Tarp's ability to influence actin dynamics is not clearly understood. Post-translational modifications like phosphorylation, binding of lipid second messengers or calcium are intrinsic mechanism by which eukaryotic cell regulate the function of many proteins including cytoskeleton regulatory proteins. For examples, L-plastin loses its ability to bundle actin in its calcium bound conformation [418]. Synapsin, another actin bundling protein, shows reduce capacity to bundle actin when phosphorylated by calmodulin dependent kinase II [419]. Also, phosphorylation of filamin by CaM kinase II decreases filamin's actin cross linking activity [420]. We have previously shown that the ability of Tarp to interacts directly with actin is restricted to its c-terminal half that harbors actin binding domain. We also demonstrated that Tarp retains the ability to nucleate actin in

its phosphorylated form [412]. It will be interesting to determine if phosphorylation alters the actin bundling activity of Tarp.

Collectively, our data demonstrates that Tarp harbors domains to distinctly bind globular and filamentous actin. Moreover, Tarps ability to bundle actin is independent of its nucleating capabilities. While Tarp from different chlamydial species shares low level of sequence homology, the actin nucleating ability is retained in all Tarp orthologs. If all Tarp orthologs also retain the ability to bundle actin and it's in vivo significance remains to be determined.

4.5 Figures

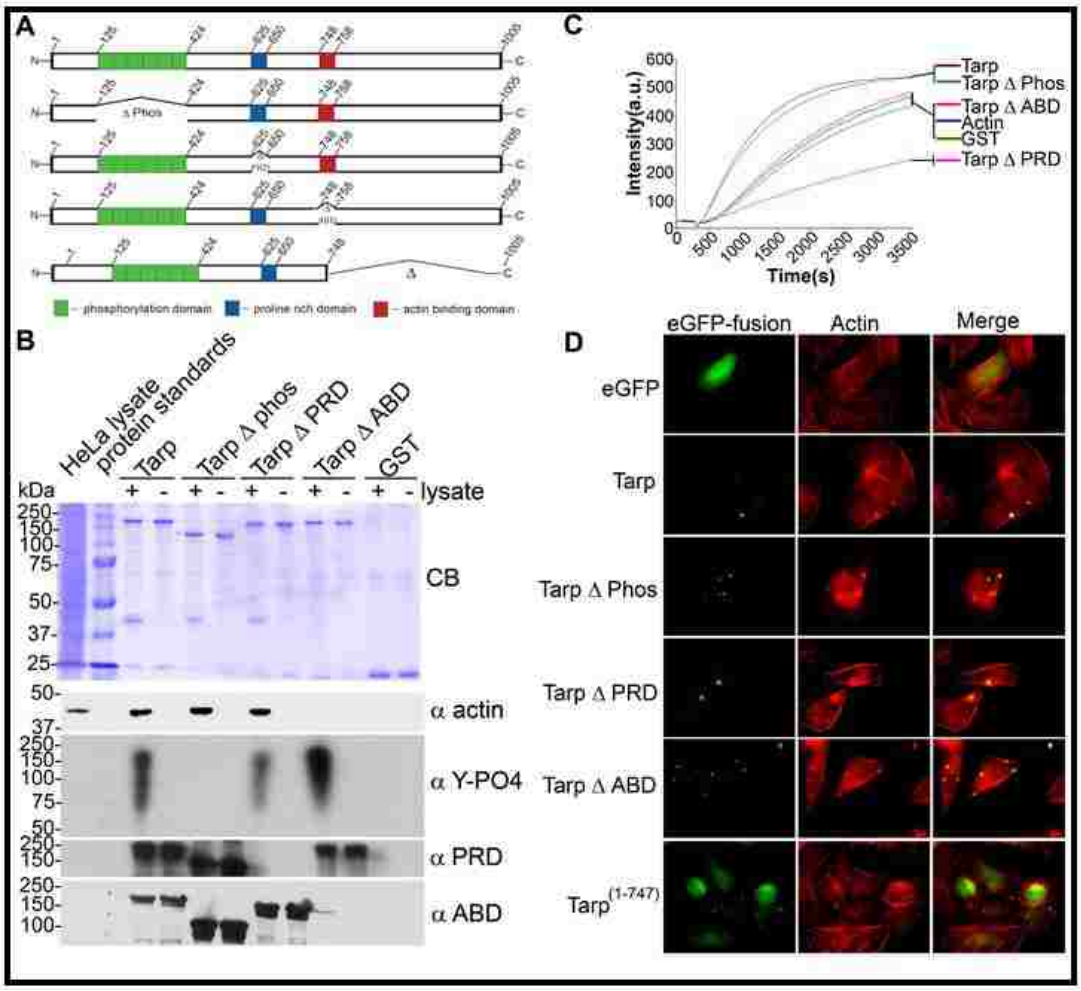

Figure 19 Tarp deletion mutant demonstrate distinct actin binding and polymerization kinetics.

A) Schematics of Tarp proteins indicating the location of the actin binding domain (red box), the proline rich domain (blue box) and the tyrosine rich phosphorylation domain (green box). Δ indicates amino acids deleted in mutant Tarp protein and numbers indicate amino acid positions encoded within the C.trachomatis tarP gene. Full length and mutant Tarp proteins were tagged with n-terminal GST tag and c-terminal histidine tag to purify recombinant protein for biochemical assays or with eGFP for

immunofluorescence assay. B) Extracts from HeLa cells were incubated with GST or GST fusions to wild-type or mutant Tarp and specifically bound proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining (CB). Specifically, Tarp proteins harboring deletions in the phosphorylation domain (Δ phos), the proline rich oligomerization domain (Δ PRD) and the actin binding domain (Δ ABD) were tested. The HeLa lysate shown in the first lane represents 1% of the material used in the + lysate pulldown lanes. Samples identical to those shown in the Coomassie-stained gel were subject to immunoblotting with actin (α actin), phosphotyrosine (α Y-PO4) and peptide antisera specific for the proline rich (α PRD) and actin binding (α ABD) Tarp domains. Molecular mass of protein standards is in kDa. C) GST or GST Tarp fusion proteins described in B were incubated with 1μM monomeric pyrene-labeled actin. A Tarp-mediated increase in actin polymerization after the addition of polymerization buffer at 300 seconds was measured as arbitrary fluorescence intensity (Intensity (a.u.) over time (Time(s)) with excitation and emission wavelengths of 365 and 407 nm respectively. GST and pyrene actin alone served as negative controls. D) eGFP-Tarp mutants colocalizes with filamentous actin in HeLa cells. Host cells expressing eGFP fusions of full length Tarp (Tarp), or deletion mutants lacking the phosphorylation domain (Tarp ∆ Phos), proline rich domain (Tarp ∆ PRD) or actin binding domain (Tarp ∆ ABD) were fixed and stained with Alexa fluor 568 conjugated phalloidin (actin). Host cells expressing eGFP alone or eGFP fusion of Tarp $^{(1\text{-}748)}$ were used as negative controls.

