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IDENTIFICATION OF NEW HOST SIGNALING PATHWAYS HIJACKED BY BACTERIAL PATHOGENS DURING INFECTION

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ABBREVIATIONS

A/E lesion - attaching and effacing lesion
Ail - Attachment and invasion locus protein
AKT - Protein kinase B (serine/threonine-specific protein kinase)
AP - clathrin–adaptor protein complex
APC - Antigen-presenting cell
ATP - Adenosine tri-phosphate
BHI - Brain heart infusion
Blebb. - Blebbistatin
Btk - Bruton's tyrosine kinase
CFU - Colony-forming unit
CK II - casein kinase II
c-Met - Hepatocyte growth factor receptor
CNS - Central Nervous System
DCs - Dendritic cells
DMEM - Dulbecco’s modified Eagle medium
DMSO - Dimethyl sulfoxide
ECM - Extracellular matrix
EHEC - Enterohemorrhagic *Escherichia coli*
ELC - Essential light chain
EMEM - Eagle’s Minimal Essential Medium
EPEC - Enteropathogenic *Escherichia coli*
ER - Endoplasmic reticulum
ERK - Extracellular-signal-regulated kinases
FAK - Focal adhesion kinase
FBS - Fetal bovine serum
GAP - GTPase activating protein
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GC – Golgi complex
GTPase - Guanosine triphosphate hydrolase
GFP - Green fluorescence protein
HGF - Hepatocyte growth factor
Hsp56 - Heat Shock Protein of 56 kDa

IFs - Intermediate filaments

ILK - Integrin-linked-kinase

InlA - Internalin A

InlB - Internalin B

Inv - Invasin

IQ motif - Isoleucine-Glutamine motif

IR - Inter repeat region

IRTKS - Insulin receptor tyrosine kinase substrate

K18 - Keratin 18

K8 - Keratin 8

K - Keratin

LB - Lysogeny broth media

LLO - Listeriolysin O

LPS - Lipopolysaccharide

LEE - Locus of enterocyte effacement

LRRs - Leucine rich repeats

MAP - Mitogen activating protein

MAPK - Mitogen-activated protein kinase

MEK - Mitogen-activated protein kinase kinase

MHC - Myosin heavy chain

MLC - Myosin light chain

MLCK - Myosin light chain kinase

MLCPase - Myosin light chain phosphatase

MOI - Multiplicity of infection

Mpl - Metalloprotease

mtDNA - Mitochondrial DNA

NF-κB - Nuclear factor-kB

NM-II - Non-muscle myosin II

NM-IIA - Non-muscle myosin isoform IIA

NM-IIB - Non-muscle myosin isoform IIB

NM-IIC - Non-muscle myosin isoform IIC

NMHC-IIA - Non-muscle myosin heavy chain isoform IIA

NMHC-IIB - Non-muscle myosin heavy chain isoform IIB

NMHC-IIC - Non-muscle myosin heavy chain isoform IIC

N-WASP - neuronal Wiskott-Aldrich syndrome protein

OCRL - Oculocerebrorenal syndrome of Lowe (Inositol polyphosphate 5-phosphatase)

PBS - Phosphate-buffered saline

PC - Phosphatidylcholine

PC-PLC/PLC-B - Phosphatidylcholine-specific phospholipase C

PI3K - Phosphatidylinositol 3-kinase

PI4K - Phosphatidylinositol 4-kinase

PI5K - Phosphatidylinositol 5-kinase

PI - Phosphoinositol

PIP - Phosphatidylinositol 1-phosphate

PIP2 - Phosphatidylinositol biphosphate

PIP3 - Phosphatidylinositol triphosphate

PIP4 - Phosphatidylinositol 4-phosphate

PI-PLC/PLC-A - Phosphatidylinositol-specific phospholipase C

PKA - Protein kinase A

PKC - Protein kinase C

PMF - Peptide mass fingerprinting

PP1 - Inhibitor of Src-family tyrosine kinases

PRK2 - protein kinase C-like 2

PTB - Phosphotyrosine binding domains

PTM - Posttranslational modification

PTK - Protein tyrosine kinase

pTyr - Tyrosine phosphorylation

pYV - virulence plasmid of *Yersinia*

RLC - Regulatory light chain

ROCK - Rho-associated kinase

RSK1 - Ribosomal S6 protein kinase 1

RTK - Receptor tyrosine kinase

SCVs - *Salmonella* containing vacuoles

SFK - Src family kinase

SH2 - Src homology-2 domain

SNX9 - Sorting nexin-9

SPI-2 - *Salmonella* pathogenicity island-2

Src - c-Src tyrosine kinase

Stx - Shiga toxin

T4SS - Type IV secretion system

TCA - Trichloroacetic acid

Tir - Trasnslocated intimin receptor

TLRs - Toll-like receptors

TRPM7 - Transient receptor potential melastatin 7

TTSS - Type III secretion system

YadA - *Yersinia* adhesin A

Yops - *Yersinia* outer proteins

YpkA - *Yersinia* protein kinase A

UTI - Urinary tract infections

WAVE - WASP family verprolin homologous protein

ABSTRACT

Listeria monocytogenes is a human food borne pathogen that may cause, in particular in immunocompromised individuals, a severe disease characterized by septicemias, meningitis, meningo-encephalitis and abortions in pregnant women. The study of the cell biology of the *L. monocytogenes* infectious process provided insights in the way bacteria manipulate the host and revealed unsuspected functions of cellular proteins. To cause infection pathogens interfere with crucial host intracellular pathways, and different pathogens often hijack the same signaling pathways. In particular, host phosphorylation cascades are preferential targets of infecting bacteria.

In this study, using *L. monocytogenes* as a pathogen model, we showed that eukaryotic cells present a variable protein phosphorylation pattern upon infection. We addressed in particular the tyrosine-phosphorylated protein profile triggered by *Listeria* infection and identified the motor protein, non-muscle myosin heavy chain IIA (NMHC-IIA) and the epithelial keratin 18 (K18), as differentially tyrosine-phosphorylated in response to *Listeria* uptake.

We demonstrated that NMHC-IIA and K18 are tyrosine-phosphorylated over the time of infection and recruited at the bacterial entry site. In addition, we were able to show that the inhibition of myosin IIA activity affected the number of intracellular *Listeria* in non-phagocytic cells. The reduction of NMHC-IIA expression using RNAi techniques resulted in an increased *Listeria* uptake. Reversely, K18 knockdown lead to a significant decrease in bacterial entry.

Together, these data unravel a novel role for the non-muscle myosin II class and for keratins in the *Listeria* cellular infection. In particular, they correlate for the first time myosin and keratin tyrosine-phosphorylation with *Listeria* entry into host cells. In addition, results obtained with EPEC, EHEC and *Yersinia* infection models further support a broader role of NMHC-IIA in bacterial infection. To better understand this role, we have identified potential NMHC-IIA-interacting partners, which are currently being characterized in the context of infection.

RESUMO

Listeria monocytogenes é uma bactéria patogénica responsável pela listeriose humana. Esta doença infecciosa afecta sobretudo indivíduos imunocomprometidos nos quais se manifesta como meningite, encefalite, septicemia ou, em mulheres grávidas, aborto ou complicações neonatais. O estudo do processo infeccioso de *Listeria* clarificou o modo como as bactérias manipulam o hospedeiro e desvendou a função de inúmeras proteínas celulares, até então desconhecida. Para causar infecção, microrganismos patogénicos interferem com mecanismos intracelulares cruciais do hospedeiro, e em algumas ocasiões, o mesmo mecanismo é sequestrado por patógenos diferentes. Neste caso, as cascatas de fosforilação intracelulares são alvos preferenciais.

Neste estudo, mostrámos que células eucariotas apresentam um padrão de fosforilação de proteínas intracelulares variável em resposta à infecção por *Listeria monocytogenes*. Analisámos especificamente o padrão de fosforilação em resíduos de tirosina que nos permitiu identificar uma proteína motora, a cadeia pesada da miosina IIA não-muscular (NMHC-IIA), e a forma epitelial da queratina 18 (K18), como sendo diferencialmente fosforiladas em tirosina em resposta à entrada de *Listeria*. Demonstrámos que a NMHC-IIA e a K18 são fosforiladas em tirosina ao longo do período de infecção e recrutadas para o local de entrada da bactéria. Mostrámos que a inibição da actividade da miosina IIA afectava o número de bactérias no interior de células não-fagocíticas. A diminuição dos níveis de expressão intracelular da NMHC-IIA, por técnicas de RNA de interferência, resultou num aumento da entrada de *Listeria* nas células hospedeiras. Em contraste, a redução da expressão de K18 teve como consequência um decréscimo na eficiência de entrada.

Em suma, estes dados permitiram desvendar a participação da classe das miosinas II não-musculares e das queratinas no processo celular de infecção por *Listeria*. Em particular, conseguimos correlacionar, pela primeira vez, a fosforilação em tirosina de miosinas e queratinas com o mecanismo de entrada de *Listeria*. Resultados obtidos com estudos de infecção por EPEC, EHEC e *Yersinia* contribuíram para reforçar o papel abrangente da NMHC-IIA na infecção por bactérias. Para melhor compreender este papel, identificamos novos potenciais

parceiros de interacção da NMHC-IIA, cujo papel no contexto de infecção por *Listeria* está a ser actualmente caracterizado.

INTRODUCTION

I. BACTERIAL PATHOGENS

Bacteria do not possess membrane-bound organelles and thus have a disorganized intracellular compartment, characteristics that include them in the group of prokaryotic organisms. They exist in different shapes and their sizes vary within the micrometer range. Bacteria can be found in a wide range of environmental niches (e.g. soil, rock, oceans, and even arctic snow), where they play an important role in the cycling of nutrients. Some species live in association with more complex organisms, such as plants and animals, including humans. Despite their traditional association with disease, relatively few bacterial species are truly pathogenic for plants and animals.

Humans live in close association with bacteria, and in most cases this interaction is important for a healthy life. In particular, symbiotic bacteria live in an intimate association with their hosts, as both co-existing organisms take important benefits from this relationship. In contrast, pathogenic bacteria can harm and directly cause disease to their hosts, through the concerted action of virulence factors

For the purpose of this thesis, bacterial pathogens from two different groups, representing both intracellular and extracellular species, were used as infection models. Their main features and mechanisms of virulence and cellular infection are described in the following sections.

I.A. *Listeria monocytogenes*

I.A.1. Overview

Listeria monocytogenes (*Lm)* was first isolated in 1926 by E.G.D. Murray and his colleagues after an outbreak in an animal care house in Cambridge (Murray et al. 1926, Pirie 1927). Human cases were first reported in 1929 but for a long time listeriosis was considered a zoonosis. The first human outbreak and the direct association to the consumption of *Lm*-contaminated food was reported in 1983 (Schlech et al. 1983). *Lm* is now recognized as a food borne pathogen mostly affecting industrialized countries due to the consumption of processed foods but becoming a worldwide concern as a result of an increase of elderly

population and immunosuppressed individuals (de Noordhout et al. 2014). Foods most frequently contaminated are industrially produced, refrigerated ready-to-eat products that are consumed without cooking or reheating. Listeriosis outbreaks were associated with the consumption of soft cheeses and dairy products, pâtés and sausages, smoked fish, salads and “delicatessen” (Swaminathan and Gerner-Smidt 2007).

Lm is a facultative anaerobic Gram-positive bacillus, included within the genus *Listeria,* which comprises 15 species (Graves et al. 2010, Leclercq et al. 2010, Bertsch et al. 2013, Lang Halter et al. 2013, Liu 2013, den Bakker et al. 2014). Together with *Lm*, nine other species are currently recognized: *L. ivanovii*, *L. seeligeri, L. innocua, L. welshimeri, L. grayi, L. marthii, L. rocourtiae, L. fleischmannii* and *L. weihenstephanensis* (Liu 2006, Graves et al. 2010, Leclercq et al. 2010, Bertsch et al. 2013, den Bakker et al. 2013, Lang Halter et al. 2013). *Lm* and *L. ivanovii* are the only pathogenic species, with *Lm* infecting humans and other animals, whereas *L. ivanovii* affecting primarily sheep and cattle. The other species live as non-pathogenic saprophytes (Glaser et al. 2001, Buchrieser et al. 2003, Nelson et al. 2004, Schmid et al. 2005, Hain et al. 2006, Steinweg et al. 2010, Buchrieser et al. 2011, Hain et al. 2012). Nonetheless, non-pathogenic *Listeria* species have been implicated in human infections, particularly in patients with a weakened immune system and/or with an underlying disease (Rocourt et al. 1986, Andre and Genicot 1987, Cummins et al. 1994, Lessing et al. 1994, Todeschini et al. 1998, Perrin et al. 2003, Snapir et al. 2006, Rapose et al. 2008, Guillet et al. 2010, Salimnia et al. 2010). More recently, five new species have been proposed to the scientific community (*L. aquatica*, *L. floridensis*, *L. cornellensis*, *L. grandesis* and *L. riparia*), which have not been associated with pathogenicity (den Bakker et al. 2014).

Lm is ubiquitously distributed in nature, being found in soil, water, plants, and food processing plants. This environmental widespreadness reflects its capacity to live under harsh conditions. Indeed, *Lm* can survive and even proliferate in high salt concentrations (up to 20%), at pH values ranging from 4.5 to 9, and in a broad temperature range (1-45 ºC). *Lm* exhibits motility at environmental temperatures (up to 30 ºC) due to the presence of flagella. However, once inside a host organism, in order to avoid detection by the immune system, expression of the immunostimulatory flagella is repressed in a thermo-

dependent manner (Way et al. 2004). These remarkable properties that allow *Lm* to be present in different environments, increase the risk of infection in humans and animals as a result of a routinely exposure to this pathogen. This makes *Lm* a serious threat to food safety and ranks it among the microorganisms that most concern the food industry (Vazquez-Boland et al. 2001, Khelef et al. 2006).

I.A.2. Listeriosis

Listeriosis manifests after ingestion of highly contaminated food (Vazquez-Boland et al. 2001, Wing and Gregory 2002). In 2009, the incidence of listeriosis in Europe was 4 cases per million inhabitants, according to a report by the European Food Safety Authority. Listeriosis is a rare disease but considered to be under-diagnosed because in some countries it is not a reportable disease, as it was the case in Portugal until this year. In Portugal, scarce reports only allow limited incidence determination of the disease during restricted periods of time. The most recent report of listeriosis in Portugal (2007) indicates an incidence of 2.3 cases per million inhabitants (Almeida et al. 2010), an increased value in comparison with reports in 2003 that established an incidence of 1.4 cases per million habitants (Almeida et al. 2006). In addition, antibiotic resistance was demonstrated to be increasing in both food and clinical *Lm* isolates in a recent Portuguese study (Barbosa et al. 2013).

Lm can be asymptomatically present in the gastro-intestinal tract of healthy humans. Although the incubation time is relatively long, varying from 1 to 67 days in healthy individuals, listeriosis can occur in less than 24 hours as febrile gastroenteritis (Goulet et al. 2013). Other form of the disease can also manifests as a cutaneous infection. Both manifestations are non-invasive forms, which usually resolve spontaneously.

Lm may also cause a more severe form of the disease (invasive listeriosis) that primarily affects immunocompromised hosts, which include risk groups such as elderly, pregnant women and newborns (Lecuit 2007, Allerberger and Wagner 2010). The majority of invasive listeriosis cases manifest in three possibly life-threatening clinical forms: (i) septicemia, (ii) meningo-encephalitis, and (iii) materno-fetal or neonatal infections. The average case-fatality rate can be as high as 30%. The major line of defense against listeriosis is cell-mediated immunity,

explaining the fact that individuals with T-cell dysfunction are prone to contract the disease (Swaminathan and Gerner-Smidt 2007).

Lm is naturally susceptible to a variety of antibiotics, such as penicilins, aminoglycosides, trimethoprim, tetracycline, macrolides, and vancomycin. Treatment of invasive listeriosis requires ampicillin, and in specific cases (bacteremia, meningitis, endocarditis), combination therapeutics with an aminoglycoside (gentamicin) has revealed to have a synergistic effect (Temple and Nahata 2000). Ampicilin is also used in clinical treatment of pregnancy-associated listeriosis (Charlier and Lecuit 2014), with a common duration of 21 days (Pereira et al. 2013).

Human invasive listeriosis develops through successive steps (Figure 1), beginning with the ingestion of contaminated food. *Lm* resists to the acidic gastric fluids and reaches the intestinal lumen, where it invades the mucus-secreting goblet cells, to cross the intestinal barrier (Lecuit et al. 2001, Nikitas et al. 2011). It is then thought to disseminate from the mesenteric lymph nodes, via lymph and blood circulation, reaching the liver and the spleen, where *Lm* can survive and replicate exponentially. A second more severe stage of the infection is achieved when *Lm* is able to reach the brain or the placenta, by crossing the blood-brain or the placental barriers, respectively. This infection outcome is responsible for the cases of meningitis or encephalitis in immunocompromised patients, abortions in pregnant women, and septicemia in infected neonates (Lecuit 2007, Allerberger and Wagner 2010).

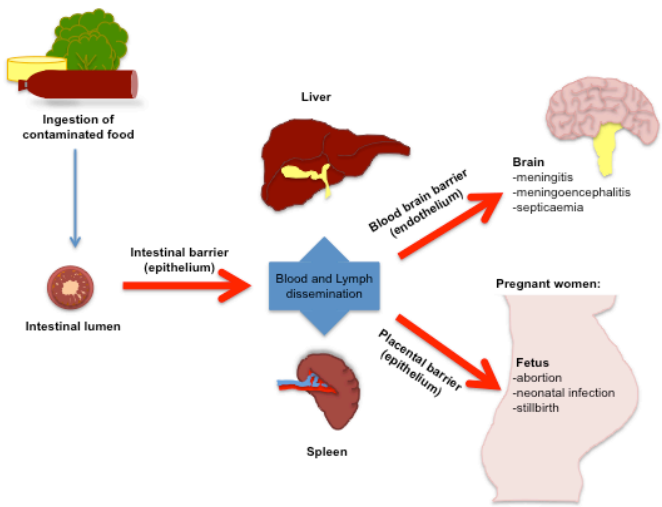

Figure 1. Successive steps of the *Lm in vivo* infection.

I.A.3. Cellular infection cycle

Lm is a facultative intracellular pathogen and the cell biology of its infectious process has been widely studied. A key feature of *Lm* is its capacity to induce its own internalization into non-phagocytic cells and to survive and multiply in the cytosol of most cell types, including enterocytes, hepatocytes, fibroblasts, endothelial cells or glial cells in CNS, and macrophages (Allerberger and Wagner 2010, Cossart 2011). In addition, *Lm* exploits the host cell cytoskeleton to propel itself through the cytoplasm by means of an actin-dependent motility. In the later steps of the cellular infection, *Lm* interacts and pushes the host cell plasma membrane outward, forming protrusions that can penetrate and be engulfed by adjacent cells. This process allows *Lm* dissemination by direct cell-to-cell spread, without re-exposure to the extracellular environment and to extracellular immune defense players, such as antibodies and the complement system (Regan et al. 2014).

This infection cycle is carried out in a step-wise mode: (i) adhesion to the host cell surface, (ii) internalization, (iii) lysis of the primary vacuole, (iv) multiplication in the host cytoplasm, (v) actin-based intracellular movement, and (vi) intercellular spread (Figure 2). *Lm* evolved a variety of molecular weapons to exploit key cellular processes in order to successfully go through these steps. Indeed, many of its currently known and characterized virulence factors interact with particular cellular components and are involved in specific steps of the cellular infection (Camejo et al. 2011).

(i) Adhesion to the host cell surface

The first step of cellular infection corresponds to the adhesion of *Lm* to the host cell surface. Several proteins expressed at the *Lm* surface (Figure 2) interact with target proteins expressed at the surface of the host cell (Bierne and Cossart 2007). *Lm* becomes therefore closely associated to the host cell membrane and is then able to induce host signaling cascades to promote its uptake by the host cell (Pizarro-Cerda et al. 2012). Previous studies have demonstrated that decreased *Lm* adherence actually culminate in diminished bacterial virulence (Milohanic et al.

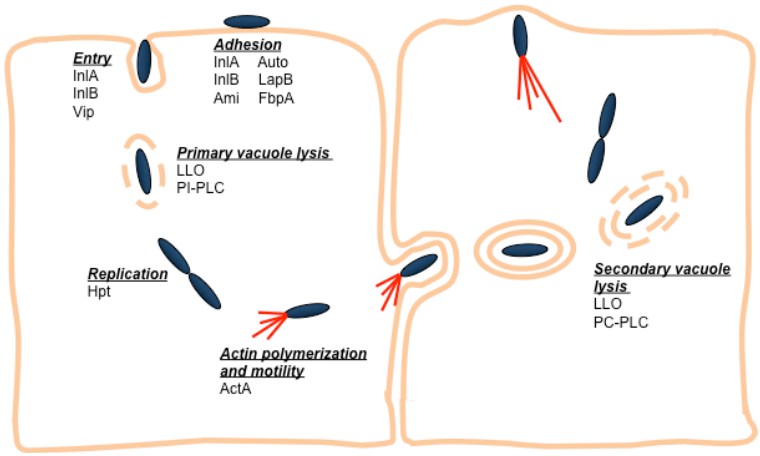

Figure 2. Schematic representation of the different steps of *Lm* cellular infection cycle. The major *Lm* virulence factors implicated in each step are indicated.

2001, Cabanes et al. 2004, Jagadeesan et al. 2010, Reis et al. 2010, Osanai et al. 2013).

(ii) Internalization

In vivo, as well as in *in vitro*-cultured cells, *Lm* can enter and survive inside professional phagocytic cells (Gray et al. 2006). Importantly, as described above, *Lm* upon adhesion induces its own internalization in non-phagocytic cells (Cossart 2011).

Lm invades non-phagocytic cells using a zipper-like mechanism, characterized by multiple intimate interactions established between the bacterium and the host cell membrane (Isberg and Van Nhieu 1994, Swanson and Baer 1995, Mengaud et al. 1996). This process is followed by a progressive invagination of the host plasma membrane leading to the consequent bacterial engulfment (Figure 2). The entry of *Lm* into host cells is a crucial step in its infection process and is therefore mediated by key virulence factors, such as the surface proteins and major *Lm* invasins, InlA and InlB (Figure 2), which will be described further on (Pizarro-Cerda et al. 2012).

(iii) Lysis of the primary vacuole

Once internalized by the host cell, *Lm* resides in a single-membrane endocytic vacuole that quickly (20 minutes) undergoes acidification. This pH

decrease promotes disruption of the vacuole by *Lm* through the secretion and activation of three bacterial proteins (LLO, PI-PLC and PC-PLC), which form pores in the vacuolar membrane and lead to the rupture of this compartment (Figure 2) (Geoffroy et al. 1987, Camilli et al. 1991, Geoffroy et al. 1991, Leimeister-Wachter et al. 1991, Mengaud et al. 1991, Goldfine and Knob 1992, Raveneau et al. 1992, Marquis and Hager 2000).

(iv) Multiplication in the host cell cytoplasm

After lysing the internalization vacuole, *Lm* is released into the host cell cytoplasm, where the abundance of cytosolic nutrients enables the bacterium to grow and multiply exponentially (Figure 2). During this phase of the *Lm* intracellular life, the expression of several genes coding for metabolic and virulence determinants is induced, namely those required for nutrient uptake and further on for actin-based motility and cell-to-cell spread (Chatterjee et al. 2006, Camejo et al. 2009).

(v) Actin-based intracellular movement

Lm is able to induce its own movement throughout the host cell cytoplasm by exploiting the host cellular machinery. Indeed, the bacterium begins recruiting and polymerizing host actin around its surface, forming an actin cloud that envelopes *Lm*. This structure is then progressively transformed into a long comet-like tail localized at one of the bacterial poles (Figure 2). The constant polyrization and depolymerization of actin in this “comet tail” generates the driving force needed for bacterial movement in the host cytoplasm (Smith and Portnoy 1997).

(vi) Intercellular spread

The *Lm* actin tail propels the bacterium randomly through the cytoplasm. Occasionally, *Lm* encounters cell periphery and, in some cases, it can push it outwards, generating a protrusion that contains the propelling bacterium at its tip (Figure 2). If this protrusion encounters the membrane of a neighboring cell, it can force its invagination and consequent engulfment by this cell, generating a double-

membrane *Lm*-containing vacuole. This secondary vacuole can then be lysed in a similar fashion as the primary vacuole, liberating *Lm* into the cytoplasm and enabling the infection of a new host cell, thus restarting the cycle.

I.A.4. Major virulence factors

Some of the *Lm* virulence factors known to be involved in the different steps of the cellular infection cycle are indicated in Figure 2. Although several other have been described and characterized I will describe in more detail some virulence factors that are representatives of each step of the infection cycle.

I.A.4.1. Internalins A and B

Internalins are key effectors for *Lm* invasion into the host cells (Bierne et al. 2007). Nonetheless, other bacterial proteins also contribute for the efficiency of internalization, revealing a complex interaction between *Lm* and eukaryotic cells during the early steps of the cellular infection cycle (Cabanes et al. 2004, Dussurget et al. 2004, Cabanes et al. 2005, Reis et al. 2010).

The internalization of *Lm* by non-phagocytic cells is mainly promoted by two members of the internalin family: internalin A (InlA) and B (InlB) (Figure 3) (Gaillard et al. 1991). The *inlA* and *inlB* genes are transcribed both individually and in an operon, and their expression is under the control of PrfA, the major transcriptional regulator of *Lm* virulence genes.

InlA is a protein of 800 amino acid residues that is required for the invasion of epithelial cells expressing its cognate receptor, E-cadherin (described below). At the N-terminus, InlA has a canonical signal peptide sequence followed by a region of 15 leucine-rich repeats (LRRs) consisting of tandem repeats of 20-22 amino acids (Schubert et al. 2001, Bierne et al. 2007) enriched in leucine residues. The LRRs are present in different prokaryotic and eukaryotic proteins, providing recognition units for protein-protein interaction (Cabanes et al. 2002). An inter-repeat region (IR) separates the LRR region from a second repeat region (B repeat). The C-terminus of InlA exhibits a cell wall-sorting LPXTG motif that allows its covalent linkage to the *Lm* peptidoglycan matrix (Figure 3). The LRR region is

both necessary and sufficient to promote *Lm* entry into epithelial cells (Lecuit et al. 1997).

InlB is a 630-amino acid protein involved in the entry of *Lm* into a broad range of cell types (Dramsi et al. 1995). Its N-terminal domain contains a signal peptide sequence that is followed by six LRRs, an IR region, and a B repeat significantly shorter than that of InlA. The C-terminal domain has three tandem repeats of about 80 amino acids, each containing the dipeptide GW (thus termed GW modules), which mediate the loose attachment of InlB to the *Lm* cell wall *via* a non-covalent interaction with lipoteichoic acids (Jonquieres et al. 1999) (Figure 3). Due to its labile association to the bacterial membrane, InlB can be released to the extracellular medium and act as a soluble factor, interfering with host cell signaling. The GW modules and the B repeat are also involved in interactions with the surface of host cells (Jonquieres et al. 2001). The LRR region of InlB is sufficient to confer invasiveness to non-invasive bacteria such as *L. innocua* (Braun et al. 1999, Jonquieres et al. 1999).

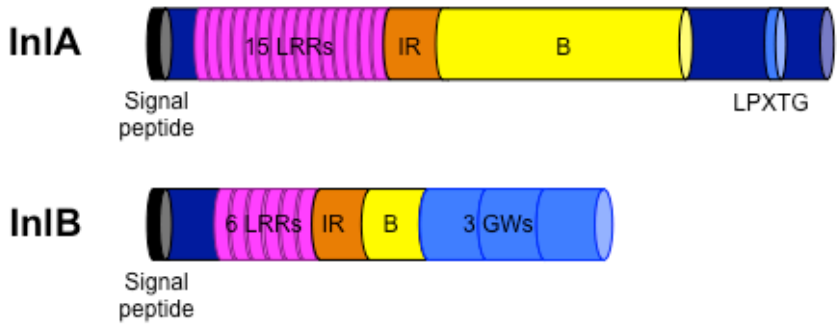

Figure 3. Schematic representation of InlA and InlB. LRRs: leucine-rich repeats; IR: inter-repeat; B: B repeat; LPXTG: cell wall sorting motif allowing the covalent anchorage to the peptidoglycan; GW: GW modules allowing the labile association to the cell wall.

I.A.4.2. Listeriolysin O and phospholipases

Listeriolysin O (**LLO**) is a cholesterol-dependent pore-forming toxin expressed by *Lm*, which plays a key role in the disruption of the primary and secondary vacuoles formed by internalization of *Lm* by host cells. Soon after internalization, the acidification of the *Lm*-containing vacuole allows LLO to reach its maximum activity (Geoffroy et al. 1987). As a consequence of pore formation,

the vacuolar pH rises and LLO is progressively inactivated (Shaughnessy et al. 2006), thus protecting further damage to other host cellular membranes. Recently, this major virulence factor has shown to be multifunctional, displaying both extracellular and intracellular roles during *Lm* infection aside from its primary function in vacuole disruption (Hamon et al. 2012, Seveau 2014). LLO triggers histone modifications prior to bacterial entry and, as a consequence, down-regulates a subset of genes that includes immunity-related genes (Hamon et al. 2007, Hamon and Cossart 2011). Moreover, LLO has been shown to interfere with SUMOylation of host proteins to favor infection (Ribet et al. 2010). Recently, an alternative *Lm* internalization pathway has been proposed, implicating LLO in this process (Vadia and Seveau 2014).

In addition to LLO, the lysis of primary and secondary vacuoles also involves two secreted phospholipases. **PlcA**, a phosphatidylinositol-specific phospholipase C (PI-PLC) is implicated in the disruption of the primary vacuole (Camilli et al. 1991, Geoffroy et al. 1991, Leimeister-Wachter et al. 1991, Mengaud et al. 1991, Goldfine and Knob 1992), while **PlcB,** a phosphatidylcholine-specific phospholipase C (PC-PLC) plays an preponderant role in the disruption of the double-membrane secondary vacuole. The activation of PlcB requires the proteolytic cleavage of its immature precursor by the metalloprotease **Mpl** (Domann et al. 1991, Raveneau et al. 1992, Marquis and Hager 2000).

I.A.4.3. Actin assembly-inducing protein

The intracellular motility of *Lm* relies on the activity of the **actin assembly-inducing protein (ActA)** (Domann et al. 1992, Kocks et al. 1992). This 639-amino acid surface protein exhibits an N-terminal signal peptide and a C-terminal transmembrane hydrophobic sequence that enable the respective targeting and anchoring of ActA to the bacterial surface. ActA, recruits and directly activates host cell proteins involved in the polymerization and depolymerization of cytoskeletal actin filaments. *Lm* initially expresses ActA around the entire surface of the bacteria and actin-clouds formed and then relocalizes at one pole of the bacterium generating actin-comet-tails to allow intracellular movement (Figure 4).

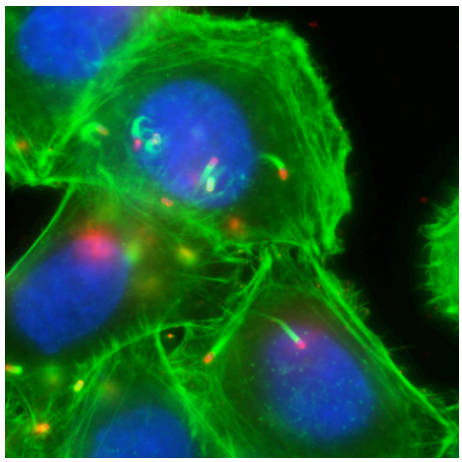

Figure 4. *Lm*-infected cells showing actin comet tails. Image from Molecular Microbiology Group: green: actin; red: *Lm*; blue: nuclei.

I.A.4.4. PrfA

Importantly, expression of the major *Lm* virulence factors is promoted by the transcriptional activator **PrfA,** a regulatory protein of 233 amino acids that belongs to the Crp/Fnr family (Sheehan et al. 1996, Khelef et al. 2006). Interestingly, the transcription of the gene encoding PrfA is thermo-regulated (Renzoni et al. 1997). At lower temperatures, the 5' UTR of the *prfA* mRNA adopts a hairpin structure that prevents access to ribosomes and thus inhibits translation. At temperatures closer to 37 ºC, the 5' UTR hairpin is destabilized, exposing the ribosome-binding site and enabling the translation of PrfA (Johansson et al. 2002). This regulatory mechanism ensures that the expression of *Lm* virulence factors is only promoted in the context of the host, at 37 ºC (Figure 5).

Host cell invasion is critical for listeriosis pathology due to its requirement for the initial crossing of the host intestinal barrier and further colonization of diverse target organs. It is therefore crucial to comprehend how *Lm* induces its

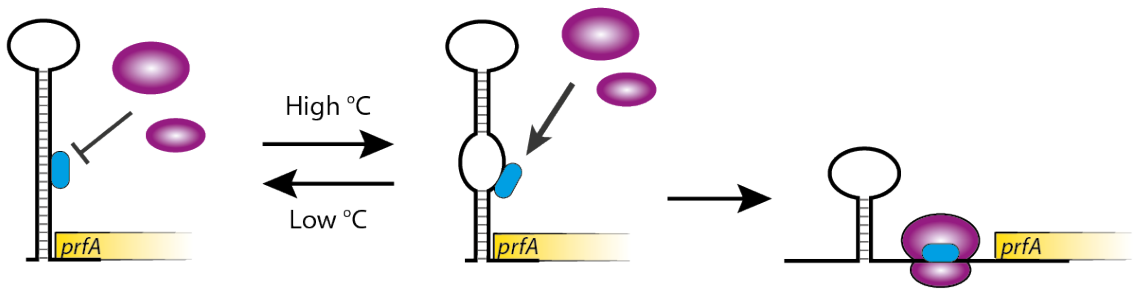

Figure 5. Thermoregulated expression of PrfA (Adapted from (Johansson et al. 2002)

own uptake by host cells. Previous studies revealed the complexity of interactions between multiple bacterial and host factors during the entry process, helping to better understand the molecular basis of the tissue tropism of *Lm* (Seveau et al. 2007). Some of these interactions will be described in more detail in Chapter II of the introduction.

I.B. Enterobacteriaceae

The Enterobacteriaceae family constitutes the largest and most heterogeneous group of clinically relevant Gram-negative rod-shaped bacteria. Its members can be found in the soil, water or vegetation, but are also part of the intestinal microbiota of animals, including humans. Although a very diverse group of bacteria, only a few of its member species are actually pathogenic (Farmer 2003).

I.B.1. *Yersinia*

The genus *Yersinia* comprises 11 species, of which only three are pathogenic for animals (including humans): *Y. enterolitica, Y. pseudotuberculosis*, and *Y. pestis*. *Yersinia* species are facultative anaerobic and non-sporulating rods or coccobacilli that can grow at temperatures ranging from 4 to 40 ºC (Nihlén 1969). Like *Lm*, *Y. enterolitica* and *Y. pseudotuberculosis* are motile at 25 ºC due to the presence of flagella that is not expressed at 37 ºC. Once inside its host *Yersinia* becomes non-motile (Boyce 1985).

Genetic studies suggest that *Y. pestis* is a subspecies that evolved from *Y. pseudotuberculosis*, while *Y. enterolitica* evolved through a different lineage (Chain et al. 2004). Therefore, *Y. pestis* showed a relatively recent evolution from a pathogen ubiquitously found in the environment and able to infect mammalian intestines, to an insect-transmitted mammalian blood-borne pathogen that causes plague, a highly fatal disease. *Y. enterolitica* and *Y. pseudotuberculosis* are either food- or water-borne pathogens that cause mainly self-limiting gastrointestinal and intestinal lymphoid system infections (Bottone 1997, Perry and Fetherston 1997, Sulakvelidze 2000, Carniel et al. 2002).

I.B.1.1. Yersiniosis

Through the ingestion of contaminated food, *Yersinia* causes a human enteric infection called yersiniosis. This disease is more common in European countries with temperate climates, although it has been reported in all continents (Bottone 1999, Smego et al. 1999). In immunocompetent individuals, symptoms range from diarrhea, abdominal pain and fever to pseudo-appendicitis. Although this gastrointestinal infection is self-limiting, symptoms can become more aggravated. Septicemia caused by *Yersinia* infection, although rare has been reported with high mortality rate (75%) (Bottone 1997, Deacon et al. 2003). In immunocompromised patients, infection by *Yersinia* can develop to chronic conditions or autoimmune disease (Laitenen et al. 1972, Laitinen et al. 1977). Upon entering the host, bacteria that survived the acidic conditions of the stomach reach the intestine and are usually restricted to the small and proximal large intestine, Peyer's patches and mesenteric lymph nodes (Young et al. 1996, Viboud and Bliska 2005). At the small intestine, *Yersinia,* attach and enter into M cells (Isberg and Barnes 2001). At the Peyer's patches, bacteria replicate extracellularly and induce strong inflammation (Grassl et al. 2003). From Peyer's patches, *Yersinia* disseminate via bloodstream inside phagocytes to mesenteric lymph nodes or other organs, such as liver and spleen, and even the central nervous system (Koornhof et al. 1999, Smego et al. 1999). At the target tissues, the extracellular replication of *Yersinia* originates microabcess lesions, in which bacteria resist to phagocytosis (Carniel et al. 2002).

I.B.1.2. Cellular infection mechanisms

Y. enterolitica and *Y. pseudotuberculosis* are well adapted to human infection. They are able to precisely regulate their virulence gene expression according to the particular conditions of the host environment, not only to potentiate their invasive capacity but also to modulate the host immune response in their own favor (Pepe et al. 1994). Pathogenic *Yersinia* spp. virulence genes are located both in the chromosome and in a 70-kb plasmid called pYV (for "plasmid involved in yersinial virulence") (Portnoy and Falkow 1981, Revell and Miller

2001). The pathogenesis of *Y. pseudotuberculosis* was shown to require the presence of this virulence plasmid (Cornelis et al. 1989).

(i) Adhesion to the host cell surface

The adhesion of *Yersinia* to host cells is mediated by bacterial adhesins and invasins either chromosomally- or plasmid-enconded, such as invasin (Inv), *Yersinia* adhesin A (YadA) and attachment and invasion locus (Ail) (Isberg and Falkow 1985, Miller and Falkow 1988, Bliska et al. 1993).

Inv, the major invasin of enteropathogenic *Yersinia*, is a chromosomally encoded 103-kDa outer membrane protein that was shown to be sufficient to confer invasive capacity to non-invasive bacteria (Isberg and Falkow 1985). This invasion-promoting protein is not present in *Y. pestis* (Miller and Falkow 1988, Rosqvist et al. 1988). Inv binds to β_1-integrins at the surface of M cells with higher affinity than fibronectin, the physiological integrin ligand (Isberg and Leong 1990, Hamburger et al. 1999).

YadA is a surface adhesin of 45 kDa encoded in the pYV virulence plasmid of both *Y. enterolitica* and *Y. pseudotuberculosis,* but absent in *Y. pestis*. This multi-functional virulence factor confers both adhesive and invasive features to enteric *Yersinia* (Balligand et al. 1985, Kapperud et al. 1987, Martinez 1989, Eitel and Dersch 2002, Heise and Dersch 2006). Compared to other *Yersinia* species, YadA from *Y. pseudotuberculosis* binds with high affinity to fibronectin already bound to β_1-integrins, promoting an uptake process similar to the one mediated by Inv (Heise and Dersch 2006).

Ail is a 17-kDa chromosomally-encoded outer membrane protein that is sufficient to cause both attachment and entry of *Y. enterolitica* into cultured cells, and also contributes to serum resistance to complement-mediated killing (Miller et al. 1989, Miller et al. 1990, Pierson and Falkow 1993, Wachtel and Miller 1995). Although Ail promotes bacterial adhesion and invasion in cultured cells, disruption of the *ail* gene showed that this protein is not involved in virulence (Wachtel and Miller 1995, Yang et al. 1996). Functional redundancy between Ail, Inv and YadA might account for this lack of phenotype *in vivo*, suggesting a crucial role for these factors in disease. In *Y. pseudotuberculosis*, Ail does not contribute to adhesion

but still confers serum resistance (Ho et al. 2012, Ho et al. 2012). Virulence of *Y. pestis* requires Ail binding for effector delivery (Kolodziejek et al. 2012).

(ii) Delivery and secretion of virulence effectors

The pYV virulence plasmid encodes a virulence effector delivery system, called type-three secretion system (TTSS). The yersinial TTSS comprises a basal body-like structure resembling that of the bacterial flagellum, which is topped by an extracellular needle-like appendage (injectisome) that protrudes outside the bacteria and contacts with the host cell membrane (Figure 6) (Thomas and Finlay 2004). Upon bacterial adhesion to host cells, the injectisome is assembled and allows the direct transport of virulence effectors from the bacteria cytoplasm into the host cytoplasm (Portnoy and Falkow 1981, Cornelis and Wolf-Watz 1997).

The TTSS or injects *Yersinia* outer proteins (Yops) directly into the host cell cytoplasm. Yops are exotoxins that mimic the activity of several host enzymes (e.g. phosphatases, proteases, kinases and acetylases) in order to subvert the host cytoskeleton and immune signaling pathways to favor infection (Table 1) (Cornelis 2002, Viboud and Bliska 2005).

Besides three *Yersinia* species (Y. enterolitica, Y. pseudotuberculosis and Y. pestis), TTSS is found in a broad number of bacterial pathogens such as *Bordetella bronchiseptica*, *Burkholderia pseudomallei*, *Chlamydia pitsitacii*, *Erwinia amylovora*, *Erwinia crysanthemi*, pathogenic *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Rhizobia* spp.,

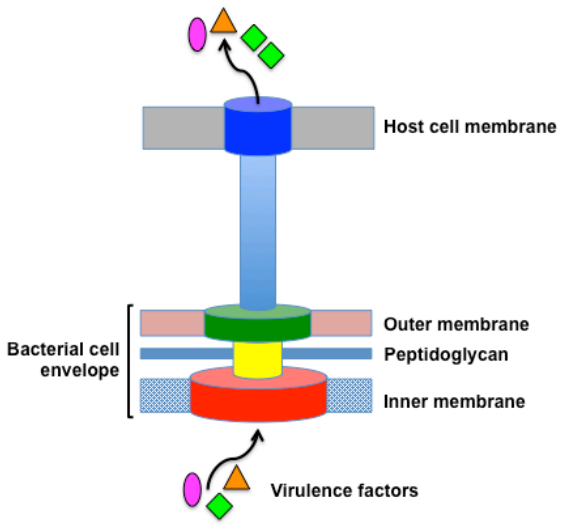

Figure 6. Schematic representation of TTSS injectisome.

Salmonella typhimurium, *Shigella flexneri* and *Xanthomonas campestri* (Hueck 1998, Yuk et al. 1998, Winstanley et al. 1999)

Table 1. *Yersinia* outer proteins (Yop) delivered to the host cell by TTSS.

Effector	Function	References
YopH	Tyrosine phosphatase	(Guan and Dixon 1990, Zhang et al. 1992)
YopE	Rho-GAP[1]	(Rosqvist et al. 1990)
YopM	Gene transcription[2]	(Leung and Straley 1989, Skrzypek and Straley 1996, Skrzypek et al. 1998, Sauvonnet et al. 2002)
YopJ	Ubiquitin-like protease	(Orth 2002)
YopO	Serine/Threonine kinase	(Fallman et al. 1997, Juris et al. 2002)
YopT	Cyseine protease	(Iriarte and Cornelis 1998)
YopK	Unknown function	(Holmstrom et al. 1995, Holmstrom et al. 1995, Holmstrom et al. 1997)
YopB	TTSS structure (pore formation)	(Rosqvist et al. 1994, Sory and Cornelis 1994, Boland et al. 1996, Hakansson et al. 1996)
YopD	TTSS structure (pore formation)	(Rosqvist et al. 1994, Sory and Cornelis 1994, Boland et al. 1996)

[1] Rho-GTPase activating protein
[2] Function unknown; recent results on trafficking towards the nucleus might influence gene expression.

(iii) Internalization

Yersinia uptake is known to be mediated by β_1 integrins. Integrins are eukaryotic heterodimeric αβ-protein receptors that mediate interactions between cells, cell-soluble protein, and cell-extracellular matrix (ECM) (Hynes 2002, Delon and Brown 2007). Depending on which α subunit it dimerizes with, the β_1 integrin subunit can bind to different ECM ligands, to other cells, and to soluble ligands. Besides cognate interactions, β_1 integrin also binds to ligands from viruses and bacteria, including *Yersinia* Inv (Hynes 1992, van der Flier and Sonnenberg 2001, Hynes 2002). At sites of adhesion, integrin clustering is triggered upon ligand binding, leading to the recruitment of signaling proteins. These signaling effectors establish an indirect connection of the actin cytoskeleton to the cytoplasmic domain of the integrin (Hynes 2002). This domain has no catalytic activity but recruits components that serve as linking or docking proteins for cytoskeleton-associated elements (Pizarro-Cerda and Cossart 2006).

Integrin engagement by Inv transmits biochemical signals and mechanical force across the cell membrane to favor invasion (Figure 7). Internalization of the activated receptor depends on downstream interactions of signaling molecules that result in rearrangements of the actin cytoskeleton beneath attached *Yersinia*.

Y. pseudotuberculosis induces binding and activation of the focal adhesion kinase (FAK) at the cytoplasmatic domain of integrin receptor (Schaller et al. 1995). FAK tyrosine phosphorylation recruits and activates Src kinase that phosphorylates downstream effector molecules involved in cytoskeletal rearrangements. This FAK-Src complex regulates invasin-mediated invasion. (Alrutz and Isberg 1998, Bruce-Staskal et al. 2002). The interaction between Inv and integrin activates the small GTPases Rac1 and Arf6 also a membrane trafficking associated protein (Honda et al. 1999, Alrutz et al. 2001). In turn, these proteins promote the recruitment of the PI-5 kinase (PIP5K) to the bacterial entry site, inducing the local production of phosphatidylinositol biphosphate (PIP2) (Wong and Isberg 2003). PIP2 is an important second messenger that mediates the subcellular localization and activation of actin-dynamics regulators (Pizarro-Cerda and Cossart 2004), such as N-WASP and its interacting partners, Nick and Grb2. In addition, *Yersinia* uptake is also modulated by a crosstalk between microtubules and Rho GTPases (McGee et al. 2001, McGee et al. 2003).

(iv) Inhibition of phagocytosis

It is important to mention that the invasive behavior of *Y. enterocolitica* and *Y. pseudotuberculosis* is only required during the initial stage of disease, to allow bacterial translocation from the lumen of the intestine to the underlying mucosal

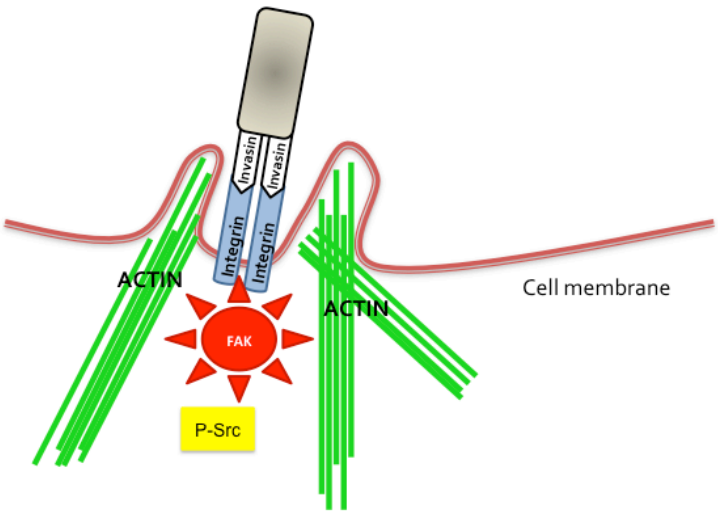

Figure 7. Schematic representation of the internalization process triggered by *Yersinia*. The interaction between *Yersinia* invasin and host cell integrins activates signaling cascades involving FAK and Src, which results in remodeling of the actin cytoskeleton to promote bacterial uptake.

tissue. *In vivo*, bacterial proliferation takes place in the extracellular space, thus to avoid phagocytosis, these species have developed an important arsenal of anti-phagocytic Yops that are injected into target host immune cells to block the potential internalization of bacteria. After crossing the intestinal epithelium, macrophages and dendritic cells of the mucosa will target *Yersinia* therefore avoidance of phagocytosis is critical for bacterial survival.