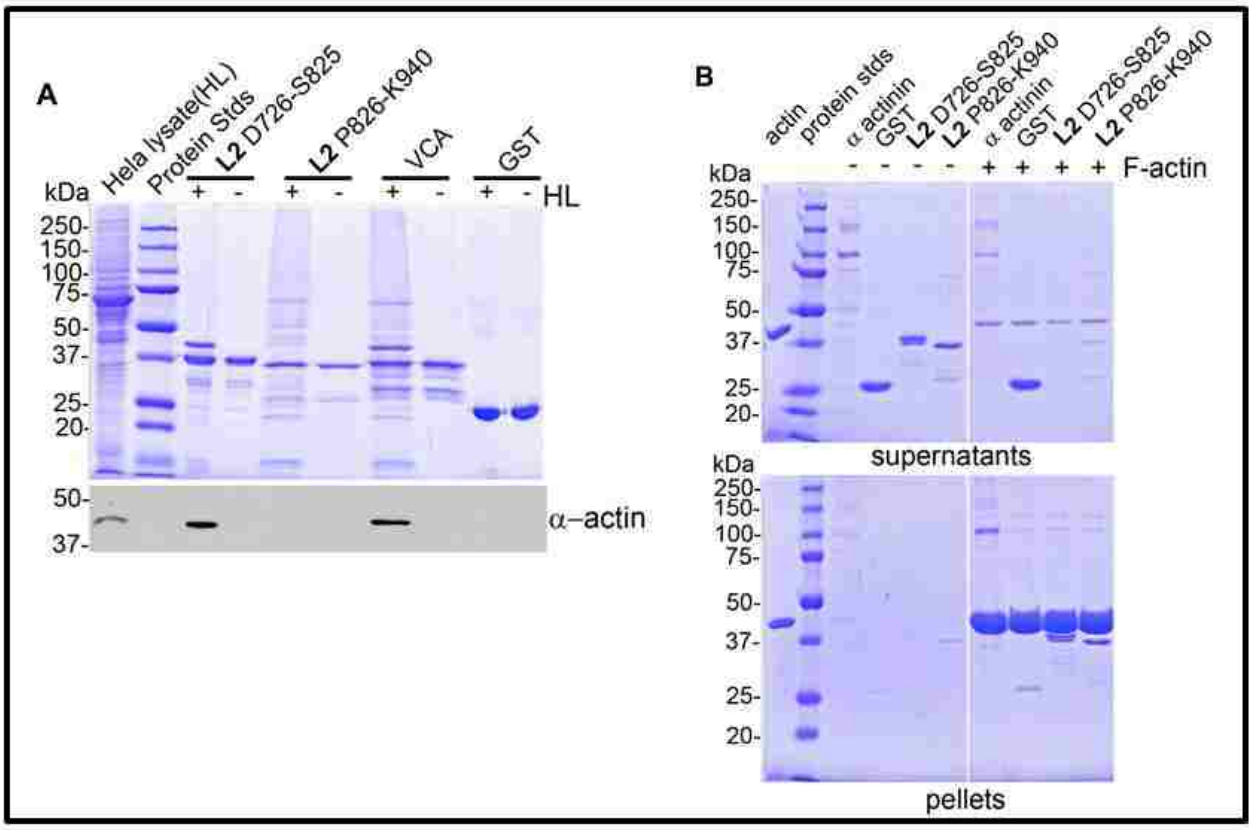

Figure 20 Tarp harbors an F-actin binding domain that is seperate from G-actin binding domain.

A) Extracts from HeLa cells were incubated with GST or GST fusions to the 100-aa fragment of Tarp containing the Tarp actin binding domain (ABD) L2 D726-S825 or the Tarp domain containing a similar sequence L2 P826-K940. Bound proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining (CB). The HeLa lysate shown in the first lane represents 1% of the material used in the + lysate pulldown lanes. Samples identical to those shown in the Coomassie-stained gel were subject to immunoblotting with actin (α- actin). B) GST-TARP fusions were incubated with filamentous actin (F-actin) and isolated by ultracentrifugation. Protein supernatants and pellets were resolved by SDS/PAGE and visualized by Coomassie blue staining. GST-L2 D726-S825 and L2 P826-K940 precipitated with F-actin in the pellet fraction, as did the positive control protein (α- actinin). The GST control did not associate with the F-actin pellet, and remained predominately in the supernatant. The actin shown in the first lane represents 2% of the material used in the + F actin lanes. Molecular mass of protein standards is in kDa.

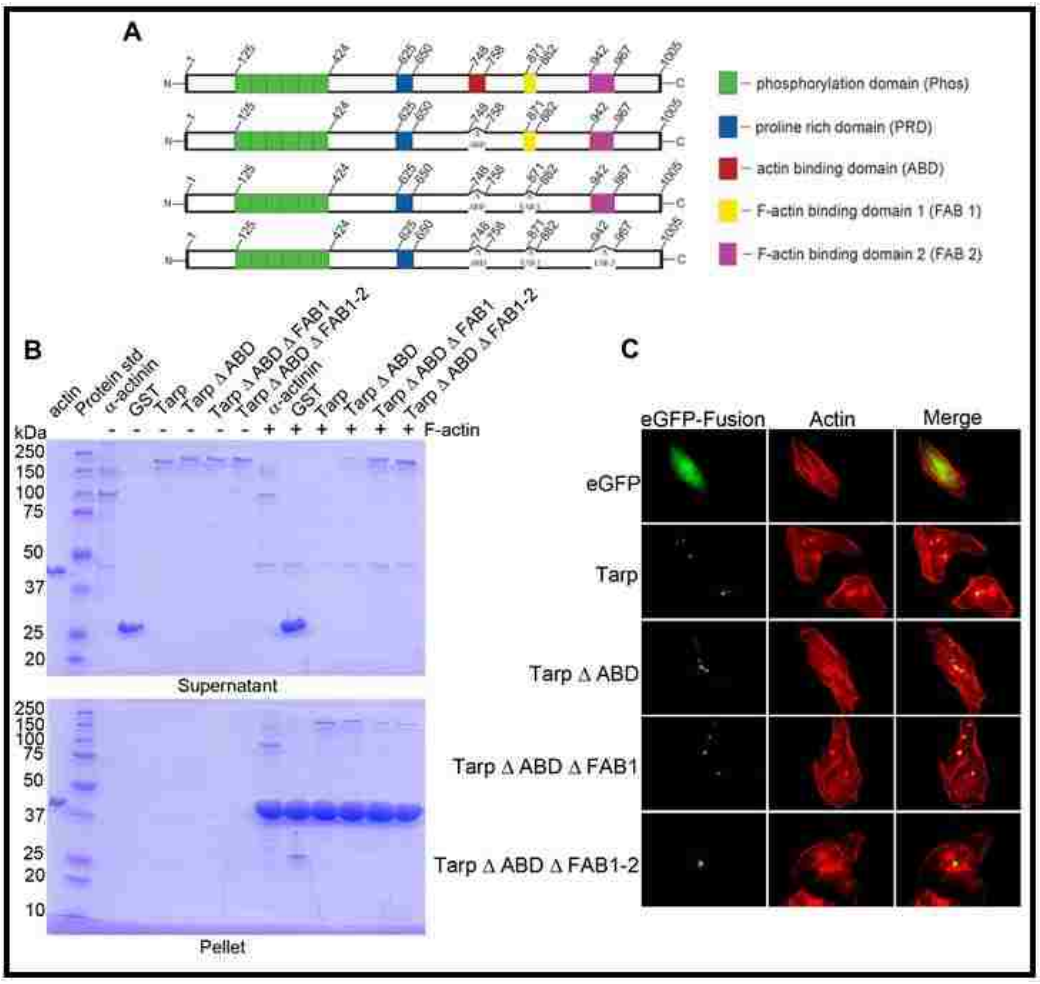

Figure 21 Tarp ability to bind filamentous actin is dependent on two F-actin binding domain and one G-actin and F-actin binding domain.