Upon injection into the host cell cytoplasm, Yop effectors inhibit signaling cascades involved in cytoskeleton dynamics by interfering with host actin-regulating proteins, such as small GTPases (Rac1, RhoA and Cdc42) (Rosqvist et al. 1990, Hakansson et al. 1996, Iriarte and Cornelis 1998, Zumbihl et al. 1999, Barz et al. 2000, Black and Bliska 2000, Andor et al. 2001, Shao et al. 2002). Delivery of anti-phagocytic Yops is also mediated by β1 integrin activation (Bovallius and Nilsson 1975, Isberg 1989, Isberg et al. 2000, Fallman et al. 2002). Phosphatase activity of YopH (Table 1) dephosphorylates FAK inhibiting inv-mediated uptake. Moreover YopE GAP function (Table 1) blocks Rac1 signaling inhibiting phagocytosis through the disruption of actin filaments (Black and Bliska 2000).

The ability to block invasion and further degradation within phagocytes relies on the expression of the virulence effectors YopH, YopE, YopO, and YopT, all encoded in the pYV virulence plasmid (Rosqvist et al. 1988, Rosqvist et al. 1990).

I.B.2. Pathogenic *E. coli*

Escherichia coli (*E. coli*) is a facultative anaerobic, motile, non-sporulating, Gram-negative bacillus, with an optimal growth at 37 °C, although some laboratory strains can replicate at temperatures up to 49 °C (Kubitschek 1990, Kubitschek 1990, Fotadar et al. 2005, Darnton et al. 2007). It is the primary facultative anaerobe of the normal intestinal microflora of humans and other mammals, adhering to the mucus layer of the large intestine. More than just a benign commensal, *E. coli* can acquire genetic elements encoding virulence factors, which enable its evolution into, often deadly, pathogenic strains. Several *E. coli* strains are known to cause intestinal and extra-intestinal diseases by tampering

with different host cellular processes, through the engagement of host signaling pathways.

E. coli identification is based on O (somatic), H (flagellar), and K (capsular) surface antigen profiles, which are specific for different disease-associated serotypes (Lawn et al. 1977, Orskov and Orskov 1977, Orskov et al. 1977). Toxins and other virulence effectors allow the discrimination of diarrheagenic pathotypes from non-pathogenic and from extra-intestinal *E. coli* (Donnenberg et al. 1998, Nataro and Kaper 1998, Nataro and Martinez 1998, Kohler and Dobrindt 2011). Pathogenic *E. coli* are responsible for three types of infections in humans: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis). Infections caused by a particular strain depend on the distribution and expression of virulence determinants, such as adhesins, invasins, toxins, as well as on the bacterial capacity to subvert host defense mechanisms.

Different diarrheagenic *E. coli* types have been identified: enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC) (Table 2) (Nataro and Kaper 1998). *E. coli* strains were also isolated from other infections, such as uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC) and sepsis-causing *E. coli* (SEPEC), which comprise the extra-intestinal group of pathogenic *E. coli* (Russo and Johnson 2000, Johnson and Russo 2002, Johnson et al. 2005, Smith et al. 2007).

Table 2. Diarrheagenic *E. coli* types, their target cells and effects.

E. coli	**Target**	**Effect**
EPEC	Small bowel enterocytes	A/E lesions causing inflammation and diarrhea
EHEC	Colon cells	A/E lesions and systemic toxin absorption causing bloody diarrhea
ETEC	Small bowel enterocytes	Enterotoxin secretion causing diarrhea
EAEC	Small and large bowel epithelia	Cytotoxin and enterotoxin secretion causing mucosal damage and diarrhea
EIEC	Colon epithelial cells	Intracellular pathogen causing inflammation, dysentery and diarrhea
DAEC	Small bowel enterocytes	Cytopathic (cellular extensions) causing inflammation and diarrhea

A/E lesions: attaching and effacing epithelial lesions

I.B.2.1. EPEC and EHEC

EPEC and EHEC have different clinical manifestations and are a group of pathogens that are evolving and are still a challenge in public health. These

pathogens share the same mechanism adhesion to intestinal cells. EPEC and EHEC strains are responsible for the high frequency of intestinal disease (Nataro and Kaper 1998). Infection outbreaks in industrialized countries demonstrated that EHEC is a zoonotic strain (commensal in ruminants) that is transmitted through fecal contamination of water - food resources (vegetables). This develops human serious clinic events as hemorrhagic colitis (HUC), hemolysis and renal failure (HUS), witch are mediated by toxins (Shiga toxins) characteristic of EHEC. EPEC is more common in developing poor countries, and is the main cause of diarrhea in small children. EPEC as never been found in animals, and its only reservoir is humans. Toxin production is not an original characteristic of these human strains (Feng 2012, Hartland and Leong 2013).

Host-pathogen interaction studies with EPEC and EHEC demonstrated that, upon colonization of intestinal epithelial cells, tightly adhered bacteria induce the formation of “attaching and effacing” (A/E) lesions (Knutton et al. 1989, Schauer and Falkow 1993, Marches et al. 2000, Donnenberg and Whittam 2001, Deng et al. 2003, Kaper et al. 2004, Mundy et al. 2005). These histopathological features are characterized by tight bacterial attachment to the epithelial cell surface resulting in the accumulation of filamentous F-actin-rich pedestal-like structures, which lift up the bacteria above the plane of the cell membrane (Staley et al. 1969, Ulshen and Rollo 1980, Moon et al. 1983). Actin-pedestals result from the destruction of the microvilli and are essential for colonization. These structures are formed in a TTSS-dependent manner upon translocation of bacterial effectors into the host cell (Figure 8) (Dean et al. 2005, Garmendia et al. 2005, Hayward et al. 2006, Dean and Kenny 2009).

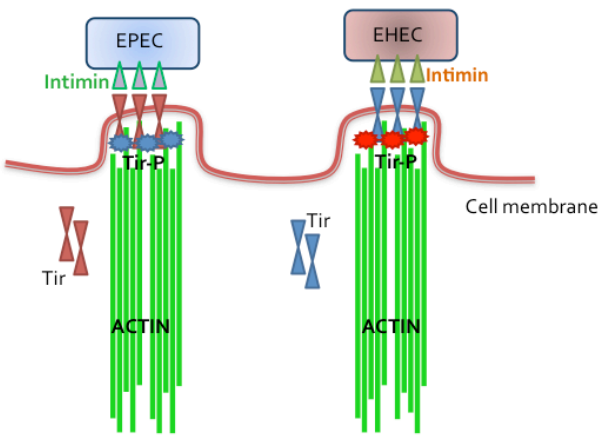

Figure 8. Schematic representation of EPEC/EHEC-induced actin pedestal formation.

I.B.2.1.1. Virulence effectors

The EPEC/EHEC TTSS is encoded in the locus of enterocyte effacement (LEE), a 35-kb pathogenicity island that is absolutely required for intestinal colonization and A/E lesion formation (Jarvis et al. 1995, Elliott et al. 1998, Perna et al. 1998). The LEE of EPEC and EHEC is highly conserved and homologous to the ones found in other pathogens that also interfere with actin polymerization, such as *Shigella*, *Salmonella* and *Yersinia* (Sperandio et al. 1998, Coburn et al. 2007). Besides TTSS components, the LEE encodes other bacterial effectors that depend on TTSS activity (Table 3). The first interaction of the bacteria with host cell is made trough contact of TTSS with enterocyte membrane by EspA-containing filamentous organelle of EPEC (Knutton et al. 1998).

Table 3. Pathogenic *E. coli* effectors and functions

Pathotype	Effector	Function	Activity/Effect
EPEC, EHEC	Intimin	Adhesin	Intimate attachment to epithelial cells; stimulates immune responses
EHEC	OmpA	Adhesin	Adhesion to epithelial cells
EHEC	Shiga toxin	Toxin	Inhibit protein synthesis; induces apoptosis
EPEC, EHEC	Tir	Receptor (Type III effector)	Nucleation of cytoskeletal proteins; loss of microvilli; GAP-like activity
EPEC	EspC	Autotransporter	Ion secretion
EHEC	EspP	Autotransporter	Cleavage of coagulator factors
EPEC, EHEC	EspF	Type III effector	Opens tight junctions; induces apoptosis
EPEC, EHEC	EspH	Type III effector	Modulates filopodia and pedestal formation
EPEC, EHEC	Map	Type III effector	Disrupts mitochondrial membrane potential
EHEC	StcE	Type II effector	Disrupts complement cascade
EHEC	Ehx	Toxin	Cell lysis
EPEC, EHEC	LifA/Efa	Toxin	Inhibits lymphocyte activation, adhesion

The translocated intimin receptor (**Tir**) through TTSS is injected into the host cytoplasm and then integrates in the host cell membrane in a hairpin-like conformation (Jerse et al. 1990, de Grado et al. 1999, Batchelor et al. 2000). There, it functions as a receptor for **intimin**, a bacterial outer membrane adhesin that is encoded in the LEE operon (Jerse et al. 1990, de Grado et al. 1999, Batchelor et al. 2000). Intimin binds to the extracellular domain of Tir, inducing clustering of the receptor, while the cytoplasmic Tir-N and Tir-C domains trigger downstream signaling events that ultimately lead to actin polymerization beneath the attached bacteria (Kenny et al. 1997, Kenny and Finlay 1997, Deibel et al. 1998, Campellone et al. 2004, Caron et al. 2006, Hayward et al. 2006). Tir-N as

been shown to bind proteins involved in actin assembly (α-actinin and cortactin), while Tir-C recruits actin polymerization components, such as the actin nucleator complex Arp2/3 and its activator N-WASP (Campellone et al. 2004, Campellone et al. 2006, Brady et al. 2007).

The Tir-intimin interaction appears as a key event in both EPEC and EHEC infection, as pedestal formation may also inhibit phagocytosis and therefore contribute to immune evasion. Tight attachment of bacteria to the host cell membrane is not a static phenomenon. The dynamic actin polymerization and depolymerization occurring beneath adhered bacteria allow their movement along the host cell membrane ("surfing") and contribute to cell-to-cell spread (Sanger et al. 1996).

The EHEC effector **EspFu** is also translocated across the host cell membrane via TTSS. Upon the establishment of a Tir-intimin interaction and further Tir clustering, EspFu is recruited at the adhesion site, where it sequesters N-WASP (Campellone et al. 2004, Lommel et al. 2004, Garmendia et al. 2005, Campellone et al. 2006). EspFu is homologous to another LEE-encoded effector, **EspF** that also binds and activates N-WASP. EspF was found in EPEC and described to nucleate a host protein-signaling complex of sorting nexin-9 (**SNX9**). EspF and SNX9 appear as an alternative signaling pathway that induces membrane-remodeling events associated with N-WASP and Arp2/3 (Alto et al. 2007, Cheng et al. 2008).

EPEC and EHEC Tir proteins are highly similar both in structure and in function, although substitution of the EPEC Tir by that of EHEC results in a strong reduction of pedestal formation (DeVinney et al. 2001, Kenny 2001, Campellone et al. 2002). Both EPEC and EHEC Tir proteins are receptor tyrosine kinases (RTKs) phosphorylated upon intimin interaction, although the main difference resides in the identity of the phosphorylated tyrosine (Y) residue. EPEC infection induces Tir Y474 phosphorylation, which in turn recruits the adaptor protein Nck that binds and activates N-WASP and Arp2/3, resulting in actin pedestal formation (Gruenheid et al. 2001, Rohatgi et al. 2001, Campellone et al. 2004). On the other hand, EHEC infection induces Tir phosphorylation at the Y458 residue. This promotes the recruitment of the insulin receptor tyrosine kinase substrate (**IRTKS**), which in turn establishes the link between Tir and EspFu. The role of IRTKS in EHEC-mediated actin pedestal formation indicates that EHEC has evolved to use

a host protein to link two secreted effectors required for N-WASP- and Arp2/3-driven actin polymerization (Campellone et al. 2002, Campellone and Leong 2003, Allen-Vercoe et al. 2006, Brady et al. 2007, Vingadassalom et al. 2009).

EHEC infection is associated with hemolytic uremic syndrome (HUS) a life-threatening infection characterized by hemolytic anemia, thrombocytopenia, and renal failure. HUS is caused by the action of **Shiga-like toxin** (**Stx**), an EHEC specific effector protein that cleaves ribosomal RNA (thus compromising protein translation) (Melton-Celsa et al. 1998). Stx is secreted in the colon and systemically disseminated to the kidneys, where it induces damage to renal endothelial cells (Andreoli et al. 2002). The O157:H7 serotype is responsible for this infection and the one that includes the most virulent strains (O'Brien et al. 1992, Karmali 2004, Karch et al. 2005).

II. POST-TRANSLATIONAL MODIFICATIONS IN BACTERIAL INFECTION

Cellular microbiology studies brought new evidence of how bacterial pathogens manipulate the host cell signaling machinery with high precision to promote and establish infection. In addition, these studies helped to characterize and understand cell biology processes. Despite their evolutionary divergence, different bacterial effectors often target the same host pathways. Several pathogens have been good models to understand what is currently known regarding protein-protein interactions, protein post-translational modifications (PTMs), and protein signaling cascades (Haglund and Welch 2011).

Control of the activity level of proteins is achieved through the introduction of PTMs, i.e. modifications of specific amino acid residues with biochemical groups, which typically results in either the activation or inactivation of these proteins. PTMs, which can be reversible or irreversible, are often elicited in response to different environmental triggers. The extreme importance of this level of protein activity regulation makes PTMs a preferential target mechanism for bacterial pathogens. The most frequent PTMs targeted directly or indirectly by bacterial pathogens have been reviewed: phosphorylation, ubiquitination, SUMOylation, AMPylation, ADP-ribosylation, acetylation, amidation and proteolysis (Ribet and Cossart 2010, Ribet and Cossart 2010). Phosphorylation, as a mechanism of protein activity regulation, will be presented in more detail regarding the context of this work.

II.A. Protein phosphorylation

Bacterial pathogens have evolved a whole array of virulence factors that hijack the host cell machinery to create a suitable niche for their survival and proliferation. In general bacterial surface proteins that mediate the attachment to host cell through the interaction with surface receptors trigger signaling events that promote close bacteria-host association and/or bacterial internalization. There are some bacterial interactions that particularly induce plasma membrane remodeling and cytoskeletal rearrangements (Mattoo et al. 2007). A strategy used by many pathogens is to interfere with the phosphorylation cascades of intracellular host signaling pathways (Bhavsar et al. 2007).

Addition of a phosphate group (from ATP) to specific amino acids (serine/threonine/tyrosine) is the hallmark of the reversible protein modification mechanism catalyzed by kinases. Sequential phosphorylation or dephosphorylation events occur within signaling cascades to activate or inactivate host kinases as well as their substrates. The dephosphorylation of these signaling components is driven by phosphatases, which hydrolyse the existing phosphoester bond.

Bacterial pathogens commonly hijack host cell phosphorylation machinery during infection. Bacterial effectors can directly phosphorylate host proteins, or requires host kinases to mediate target phospohorylation. In addition, there are examples of bacterial effectors that also exhibit phosphatase activity (Bliska et al. 1991, Viboud and Bliska 2005, Navarro et al. 2007).

II.A.1. Protein phosphorylation in *Listeria monocytogenes* infection

As previously mentioned, InlA and InlB are the major *Lm* surface-associated proteins that promote bacterial entry into non-phagocytic cells upon interaction with their specific host cell surface receptors. While InlA interacts with only one receptor, the E-cadherin, InlB interacts with several other receptors among which hepatocyte growth factor receptor (c-Met) is the major representative (Mengaud et al. 1996, Braun et al. 2000, Shen et al. 2000, Jonquieres et al. 2001). The signaling cascades triggered downstream the InlA/E-cadherin and InlB/c-Met interactions, during *Lm* entry into host cells, have been characterized in detail (Bierne and Cossart 2002, Cossart et al. 2003, Sousa et al. 2004, Sousa et al. 2005, Hamon et al. 2006, Sousa et al. 2007, Bonazzi et al. 2008, Pizarro-Cerda et al. 2012).

c-Met belongs to the family of RTKs, an important family of transmembrane signaling receptors, expressed in a variety of cells. Upon stimulation by InlB, c-Met phosphorylation takes place, together with phosphorylation of Cbl, Shc and Gab1 (Ireton et al. 1996, Shen et al. 2000). The recruitment and activation of Gab1 promoted by InlB can take place through two redundant pathways, both of which require either phosphorylation of c-Met tyrosine residues and Gab1 binding to the activated c-Met multi-docking site, or activation of PI3K, leading to recruitment of phosphatidylinositol triphosphate (PIP3) and Gab1 (Basar et al. 2005). Gab1, Shc

and Cbl are also involved in the activation of PI3K (Ireton et al. 1996), which in turn is involved in activation of the small GTP-binding protein Rac1 and induction of actin polymerization through the activity of WAVE and Arp2/3 complex for membrane remodeling and bacterial internalization (Figure 9) (Bierne et al. 2005).

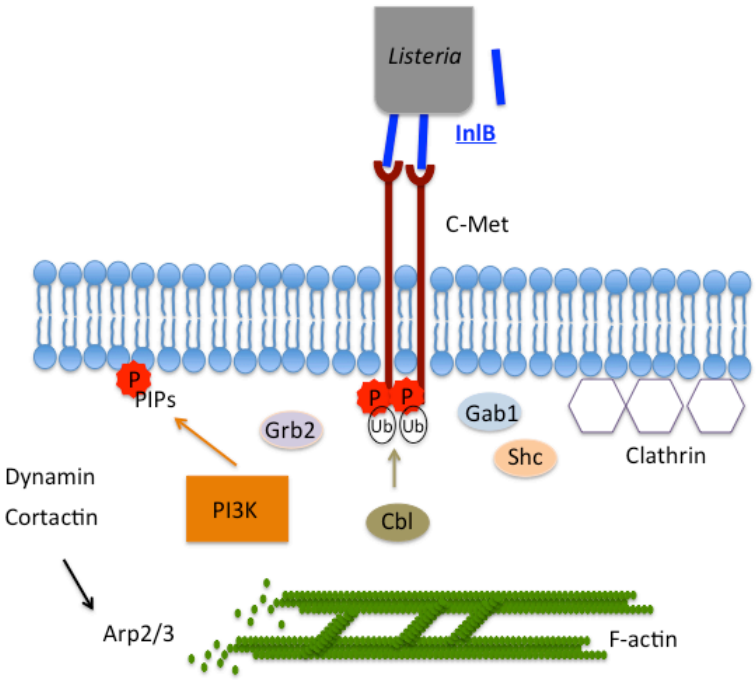

Figure 9. Schematic representation of the host factors that mediate the InlB/c-Met-dependent *Lm* uptake.

The structure of InlB and its labile association to the cell wall of *Lm* allows the bacteria to express this internalin in both *Lm*-bound and soluble forms. The interaction between *Lm*-bound and soluble InlB with c-Met induces strong actin polymerization at the site of bacterial attachment. Moreover the stimulation of c-Met by soluble InlB promotes a spread membrane ruffling and further cell scattering (Shen et al. 2000).

PIP2 and PIP3 are two plasma membrane phosphoinositide species that function as modulators of actin dynamics during *Lm* invasion. The InlB/c-Met interaction is also responsible for the production of phosphatidylinositol 4-phosphate (PIP4) by PI-4 kinases, which are activated in a PI3K-independent mechanism of host cell invasion by *Lm* (Pizarro-Cerda et al. 2007). Besides lipid kinases, host lipid phosphatase OCRL dephosphorylates PIP2 and PIP3.

Moreover, OCRL phosphatase activity at the plasma membrane restricts *Lm* invasion by altering actin dynamics at bacterial entry sites (Kuhbacher et al. 2012).

During InlA/E-cadherin-mediated entry, Src, a non-receptor tyrosine kinase is activated. Cortactin, a Src substrate is immediately recruited and the Arp2/3 complex is in turn activated. In addition, the small GTPase Rac1 is also required for this process (Sousa et al. 2007). These data suggest a model in which activated Src kinase, together with Rac1, promotes recruitment and phosphorylation of cortactin and activation of Arp2/3 complex at the bacterial entry site, where active actin polymerization takes place. Subsequentially, it has been shown that InlA triggers two successive E-cadherin post-translational modifications: Src-mediated tyrosine phosphorylation and ubiquitination by the ubiquitin ligase Hakai (Figure 10) (Bonazzi et al. 2008).

Several studies have demonstrated that *Lm* entry into cells involves the clathrin-dependent endocytic machinery (Veiga and Cossart 2005, Veiga et al. 2007). Moreover, both InlA and InlB trigger clathrin tyrosine phosphorylation that is required for bacterial internalization (Bonazzi et al. 2011).

As mentioned before, bacterial effectors directly or indirectly induce host protein phosphorylation. Interestingly, these effectors can be themselves post-translationally modified. In the context of *Lm* infection, studies have revealed that some virulence factors can be phosphorylated either by bacterial kinases (e.g. *Lm* activates its superoxide dismutase through phosphorylation, enabling its protective

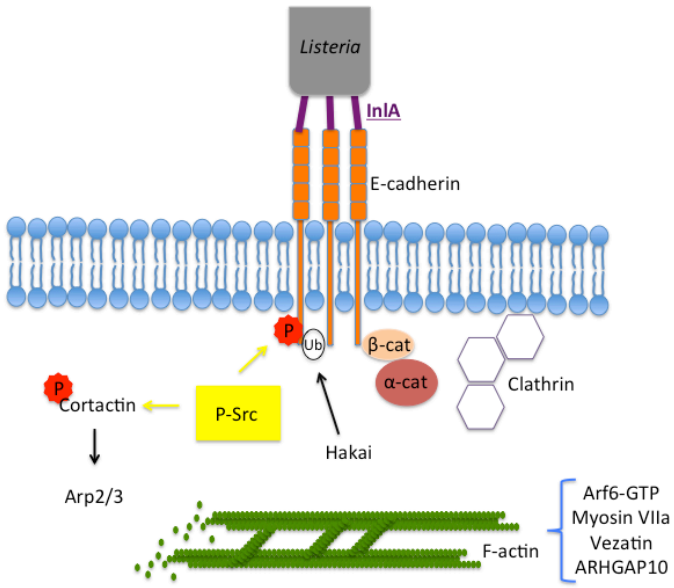

Figure 10. Schematic representation of the host factors that mediate InlA/E-cadherin-dependent *Lm* uptake.

role against reactive oxygen species produced by the host innate immune system (Archambaud et al. 2006)) or by host kinases. Examples of the latter case include the phosphorylation of LLO, to prevent its pore-forming activity to cause further host cell damage, and of ActA, to mimic the actin nucleation-promoting activity of host cell proteins (Schnupf et al. 2006, Chong et al. 2009).

II.A.2. Protein phosphorylation in other bacterial infections

Several studies have demonstrated the importance of phosphorylation signaling events in the establishment of infections. Some examples will be presented in more detail.

The effector YpkA produced by *Yersinia spp.* has structural and functional similarities to serine/threonine kinases. This effector is autophosphorylated and activated in the host cell, where it disrupts the actin-based cytoskeletal system (Barz et al. 2000, Dukuzumuremyi et al. 2000, Juris et al. 2000, Prehna et al. 2006, Wiley et al. 2009, Edelmann et al. 2010, Pha et al. 2014). Host kinase Hri (eIF2α kinase) is highly phosphorylated by YpkA and this event was shown to play a protective role against YpkA-derived cell toxicity (Wiley et al. 2009). YopM, another *Yersinia* effector, has been shown to simultaneously bind to and activate two host protein kinases, protein kinase C-like 2 (PRK2) and ribosomal S6 protein kinase 1 (RSK1) (McDonald et al. 2003, Hentschke et al. 2010). As mentioned, to promote invasion, *Yersinia* induces the activation of several other host kinases, such as FAK, Src and PI3K (Alrutz and Isberg 1998, Cossart and Sansonetti 2004).

As previously mentioned, EPEC and EHEC make use of a TTSS for the delivery of effector molecules to generate actin- and keratin-rich pedestals beneath adherent bacteria (Patel et al. 2006). During this process, the intimin receptor (Tir) translocates into host cell and inserts into the plasma membrane. Tir becomes phosphorylated on tyrosine residues by host kinases Fyn, Abl and PKA, leading to the generation of actin filaments beneath the attached bacteria and the consequent formation of the pedestal structure (Gruenheid et al. 2001, Backert et al. 2008, Brandt et al. 2009). EPEC and EHEC actin pedestals rely on Tir phosphorylation as an upstream command signal for the actin polymerization

machinery (Nieto-Pelegrin and Martinez-Quiles 2009, Vingadassalom et al. 2009, Crepin et al. 2010, Vingadassalom et al. 2010). Recent studies demonstrated that the secreted effector EspZ augments FAK and AKT phosphorylation, contributing to cytotoxicity protection (Shames et al. 2010). Furthermore, these extracellular pathogens appear to block bacterial uptake by a mechanism dependent on caveolin-1 tyrosine phosphorylation (Boettcher et al. 2010).

Both *Salmonella* and *Shigella* interact with host cells by a TTSS that delivers bacterial effectors directly into the host cytoplasm, in subdomains enriched in signaling molecules, such as tyrosine kinases of the Src family (Cossart and Sansonetti 2004). SopB, a *Salmonella* TTSS effector, functions as a phosphoinositide phosphatase that catalyzes the dephosphorylation of host phosphatidylinositol species, stimulating actin rearrangements (Norris et al. 1998, Terebiznik et al. 2002). SptP, another *Salmonella* TTSS-secreted protein, has two functions: as a tyrosine phosphatase, it regulates MAPK activity induced by bacterial internalization; and as a GAP, it controls the activity of Cdc42 and Rac, resulting in the shrinking of the entry focus by blocking further actin polymerization (Stebbins and Galan 2000). Recently, SteA has been shown to control SCV membrane dynamics (Domingues et al. 2014). Constitutive expression in HeLa cells lead to the induction of genes related to host cell processes associated with serine/threonine kinase signaling pathways (Cardenal-Munoz et al. 2014).

In *Shigella* infection activation of c-Src is followed by cortactin tyrosine phosphorylation and its recruitment to the cell membrane. Moreover, IpaC induces the activation of GTPases Cdc42 and Rac1, altogether these signaling events lead to massive actin polymerization at the vicinity of the bacterial entry site (Dehio et al. 1995, Adam et al. 1996, Tran Van Nhieu et al. 1999). The Abl family of tyrosine kinases is also involved in *Shigella* entry through phosphorylation of the adaptor molecule Crk (Bougneres et al. 2004, Backert et al. 2008, Vepachedu et al. 2009). The *Shigella* outer protein OspF induces apoptosis of DCs by binding and dephosphorylating phospho-Erk (Kim et al. 2008). Moreover, upon interaction with ILK, OspE decreases FAK and paxilin phosphorylation as a way to prevent bacterial-cell detachment (Kim et al. 2009). Recently, it has been described that *Shigella* actin-based motility inside intestinal epithelial cells is regulated by the host Bruton's tyrosine kinase (Btk). Btk inhibition effectively impaired *Shigella* spread from cell to cell (Dragoi and Agaisse 2014).

Rickettsia conorii recruits the Arp2/3 complex to the site of entry and its invasion is dependent on PI3K and Src-family kinases. In fact, c-Src and its downstream target, cortactin, colocalize at entry sites. *R. conorii* internalization correlated with the tyrosine phosphorylation of several other host proteins, including FAK (Martinez and Cossart 2004).

Through a type four secretion system (T4SS), *Helicobacter pylori (H. pylori)* translocates into the host cytoplasm one of its main virulence factors, CagA, which modulates cellular functions related to cytoskeleton, cell-to-cell adhesion and intracellular signal transduction (Hayashi et al. 2012, Kaplan-Turkoz et al. 2012, Mueller et al. 2012). Once inside the host cell, CagA interacts with host tyrosine kinases, becoming tyrosine-phosphorylated by the action of kinases from the Src and Abl family. After phosphorylation by c-Src and Fyn, CagA remains at the plasma membrane, where it interacts with a number of proteins and triggers signals resembling the activation of the RTKs such as c-Met (Amieva and El-Omar 2008, Backert and Selbach 2008, Franke et al. 2008). Recently, the CagA phosphorylation status has been shown to mediate SHP2/ERK and JAK/STAT3 pathway balance during infection of epithelial cells (Lee et al. 2010). *H. pylori* stimulates an inflammatory response by phosphorylating IκBα, which is counter-regulated by the interference of Muc1 cell receptor with the IKKγ in IKK→ IκBα→NFκB activation pathway (Guang et al. 2010). Integrin-linked kinase (ILK) is involved in an alternative inflammatory signaling pathway in response to lipopolysaccharide (LPS), causing activation of NF-κB and phosphorylation of p65 at the S536 residue. This alternative activation through S536 phosphorylation also occurs during *H. pylori* infection of macrophages and gastric cells. ILK-mediated phosphorylation of p65 is dependent on the PI3K/Akt pathway during *H. pylori*-induced inflammatory response (Ahmed et al. 2014).

III. MYOSINS

The intracellular architecture is supported by an interconnected network of filaments that confer morphological organization and stability to the cell during processes such as movement and division. The eukaryotic cytoskeleton comprises actomyosin filaments, microtubules and intermediate filaments (Hartman and Spudich 2012).

To properly exert their multiple physiological functions, some cellular components must be anchored to pre-existing molecular framework structures. Intracellular transport leads to physiological asymmetries both in cell morphology and macromolecular complexes composition. For long-range transportation, cells take advantage of motor proteins that convert the energy of ATP hydrolysis into mechanical work known as myosins (O'Connell et al. 2007).

Structural and functional studies of the actomyosin cytoskeleton were first focused on actin (Conti and Adelstein 2008), until the myosin protein family was characterized, in particular the non-muscle myosin II subfamily (Kuhne 1860).

III.A. Structure, classification and function

Myosins are actin-based molecular motors that contribute to cellular organization in a variety of ways (O'Connell et al. 2007). Typically, myosins are composed by a pair of heterotrimeric protein complexes, each consisting of a 230-kDa myosin heavy chain (MHC), a regulatory light chain (RLC) that modulates the myosin motor activity, and an essential light chain (ELC) that stabilizes myosin structure. The MHC contains three domains: (i) the N-terminal head or motor domain, responsible for actin binding and conversion of ATP energy into mechanical force; (ii) a hinged neck region containing one or more isoleucine-glutamine (IQ) motifs; and (iii) a C-terminal tail domain (Figure 11) (Sellers 2000, Berg et al. 2001, Krendel and Mooseker 2005, O'Connell et al. 2007). The neck regions are stabilized by the binding of the light chains, which allows these regions to swing like a lever arm (Howard 1997). Besides providing a certain degree of rigidity to the neck regions, the RLC also participate in the regulation of the ATPase and motor activities of the MHC component. Regulatory mechanisms of

RLC involve direct binding of calcium ions to the light chains or the post-translational modification of the light chains by phosphorylation.

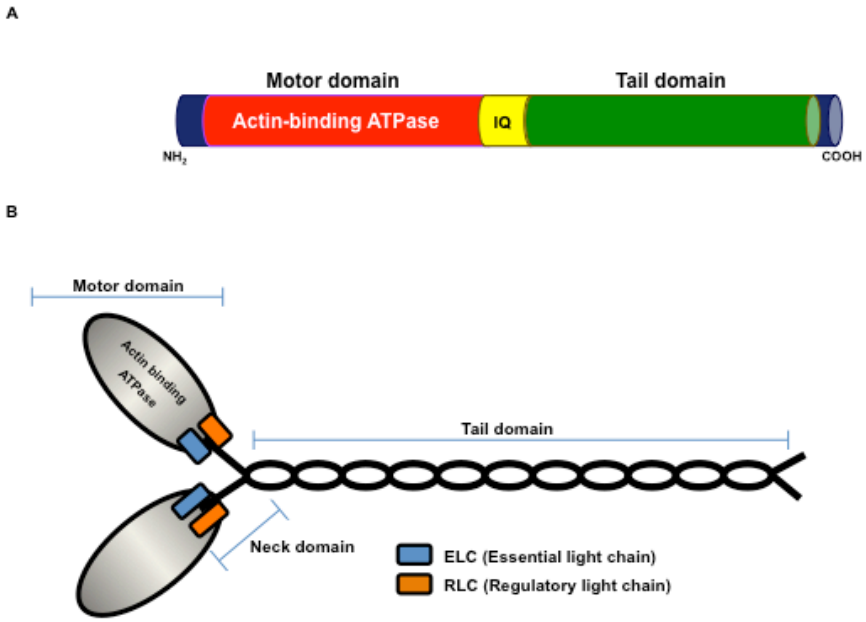

Figure 11. Myosin domains and structure. (A) Schematic representation of the myosin heavy chain (MHC): N-terminal motor/head domain (actin binding and ATP hydrolysis sites); neck region containing isoleucine-glutamine (IQ) motifs and a C-terminal tail domain. (B) Schematic representation of the myosin heterohexameric structure. Typical myosins are composed by two heterotrimers of one MHC and two myosin light chains (one RLC and one ELC). Myosin light chains interact with the MHC neck domain.

Together with the neck or regulatory domain, the myosin head domain forms the motor region that is sufficient for mechanical force generation along actin filaments (Bahler 2000).

The tail domain directs the interaction of a given myosin with its cargo and is responsible for class-specific properties and dimerization of MHCs. In addition, MHC tails also contain some conserved subdomains, which are responsible for protein-protein interactions in signaling cascades or additional functions, such as kinase activity or lipid binding (Lu et al. 2014). Several tail-binding proteins have been identified which have provided important insights into specific myosin functions. Functional diversity across myosins is also manifested by significant differences in the motor properties.

Sequence analysis has allowed the identification of a large number of myosin sub-families (Odronitz and Kollmar 2007), although their classification is nowadays based more on the characterization of their biochemical and mechanical properties. Based on their motor properties, it has been proposed for

myosins to be classified into four different groups: (1) fast movers, (2) slow/efficient force holders, (3) strain sensors, and (4) gates (Bloemink and Geeves 2011). To date, the myosin superfamily consists of at least 37 different classes based on the sequence comparison of both myosin head and tail domain organization (Krendel and Mooseker 2005, Odronitz and Kollmar 2007, Conti and Adelstein 2008).

Myosins are fundamental for eukaryotic cell motility (Even-Ram et al. 2007), and also assume an important role in cytokinesis (class II myosins) and organelle/particle trafficking (classes I and V) (Thompson and Langford 2002). Myosins are also implicated in cell polarization (class V) (Yin et al. 2000) and intracellular signal transduction pathways (classes I, III, VI, VII and IX), in which they modulate the activity of other proteins and position signaling activities at the cytoskeleton-membrane interface (Bahler 1996, Bahler 2000). Additionally, myosins have been described to play important roles in cell migration (Vicente-Manzanares et al. 2009), phagocytosis (classes II, IXb and X) (Groves et al. 2008), growth cone extension and maintenance of cell shape (classes I and II) (Berg et al. 2001), and actin polymerization (class I), revealing the molecular basis of actin-based cell motility (Evangelista et al. 2000, Lechler et al. 2000, Lee et al. 2000). Several human pathologies have been associated with mutations within myosin genes (Redowicz 2002).

III.B. Non-muscle myosin IIA (NM-IIA)

The myosin II subfamily was the first class of myosins to be described, being referred to as “conventional myosins”, with all other classes considered to be “unconventional” (Kalhammer and Bahler 2000, Billington et al. 2013). Myosin II members include both skeletal, cardiac and smooth muscle myosins, and non-muscle myosin (NM-II). Typically, NM-II molecules by MHC interactions form bipolar filaments that bind through motor domain to actin cables that slide in an antiparallel mode. In an actin-dependent manner, NM-II converts chemical energy from ATP hydrolysis to mechanical energy required to generate movement on top of actin fibers (Conti and Adelstein 2008, Vicente-Manzanares et al. 2009, Hartman and Spudich 2012).

In mammals, NM-II heavy chains (NMHC-II) have three isoforms (NMHC-IIA, NMHC-IIB and NMHC-IIC), each encoded by an independent gene (*MYH9*, *MYH10* and *MYH14*, respectively). For this reason, the corresponding NM-II variants are accordingly named NM-IIA, NM-IIB and NM-IIC (Wang et al. 2011, Billington et al. 2013). The three NM-II isoforms exhibit 60-80% amino acid homology, which is higher between motor domains and lower between the non-helical terminal tail sequence (Wang et al. 2011, Billington et al. 2013). Each isoform has a differential tissue/organ expression, function and distribution. Nevertheless, there is still some degree of functional and spatial redundancy, suggesting overlap of cellular functions (Maupin et al. 1994, Kelley et al. 1996, Kolega 1998, Bao et al. 2005, Vicente-Manzanares et al. 2007).

The work presented on this thesis has focused mainly on NM-II isoform A (NM-IIA).

III.B.1. NM-IIA structure

Different cell types present different modes of NM-IIA activation, either through actin-associated proteins (troponin and tropomyosin), in the case of skeletal and cardiac muscle cells, or through phosphorylation of NM-IIA RLCs, as described in vertebrate non-muscle and smooth muscle cells (Adelstein and Conti 1975, Amano et al. 1996, Kimura et al. 1996).

NMHC-IIA displays the archetypal myosin heavy chain structure. It contains a globular head domain comprising both ATP- and actin-binding sites (Vicente-Manzanares et al. 2009, Billington et al. 2013). This domain displays an actin-dependent Mg^{2+}-ATPase activity that converts ATP hydrolysis energy into mechanical force required for NM-IIA sliding on top of actin filaments (Billington et al. 2013). Following the head domain, an extended alpha-helical neck region that contains a variable number of calmodulin or calmodulin-like light chain-binding sites (Sweeney and Houdusse 2010). It acts as a lever arm that amplifies the head domain rotation, upon ATP hydrolysis (Vicente-Manzanares et al. 2009, Hartman and Spudich 2012). The neck region of NM-IIA binds very tightly, although not covalently, two RLCs and two ELCs.

The cellular role and localization of a particular myosin are encoded in the tail domain. In the case of NM-IIA, it was shown that the C-terminal 179-190 amino

acid residues determine its intracellular location (Bao et al. 2005, Vicente-Manzanares et al. 2007). The NM-IIA C-terminal domain is a long (1100 amino acids) α-helical region that is responsible for the antiparallel MHC dimerization in NM-II molecules. This bipolar filament formation confers the capacity to cross-link distinct actin filaments and exert tension (Wang et al. 2011, Billington et al. 2013).

III.B.2. Regulation of NM-IIA activity

NM-IIA regulation is different from other myosins and also varies from lower to higher eukaryotes (Somlyo and Somlyo 2003, Heissler and Manstein 2013). The RLCs regulate the Mg^{2+}-ATPase activity and are important for myosin filament dimerization, in a process that requires RLC phosphorylation, particularly on Thr18 and Ser19 residues (Vicente-Manzanares et al. 2009, Wang et al. 2011, Heissler and Manstein 2013). Two particular kinases, Rho-associated kinase (ROCK) and myosin light chain kinase (MLCK) have been described to phosphorylate NM-IIA RLCs (Figure 12) (Kamm and Stull 2001, Matsumura 2005, Sandquist et al. 2006).

NM-IIA filament assembly is also regulated by phosphorylation of MHCs coiled-coil and tail domains. Phosphorylations occur at thr1800, ser1803 and ser1808 by transient receptor potential melastatin 7 (TRPM7) (Clark et al. 2008), at ser1916 by PKCβ (Conti et al. 1991, Ludowyke et al. 2006) and at ser1943 by

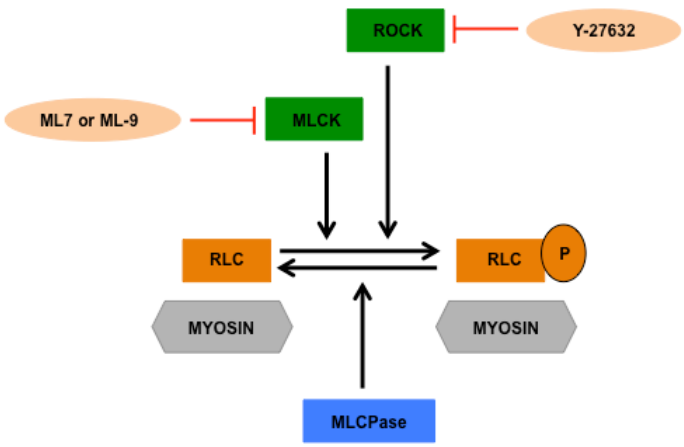

Figure 12. Schematic diagram of myosin motor activation by RLC phosphorylation. Myosin activation requires phosphorylation of its regulatory light chain (RLC) by the myosin light chain kinase (MLCK) or by the Rho-associated kinase (ROCK). Dephosphorylation of RLC is catalyzed by the myosin light chain phosphatase (MLCPase). Various inhibitors interfere with myosin activation: ROCK is inhibited by Y-27632 and MLCK is inhibited by ML-7 or ML-9. Adapted from (Fabian et al. 2007).

casein kinase II (CK II) (Dulyaninova et al. 2005, Li and Bresnick 2006). In each case phosphorylation of the MHC compromises myosin filament assembly or stability interfering with cell motility.

III.B.3. Cellular functions of NM-IIA

NM-IIA has been associated to different cell processes, based on its canonical protein function. It has been demonstrated that NM-IIA has an essential role in cell polarization during mitosis (Conti and Adelstein 2008), and cell migration studies implicate this isoform in membrane protrusion activity (Even-Ram et al. 2007, Vicente-Manzanares et al. 2007). Cell-to-cell adhesions and contractile activity were also shown to require NM-IIA activation (Vicente-Manzanares et al. 2007, Vicente-Manzanares et al. 2009, Smutny et al. 2010).

During cytokinesis, NM-IIA binds to actin filaments to generate the force and tension required at the constriction ring (Guha et al. 2005). NM-IIA functions are strongly related to cell membrane tension and reshape. Other studies have been consistently describing NM-IIA role in vesicle trafficking and membrane repair (Togo and Steinhardt 2004, Lin et al. 2012, Petrosyan et al. 2012). Moreover, actomyosin function in mitochondrial DNA (mtDNA) maintenance and transmission has been shown implicate interactions of mtDNA with NM-IIA. NM-IIA in this study was present at mitochondrial nucleoprotein complexes (Reyes et al. 2011).

III.C. Myosins and bacterial infection

Myosins are among the host cytoskeleton proteins hijacked by bacterial pathogens during infection.

Neisseria gonorrhoeae infection of mucosal epithelial tissues involves bacterial adherence to the host cell, followed by invasion, trans-epithelial trafficking and, finally, exocytosis. Host cell factors, necessary for gonococcal adherence, entry and transcytosis include membrane receptors that interact with neisserial opacity-associated (Opa) proteins (Wang et al. 1998). However, rearrangements of the host cytoskeleton are also required for the success of *Neisseria* infection (Pujol et al. 2000, Wang et al. 2008). It has been shown that

myosin I is involved in *Neisseria* invasion and transcytosis, but not in adherence. It was also suggested that cell invasion by Opa-expressing gonococci may be driven by myosin I molecules sliding along actin filaments to the basal side of the epithelium (Wang et al. 2008). However, the molecular basis of myosin I function in the alterations of the cytoskeleton upon *Neisseria* infection is not yet completely understood.

Shigella flexineri was found to disseminate through the intestinal epithelium via protrusions that extend from infected cells and are engulfed by neighboring cells (Rathman et al. 2000). It has been shown that phosphorylation of myosin II by its upstream regulator, MLCK, leads to the formation of the bacterium-containing protrusion and can also contribute to its engulfment by adjacent cells (Rathman et al. 2000). The myosin IXb has been identified as a regulator of *Shigella* infection, being recruited to *Shigella*-induced cellular projections essential for bacterial entry. Myosin IXb has a GAP subdomain in its tail domain that, together with its actin-binding site, establishes a connection between Rho-like GTPases and the cytoskeleton. *Shigella* invasion efficiency is probably a result of tightly regulated Rho-dependent cytoskeletal rearrangements, underlining the sophisticated use of the motor and GAP activities of a class IX myosin by *Shigella,* during infection of epithelial cells (Graf et al. 2000).

Previous studies suggested that myosins have a role in the dynamics of *Salmonella*-containing vacuoles (SCVs) within infected host cells. Furthermore, vacuole positioning is controlled by specific bacterial effectors, through the activation of myosin II (Wasylnka et al. 2008). *Salmonella* invasion has also been shown to be a myosin II-mediated process complementary to the Arp2/3-dependent pathway. The TTSS effector SopB is implicated in the regulation of this myosin II-dependent invasion pathway (Hanisch et al. 2011). *Salmonella* persists and replicates in SCVs, which recruit and accumulate F-actin through the kinase activity of the SPI-2 effector SteC. It has recently been described that SteC promotes actin rearrangements by activating a signaling pathway involving the MAP kinases MEK and ERK, MLCK and NM-IIB (Odendall et al. 2012).

EPEC destroys intestinal microvilli and suppresses phagocytosis by injecting TTSS effectors into infected target cells. One of these effectors, EspB, was shown to bind to host myosins, and recent studies have identified myosin Ic

as one of these EspB-binding myosins. By binding to myosin Ic, EspB inhibits its interaction with actin, thus suggesting that EspB inhibits actomyosin-associated processes, which facilitates efficient EPEC infection (Iizumi et al. 2007).

Previous work by Sousa and colleagues enabled the identification of two novel host proteins involved in *Lm*-induced uptake by host cells: myosin VIIa (unconventional myosin) and its ligand vezatin. Vezatin is a transmembrane protein that binds to myosin VIIa and recruits it to adherens junctions (Kussel-Andermann et al. 2000). Both proteins are recruited to the *Lm* entry site, where they localize with actin, suggesting that vezatin acts as the molecular link between myosin VIIa and the E-cadherin/catenins/actin complex (Figure 10). It was also demonstrated that myosin VIIa is required for InlA/E-cadherin-mediated internalization, but not for entry mediated by the other *Lm* invasin, InlB (Sousa et al. 2004). Another unconventional myosin, myosin VI, is recruited to the fully assembled clathrin-actin machinery complex located at the *Lm* entry site, where it likely plays a role in generating the pulling force required for bacterial internalization (Bonazzi et al. 2011).

PROJECT PRESENTATION

Infectious diseases are major threats to human health worldwide, and tremendous effort has gone into understanding various infectious agents and their mechanisms of virulence. In particular, the mechanism through which invasive bacteria exploit mammalian host cell components to induce their entry into cells has received special attention in the last two decades. Model organisms have emerged and helped understanding the various mechanisms that are used. Among those, *Lm* is one of the most documented. Investigations on InlA- and InlB-mediated entry pathways have repeatedly shown that *Listeria* fully usurps the host cell machinery. Moreover, they have also shown that unknown cellular components discovered during the study of *Listeria* invasion play a role either in E-cadherin-mediated cell–cell adhesion or c-Met signaling.

Phosphorylation cascades have been extensively involved in cell signaling processes. Protein phosphorylation is an essential regulatory mechanism allowing the activation or deactivation of protein functions in response to an external stimulus. Importantly, phosphorylation events have been described as key regulators of cellular processes such as metabolic pathways, cell cycle, molecule transport and secretion, cytoskeleton organization and cell adhesion. The uncontrolled protein phosphorylation/dephosphorylation leads to cellular dysfunctions associated to serious diseases.

Given the central role of phosphorylation events in the normal cellular processes, the phosphorylation cascades are targeted by bacterial pathogens in order to hijack the host cell machinery. *Lm*, as other human pathogens, induces the phosphorylation of its cellular receptors and triggers a series of phosphorylation events that contribute to the bacterial internalization. At the time when I started my PhD, a recent publication from our laboratory showed that shortly after the incubation of *Lm* with epithelial cells Src kinase was activated. E-cadherin-mediated *Lm* internalization required the small GTPase Rac1 and Src-tyrosine kinase activity to recruit cortactin and activate Arp2/3 complex, regulating the transient actin polymerization required at the bacterial entry site. In this study, in response to *Lm,* the only Src kinase substrate identified to be phosphorylated*,* was cortactin (Sousa et al. 2007). Given that, the project of my PhD was focused in the identification of other potential targets of tyrosine phosphorylation and their role in *Lm* infection. I specifically focused on tyrosine phosphorylation cascades and searched for host cellular proteins showing a different tyrosine

phosphorylation status in infected as compared to non-infected cells. The role of identified proteins in the infectious process has been addressed. Moreover, I also aimed to investigate whether these tyrosine phosphorylations are important for bacterial pathogenesis and if they are dependent on already known pathogen virulence factors. The respective kinases that regulate the identified phosphorylation events were also part of the objectives of this study. In addition, the function of the phosphorylated residues in the identified proteins was important to determine the significance of such post-translational modification in both infection and normal cell biology processes, as well as their integration in contextualized signaling pathways. Given that different pathogens often hijack the same eukaryotic pathways, I intend to investigate whether the identified proteins were specifically targeted during *Lm* infection or exploited by other human pathogens. Although *Lm* was the pathogen model, in collaboration studies, I would also study the identified signaling events in *Yersinia* and pathogenic *E. coli (*EPEC and EHEC) infection. Globally my PhD project aimed to reveal new host intracellular pathways that are exploited by the pathogens in order to cause and establish infection.