Tarp deletion mutants expressed as either GST or eGFP fusions were examined for their ability to associate with filamentous actin. **A)** Schematic of the GST- or eGFP-Tarp fusion proteins indicating the location of the actin binding domain (red box), the proline rich domain (blue box), the tyrosine rich phosphorylation domain (green boxes) and the newly characterized F-actin binding domains (yellow and purple boxes). □ indicates amino acids deleted in the mutant Tarp proteins and numbers indicate amino acid positions encoded within the *C. trachomatis* tarP gene. **B)** GST-Tarp fusions were incubated with filamentous actin (F-actin) and isolated by ultracentrifugation. Protein supernatants and pellets were resolved by SDS/PAGE and visualized by Coomassie blue staining. GST-Tarp precipitated with F-actin in the pellet fraction, as did the positive control protein (α actinin). GST-Tarp fusions harboring deletions in ABD, FAB1 and FAB2 domains precipitated with F-actin to a lesser extent as compared to GST-

Tarp. The GST control did not associate with the F-actin pellet, and remained predominately in the supernatant. The actin shown in the first lane represents 2% of the material used in the + F actin lanes. Molecular mass of protein standards is in kDa. **C)** Tarp mutants ectopically expressed as enhanced green fluorescence protein fusions were examined for their ability to localize with actin filaments. Host cells expressing eGFP alone, eGFP fusions of full length Tarp (Tarp), or single deletion mutants lacking actin binding domain (Tarp ∆ ABD), double deletion mutant lacking actin binding domain and first F-actin binding domain (Tarp ∆ ABD ∆ FAB1) or triple deletion mutant lacking actin binding domain and both F-actin binding domains (Tarp ∆ ABD ∆ FAB1-2) were fixed and stained with Alexa fluor 568 conjugated phalloidin (actin).

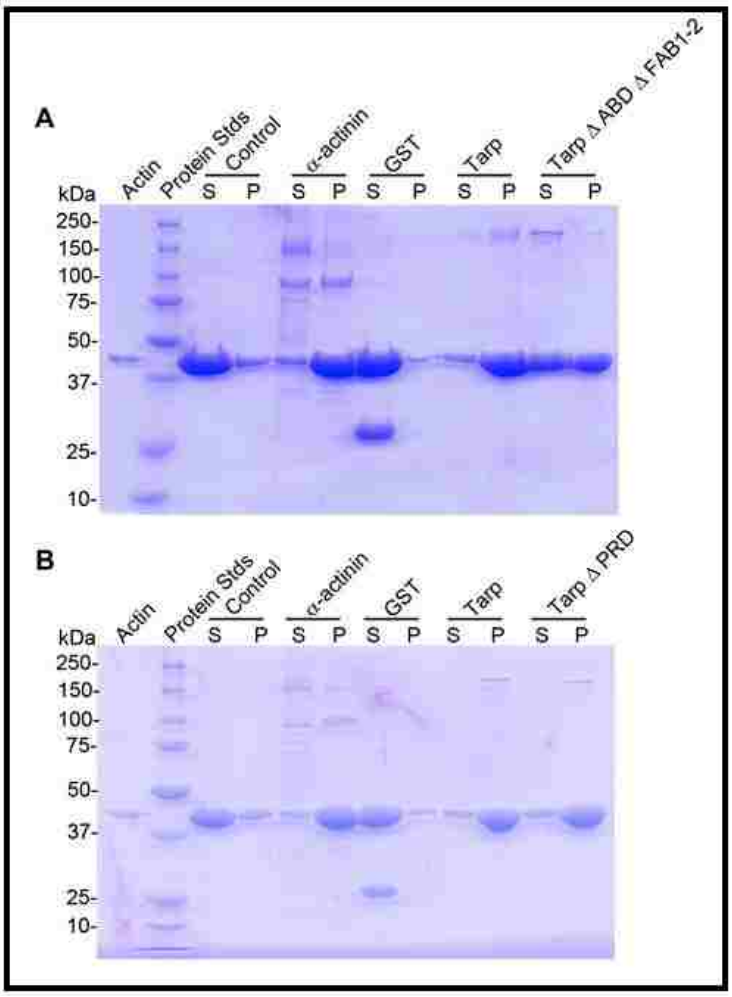

Figure 22 Tarp bundels actin filaments.

A) Purified recombinant Tarp (GST removed) were incubated with filamentous actin (F-actin) and isolated by low speed centrifugation. Protein supernatants and pellets were resolved by SDS/PAGE and visualized by coomassie blue staining. Tarp associated with actin filaments to form actin bundles which appeared in the pellet fraction, as did the positive actin bundling control protein (a-actinin). Neither the Tarp mutant, harboring three actin binding domain deletions, nor the GST control associate with actin, and remained predominately in the F-actin supernatant. Molecular mass of protein standards is in kDa. B) Similar experiment as described above was performed using Tarp mutant harboring proline rich domain deletion (Tarp Δ PRD). Purified Tarp Δ PRD (GST removed) retained the ability to cosediment with bundled F-actin.

4.6 Supplemental Information

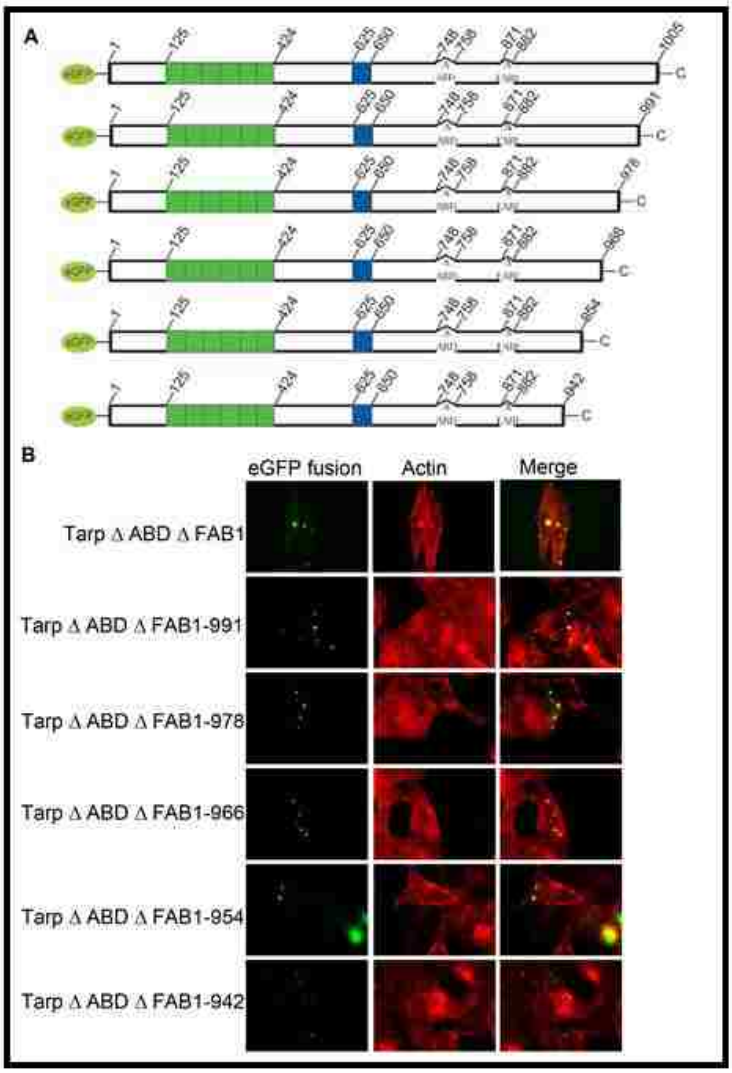

Figure 23 Tarp harbors a second site near its C-terminal that distinctly binds F-actin.

A) Schematics showing series of truncated mutants of eGFP- Tarp Δ ABD Δ FAB1 fusions having deletions at C-terminal domain at increments of approximately 12 amino acids. Phosphorylation domain and proline rich domain are represented by green and blue boxes respectively. Δ indicates amino acids deleted in the mutant Tarp proteins and numbers indicate amino acid positions encoded within the *C. trachomatis* tarP gene. B) eGFP- Δ ABD Δ FAB1 Tarp mutants as depicted above were ectopically

expressed in hela cells for their ability to form actin aggregates and colocalize with alexa fluor 568 conjugated phalloidin (actin). eGFP- Δ ABD Δ FAB1 Tarp mutants harboring deletions downstream to 966 amino acid of Tarp protein retained the ability to colocalize with phalloidin stained actin, whereas eGFP- Δ ABD Δ FAB1 Tarp mutants harboring deletions upstream to 966 amino acid of Tarp protein did not co-localize with phalloidin stained actin.

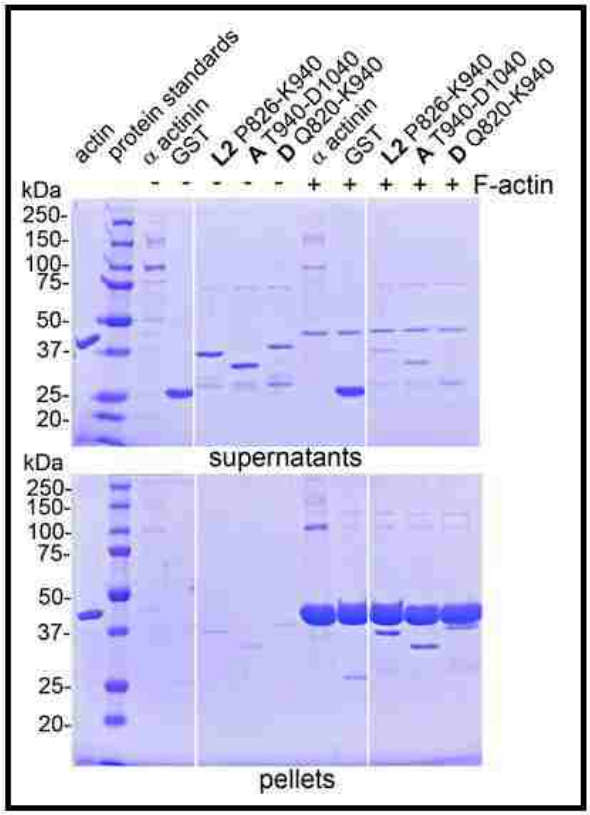

Figure 24 Tarp orthologs from C. trachomatis serovar A and D harbor domains that distinctly binds F-actin.

GST-A T940-D1040 and GST-D Q820-K940 from *Chlamydia trachomatis* serovar A and D, harboring domain that is similar to FAB1 of *Chlamydia trachomatis* L2 Tarp, were incubated with filamentous actin (F-actin) and isolated by ultracentrifugation. Protein supernatants and pellets were resolved by SDS/PAGE and visualized by Coomassie blue staining. GST- L2 P826-K940, GST-A T940-D1040 and GST-D Q820-K940 precipitated with F-actin in the pellet fraction, as did the positive control protein (α-actinin). The GST control did not associate with the F-actin pellet, and remained predominately in the supernatant. The actin shown in the first lane represents 2% of the material used in the + F actin lanes. Molecular mass of protein standards is in kDa.

CHAPTER FIVE: CONCLUSION

5.1 Conclusion

Intracellular bacterial pathogens represent a diverse group of microorganisms that have adapted to survive within eukaryotic host cells. Although these pathogens exhibit differences in their lifestyle and pathogenesis, they all share one common goal and that is to invade the host cells. To accomplish this goal, each organism is equipped with a unique set of proteins that have distinct functional capabilities which subvert the host cytoskeleton machinery. Unraveling the functions of these proteins and their interaction with the host will be the most critical step in developing new strategies to fight intracellular infections. My research was a step towards understanding the invasive strategies employed by two entirely different pathogens, *L. monocytogenes* and *C. trachomatis*. Below I will discuss the important findings of our research and its implications for future studies.

PI 3-kinase is a ubiquitously expressed eukaryotic protein that plays a critical role in cell motility, survival, and proliferation [317, 318]. Increasing evidence is accumulating which suggests that the PI 3-kinase pathway is commonly exploited by many intracellular pathogens in promoting their own entry [365, 366, 368-371]; however, there have been no major efforts in elucidating the mechanisms by which PI 3-kinase activity can promote pathogen uptake. At best, the evidence has accumulated only in a piecemeal fashion. Using *L. monocytogenes* as a model, we performed a systematic RNAi-based screen to identify PI 3-kinase regulated host genes that might play a role in

pathogen uptake. Our study led to identification of at least 10 genes that are critical in *Listeria* entry which includes Rab5c, ARNO, ARAP2, GIT1, SWAP70, DAPP1, mTOR, PLC-γ, PDK1 and PKC-ζ. In the future, it will be important to apply similar screening strategies on other pathogens whose entry also depends on the PI 3-kinase pathway. Such comparisons may lead to identification of certain key signaling proteins that are redundantly exploited by bacterial pathogens, and may serve as targets for adjuvant therapies.