RESULTS

Note: This chapter is divided in three sections. The results presented in section I correspond to the work developed as the main research line of this PhD project and published at Journal of Biological Chemistry (J. Biol. Chem. 2015 290: 8383-8395. First Published on January 29, 2015). Sections II and III correspond to the results obtained in complementary projects in which I participated.

I. SRC-DEPENDENT TYROSINE PHOSPHORYLATION OF NON-MUSCLE MYOSIN HEAVY CHAIN-IIA RESTRICTS *LISTERIA MONOCYTOGENES* CELLULAR INFECTION

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Running title: *Src kinase phosphorylates NMHC-IIA upon bacterial infection*.

Keywords: post-translational modification, phosphotyrosine signaling, Src, myosin, host-pathogen interactions, Gram positive bacteria, non-muscle myosin IIA, intracellular bacterial pathogen, *Listeria monocytogenes*, cellular infection

Background: Non-muscle myosin IIA is involved in force generation, movement and membrane reshaping. Its activity is regulated by phosphorylation of the light chain.

Results: NMHC-IIA head domain is tyrosine phosphorylated by Src and modulates *Listeria* intracellular levels.

Conclusion: Tyrosine phosphorylation of NMHC-IIA affects the outcome of infection.

Significance: This novel post-translational modification of NMHC-IIA possibly affects its functions.

SUMMARY

Bacterial pathogens often interfere with host tyrosine phosphorylation cascades to control host responses and cause infection. Given the role of tyrosine phosphorylation events in different human infections and our previous results showing the activation of the tyrosine kinase Src upon incubation of cells with *Listeria monocytogenes* (*Lm*), we searched for novel host proteins undergoing tyrosine phosphorylation upon *Lm* infection. We identify the heavy chain of the non-muscle myosin IIA (NMHC-IIA) as being phosphorylated in a specific tyrosine residue in response to *Lm* infection. We characterize this novel post-translational modification event and show that, upon *Lm* infection, Src phosphorylates NMHC-IIA in a previously uncharacterized tyrosine residue (Y158) located in its motor domain near the ATP-binding site. In addition, we found that other intracellular and extracellular bacterial pathogens trigger NMHC-IIA tyrosine phosphorylation. We demonstrate that NMHC-IIA limits intracellular levels of *Lm* and this is dependent on the phosphorylation of Y158. Our data suggest a novel mechanism of regulation of NMHC-IIA activity relying on the phosphorylation of Y158 by Src.

INTRODUCTION

Listeria monocytogenes (*Lm*) is a human intracellular foodborne bacterial pathogen that causes serious disease in immunocompromised individuals. Within the host it finds suitable replication niches in the liver and spleen, disseminates and can reach the central nervous system. In pregnant women, *Lm* targets the fetus, eliciting fetal infection and abortions (Allerberger and Wagner 2010). The ability of *Lm* to cause disease relies on its capacity to invade nonphagocytic cells, replicate therein and spread to the entire organism overcoming the intestinal, blood-brain and fetoplacental barriers (Lecuit 2005). Through the expression of bacterial factors *Lm* establishes a crosstalk with host cells favoring the progression of the cellular infection (Camejo et al. 2011). In epithelial cells, *Lm* invasion is mainly driven by the bacterial surface proteins InlA and InlB that bind E-cadherin and c-Met, respectively, at the surface of host cells (Mengaud et al. 1996, Shen et al. 2000). This engagement of host cell receptors triggers tyrosine phosphorylation-mediated signaling, resulting in the local activation of the Arp2/3 complex that initiates actin polymerization at the site of *Lm* attachment (Bierne et al. 2001, Sousa et al. 2007), causing membrane invagination that supports bacterial entry. InlB interaction with the receptor tyrosine kinase c-Met stimulates its auto-phosphorylation, induces the tyrosine phosphorylation and recruitment of adaptor proteins, and the activation of phosphoinositide 3-kinase (PI3K) (Ireton et al. 1996, Ireton et al. 1999, Shen et al. 2000). Phosphatidylinositol (3,4,5)-triphosphate generated by PI3K accumulates at the cell membrane during *Lm* infection (Ireton et al. 1996) and plays a crucial role in the recruitment of molecules controlling actin polymerization, such as Rac1 and WAVE2 (Bierne et al. 2001, Seveau et al. 2004, Bierne et al. 2005, Kuhbacher et al. 2012). In turn, InlA binding to E-cadherin induces the activation of Src tyrosine kinase that subsequently phosphorylates cortactin, E-cadherin and the clathrin heavy chain (Sousa et al. 2007, Bonazzi et al. 2008, Bonazzi et al. 2011). While cortactin and clathrin tyrosine phosphorylations are critical events for actin polymerization and recruitment at the *Lm* entry site (Sousa et al. 2007, Bonazzi et al. 2011), E-cadherin phosphorylation leads to its ubiquitination, internalization and further degradation (Bonazzi et al. 2008). The combined action of these events leads to the internalization the *Lm* into epithelial cells.

In this study we aimed to identify new cellular proteins undergoing tyrosine phosphorylation in response to *Lm* infection and address whether such post-translational modification would regulate cellular infection. The tyrosine phosphorylated proteins were recovered from *Lm*-infected epithelial cells and subjected to mass spectrometry identification. We identified the non-muscle myosin heavy chain IIA (NMHC-IIA) as one of the enriched tyrosine phosphorylated proteins recovered upon *Lm* infection.

NMHC-IIA is an actin-binding protein with motor and contractile properties, involved in cellular processes requiring force generation, cell movement and membrane reshaping (Vicente-Manzanares et al. 2009). In infection, NMHC-IIA is critical for viral entry (Arii et al. 2010, Valiya Veettil et al. 2010) and supports invasion (Hanisch et al. 2011) and dissemination (Hybiske and Stephens 2007) of various bacteria. While the serine/threonine phosphorylation of the regulatory light chain is a well-known mechanism to regulate non-muscle myosin IIA activity (Vicente-Manzanares et al. 2009), our knowledge on the regulation of the heavy chain is limited and NMHC-IIA tyrosine phosphorylation has never been characterized. Here we show that NMHC-IIA undergoes tyrosine phosphorylation in response to several bacterial pathogens. Our data indicate that upon *Lm* cellular infection NMHC-IIA was phosphorylated in tyrosine residue 158 by the host Src kinase. In the presence of blebbistatin, a chemical inhibitor of Myosin II activity, the percentage of cells showing *Lm*-associated actin foci was increased and correlated with higher levels of intracellular *Lm*. In addition, increased numbers of intracellular *Lm* were also found in cells depleted for NMHC-IIA as well as in conditions where NMHC-IIA tyrosine phosphorylation is prevented. These results show the involvement of NMHC-IIA in *Lm* infection and point to the regulatory role of its phosphorylation in tyrosine 158 which could affect NMHC-IIA activity. Our findings describe a novel post-translational modification of NMHC-IIA with important implications in bacterial infection. Taking into account the central role of NMHC-IIA in key cell biology processes, our data also suggest the existence of a new mechanism of NMHC-IIA regulation that could be of critical importance in the canonical functions of non-muscle Myosin IIA.

I. EXPERIMENTAL PROCEDURES

Bacterial strains and cell lines. *Listeria* and *E. coli* strains were grown aerobically at 37 ºC, with shaking, in brain-heart infusion (BHI) and Lysogeny Broth (LB) media, respectively. *Yersinia* was grown aerobically at 26 ºC, with shaking, in LB media. When required antibiotics were added to growth media. Details are provided in Table 1. Caco-2 cells (ATCC HTB-37) were cultivated in MEM with L-glutamine, supplemented with non-essential amino acids, sodium pyruvate, and 20% fetal bovine serum (FBS). HeLa (ATCC CCL-2), HEK293 (ATCC CRL-1573) and COS-7 (ATCC CRL-1651) cells were cultivated in DMEM with glucose (4.5 g/l) and L-glutamine, supplemented with 10% FBS. Cells were maintained at 37 ºC in a 5% CO_2-enriched atmosphere. Cell culture media and supplements were from Lonza.

Table 1. List of bacterial strains used in this study and the corresponding growth conditions.

Bacterial strains	Growth Media	T (°C)
L. monocytogenes (EGDe)	BHI (Difco Laboratories)	37
L. innocua (CLIP 11262)	BHI	37
L. innocua-inlB	BHI Erythromycin 5µg/ml	37
E. coli DH5α	LB (Difco Laboratories)	37
EPEC	LB Ampicilin 100 µg/ml (Amp100)	37
EHEC	LB	37
E. coli K12-*inv*	LB Amp100	37
E. coli K12-Δ*inv*	LB Amp100	37
Y. *pseudotuberculosis*	LB Amp100	26

Table 2. List of plasmids used in this study.

Plasmid	Description	Source
GFP-NMHCIIA-WT	pEGFP-C3:CMV-GFP-NMHC IIA	Addgene (#11347)
GFP-NMHCIIA-Y158F	pEGFP-C3:CMV-GFP-NMHC IIA (Y158F)	This study
GFP-NMHCIIA-Y190F	pEGFP-C3:CMV-GFP-NMHC IIA (Y190F)	This study
GFP-NMHCIIA-WT-siRes	pEGFP-C3:CMV-GFP-NMHC IIA siRNA resistant	This study
Src-KD	pcDNA3-Src Kinase Dead (A430V)	Sarah J. Parsons, Univ. of Virginia

Plasmids, antibodies and reagents. Plasmids used are listed in Table 2. Plasmids GFP-NMHC-IIA-Y158F and GFP-NMHC-IIA-Y190F were generated using GFP-NMHC-IIA-WT from Addgene (Wei and Adelstein 2000) and the QuickChangeII XL site-directed mutagenesis kit (Agilent Technologies). For NMHC-IIA rescue assays, a plasmid encoding siRNA-resistant GFP-NMHC-IIA-

WT transcripts was generated. Oligonucleotide sequences are provided in Table 3. Antibodies are listed in Table 4. F-actin was labeled with Alexa Fluor 647- or 555-conjugated phalloidin (Invitrogen). Chemical inhibitors Y-26732 (Sigma-Aldrich), Blebbistatin and PP1 (Calbiochem) were handled as recommended. FluoSpheres-carboxylate modified microspheres were from Invitrogen (F-8814).

Table 3. Sequences of siRNA duplexes, shRNAs and primers used in this study.

siRNA duplexes			
Name	**Oligo Sequence (5'-3')**		**Source**
NMHCIIA-si#1 (Pool of 3 siRNAs)	A	Sense: CAUCUACUCUGAAGAGAUUtt	Santa Cruz Biotechnology (#sc-61120)
		Antisense: AAUCUCUUCAGAGUAGAUGtt	
	B	Sense: GAAGAUCAAUCCAUCUUGUtt	
		Antisense: ACAAGAUGGAUUGAUCUUCtt	
	C	Sense: CCAAAGAGAACGAGAAGAAtt	
		Antisense: UUCUUCUCGUUCUCUUUGGtt	
NMHCIIA-si#2	Sense: GAAGAUCAAUCCAUCUUGUtt		Santa Cruz Biotechnology (#sc-61120B)
	Antisense: ACAAGAUGGAUUGAUCUUCtt		
NMHCIIB-si	Sense: GCAAUACAGUGGGACAGUUtt		Sigma-Aldrich (#00072460)
	Antisense: AACUGUCCCACUGUAUUGCtt		
shRNAs Sequence (5'-3') and Source			
Src	CCGGGTGGCTTACTACTCCAAACATCTCGAGATGTTTGGAGTAGTAAGCCACTTTTT **Sigma-Aldrich (TRCN0000023597)**		
Control	CCGGGCGCGATAGCGCTAATAATTTCTCGAGAAATTATTAGCGCTATCGCGCTTTTT **Sigma-Aldrich (SHC016)**		
Primers sequences (5'-3')			
NMHC-IIA-Y158F	Fw: CTATGCCATCACAGACACCGCCTTCAGGAGTATGATGCAAGAC		
	Rev: GTCTTGCATCATACTCCTGAAGGCGGTGTCTGTGATGGCATAG		
NMHC-IIA-Y190F	Fw: CACCAAGAAGGTCATCCAGTTTCTGGCGTACGTGGCGTCTCG		
	Rev: CGAGGACGCCACGTACGCCAGAACTGGATGACCTTCTTGGTG		
NMHC-IIA-WT-siRes	Fw: GATGCAAGACCGAGAGGATCAATCCATACTGTGCACTGGTGAATC		
	Rev: GATTCACCAGTGCACAGTATGGATTGATCCTCTCGGTCTTGCATC		
c-Src	Fw: CTGTTCGGAGGCTTCAACTC		
	Rev: CCACCAGTCTCCCTCTGTGT		
HPRT1	Fw: GGCGTCGTGATTAGTGATG		
	Rev: CACCCTTTCCAAATCCTCAG		

Determination of intracellular bacteria. The levels of intracellular bacteria were determined as described (Reis et al. 2010). When indicated cells were incubated with serum-free medium containing blebbistatin, PP1 or DMSO. Cells were challenged with pre-washed *Lm* at a multiplicity of infection (MOI) of 50 or with *Yp* (MOI 10) for 60 min, treated with 20 µg/ml gentamicin for 90 min, washed in PBS, lysed with 0.2% Triton X-100 and serial dilutions were plated for CFU

counting. For immunofluorescence scoring, cells infected with *Lm* (MOI 50) were treated with 100 µg/ml gentamicin for 10 min, and washed with 20 µg/ml gentamicin prior fixation.

Table 4. List of antibodies used in this study.

Antigen	Species	Applications	Reference	Source
Phosphotyrosine	Mouse	IP (1:300) WB (1:1000)	4G10, 05-321	Millipore
Phosphotyrosine	Mouse	WB (1:1000)	PY20, P4110	Sigma-Aldrich
Actin	Mouse	WB (1:5000)	AC-15, A5441	Sigma-Aldrich
NMHC-IIA	Mouse	IF (1:1000)	ab55456	Abcam
GFP	Mouse	IP (1:100) WB (1:500)	B2, sc-9996	Santa Cruz Biotechnology
NMHC-IIA pY158	Rabbit	WB (1:500)	AP3775a	Abgent
Listeria	Rabbit	IF (1:200)	ab35132	Abcam
NMHC-IIA	Rabbit	IP (1:100) WB (1:5000)	M8064	Sigma-Aldrich
c-Src	Rabbit	WB (1:500)	GD11, 05-184	Millipore
c-Src	Rabbit	WB (1:1000)	ab109381	Abcam
NMHC-IIB	Rabbit	WB (1:1000)	M7939	Sigma-Aldrich
Anti-rabbit or anti-mouse HRP	Goat	WB	BI2413C BI2407	PARIS
Anti-rabbit or anti-mouse Alexa Fluor 488	Goat	IF	A11034 A11001	Invitrogen
Anti-rabbit or anti-mouse Cy3	Goat	IF	111-165-144 115-165-146	Jackson ImmunoResearch
Anti-rabbit or anti-mouse Cy5	Goat	IF	111-175-144 115-175-146	Jackson ImmunoResearch

Immunoprecipitation assays. HeLa or Caco-2 cells grown until confluence were washed twice with warm phosphate-buffered saline (PBS), serum-starved (5 h) and left non-infected (NI) or incubated with pre-washed *Lm* (Reis et al. 2010) at MOI 200 for different periods of time, or with *E. coli* (EPEC, EHEC or K12-*inv* strains) at MOI 200 for 4 h as described (Campellone et al. 2007). When indicated, cells were treated with 10 µM PP1 or 50 µM Y-27632 30 min before infection. After washing twice with ice-cold PBS, cells were lysed in 1 ml of lysis buffer [1% Igepal CA-630 (Sigma-Aldrich), 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM AEBSF (Interchim), PhosSTOP and cOmplete Protease Inhibitor Cocktail (Roche Pharmaceuticals)] and lysates recovered after centrifugation (15,000 × g, 15 min, 4 ºC). Cell lysates (500 µg) were incubated overnight (4 ºC) with 1 µg of anti-phosphotyrosine 4G10 or 5 µg of anti-NMHC-IIA antibodies. Immune complexes were captured with 50 µl of PureProteome Protein A or G magnetic beads (Millipore). Immunoprecipitated fractions were resolved by SDS-PAGE and gels were silver-stained using the ProteoSilver^{TM} Plus Silver Staining Kit (Sigma-Aldrich) or processed for immunoblotting. For kinase assay, HEK293 cells were

harvested and lysed 24 h post-transfection, GFP fusion proteins were immunoprecipitated with anti-GFP conjugated agarose beads (sc-9996 AC, Santa Cruz Biotechnology) and eluted in 0.2 M glycine, pH 2.5.

Protein identification by mass spectrometry (MS). Protein identification was performed by MALDI TOF/TOF mass spectrometry as described (Osorio and Reis 2013). Protein bands were excised from SDS-PAGE gels, reduced with dithiothreitol, alkylated with iodacetamide and in gel digested with trypsin. Peptides were extracted, desalted, concentrated using ZipTips (Millipore), crystallized onto a MALDI sample plate and analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems). Peptidic mass spectra were acquired in reflector positive mode at a 700-4000 m/z mass window and proteins identified by Peptide Mass Fingerprint using the Mascot software (Matrix Science, UK) integrated in the GPS Explorer software (ABSCIEX, CA) and searched against the SwissProt/UniProt *Homo sapiens* protein sequence database. The maximum error tolerance was 35 ppm and up to two missed cleavages were allowed.

Phosphopeptide analysis by MS. Bands corresponding to NMHC-IIA, from anti-NMHC-IIA IPs of NI and *Lm*-infected HeLa cells, were processed as described above. Phosphopeptides were selectively enriched by titanium dioxide chromatography (TiO2 Mag Sepharose, GE Healthcare). MALDI matrix used was THAP/DAC (2',4',6'-trihydroxyacetophenone monohydrate 9 mg/mL, diammonium citrate 5 mg/mL, in water/Acetonitrile 50:50, v/v). Mass spectra were acquired in a 4800 Plus MALDI TOF/TOF Analyzer mass spectrometer (AB SCIEX) both in reflector negative and MS/MS modes.

Immunoblotting. Proteins were resolved in SDS-PAGE gels and transferred onto Nitrocellulose membranes (Hybond ECL, GE Healthcare Life Sciences). Membranes were blocked with 5% skimmed milk in buffer A (150 mM NaCl, 20 mM Tris-HCl pH 7.4 and 0.1% Triton X-100) for 1 h at room temperature or overnight at 4ºC. Primary and secondary antibodies were diluted in 2.5% skimmed milk in buffer A. Membranes used for anti-phosphotyrosine detection were blocked with Western Blocker solution (Sigma Aldrich), also used to dilute primary and secondary antibodies.

Immunofluorescence analysis. Cells were fixed in 3% paraformaldehyde (15 min), quenched with 20 mM NH_4Cl (1 h), permeabilized with 0.1% Triton X-100 (5 min) and blocked with 1% BSA in PBS (30 min). Antibodies were diluted in PBS

containing 1% BSA. Coverslips were incubated 1 h with primary antibodies washed three times in PBS and incubated 45 min with secondary antibodies and Phalloidin Alexa-555 or 647. DNA was counterstained with DAPI (Sigma-Aldrich). Coverslips were mounted onto microscope slides with Aqua-Poly/Mount (18606, Polysciences). Images were collected with a confocal laser-scanning microscope (Zeiss Axiovert LSM 510 or Leica SP2 AOBS SE) and processed using Adobe Photoshop software.

Transfection and lentiviral transduction. The lentiviral shRNA expression plasmids Mission pLKO.1-puro (control) and Mission shRNA-cSrc (Sigma-Aldrich), were used in combination with the envelope plasmid pMD.G, and packaging plasmid pCMVR8.91. Packaging, envelope, and shRNA vector plasmids were co-transfected into HEK293 cells. Viral supernatants harvested after 72 h, filtered and incubated with target HeLa cells for 48 h at 37°C. Puromycin was used to select for individual clones. The knock-down was verified by immunoblot and/or real-time RT-PCR.

Transfection of siRNA duplexes and plasmid DNA. HeLa cells seeded in 24- or 6-well plates were transfected with 60 nM of control siRNA-D (sc-44232 Santa Cruz Biotechnology) or specific siRNAs for NMHC-IIA or NMHC-IIB depletion, using Lipofectamine RNAiMax (Invitrogen) following manufacturer's instructions. Assays were performed 48 h later. Sequences of siRNAs are provided in Table 4. For transient protein expression, HeLa cells were seeded in 24-well plates ($1x10^5$ cells/well), 6-well plates ($4x10^5$ cells/well), or 10 cm dishes ($3x10^6$ cells/dish), and transfected at 90% confluency with 500 ng, 2.5 µg or 15 µg of plasmid DNA, respectively, using Lipofectamine 2000 (Invitrogen). Assays were performed 24 h later. For rescue assays, HeLa cells were transfected with NMHC-IIA-si#2 and 24 h later transiently transfected with plasmids encoding siRNA-resistant GFP-NMHC-IIA-WT.

Kinase Assay. Kinase assays were performed using the Src Assay Kit (17-131, Millipore), following manufacturer's instructions. Anti-GFP-immunoprecipitated fractions from HEK293 cells expressing GFP-NMHC-IIA variants were incubated (10 min, 30 ºC) with 10 units of recombinant Src (14-117, Millipore), in 30 µl of kinase reaction buffer supplemented with 9 µl of Manganese/ATP Cocktail and 10 µCi γ^{32}P-ATP (PerkinElmer). Reactions including a Src-specific substrate or lacking Src were used as controls. Reactions were

precipitated with 40% TCA and spotted onto P81 phosphocellulose paper squares, washed three times with 0.75% phosphoric acid, once with acetone and transferred to microtubes containing UniverSol liquid scintillation cocktail (MP Biomedicals). Incorporation of ^{32}P was determined in a Wallac 1450 MicroBeta TriLux liquid scintillation counter (PerkinElmer), as counts per minute (cpm). Radioactivity measurements were performed in duplicate in two independent assays.

Statistical analyses. Statistical analyses were performed with Prism 6 software (GraphPad Software, Inc.). One-way ANOVA with post hoc testing analyses were used for pair-wise comparison of means from at least three unmatched groups. Two-tailed Student's t-test was used to compare means of two samples and one-sample t-test to compare with samples arbitrarily fixed to 100. Differences were not considered statistically significant for p value ≥ 0.05.

I. RESULTS

I.A. *NMHC-IIA is tyrosine-phosphorylated in response to bacterial infection.*

To identify new host proteins undergoing tyrosine phosphorylation (pTyr) in response to *Lm* and which could affect *Lm* cellular infection, we compared the pTyr protein profiles of *Lm*-infected and non-infected (NI) HeLa cells. Cell extracts were collected at different time points post inoculation and subjected to immunoprecipitation (IP) using anti-phosphotyrosine antibodies (anti-pTyr). IP fractions were resolved by SDS-PAGE followed by silver staining. Bands showing variable intensities in *Lm*-infected versus NI cells were excised and processed for mass spectrometry identification. A band corresponding to a ≈250 kDa protein and displaying increased intensity throughout the infection (Fig. 1A) was identified as the human non-muscle myosin heavy chain IIA (NMHC-IIA) (data not shown).

To validate this result, HeLa and Caco-2 cells were incubated with *Lm* for different time periods and the presence of NMHC-IIA in anti-pTyr IP fractions was assessed by immunoblot using NMHC-IIA specific antibodies. We detected a time-dependent increase of NMHC-IIA in IP fractions from *Lm*-infected cells (Fig. 1B). Levels of NMHC-IIA in pTyr fraction increased 3.5-fold after 60 minutes of *Lm* incubation with HeLa cells and 15-fold in Caco-2 cells upon 20 minutes of *Lm* infection (Fig. 1B). Levels of NMHC-IIA in whole cell lysates (WCL) were not affected by infection (Fig. 1B), showing that increased levels of NMHC-IIA in IP samples are not related to an augmentation of NMHC-IIA expression. Incubation of HeLa cells with the non-pathogenic species *Listeria innocua* (*Li*) for 60 minutes only induced a small enrichment of NMHC-IIA in the anti-pTyr IP fractions as compared to *Lm* (Fig. 1C). In addition, NMHC-IIA was barely detected in IP fractions from HeLa cells stimulated by *E. coli* DH5a or latex beads (Fig. 1C). Altogether, these results indicate that the enrichment of NMHC-IIA in the pool of pTyr proteins is associated to the pathogenic features of *Lm* and is not a broad cellular response to any extracellular stimuli.