One of the most interesting outcomes of our screen was the identification of several PKC isoforms involved in *Listeria* entry. As we could confirm the role of PKC- ζ in *Listeria* entry, we further pursued studying the role of PKC-δ. We confirmed the involvement of PKC-δ by showing that multiple siRNAs that deplete the PKC-δ protein also inhibit entry of *Listeria*. In addition, we also established that InlB treatment causes increased phosphorylation of PKC-δ, and that PKC-δ is needed for F-actin recruitment during InlB-mediated entry. Importantly, experiments with a PI 3-kinase inhibitor indicate that phosphorylation of PKC-δ caused by InlB is PI3-kinase-dependent (data not shown). Taken together, our results indicate that PKC-δ is needed for cyoskeletal changes and entry of *Listeria*, and suggest that PKC-δ acts downstream of PI 3-kinase.

Among many different PKC isoforms, our screen included only PKC-δ, PKC-1/γ and PKC-ζ because these are the only PKC isoforms that can be regulated directly by PIP3, a product of PI 3-kinase activity or by Grb2/Src kinase pathway [342, 421-423]. It is possible that in addition to atypical and novel PKCs, the conventional PKC (cPKC)

isoforms are also involved in internalization of *Listeria*. This consideration is based on the fact that Type IA PI 3-kinase (PI3K) can indirectly regulate cPKCs via activation of PDK1 or PLC-γ, which were also identified to play roles in *Listeria* entry based on our RNAi screen [424, 425]. PIP3 activates PDK1 by binding to its PH domain [426]. PDK1, in turn, controls the catalytically active conformation of PKCs by phosphorylation of a critical threonine residue in the activation loop [425]. Another way that PIP3 can regulate the activity of cPKC is through activation of Phospholipase C (PLC)-γ1 [424]. The end product of PLC-γ1, DAG, is a required cofactor for PKC activation and membrane recruitment [422]. In addition , IP3, a byproducts of PLC-γ1 activity cause fluxes of calcium, which is also an important cofactor for cPKC activity [427]. Thus it will be important in the future to study the role of conventional PKC isoforms in the entry of *Listeria.* In this regard, another interesting question will be the mechanism by which PI 3-kinase regulates these PKC isoforms; via PDK1 mediated phosphorylation at critical serine/threonine residues, lipid messenger induced conformational changes, or both? In addition to their kinase activity, PKCs also possess protein-protein interaction motifs suggesting that PKCs might function as adaptor proteins [428]. It will also be important to know whether the kinase activity of PKCs is necessary for entry of *Listeria* or if their function involves protein-protein interaction.

Upon activation, PKC mediates its effect by phosphorylation of various downstream target proteins, resulting in their activation and translocation to different cellular compartments [146]. Various substrates of PKCs include, but are not limited to;

proteins involved in phagocytosis, membrane trafficking, cell motility, migration, and cytoskeletal rearrangements [146]. Preliminary data suggest that PKC isoforms are involved in cytoskeletal rearrangements during InlB mediated entry, because siRNA inhibition of PKC-δ resulted in decreased F-actin recruitment around InlB coated microspheres. If other PKC isoforms also regulate F-actin changes, further analysis will be needed. We also observed recruitment of Myristoyleted Alanine rich C-Kinase Substrate (MARCKS), an actin cross-linking protein and a known target of PKCs, around InlB coated beads; however, we didn't confirm if a PKC isoform was responsible for recruitment of MARCKS. There are many other actin regulatory proteins that could also be the target of PKC activity in *Listeria* entry (**see Table 4**). Identification of PKC isoforms and their substrates involved in the internalization of *Listeria* will be helpful to understand the molecular mechanism by which type IA PI 3-kinase promotes bacterial entry.

Rab5c is another interesting protein identified in our screen for *Listeria* entry. Rab5c could potentially control endocytic machinery or Rac dependent actin polymerization during Listeria entry [345, 347]. Rab5c is known to interact directly with both the p85α and p110 subunits of PI 3-kinase [147, 149, 346]. Such interaction stimulates intrinsic Rab GTPase activity which causes inactivation of Rab by GTP hydrolysis. The other potential pathway that controls Rab5c activity is the p38 map kinase pathway, which stimulates GDP/GTP exchange by inhibiting the activity of the GDP dissociation inhibitor (GDI)[429]. Interestingly, Rab5c/Rac induced membrane

ruffling following HGF stimulation of the Met receptor in HeLa cells was found to be independent of the PI 3-kinase pathway[347]. Moreover, Rab5c has also been shown to regulate endocytic trafficking via the p38 map kinase pathway[429]. Since components of the endocytic pathway are involved in *Listeria* entry, the interesting question will be to know if Rab5c activity is regulated via the PI 3-kinase pathway, the p38 Map kinase pathway, or both given the fact that both pathways are active in *Listeria* entry [142, 158, 430].

Recently, the role of β1 and β3 integrin receptors have been implicated in InlB mediated entry as antibody mediated blocking of integrin receptors abrogated *Listeria* entry[355]. Also, incubation of HeLa cells with a ΔInlA mutant *Listeria* strain resulted in increased Focal adhesion kinase (FAK) phosphorylation, suggesting a possible contribution of integrin/FAK signaling in InlB mediated entry [355]. Interestingly, FAK and integrin linked kinases (ILK) were found to be dispensable for *Listeria* entry in our screen, and how integrin promotes InlB mediated entry is not yet known. Recently, the role of the β1 integrin receptor in PI 3-kinase dependent entry of the vaccinia virus has been demonstrated [431]. There is also a considerable amount of evidence that suggests cross talk between integrin and growth factor receptors, including Met [432, 433]. In the future, it will be interesting to test the contribution of the integrin receptor on Met/PI 3-kinase activation during entry of *Listeria*.

Signaling via the PI 3-kinase pathway is also implicated in the developmental cycle of many chlamydial species. For example, it plays a critical role in invasion of *C.*

pnuemoniae and *C. caviae* [298, 366]. Evidence is also accumulating that indicates PI 3-kinase signaling plays a role in post entry events of *C. trachomatis* and *C. pneumonia* [258, 366]. Despite such significant involvement, efforts are lacking to dissect the components of the PI 3-kinase pathways involved in chlamydial biology. The RNAi screening strategy we employed to dissect out molecular mechanisms of *Listeria* entry has proven to be a valuable tool. Of note, the library we created comprises proteins such as AKT, mTor, PDK1, and several AGC kinases that are known to play a multifunctional role in many physiological processes, including cell motility, survival, and lipid metabolism. It is likely that some of these components are also usurped by different chlamydial species for either invasion or intracellular development. Employing a similar RNAi screen, as in the case of *Listeria*, will significantly enhance our understanding of the role PI 3-Kinase plays in chlamydial biology.

Unlike *Listeria*, chlamydial species have conserved genes for the type III secretion apparatus [224, 240]. *Chlamydia* uses this needle like apparatus to deliver effector proteins that play important roles in various phases of the chlamydial development cycle. Tarp is one such effector protein delivered by chlamydia into the host cytoplasm prior to its invasion [243]. Tarp has the capability to nucleate actin, which was found to be essential for chlamydia entry [64, 307]. Thus, the most distinguishing difference between *Listeria* and *Chlamydia* invasion is that the cytoskeletal rearrangements necessary for *Listeria* invasion are dependent on a host cell actin nucleator, the Arp2/3 complex[150]; whereas, the *Chlamydia* invasion requires

the activity of both the Arp2/3 complex and the Tarp [296, 307]. Why a bacterium like *Chlamydia* needs a host cell actin nucleator when it has one of its own is unknown. Evidence suggests that certain bacteria like entero-invasive *E. coli* require prior cytoskeletal rearrangements for efficient delivery of effector proteins [402]. However, this does not seem to be the case for *Chlamydia*, as delivery of the effector protein, Tarp, was not hampered in host cells that were inhibited for Arp2/3 complex activity. Using an *in vitro* actin polymerization assay, we provided evidence suggesting that Tarp and the Arp2/3 complex co-operate to polymerize actin. Use of multiple nucleators, and their cooperative activity, might be a common strategy for obligate intracellular organisms like *Chlamydia* to promote efficient entry, as their survival is completely dependent on their ability to invade the host cell. This can be reflected by the fact that the efficiency of *Chlamydia* to invade host cells is at least 10 times greater than *Listeria,* which can survive in an extracellular environment [294, 434].

The molecular mechanism of the Arp2/3 complex activation during chlamydial entry is the most important unresolved question in its biology. Generally, the activation of the Arp2/3 complex during bacterial entry is a function of either adhesin/receptor interactions or effector proteins. Previously, the role of phosphorylated Tarp was implicated in the Arp2/3 complex activation [296]. However, we provided evidence against the ability of Tarp to activate the Arp2/3 complex. We demonstrated that neither Tarp acts as a nucleation promoting factor, nor does it initiate intracellular signaling events responsible for activation of the Arp2/3 complex. Whether *Chlamydia* secretes

some unknown effector protein or activates host surface receptors to cause the Arp2/3 complex activation will be the most interesting scientific pursuit in the future. Several reports have been published which claim the role of different host receptors involved in chlamydial entry, including PDGF, FGF, CFTR, and PDI [237-239, 291]. However, the inhibition of main signaling pathways downstream to these receptors, including the PI 3-kinase and MAP kinase pathways, had no effect on *C. trachomatis* entry. This suggests that some other independent pathway might be responsible for activation of the Arp2/3 complex. Recently, IQGAP, an adaptor protein that can link growth factor receptors directly to N-WASP/Arp2/3 mediated cytoskeleton changes, has been identified [409]. This might explain the activation of the Arp2/3 complex during chlamydial entry; however, such claims need further scientific evaluations.

The most significant finding of our research on *Chlamydia* was the identification of two F-actin binding (FAB) domains in the c-terminal half of *C. trachomatis* L2 Tarp. Apart from Tarp's ability to nucleate actin, the presence of FAB domains impart Tarp with capabilities to bundle actin, at least in an *in vitro* assay. The function of Tarp to nucleate actin was found to be essential for chlamydial entry as microinjection of host cells with antibodies targeting the ABD abrogated entry [307]. Whether the bundling activity of Tarp is also essential for entry of *Chlamydia* is yet to be studied. Recently, a *Salmonella* effector protein, SipC, was found to possess actin nucleation and bundling activity similar to Tarp [415]. *In vivo* studies using SipC mutant strains of *Salmonella* demonstrated that both nucleation and bundling activity of SipC are essential for

efficient *Salmonella* invasion [8]. Similar studies in *Chlamydia* would require generation of mutant strains harboring in frame deletions of the FAB domains. However, unlike *Salmonella* or other microorganisms for which we have established scientific methods of genetic manipulations, the transformation strategies for *Chlamydia* are in its infancy [435]. Therefore it has not been possible to target any single gene of the chlamydial genome. Nonetheless, many labs, including ours, are focusing on this aspect of chlamydial research. We have developed shuttle vectors that can be transformed into *Chlamydia* and are currently directing our resources to transform *Chlamydia* with a vector harboring different domain deletion mutants of Tarp, including the FAB domains. Another strategy that could also be employed for studying the function of the FAB domains is to functionally target the domains using antibodies specific to these domain. Currently, there are no monoclonal antibodies that can specifically recognize either FAB domain of Tarp. Thus functional inhibition of the FAB domains would require generation of FAB specific antibodies.

Upon genomic analysis, different chlamydial species share 99% sequence identity [281, 305]. The differences in pathogenesis of chlamydial infections, such as host specificity and tissue tropism, are believed to be due to variations within the genes representing 1% of the genome. Tarp is one such gene that exhibits high level of variability within different chlamydial species and serovars; however, certain domains, like the actin binding domain and proline rich domain, are found to be conserved among all Tarp orthologs [306]. Thus, these domains represent the important aspects of

chlamydial biology as confirmed previously by *in vivo* analysis. Interestingly, the secondary structure analysis of all Tarp orthologs also reveals the presence of α-helices similar to the FAB1 and FAB2 domains found in L2 Tarp. Moreover, Tarp orthologs within the trachoma group share 100% sequence homology for FAB1 and FAB2 domains (**see figure 25**). While our study confirmed the significance of the FAB domains in L2 Tarp to bundle F-actin, it will be important in the future to analyze if such capabilities are retained in different Tarp orthologs. Conservation of Tarp's function would point towards the importance of FAB domains in the pathogenesis and overall biology of *Chlamydia*.

Unlike the PRD, ABD, and potentially the FAB domains, which seem to be conserved among different Tarp orthologs, the PhosD is present only in Tarp orthologs that belong to the trachoma group of chlamydial species [306]. So far, the role of the PhosD in Tarp has remained elusive in chlamydial biology. Previous observations have linked the PhosD with cytoskeleton regulation during invasion, but our recent evidence has refuted such claims. Since Tarp remains bound to the inclusion membrane, it will be interesting to know if the PhosD plays a significant role in intracellular development of *C. trachomatis* [243]. Recent evidence suggests that the PhosD can serve as a hub for host signaling proteins. Biochemical *in vitro* analysis revealed the ability of phosphorylated Tarp to bind several SH2 domain containing proteins including PI 3-kinase and adaptor proteins like SHC1 and Nck1/2 [312, 398]. The SHC1 adaptor protein linked MAPK/ERK pathway is critical for host cell survival following chlamydial

infection [398]. Moreover, unpublished data from our lab also implicates the important role of PI 3-kinase/AKT signaling in inclusion development. Whether Tarp or some other chlamydial proteins initiate such signaling events *in vivo* is not yet known. One possible approach to determine the effects of Tarp phosphorylation would be to transfect full length and phosphorylation domain deletion mutants of Tarp in eukaryotic cell lines to monitor the effects on activation of key signaling molecules such as AKT, ERK1/2, etc. However, confirmatory evidence would require genetic approaches to study the effects of the PhosD deletion on overall development of *C*. *trachomatis.*

Certainly, Tarp seems to be one of the most critical virulent genes acquired by pathogenic chlamydial species. Current evidence indicates the role of Tarp in many different aspects of chlamydial biology including invasion, intracellular development, and pathogenesis. While I am curious to learn more about the functions of Tarp, I will desperately await the day we know 'the life of *Chlamydia* without Tarp'.