To investigate whether the same response could be induced upon infection with other human bacterial pathogens, HeLa cells were incubated for 4 h with: the extracellular pathogenic *E. coli* EPEC and EHEC; or the invasive *E. coli* K12 expressing the *Yersinia pseudotuberculosis* (*Yp*) invasin (K12-*inv*) (Isberg and

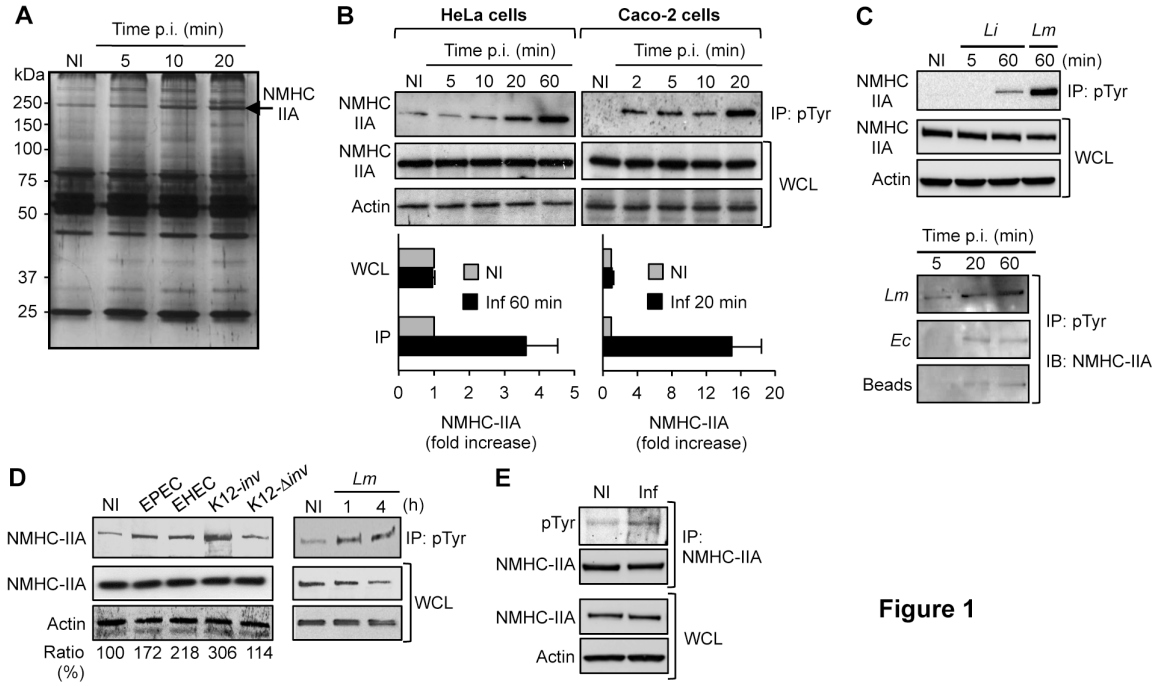


Figure 1

Figure 1. NMHC-IIA is tyrosine-phosphorylated in response to human bacterial pathogens. (A) Silver-stained acrylamide gel showing the tyrosine phosphorylation profiles of non-infected (NI) and Lm-infected HeLa cells, for the indicated periods of time. Total tyrosine-phosphorylated proteins were immunoprecipitated with an anti-pTyr antibody. Molecular weight standards are indicated. Arrow shows a protein band with increased intensity over the time of infection and identified by mass spectrometry analysis as NMHC-IIA. (B) HeLa and Caco-2 cells were left non-infected (NI) or incubated with Lm and harvested at indicated time points post-infection (p.i.). Tyrosine-phosphorylated proteins were immunoprecipitated (IP: pTyr) from whole cell lysates (WCL) and NMHC-IIA was detected by immunoblot (NMHC-IIA) in IP fractions and WCL. Detection of actin protein levels served as loading control. Bottom panels show quantifications of NMHC-IIA signals from at least 3 independent experiments in WCL and IP fractions of NI and Lm-infected HeLa (60 min p.i.) and Caco-2 (20 min p.i.) cells. (C) HeLa cells were left NI or incubated with either Lm, L. innocua (Li) (top panels), E. coli DH5a (Ec) or latex beads (bottom panels). Tyrosine-phosphorylated proteins were immunoprecipitated from WCL recovered at different time points and NMHC-IIA was analyzed by immunoblot in IP fractions and WCL. (D) HeLa cells were left NI or incubated, for 4 h, with pathogenic E. coli (EPEC and EHEC) and E. coli K12 expressing a functional (inv) or mutated variant (Δinv) of Y. pseudotuberculosis invasin. Cells were also incubated with Lm for 1 and 4 h (right panel). Tyrosine-phosphorylated proteins were immunoprecipitated and NMHC-IIA detected by immunoblot in IP fractions and WCL. Quantifications of NMHC-IIA signals for each IP fraction related to WCL are indicated (ratio). Values represent the mean of three independent experiments. (E) NMHC-IIA was immunoprecipitated (IP: NMHC-IIA) from WCL of NI and Lm-infected (Inf, 60 min) HeLa cells. Tyrosine-phosphorylated proteins (pTyr) and NMHC-IIA were detected in immunoprecipitates. As control, NMHC-IIA and actin were also detected in WCL.

Falkow 1985), an infection model allowing the study of signaling pathways triggered downstream the invasin-integrin interaction. As co mpared to NI conditions, NMHC-IIA appeared slightly increased in anti-pTyr IP fractions from EPEC- and EHEC-infected cells. Strikingly, K12-*inv* induced a robust enrichment

of NMHC-IIA in IP samples that is abolished in cells incubated with bacteria harboring a disrupted invasin-encoding gene (K12-Δ*inv*, Fig. 1D). For comparison

cells were also incubated with *Lm* for 1 and 4 h (Fig. 1D). These results indicate that the enrichment of NMHC-IIA in the pool of pTyr proteins is an event triggered by several human bacterial pathogens.

Our data suggest that bacterial infection either induces the direct NMHC-IIA pTyr or stimulates its interaction with a protein that undergoes itself pTyr. To address this issue, endogenous NMHC-IIA was immunoprecipitated from NI and *Lm*-infected HeLa cells and pTyr proteins were detected by immunoblot. A band showing a consistent 1.5-fold increase in intensity in infected samples was detected at the molecular weight of NMHC-IIA (Fig. 1E). Immunoprecipitated levels of NMHC-IIA were similar in NI and *Lm*-infected cells. These results support a direct pTyr of NMHC-IIA triggered by *Lm* infection.

I.B. *NMHC-IIA-pTyr induced by Lm cellular infection requires the activity of Src tyrosine kinase.*

Considering our previous findings revealing the key role of the tyrosine kinase Src during *Lm* invasion (Sousa et al. 2007), we addressed the role of this kinase in NMHC-IIA-pTyr in the context of *Lm* infection. Prior to *Lm* incubation, HeLa cells were treated with PP1, an inhibitor of Src family kinases, or with Y-27632, an inhibitor of the serine/threonine kinase ROCK that regulates NMHC-IIA activity through the phosphorylation of the regulatory light chain of myosin II and limits *Lm* internalization (Kirchner and Higgins 2008). Given that NMHC-IIA-pTyr is hardly detected by using anti-pTyr antibodies in immunoblot, cell lysates were subjected to anti-pTyr IP assay and NMHC-IIA was detected in IP fractions. The increase in NMHC-IIA-pTyr induced by *Lm* infection of non-treated cells (NT) was abolished in PP1-treated cells while being not affected by Y-27632 treatment (Fig. 2A), suggesting that NMHC-IIA-pTyr requires Src kinase activity and occurs independently from ROCK activity. In addition, we interfered with Src activity by overexpressing a Src kinase-dead variant (Src-KD) (Wilson et al. 1989). Levels of NMHC-IIA-pTyr induced by *Lm* infection were assessed in HeLa cells non-transfected (NT), transfected with an empty plasmid (Ctr) or overexpressing Src-KD. In contrast to NT and Ctr cells showing increased levels of NMHC-IIA-pTyr

upon *Lm* infection, in cells overexpressing Src-KD the NMHC-IIA-pTyr was almost undetectable (Fig. 2B).

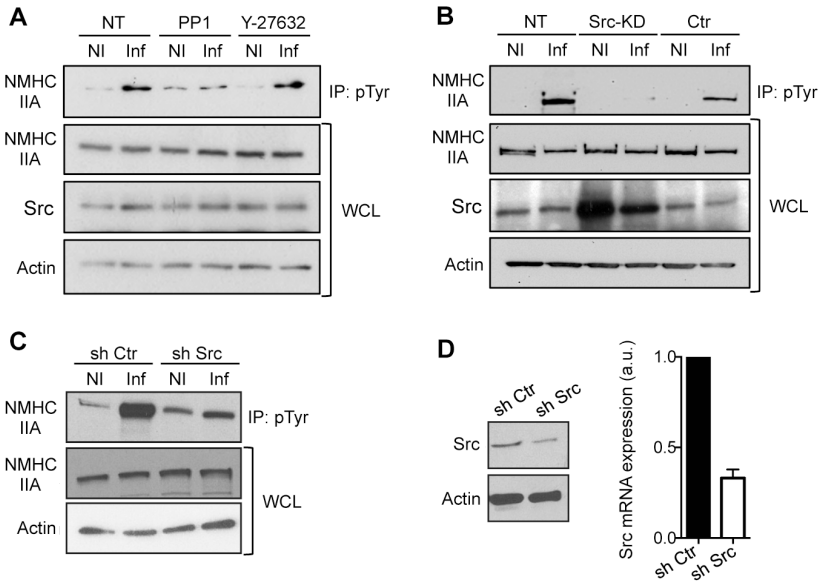


Figure 2. The activity of Src kinase is required for NMHC-IIA tyrosine phosphorylation upon *Lm* cellular infection. (A) HeLa cells pre-treated with PP1 (10 µM) or Y-27632 (50 µM) during 30 min, were left NI or incubated with *Lm* for 1 h (Inf) in the presence of the same concentrations of drugs. Non-treated (NT) HeLa cells were used as control. (B) HeLa cells non-transfected (NT), transfected with a control empty plasmid (Ctr) or with a Src kinase dead (Src-KD)-encoding plasmid. (C) HeLa cells stably expressing an shRNA control (sh Ctr) or a specific shRNA targeting Src expression (sh Src). Cells in B and C were left NI or incubated with *Lm* for 1 h (Inf). In A, B and C, total tyrosine-phosphorylated proteins were immunoprecipitated and NMHC-IIA was detected by immunoblot in IP fractions and WCL. Detection of actin levels served as loading control. Src protein levels were also confirmed by immunoblot. (D) Efficiency of Src depletion in sh Src HeLa cells was assessed by immunoblot using actin protein detection as loading control (left panel) and by qRT-PCR (right panel). Src mRNA expression is represented relative to the expression of control HPRT1. In sh Ctr cells, the relative expression was arbitrarily fixed to 1.

To further confirm these data, we targeted the expression of endogenous Src by using specific shRNAs. We observed that, *Lm*-induced NMHC-IIA-pTyr occurred in shRNA control (sh Ctr) and was clearly diminished in shRNA Src expressing (sh Src) HeLa cells, in which Src expression is reduced by 60% (Fig. 2C and 2D). Altogether, these data demonstrate that Src activity is required for NMHC-IIA-pTyr triggered by bacterial infection.

I.C. *Host Src kinase phosphorylates NMHC-IIA in tyrosine residue 158.*

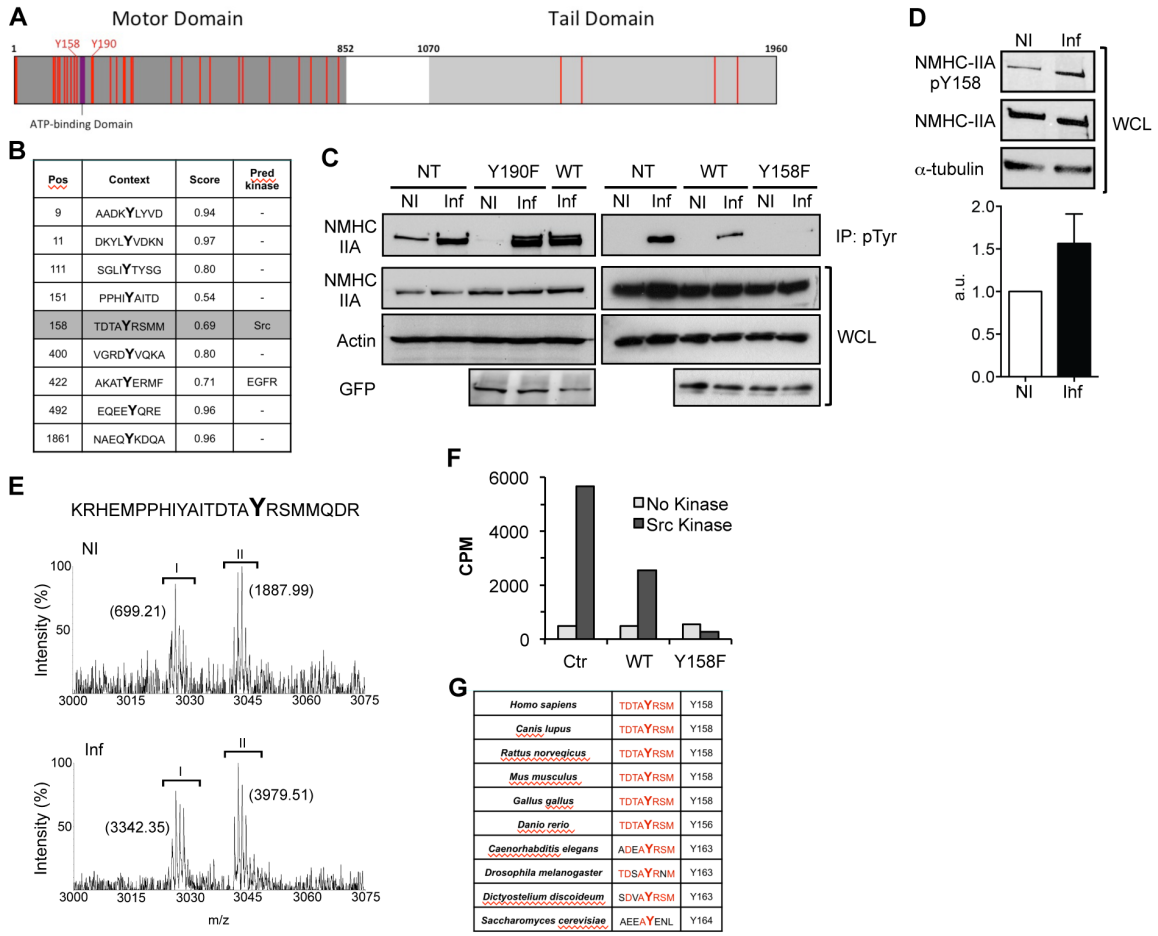
The NMHC-IIA amino acid sequence includes 34 tyrosine residues, most of which located in the myosin motor domain (Fig. 3A). To identify the NMHC-IIA tyrosine residues phosphorylated by Src upon *Lm* infection, we used combined *in*

silico approaches (NetPhos 2.0 and NetPhosK). Nine tyrosine residues were predicted as potentially phosphorylated, among which only the tyrosine in position 158 (Y158) was a putative substrate for Src kinase (Fig. 3B). To assess these *in silico* predictions and taking into account that *Lm*-induced NMHC-IIA-pTyr requires Src kinase activity (Fig. 2), we determined if NMHC-IIA-pTyr occurs upon *Lm* infection of cells ectopically expressing either GFP-tagged NMHC-IIA-Y158F (in which Y158 residue was replaced by a phenylalanine), NMHC-IIA-Y190F (harboring the same amino acid substitution in position 190, randomly selected and unrelated to *in silico* predictions) or NMHC-IIA-WT (wild type NMHC-IIA). *Lm* infection of non-transfected (NT), NMHC-IIA-WT- and NMHC-IIA-Y190F-overexpressing cells generated increased levels of NMHC-IIA-pTyr as compared to NI cells, while the overexpression of NMHC-IIA-Y158F largely limited *Lm*-induced NMHC-IIA-pTyr (Fig. 3C). Exogenous NMHC-IIA-WT and NMHC-IIA-Y190F were occasionally detected in anti-pTyr IP fractions of *Lm*-infected cells (data not shown). Levels of endogenous NMHC-IIA were comparable in the different conditions and GFP fusion proteins were expressed similarly in NI and infected cells (Fig. 3C). These results corroborate *in silico* predictions and suggest the central role of Y158 in NMHC-IIA-pTyr triggered upon infection. To validate our results, total lysates from NI and *Lm*-infected cells were probed with an antibody raised against a peptide comprising phosphorylated Y158 residue of NMHC-IIA (pY158). In agreement with our data, levels of NMHC-IIA-pY158 were 1.5-fold increased in *Lm*-infected cells (Fig. 3D). In addition, samples enriched in NMHC-IIA phosphopeptides from NI and *Lm*-infected cells were analyzed by mass spectrometry. A phosphopeptide spanning Y158 (amino acid 142 to 165, KRHEMPPHIYAITDTA**Y**RSMMQDR) was detected at *m/z* 3025.37 $[M-H]^-$ (Fig. 3E, cluster I) and at 3041.36 $[M-H]^-$ with an oxidized methionine (Fig. 3E, cluster II). In infected samples, the area of cluster I that is correlated with the abundance of the corresponding phosphopeptide, was 4.8-fold increased. Cluster II appeared 2.1-fold more abundant in *Lm*-infected samples as compared to NI. Cluster I was further characterized and validated by MS/MS sequencing. Altogether our data show that phosphorylation occurs in position Y158.

We further evaluate whether NMHC-IIA-pTyr occurs specifically on Y158 through Src activity, performing an *in vitro* kinase assay. GFP-NMHC-IIA-WT or Y158F ectopically expressed in HEK293 cells were highly enriched through

immunoprecipitation using an anti-GFP antibody and incubated with purified Src kinase and γ-^{32}P-ATP. A synthetic peptide substrate for Src was used as positive control. In the absence of kinase, the control peptide (Ctr) and IP fractions of NMHC-IIA-WT and Y158F showed residual levels of γ-^{32}P-ATP incorporation. In the presence of Src kinase, the NMHC-IIA-WT enriched IP fraction and the control peptide became radiolabeled while the radioactivity incorporation in the NMHC-IIA-Y158F enriched sample remained at a basal level (Fig. 3F).

Altogether these results strongly suggest that Y158 of NMHC-IIA is a substrate for Src kinase, becoming phosphorylated in response to *Lm* infection, and put forward the putative role of this event in cellular infection. In addition, Y158 appears extremely conserved among species ranging from *Saccharomyces cerevisiae* to *Homo sapiens* (Fig. 3G), pointing the broad importance for Y158 in the regulation of highly conserved canonical functions of NMHC-IIA.



Pos	Context	Score	Pred kinase
9	AADKYLYVD	0.94	-
11	DKYLYVDKN	0.97	-
111	SGLIYTYSG	0.80	-
151	PPHIYAITD	0.54	-
158	TDTAYRSMM	0.69	Src
400	VGRDYVQKA	0.80	-
422	AKATYERMF	0.71	EGFR
492	EQEEYQRE	0.96	-
1861	NAEQYKDQA	0.96	-

Figure 3. NMHC-IIA tyrosine residue in position 158 is phosphorylated in response to *Lm* infection. (A) Schematic representation of NMHC-IIA showing the distribution of tyrosine residues (red bars). Tyrosine residues in position 158 (Y158) and 190 (Y190) are highlighted. ATP-binding site, motor and tail domains are indicated. (B) *In silico* predictions obtained from NetPhos 2.0 and

NetPhosK servers, for tyrosine phosphorylation potential (score) and putative kinase involved. The position and amino acid environment of tyrosine residues showing a phosphorylation potential above the threshold (score >0.5) are indicated. (C) HeLa cells expressing the wild type GFP-NMHC-IIA (WT) and the mutants GFP-NMHC-IIA-Y158F (Y158F) or GFP-NMHC-IIA-Y190F (Y190F) were left NI or incubated with *Lm* for 1 h (Inf). NMHC-IIA was detected by immunoblot in anti-pTyr immunoprecipitates and WCL. Detection of GFP indicates similar expression levels of NMHC-IIA constructs and actin levels served as loading control. (D) HeLa cells were left NI or incubated with *Lm* for 1 h (Inf). Total cell extracts were used in immunoblot using an antibody raised against NMHC-IIA-pY158. Total levels of NMHC-IIA were detected using an anti-NMHC-IIA antibody and a-tubulin levels were used as loading control. Bottom panel show quantification of NMHC-IIA-pY158 signals from 3 independent experiments in NI and *Lm*-infected HeLa cells. (E) Mass spectra from NMHC-IIA acquired after phosphopeptide enrichment from NI and Inf HeLa cells. Two peak clusters marked as I (monoisotopic peak at *m/z* 3025.37 $[M-H]^-$) and II (monoisotopic peak at *m/z* 3041.36 $[M-H]^-$ with oxidized methionine) were detected. The corresponding NMHC-IIA peptide (aa 142 to 165) is indicated and Y158 highlighted. The area of the clusters in NI and Inf conditions is indicated between parentheses. (F) Anti-GFP IP fractions obtained from WCL of HEK293 cells expressing either GFP-NMHC-IIA-WT (WT) or GFP-NMHC-IIA-Y158F (Y158F) were used in *in vitro* Src kinase assays. A synthetic peptide was used as positive control (Ctr). Incorporation of radiolabeled γ-^{32}P-ATP was measured in CPM (counts per minute) for each condition. Results are representative of two independent experiments. (G) Comparative analysis of the NMHC-IIA amino acid sequence from different species, focused in the region encompassing the tyrosine on position 158.

I.D. *Inhibition of NMHC-IIA activity affects intracellular levels of Lm.*

To assess the role of NMHC-IIA activity in cellular infection, we measured intracellular levels of *Lm* following chemical inhibition of NMHC-IIA. Blebbistatin (Blebb), a specific inhibitor of myosin II activity (Straight et al. 2003), was added (10 or 100 μM) to HeLa and Caco-2 cells and *Lm* infection efficiency was quantified by gentamicin protection assays. As control we used an inactive form of blebbistatin [(+)Blebb]. *Lm* intracellular levels were increased by 2- to 8-fold, in a dose-dependent manner in both cell lines, following treatment with the active [(-)Blebb] as compared to the inactive enantiomer of blebbistatin (Fig. 4A). Untreated and inactive blebbistatin-treated cells showed similar levels of intracellular *Lm* (data not shown). Our data are in agreement with a previous report showing that blebbistatin treatment of L2 cells increases *Lm* adhesion and invasion (Kirchner and Higgins 2008). Recruitment of NMHC-IIA and formation of actin foci at *Lm* entry sites were both detected in control (DMSO) and active blebbistatin-treated (Blebb) HeLa cells (Fig. 4B). While the percentage of *Lm*-associated cells remained similar in both conditions, the percentage of cells showing *Lm*-actin foci increased in the presence of active blebbistatin (Fig. 4C). Together, our results indicate that the ATPase activity of NMHC-IIA is not required for its localization to the sites of *Lm* uptake and does not influence the interaction of *Lm* with host cells.

However, inhibition of NMHC-IIA ATPase activity fosters the formation of *Lm*-actin foci, which correlates with increased rates of intracellular bacteria.

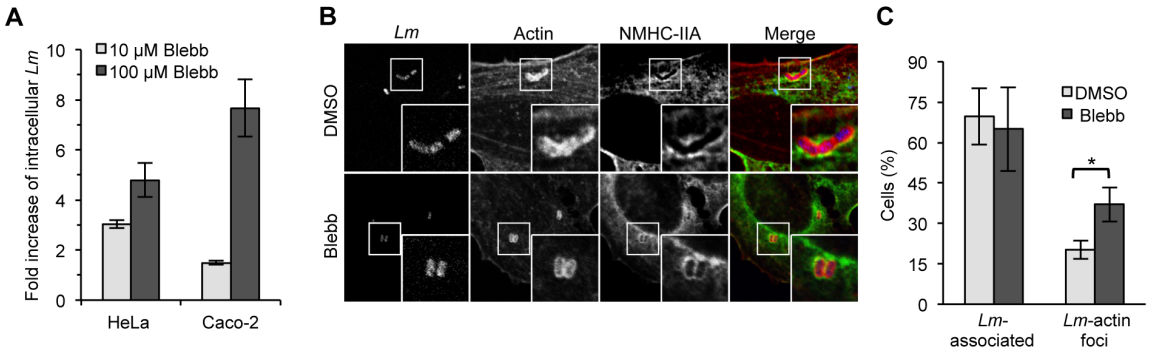


Figure 4. *Lm* intracellular levels increased upon inhibition of NMHC-IIA activity. (A) Levels of intracellular *Lm* were assessed by gentamicin protection assay and CFU counting, in HeLa and Caco-2 cells treated with 10 or 100 µM blebbistatin (Blebb). Graph shows the fold-increase of intracellular *Lm* determined as the ratio of intracellular bacteria in cells treated with the active *versus* the inactive enantiomer of blebbistatin. (B) Single confocal sections of HeLa cells infected with *Lm* in the presence of DMSO (control) or 50 µM active Blebbistatin. Infected cells were immunolabelled for NMHC-IIA (green) and *Lm* (blue) and stained for actin (red). (C) Immunofluorescence scoring of DMSO- and active Blebbistatin-treated HeLa cells associated to *Lm* and showing *Lm*-associated actin foci. Results are means±SD from three independent experiments, each done in duplicate. Statistically significant differences are indicated: * $p<0.05$.