In conclusion, based on the findings from this research we are proposing the revised models of *Listeria monocytogenes* and *Chlamydia trachomatis* entry (**See figure 26 and 27**). I strongly believe that the work done in thesis will serve as a frame work for future research to extend our knowledge about the entry mechanisms used by these organisms. With this knowledge I hope we will be able to develop strategies in preventing infections from these organisms.

5.2 Figures

Table 4 Known downstream targets of PKC

Protein	Gene	Accession no.	Function	Biologic activity
MARCKS	MARCKS	Nm_002356	Actin binding protein	Cell motility, Phagocytosis, membrane trafficking
Coronin 1A	CORO 1A	Nm_007074	Actin binding protein	Cell motility, Phagocytosis, membrane trafficking
Coronin 1B	CORO 1B	Nm_020441	Actin binding protein	Cell motility, Phagocytosis, membrane trafficking
Coronin 1C	CORO 1C	Nm_014325.2	Actin binding protein	Cell motility, Phagocytosis, membrane trafficking
Coronin 2A	CORO 2A	Nm_003389.3	Actin binding protein	Cell motility, Phagocytosis, membrane trafficking
Adducin 1	ADD1	Nm_001119.3	Actin capping protein	Actin cytoskeleton
Adducin 2	ADD2	Nm_017482.2	Actin capping protein	Actin cytoskeleton
Adducin 3	ADD3	Nm_016824.3	Actin capping protein	Actin cytoskeleton
Fascin 1	FSCN1	Nm_003088.2	Actin bundling protein	Cell motility and morphogenesis
Fascin 2	FSCN2	Nm_012481.2	Actin bundling protein	Cell motility and morphogenesis
Fascin 3	FSCN3	Nm_020369.1	Actin bundling protein	Cell motility and morphogenesis
Ezrin	EZR	Nm_003379.4	Actin crosslinking protein	Cell motility and morphogenesis
Radixin	RDX	Nm_002906.3	Actin crosslinking protein	Cell motility and morphogenesis
Moesin	MSN	Nm_002444.2	Actin crosslinking protein	Cell motility and morphogenesis
AFAP-110	AFAP1	Nm_001134647.1	Binds Src Kinase	Needs for podosome formation
Vinculin	VCL	Nm_003373.3	Localize to focal adhesion	Regulate focal adhesion formation

References:[436-454]

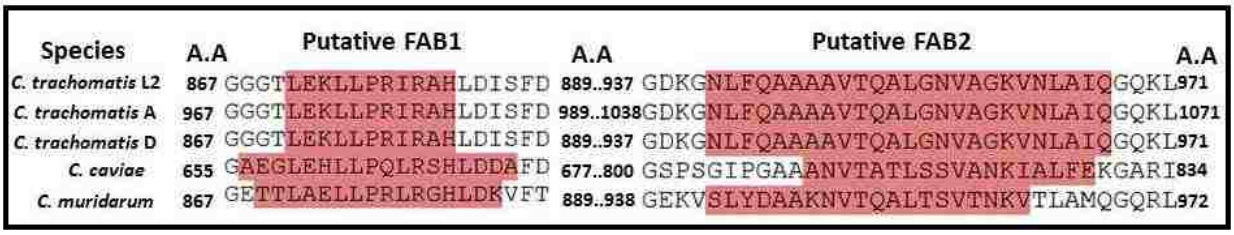

Figure 25 Putative FAB domains in C. trachomatis serovar A and D, C. caviae and C. muridarum.

Secondary structure analysis was done to identify the presence of additional alpha helices in C-terminal domain of Tarp orthologs except for the known actin binding domains. Number represents the amino acid position. Amino acids highlighted in pink box represent alpha helix on secondary structure analysis. Sequence alignment of Tarp orthologs *C. trachomatis* serovar L2, A and D, *C. caviae* and *C. muridarum* was done using Clustal W. version 1.82, multiple-sequence alignment software (http:// www.ebi.ac.uk/clustalw). Note the presence of conserved FAB1 and FAB2 domain with 100% sequence homology within Tarp orthologs from C. trachomatis serovar L2, A and D. Alpha helixes are also identified in *C. caviae* and *C. muridarum* Tarp at the corresponding locations with ~65% sequence homology to FAB1 and FAB2 in *C. trachomatis* L2 Tarp.

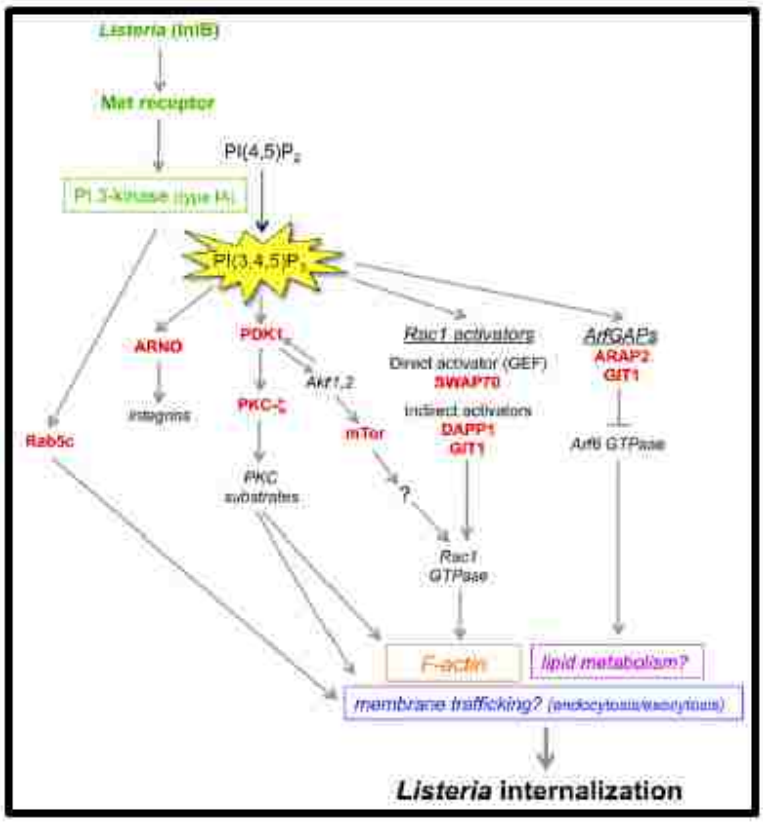

Figure 26 Potential mechanism of control of Listeria entry by type 1A PI 3-kinase. Infection of human cells with *Listeria* expressing InlB results in activation of the host Met receptor and of type IA PI 3-kinase [61, 130, 142]. The RNAi-based screen described in this work led to the identification of nine human proteins involved in PI 3-kinase signaling that play important roles in *Listeria* entry. Based on biological functions of these nine proteins reported in the scientific literature, a diagram was constructed depicting some of the possible ways that the host proteins could participate in bacterial uptake. Rab5c, a protein that interacts with regulatory and catalytic subunits of type IA PI 3-kinase, could promote *Listeria* entry by controlling the host endocytic machinery [158, 345]. ARNO, an activator of Arf GTPases that binds directly to the PI 3-kinase product PI(3,4,5)P_3, might help maintain proper plasma membrane levels of integrins, a class of receptor recently found to enhance InlB-mediated entry [355]. The serine/threonine kinase PKC-□ could promote *Listeria* internalization by controlling the actin cytoskeleton and/or delivery of membrane through exocytosis (6, 48, 67). PKC-□ is indirectly regulated by PI 3-kinase through the master kinase PDK1, which is a direct target of PI(3,4,5)P_3 [338]. mTor, a serine/threonine kinase indirectly controlled by type IA PI 3-kinase, might promote bacterial uptake through activation of the host proteins Rac1 and/or PKC-□ (not depicted) (69, 85) Apart from mTor, three other human

proteins identified in the RNAi screen have the potential to control *Listeria* entry though activation of Rac1 GTPase. These proteins, SWAP70, DAPP1, and GIT1, each bind directly to $PI(3,4,5)P_3$. SWAP70 is a direct activator of Rac1, and stimulates nucleotide exchange on the GTPase [331]. DAPP1 and GIT1 lack recognizable guanine nucleotide exchange factor (GEF) domains, and likely indirectly activate Rac1. In addition to being an indirect activator of Rac1, GIT1 is also a GTPase Activating Protein (GAP) that inhibits Arf6 GTPase [351]. Along with ARAP2, another Arf6 GAP needed for *Listeria* entry [323], GIT1 might restrain activation of Arf6, which would otherwise interfere with bacterial uptake. Constitutively activated Arf6 alleles inhibit *Listeria* internalization [323], and also induce the redistribution of cholesterol from the plasma membrane to internal membrane compartments [350]. Since plasma membrane cholesterol is critical for InlB-mediated entry, it is possible that GIT1 and/or ARAP2 promote *Listeria* uptake by maintaining proper localization of cholesterol and/or other lipids [323].

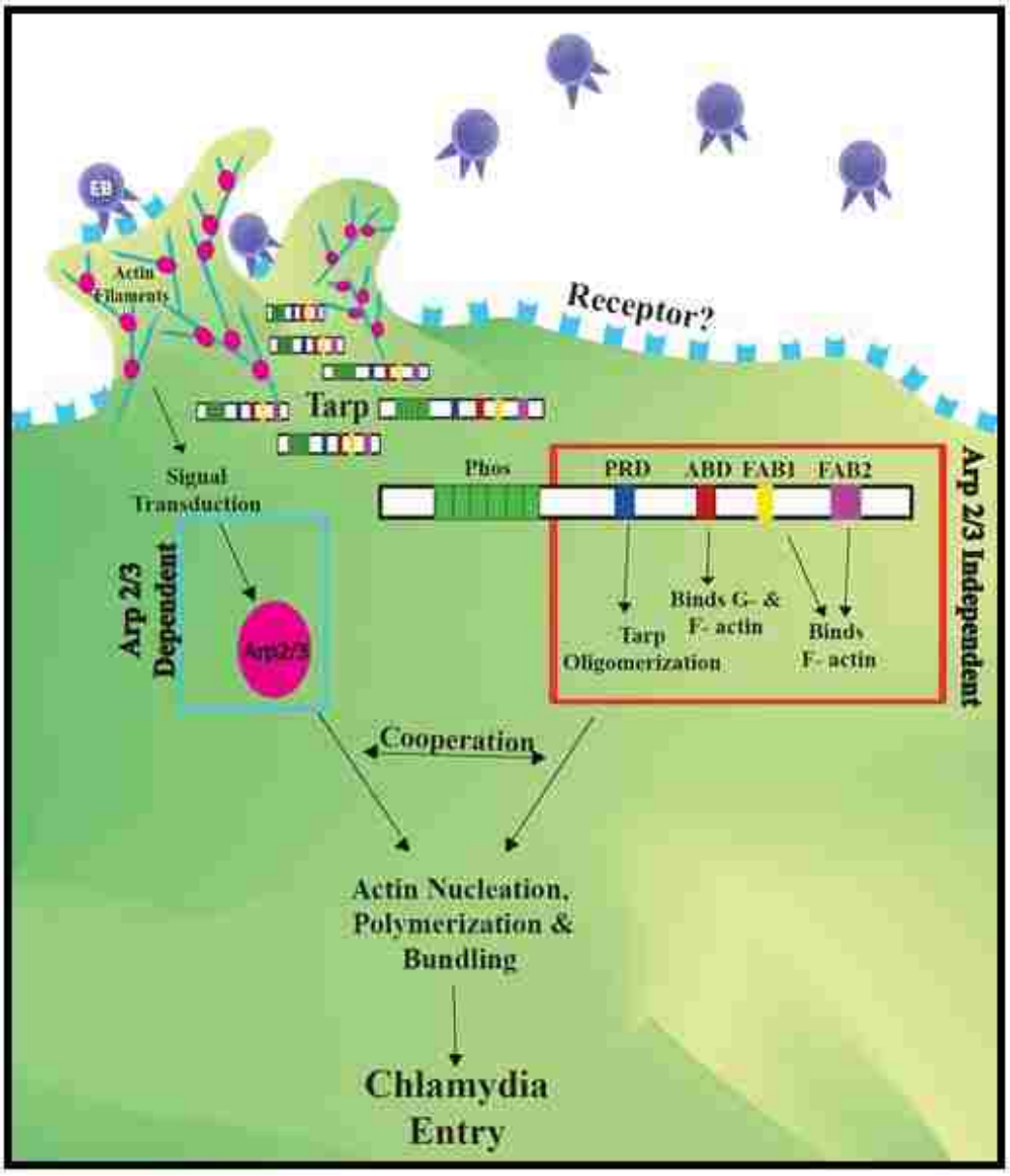

Figure 27 Revised model for entry of C. trachomatis in non-phagocytic cells.
EB translocate Tarp into host cell cytoplasm. Tarp, via its actin binding domain (red box) and proline rich oligomerization domain (blue box), directly polymerizes actin to generate linear F-actin. Tarp via its FAB domains binds and bundles F-actin. Interaction of EB with host cell surface receptor causes activation of Arp2/3 complex. Arp2/3 complex uses linear bundles of actin filaments to form complex array of actin network to promote efficient uptake of EBs into non-phagocytic cells.

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Title: Identification of Components of the Host Type IA Phosphoinositide 3-Kinase Pathway That Promote Internalization of Listeria monocytogenes

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