I.E. *Reduced expression of NMHC-IIA increases the level of intracellular Lm.*

To further address the role of NMHC-IIA in *Lm* cellular infection, levels of adherent and intracellular *Lm* were quantified by gentamicin protection assays in NMHC-IIA-depleted HeLa cells, using two siRNAs (si#1 and si#2). In accordance with data described above, levels of intracellular *Lm* increased 2-fold in NMHC-IIA-depleted (IIA-si#1 and IIA-si#2) as compared to control siRNA-transfected cells (Ctr) (Fig. 5A). NMHC-IIA depletion assessed by immunoblot reached 85% in si#1-transfected cells and 65% when using si#2 (Fig. 5A). Levels of adhered *Lm* were also augmented in NMHC-IIA-depleted cells (data not shown). Immunofluorescence analysis of *Lm*-infected NMHC-IIA-depleted cells revealed a 2-fold increase in the percentage of cells associated to *Lm* and a 3-fold increase in the percentage of cells showing *Lm*-associated actin foci (Fig. 5B). The number of bacteria and actin foci per cell were also increased in NMHC-IIA-depleted cells (Fig. 5C), correlating with increased levels of intracellular bacteria. Our data indicate that, while *Lm* association to cells does not require NMHC-II activity it is modulated by NMHC-IIA itself probably through the interaction with other proteins.

To discard the hypothesis that increased levels of intracellular *Lm* detected in NMHC-IIA-depleted cells could result from the overexpression of the isoform B of non-muscle myosin heavy chain (NMHC-IIB), we confirmed that expression levels of NMHC-IIB were similar in NMHC-IIA-depleted cells and control cells (Fig. 5D). In addition, we found that *Lm* intracellular levels decreased 3-fold in NMHC-IIB-depleted HeLa cells (Fig. 5E), suggesting that NMHC-IIA and IIB play opposite roles in *Lm* infection and thus undermining the possibility of their mutual functional replacement.

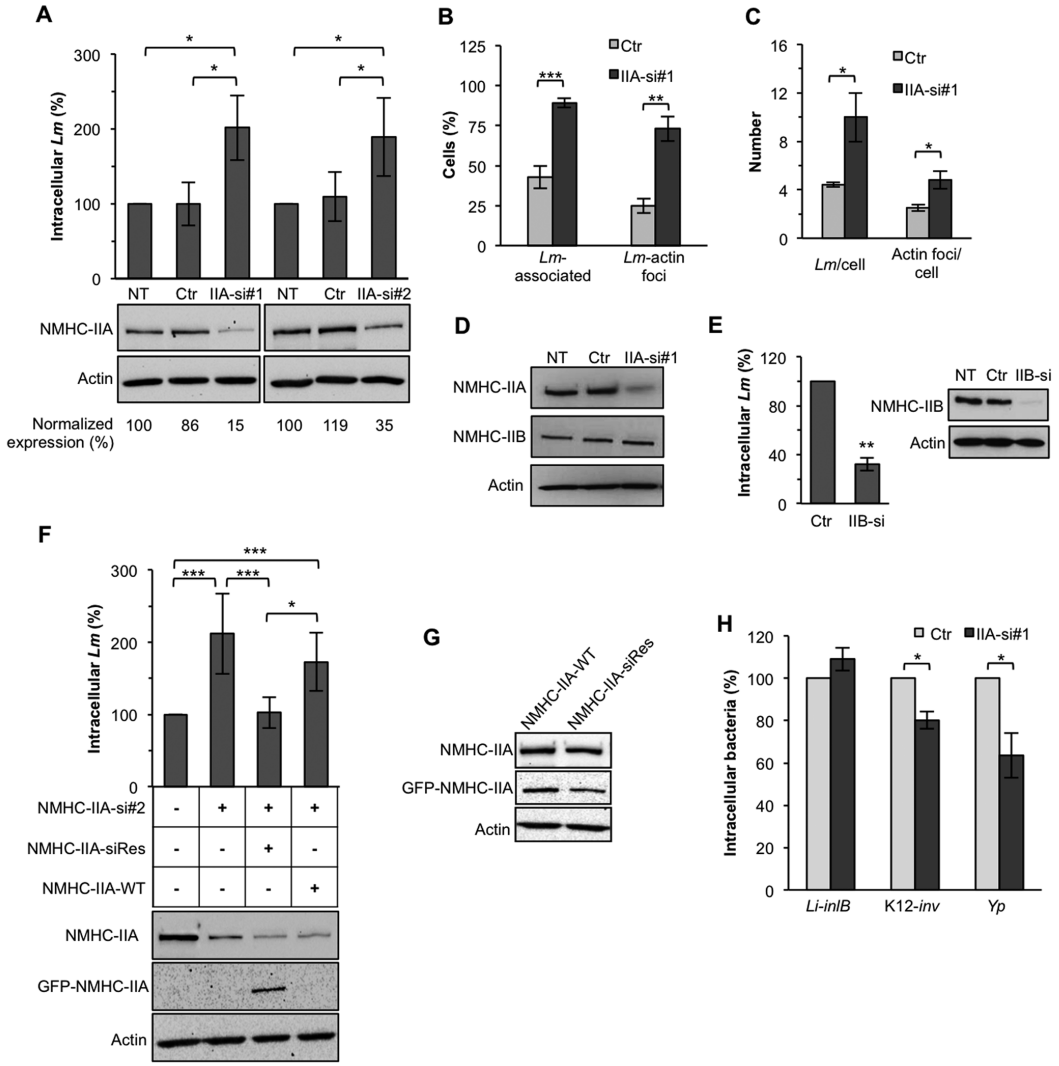


Figure 5. Depletion of NMHC-IIA facilitated *Lm* cellular infection. (A) Intracellular levels of *Lm* assessed by gentamicin protection assay in HeLa cells non-transfected (NT) or transfected with either control siRNA (Ctr) or NMHC-IIA-specific siRNAs (si#1 and si#2). Efficiency of NMHC-IIA knockdown was assessed by immunoblot and quantified. Indicated values (Normalized expression) are relative to actin and NMHC-IIA expression levels in NT cells. (B) Percentage of control (Ctr) or NMHC-IIA-depleted cells (IIA-si#1) associated to *Lm* and showing *Lm*-associated actin foci, evaluated by immunofluorescence scoring. (C) Number of bacteria and actin foci per cell in control and NMHC-IIA-depleted conditions. (D) Depletion of NMHC-IIA does not affect the expression of NMHC-IIB. NMHC-IIB expression levels were evaluated by immunoblot in NMHC-IIA-depleted (IIA-

si#1) as compared to control (NT and Ctr) cells. Actin was used as loading control. (E) Intracellular levels of *Lm* were assessed by gentamicin protection assay in HeLa cells transfected with either control siRNA (Ctr) or NMHC-IIB-specific siRNA (IIB-si). Efficiency of NMHC-IIB knockdown was assessed by immunoblot using actin protein detection as loading control. (F) Expression of NMHC-IIA was restored in si#2-depleted cells through the expression of a siRNA-resistant GFP-NMHC-IIA (NMHC-IIA-siRes). Intracellular levels of *Lm* assessed by gentamicin protection assay in HeLa cells expressing different levels of NMHC-IIA are shown. Non-treated and NHMC-IIA-depleted cells expressing a wild type GFP-NMHC-IIA (NMHC-IIA-WT) were used as controls. Endogenous NMHC-IIA silencing and GFP-NMHC-IIA expression was evaluated by immunoblot. Detection of actin levels served as loading control. (G) Western blot showing expression levels of endogenous (anti-NMHC-IIA, M8064, Sigma-Aldrich) and ectopically expressed NMHC-IIA (anti-GFP, B2, Santa Cruz Biotech) in HeLa cells transfected either with GFP-NMHC-IIA-WT or GFP-NMHC-IIA-siRes expression vectors. (H) Intracellular levels of *L. innocua* expressing *inlB* (*Li-inlB*), *E. coli* K12 expressing the invasin (K12-*inv*) and *Y. pseudotuberculosis* (Yp) were assessed by gentamicin protection assay in HeLa cells transfected with either control siRNA (Ctr) or NMHC-IIA-specific siRNA (IIA-si#1). In panels A and F, the number of intracellular *Lm* in NT cells was normalized to 100% and those in siRNA-transfected cells were expressed as relative values to NT cells. In panels E and H, numbers of intracellular bacteria were normalized to 100% in Ctr cells and expressed as relative values in the other conditions. Results shown in panels A, B, C, E, F and H are means±SEM of at least three independent experiments, each done in triplicate. Statistically significant differences are indicated: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

To definitively reinforce our findings and exclude potential uncontrolled off-target effects, we performed gentamicin protection assays following gene rescue experiments. We created a siRNA-resistant GFP-NMHC-IIA construct (NMHC-IIA-siRes) by introducing silent point mutations within the si#2 target sequence. We found that increased levels of intracellular *Lm* detected upon NMHC-IIA depletion (IIA-si#2) dropped to control levels in NMHC-IIA-depleted cells expressing NMHC-IIA-siRes (Fig. 5F). In contrast, the expression of NMHC-IIA-WT in NMHC-IIA-depleted cells did not restore control levels of intracellular *Lm*. Immunoblot analysis confirmed that the expression of endogenous NMHC-IIA was diminished in the presence of si#2 and that ectopically expressed NMHCH-IIA was only detected in NMHC-IIA-siRes-transfected cells (Fig. 5F). However, in absence of si#2, both NMHC-IIA-WT and siRes variants are expressed at similar levels (Fig. 5G). Together these results confirm that the increase in *Lm* intracellular levels observed in NMHC-IIA-depleted cells is specifically due to NMHC-IIA depletion.

To analyze whether the role of NMHC-IIA on intracellular levels of bacteria was specific for *Lm* or could be broadened to other bacterial infectious processes, we performed gentamicin protection assays using *Li* expressing InlB (*Li-inlB*), the major internalin driving *Lm* entry in HeLa cells (Pizarro-Cerda et al. 2012); K12-*inv* and *Yp*. Numbers of intracellular *Li-inlB* were not significantly different in NMHC-IIA-depleted and Ctr cells (Fig. 5H). In contrast, levels of intracellular K12-*inv* and *Yp* were significantly lower in NMHC-IIA-depleted cells (Fig. 5H). Our data indicate

that NMHC-IIA is specifically triggered by pathogenic *Lm* and is independent of an InlB-mediated uptake. By the contrary, the invasin-mediated uptake requires NMHC-IIA. Interestingly, NMHC-IIA and IIB were shown to be required for SopB-mediated invasion of *Salmonella* (Hanisch et al. 2011). Our findings, together with published reports, reveal that NMHC-IIA plays opposite roles in different infection models: while it is required for an utmost *Yp* and *Salmonella* infection, it has a restrictive role in *Lm* cellular infection.

I.F. *The function of NMHC-IIA in Lm infection relies on the phosphorylation of its tyrosine 158.*

We reported above two important observations: 1) NMHC-IIA is tyrosine phosphorylated by Src kinase upon *Lm* incubation with cells, and 2) *Lm* intracellular levels are increased in conditions of NMHC-IIA depletion or inhibition of its activity, demonstrating that NMHC-IIA activity limits *Lm* infection. To investigate whether both findings could be interconnected we evaluated levels of intracellular bacteria under conditions where NMHC-IIA-pTyr does not occur. We used cells with compromised Src activity (PP1 treatment and Src-KD overexpression) and cells expressing an NMHC-IIA non-phosphorylable variant (NMHC-IIA-Y158F). Levels of intracellular *Lm* showed a 2.5-fold increase in PP1-treated HeLa cells as compared to control DMSO-treated cells (Fig. 6A). In agreement, we observed an increase in *Lm* intracellular levels in cells expressing Src-KD (Fig. 6B). Inversely, intracellular levels of K12-*inv* decreased 2-fold in PP1-treated cells (Fig. 6C), as previously reported (Alrutz and Isberg 1998). Increased levels of intracellular *Lm* detected in conditions of Src inactivation and thus in absence of NMHC-IIA-pTyr, correlates with our data showing that reduced levels or inactivation of NMHC-IIA resulted in increased numbers of intracellular *Lm*. Our data also suggest an association between the role of NMHC-IIA in *Yp* invasin-mediated uptake and invasin-triggered NMHC-IIA-pTyr.

To further confirm the role of NMHC-IIA-pTyr in the *Lm* cellular infection, we evaluated intracellular levels of *Lm* in HeLa and COS-7 cells transiently expressing either the GFP-NMHC-IIA-WT (WT) or the non-phosphorylable variant GFP-NMHC-IIA-Y158F (Y158F). Contrarily to HeLa cells, COS-7 cells naturally lack NMHC-IIA expression, therefore allowing the overcoming of effects from the

endogenous protein. Equivalent expression levels of both constructs were verified by flow cytometry and immunoblot (data not shown). *Lm* intracellular rates were determined by gentamicin protection assays in cell populations containing about 50% of transfected cells. As compared to NMHC-IIA-WT, the expression of NMHC-IIA-Y158F led to increased levels of intracellular *Lm* in both cell lines (Fig. 6D). Thus, NMHC-IIA-Y158F expression recapitulates the increase of intracellular *Lm* in NMHC-IIA-depleted or inactivated cells. Furthermore, both GFP-NMHC-IIA-WT and GFP-NMHC-IIA-Y158F showed the same localization and accumulate at the site of *Lm* entry in HeLa cells (Fig. 6E).

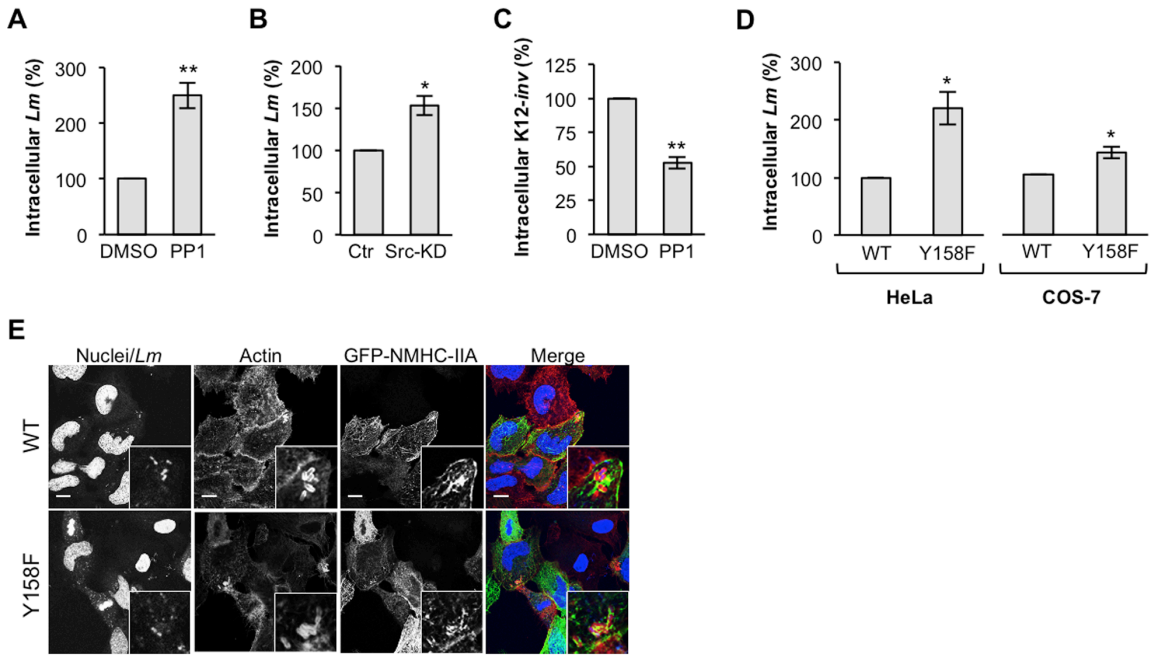


Figure 6. NMHC-IIA phosphorylation in tyrosine 158 is required to limit *Lm* cellular infection. (A and B) Intracellular levels of *Lm* assessed by gentamicin protection assays in the presence of 10 μM PP1 (A) or in HeLa cells expressing Src-KD (B). (C) Levels of intracellular K12-*inv* were assessed by gentamicin protection assay in HeLa cells treated with 10 μM of PP1. (D) Intracellular levels of *Lm* were assessed by gentamicin protection assays in HeLa and COS-7 cells expressing either GFP-NMHC-IIA-WT (WT) or GFP-NMHC-IIA-Y158F (Y158F). Results shown in panels A, B, C and D are means±SEM of three independent experiments, each done in triplicate Numbers of intracellular bacteria were normalized to 100% in control cells and expressed as relative values in the other experimental conditions. Statistically significant differences are indicated: * $p<0.05$, ** $p<0.01$. (E) Single confocal section of COS-7 cells ectopically expressing either GFP-NMHC-IIA-WT or Y158F variants incubated with *Lm* for 1 h and stained for actin (phalloidin, red) and DNA (DAPI, blue) (scale bar 10 μm).

These results indicate that, whereas NMHC-IIA subcellular localization and recruitment to the site of bacterial uptake are unrelated to Y158, the phosphorylation of this specific NMHC-IIA tyrosine plays a key role in restraining *Lm* infection.

I. DISCUSSION

Pathogens interfere with host phosphorylation cascades to foster adhesion, invasion and intracellular survival. Here, we searched for new host proteins undergoing tyrosine phosphorylation upon *Lm* infection. We showed that NMHC-IIA is tyrosine phosphorylated in response to *Lm* as well as to other human bacterial pathogens such as EPEC, EHEC and K12-*inv*. In *Lm* infection, this previously unknown tyrosine phosphorylation event is triggered by Src kinase on residue Y158 of NMHC-IIA, and limits intracellular bacterial levels.

Myosin II activity is regulated by phosphorylation events in serine and threonine residues of the regulatory light chain (Vicente-Manzanares et al. 2009). NMHC-IIA also undergoes serine and threonine phosphorylations, which regulate the assembly of myosin II filaments *in vitro* and are thought to control subcellular localization of NMHC-IIA and contractility that depends on the actin-crosslinking activity of NMHC-IIA (Vicente-Manzanares et al. 2009). While NMHC-IIA was detected in studies aiming to unravel the global phosphotyrosine signaling in cancer tissues (Rikova et al. 2007, Guo et al. 2008), its tyrosine phosphorylation has never been characterized. Our data constitutes the first report showing and characterizing NMHC-IIA-pTyr. Our preliminary *in silico* analysis suggest an important and broad role for NMHC-IIA pTyr in position 158: 1) Y158 is highly conserved among species ranging from *Saccharomyces cerevisiae* to *Homo sapiens*, 2) an *in silico* study suggested that the residue Y163 of muscle myosin heavy chain (matching Y158 in NMHC-IIA) could be phosphorylated (Harney et al. 2005), 3) Y158 is located in the motor domain of NMHC-IIA nearby the ATP-binding pocket and 4) analysis of the crystal structure of myosin motor domain (Dominguez et al. 1998) showed that Y158 is exposed at the surface of the protein and is thus accessible for phosphorylation. Thus, we hypothesize that the phosphorylation of NMHC-IIA Y158 could modulate NMHC-IIA activity most probably by affecting its ability to bind and/or hydrolyze ATP. However at this point any other mechanism could be envisaged. In addition it is likely that NMHC-IIA-pTyr in Y158 occurs in specific physiological conditions engaging NMHC-IIA activity and thus plays a role in the regulation of the highly conserved canonical functions of NMHC-IIA. The functional and structural outcomes of such modification are now critical to elucidate.

Our data suggest that, upon infection, only a small pool of NMHC-IIA becomes phosphorylated in Y158, probably concentrated in a restricted subcellular localization and/or interacting with specific partners, which would impact infection. Yet, we observed that both NMHC-IIA-WT and Y158F concentrated around bacteria at the entry site. We also found that phosphorylation of Y158 does not affect the phosphorylation of the myosin regulatory light chain (our unpublished data), that is achieved by MLCK and is required for activation of myosin II motor activity (Vicente-Manzanares et al. 2009). Interestingly, Src was previously shown recruited to membrane blebs where it associates with MLCK and myosin II (Barfod et al. 2005, Barfod et al. 2011). In response to cell swelling, Src and MLCK form a complex in which Src activates MLCK and both regulate a compensatory membrane retrieval that requires myosin II (Barfod et al. 2011). It is thus conceivable that Src and MLCK could work together to fine-tune the activity of myosin II in the context of infection.

Myosin II isoforms were recently involved in viral and bacterial infections either promoting or limiting pathogen progression. However their role in such processes is still mainly descriptive. NMHC-IIA is required for KSHV and HSV1 entry into cells (Arii et al. 2010, Valiya Veettil et al. 2010, Chakraborty et al. 2012), facilitates *Salmonella* invasion and regulates its intracellular growth (Wasylnka et al. 2008, Hanisch et al. 2011) and promotes *Chlamydia* dissemination (Hybiske and Stephens 2007). Conversely, myosin II limits bacterial cell-to-cell spread by restraining *Lm* protrusion formation (Rajabian et al. 2009) and participating in the formation of *Shigella*-associated septin cages (Mostowy et al. 2010). NMHC-IIB is involved in the formation of actin-rich structures that accumulate near the *Salmonella*-containing vacuole and restrain bacterial intracellular multiplication (Odendall et al. 2012). Altogether, these data suggest that the different outcomes associated with myosin II function during infection are probably related to the cellular machinery engaged in the various infectious processes. Our results indicate that NMHC-IIA activity limits *Lm* infection most probably hindering cellular invasion by interfering with the formation of *Lm*-induced actin foci. NMHC-IIA-depleted or inactivated cells were reported to lose cytoplasm cohesion and show increased membrane activity and plasticity (Cai and Sheetz 2009, Cai et al. 2010). These phenotypes could thus suggest that the increased numbers of intracellular *Lm* observed in such cells would be greatly due to the disruption membrane

rigidity. However, if this was the case, cells displaying low NMHC-IIA activity should be more permissive to any extracellular pathogen, which was not observed in KSHV (Valiya Veettil et al. 2010), HSV1 (Arii et al. 2010) and *Salmonella* (Hamon and Cossart 2011) infections. In addition, we show here that NMHC-IIA sustains invasin-mediated *Yp* infection and the invasion rate of *Li* expressing InlB was not significantly increased by NMHC-IIA-depletion, thus excluding a non-specific cell invasion mechanism.

NMHC-IIA participates in cellular processes associated to phosphotyrosine signaling, which are largely usurped by bacteria, namely *Lm* and *Yp* (Veiga and Cossart 2006), during infection. It 1) regulates protrusion formation and cell migration through the generation of actin retrograde flow (Cai et al. 2006, Giannone et al. 2007), 2) is required for integrin-mediated adhesion maturation (Choi et al. 2008), 3) controls cell-cell adhesion promoting E-cadherin clustering and stabilizing cellular junctions (Smutny et al. 2010), and 4) it governs the polarization of epithelial cells generating forces to maintain the epithelia (Bertet et al. 2004). Whether NMHC-IIA is pTyr in these processes is unknown.

In intercellular junctions, NMHC-IIA is critical for the E-cadherin localization (Smutny et al. 2010) and Src activation is required for actin polymerization at cell-cell contacts (McLachlan et al. 2007), as it is during E-cadherin-mediated *Lm* invasion (Sousa et al. 2007). Interestingly, Src activation and recruitment of c-Cbl are key events to control c-Met signaling (Organ and Tsao 2011). Our data show that Src activity restricts intracellular levels of *Lm* in HeLa cells in which *Lm* uptake is mainly mediated by c-Met and rise the hypothesis that Src is acting through the tyrosine phosphorylation of NMHC-IIA to inhibit entry. Remarkably, in KSHV infection, which depends on integrin and Src activation (Chandran 2010), NMHC-IIA interacts with the ubiquitin ligase c-Cbl (Valiya Veettil et al. 2010). The complex c-Cbl- NMHC-IIA associates with the receptor tyrosine kinase EphA2 that amplifies Src signaling to promote viral macropinocytosis (Chakraborty et al. 2012). It is thus possible that c-Cbl, which is required for *Lm* infection (Veiga and Cossart 2005), associates with NMHC-IIA and c-Met to modulate *Lm* infection through tyrosine phosphorylation events. To invade cells, *Yp* binds β1-integrin (Isberg 1990), which *via* its cytoplasmic tail interacts with NMHC-IIA to regulate cell migration (Rivera Rosado et al. 2011). As in adhesion and cell migration processes (Destaing et al. 2011), during *Yp* infection the engagement of β1-integrin leads to the activation of

Src kinase (Bruce-Staskal et al. 2002) that could also act on NMHC-IIA triggering its tyrosine phosphorylation at the site of bacterial attachment thereby promoting *Yp* infection.

Our data open new perspectives in the regulatory mechanisms governing NMHC-IIA functions in infection and physiological cellular processes. Further work should reveal whether NMHC-IIA-pTyr affects its motor activity, binding partners and/or the formation of actomyosin filaments.

II. NMHC-IIA-INTERACTING PROTEINS

The proposed restrictive function of NMHC-IIA in *Lm* infection requires further understanding of its role and possible integration in a signaling cascade that would be activated during infection. To address this issue, we undertook two approaches to identify novel NMHC-IIA protein partners: a high-throughput approach and a candidate-based approach. The high-throughput approach was based on a yeast two-hybrid (Y2H) screen (Fields and Song 1989) that was performed by HYBRIGENICS Services with the aim of revealing unknown protein partners.

The candidate-based approach was achieved by biochemical techniques, namely immunoprecitpitations, to confirm interactions with suspected partners.

The Y2H principle is based on the reconstitution of a functional transcription factor (TF) (Figure 13A) when two proteins interact. The process occurs in genetically modified yeast strains, in which the TF-mediated expression of a reporter gene leads to growth on a selective medium and detection of positive colonies. Examples of reporter genes are HIS3, which encodes a protein involved in histidine biosynthesis, to select positive clones on a medium without histidine; and *lacZ,* that encodes a β-galactosidase that signals for positive colonies through the generation of a blue precipitate in the medium, resulting from the hydrolysis of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

To setup a Y2H screen, the coding DNA sequence of the protein of interest (“bait”) is fused to the coding sequence of the TF DNA binding domain (DBD) (the fusion protein is named “bait”). In parallel, a library of cDNA fragments is fused to the TF activation domain (AD) coding sequence (fusion proteins called “prey”) (Figure 13B). Both bait and prey plasmids are introduced in particular yeast cells that will be mated and plated in selective medium allowing the detection of positive colonies (Figure 13C). When interaction occurs between bait and prey proteins, the DBD and AD are brought together and a functional TF is reconstituted upstream of the reporter gene leading to its transcription (Figure 13B). Common protein fusions use the DBD and AD of Gal4, a yeast transcription activator. The bacterial DNA-binding protein LexA is also used as a DBD in combination with the AD of Gal4.

For the purpose of our screen, we selected the C-terminal domain of NMHC-IIA (Leu837 to Glu1960) as the bait protein fragment (Figure 14) based on several criteria: 1) the motor domain function and its actin-interacting partners are well-described; 2) myosins from different classes share high homology through motor domains, increasing the probability of common binding partners; 3) the tail fragment subdomains are responsible for functional diversity among myosins and tail domain interactions with different cytoplasmic proteins have been described; and 4) the tail domain dictates the cellular localization of a particular myosin through its interaction with specific partners (Kussel-Andermann et al. 2000, Dahan et al. 2012, Majewski et al. 2012, Ramagopal et al. 2013). As a source of potential interacting prey proteins, we selected a library of human placental proteins, based on the fact that the placenta is a fast-growing and proliferative organ with high protein diversity and expression levels, and is particularly relevant for *Lm* pathology (Mylonakis et al. 2002, Lecuit et al. 2004, Lecuit 2005).

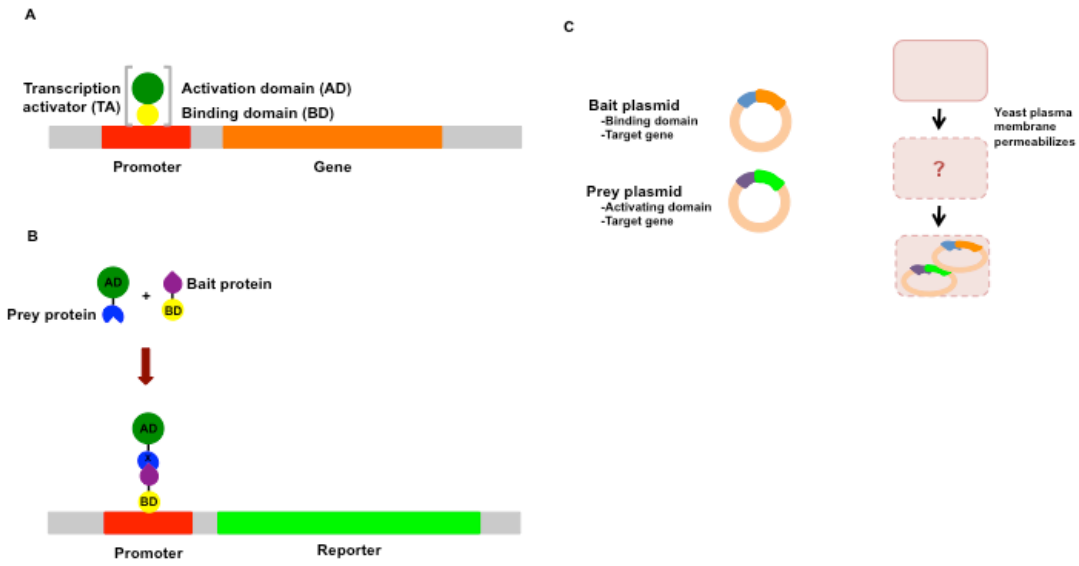


Figure 13. Schematic diagram of the Y2H system. (A) Transcription factor (TF)-mediated activation of gene expression in physiological conditions. (B) A positive interaction between bait and prey proteins leads to reconstitution of a functional TF and promotes reporter gene expression. (C) Bait and prey plasmids are transformed into a yeast cell and if an interaction occurs, a yeast colony will grow in the selective medium.

II. EXPERIMENTAL PROCEDURES

Y2H screening. A DNA fragment containing the coding sequence for the NMHC-IIA tail domain (Leu837 to Glu1960) was fused with the Gal4 DBD coding sequence to generate our bait plasmid. Similarly, prey plasmids were generated from fusions of the Gal4 AD coding sequence with fragments from a randomly primed human placental cDNA library. Yeast cells containing a bait plasmid were mated with a population of yeast cells, each containing a unique prey plasmid. Upon growth of bait and prey plasmid-transformed yeast cells in selective media, positive clones were identified through both LacZ activity and HIS3 expression. The positive prey fragments were identified by PCR and DNA sequencing at their 5' and 3' sites and the obtained DNA sequences were used to identify the corresponding gene in the GenBank database (NCBI), using BLASTN (Rain et al. 2001, Formstecher et al. 2005).

***Cell lines and culture media*.** Jeg-3 cells were grown in Eagle's minimal essential medium (EMEM) with L-glutamine supplemented with 2 mM non-essential amino acids, 2 mM sodium pyruvate and 10% fetal bovine serum (FBS). HeLa and HEK293 cells were cultivated in Dulbecco's modified Eagle medium (DMEM) with glucose (4.5 g/L), L-glutamine and 10% FBS at 37ºC in a 5% CO_2 atmosphere. All media and reagents were purchased from Lonza.

Plasmids and antibodies. GFP-NMHC-IIA-WT (pEGFP-C3:CMV-GFP-NMHC IIA) was obtained from Addgene. Mouse monoclonal anti-actin AC-15 (A5441) and rabbit polyclonal anti-NMHC-IIA (M8064) were purchased from Sigma; mouse monoclonal anti-NMHC-IIA (ab55456) was purchased from Abcam; rabbit polyclonal anti-β-Adaptin (H-300; sc-10672) was obtained from Santa Cruz Biotechnology; and mouse monoclonal anti-Hsp56 (ADI-SRA-1400) was purchased from Enzo Life Sciences. HRP-conjugated secondary antibodies were from P.A.R.I.S (Cambridge Bioscience).

Immunoprecipitation (IP). HeLa cells were washed in phosphate-buffered saline (PBS) and lysed for 30 minutes on ice with lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM AEBSF (Uptima-Interchim), cOmplete Protease Inhibitor Cocktail (Roche Pharmaceuticals), 1% NP-40]. Lysates were centrifuged at 15,000 g for 10 min at 4°C and the supernatants were cleared with a 20% (v/v) suspension of Protein G Sepharose beads (GE Healthcare) or Protein

G magnetic beads (Millipore) for 1 h at 4°C. Cleared lysates were immunoprecipitated with anti-NMHC-IIA, anti-β-Adaptin or anti-Hsp56 (10 µg per mg of total protein extracts) overnight at 4°C with agitation. Immune complexes were captured with 60 µl of a 20% (v/v) suspension of Protein G Sepharose or magnetic beads for 3h at 4°C and washed twice in wash buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM AEBSF, cOmplete Protease Inhibitor Cocktail, 0.2% NP-40). Immunoprecipitated complexes were recovered in Laemmli buffer (0.25 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 10% β-mercaptoethanol, 0.008% bromophenol blue). Proteins were denatured at 95ºC for 10 min and resolved by SDS–PAGE.

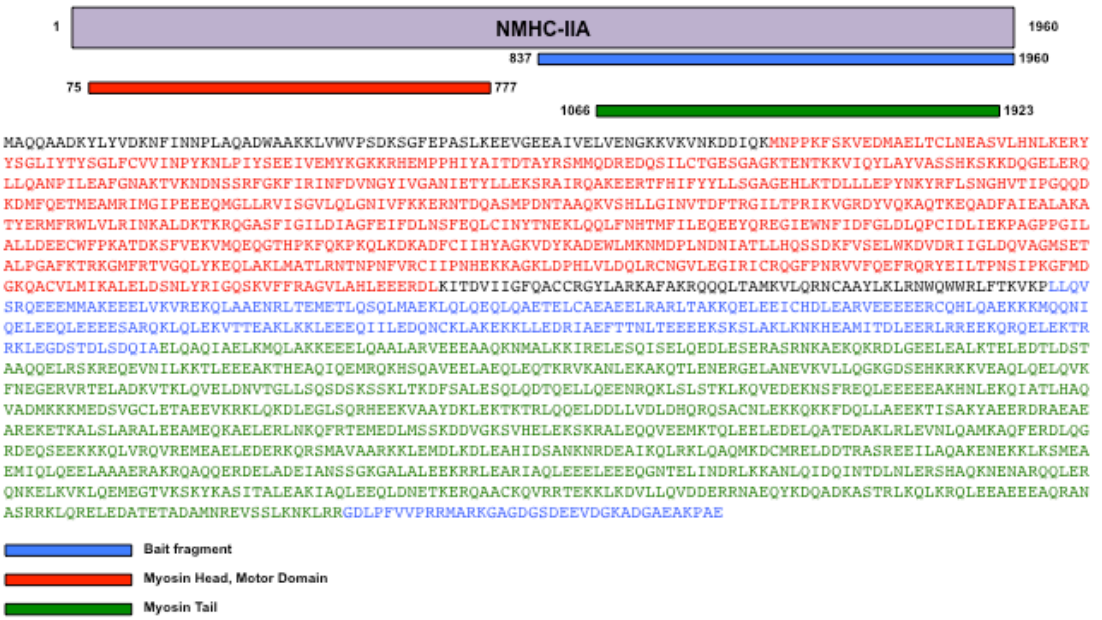


Figure 14. Schematic representation and amino acid sequence of NMHC-IIA. Fragment used as bait for Y2H (L837 to E1960) is depicted in blue. Motor and tail domains are highlighted in red and green, respectively.

II. RESULTS

II.A. Selection of candidate prey proteins

Our Y2H screen allowed the analysis of 232 million interactions, of which 97 were positive and validated by HYBRIGENICS. From these 97 positive interactions, we selected five prey protein candidates for confirmation of NMHC-IIA interaction (Table 4 and Figure 15). Three were selected based on their high interaction reliability rating: ZNF12, SMPD4 and AFF1. For the purpose of this work, two other candidate proteins, β-adaptin and Hsp56, although low-rated, were also selected considering that they have been described in the literature to be associated with NMHC-IIA function and relevant in the context of infection.

Table 4. Prey proteins selected from Y2H screen for further study.

Target protein	Rating[1]	Interaction domain		Frame	Number of hits
		Nucleotide	Amino acid		
ZNF12	A	33-2891	422-597	In-frame	23
SMPD4	B	57-745	33-243	In-frame	4
AFF1	C	2331-3143	872-1046	In-frame	2
β-Adaptin	D	711-1430	238-476	In-frame	1
Hsp56	D	57-1150	20-383	In-frame	1

[1] Ratings range from A to F: A, B, C and D represent very high, high, good and moderate confidence interactions, respectively. D-rated interactions correspond to weakly detected interactions usually identified through one unique prey fragment (just one hit in the Y2H). Ratings E and F are considered very low confidence interactions and were not considered in this study (Rain et al. 2001).

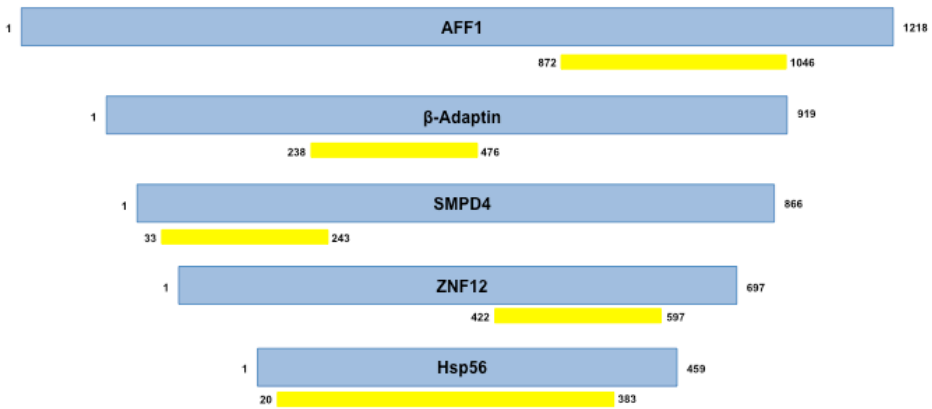


Figure 15. Schematic representation of the Selected Interaction Domains (SID) of the selected prey proteins. Amino acid sequences of the five selected Y2H targets are depicted in blue and prey protein domains that interacted with the bait fragment is depicted in yellow.

II.B. Validation of NMCH-IIA interactions with β-adaptin and Hsp56

The β-adaptin and Hsp56 interactions with NMHC-IIA positively identified in a yeast system needed to be further validated by other methods in human cell lines. We first aimed to verify whether both proteins were endogenously expressed in cells lines used in our laboratory, some of which relevant for infection. To do that, we performed WB on total extracts from HeLa, HEK-293 and JEG-3 cells and detected the proteins using specific antibodies. We observed that both β-adaptin (105 kDa) and Hsp56 (56 kDa) were expressed in the three cell lines (Figure 16).

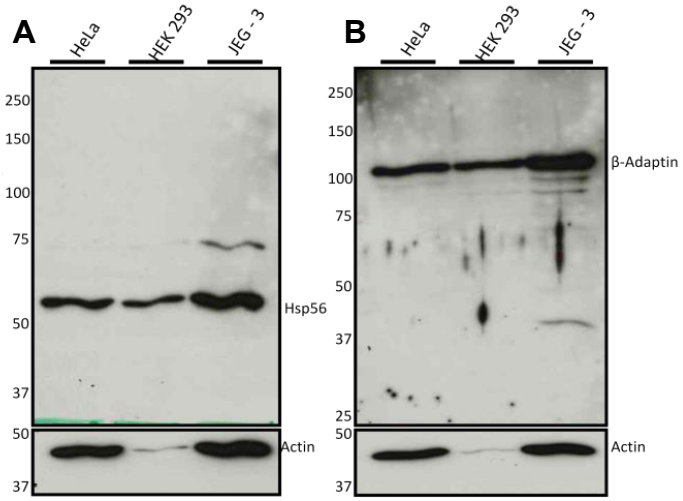


Figure 16. Expression of Y2H target proteins in human cell lines. WB analysis of (A) Hsp56 and (B) β-adaptin expression in HeLa, HEK293 and Jeg-3 cells. Actin used as control.

We then performed immunoprecipitation (IP) assays for further validation of the selected NMHC-IIA interactions. Although IP of NMHC-IIA from non-infected HeLa cell extracts was not highly efficient, WB analysis confirmed that both β-adaptin (105 kDa) and Hsp56 (56 kDa) co-immunoprecipitate with NMHC-IIA (Figure 17A). Reversely, IP of Hsp56 and β-adaptin revealed that NMHC-IIA co-eluted with the immunoprecipitated proteins: NMHC-IIA was detected at 1/100 Hsp56 IP (Figure 17B) and 1/50 β-adaptin (Figure 17C) conditions. These results confirm biochemically the NMHC-IIA-β-adaptin and NMHC-IIA-Hsp56 interactions. Further evaluation of these interactions is currently ongoing in the context of infection, and it would be interesting to verify if bacterial infection potentiates or not this phenotype.

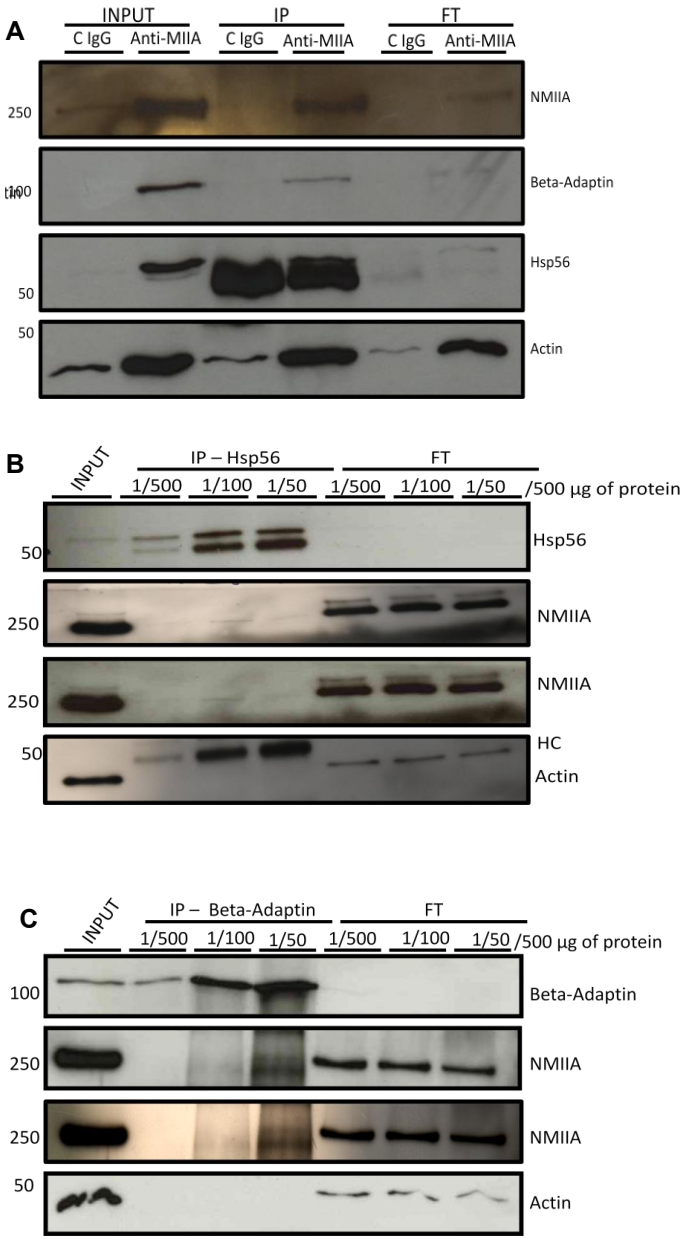


Figure 17. Interaction of NMHC-IIA with Hsp56 and β-adaptin. (A) Immunoprecipitation (IP) of NMHC-IIA in HeLa cell extracts and WB detection of β-adaptin and Hsp56 in the co-immunoprecipitates (co-IP). (B) IP of Hsp56 or (C) β-adaptin in HeLa cell extracts and WB detection of NMHC-IIA in the co-IP. Actin was used as loading control. IP efficiency was evaluated by comparison with total cell extracts (INPUT). FT: IP flow-through fraction.

II.C. Candidate-based approach (Gp96-MIIA)

Previous work in our lab identified Gp96 as a host receptor for *Lm* virulence factor Vip (Cabanes et al. 2005). We have also shown that *Lm* infection increased the cell surface expression of Gp96 and we characterized the interaction between Vip and Gp96 at the molecular level (Martins et al. 2012) (see also Annex I of this thesis). Considering these results, we wanted to see if NMHC-IIA would also

interact with Gp96 during infection. Interestingly, we observed that upon infection, there was an increase in the amount of Gp96 immunoprecipitated with NMHC-IIA (Figure 18), suggesting that this protein association is potentiated by *Lm* infection. Other experiments have shown that NMHC-IIA interaction with Gp96 is triggered by *Lm* infection in a LLO- and Ca^{2+}-dependent manner (Mesquita, F.S. unpublished results) but independent on Vip. Recently, mass spectrometry analysis of Gp96 immunoprecipitated protein fraction allowed the identification of a protein as being NMHC-IIA.

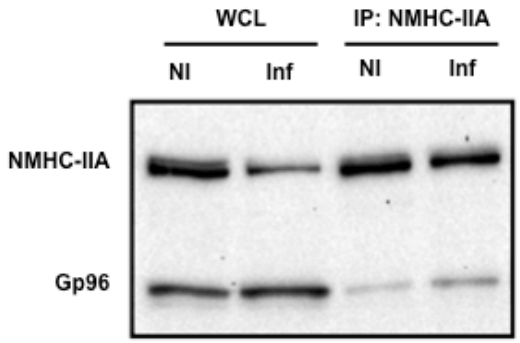


Figure 18: Gp96 co-immunoprecipitates with NMHC-IIA. HeLa cells were left non-infected (NI) or incubated with *Lm* and harvested 60 minutes post-infection (Inf). NMHC-IIA was immunoprecipitated (IP: NMHC-IIA) from whole cell lysates (WCL) and NMHC-IIA and Gp96 were detected by immunoblot in WCL and IP: NMHC-IIA fractions.

II. DISCUSSION

Based on the results presented above we can propose three valid candidates as NMHC-IIA protein interactors: β-adaptin, Hsp56 and Gp96.

β-adaptin

Adaptins are subunits of the clathrin–adaptor protein (AP) complex, a family of heterotetrameric protein complexes that mediate the sorting of membrane proteins in both exocytic and endocytic pathways connecting the *trans*-Golgi complex (GC), endosomes, lysosomes, and the plasma membrane (Boehm and Bonifacino 2001, Nakatsu and Ohno 2003). Each AP complex has four subunits: two large subunits (one from α1 to α4 and another from β1 to β4), one medium subunit (μ1 to μ4) and one small subunit (σ1 to σ4) (Figure 16). To date four AP complexes have been described: AP1, AP2, AP3 and AP4. The AP2 acts specifically at the plasma membrane in endocytic processes while AP1, AP3 and potentially AP4 are associated with the formation of clathrin-coated vesicles from intracellular membranes (Blondeau et al. 2004, Robinson 2004).

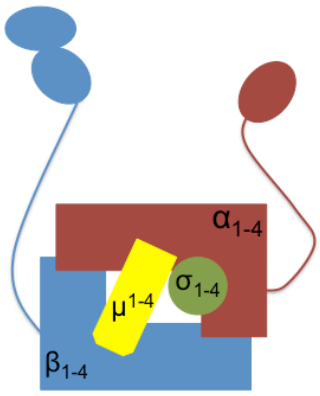


Figure 16. Schematic representation of a clathrin-adaptor protein complexes 1 to 4. Each complex is composed by four subunits: two large subunits (α and β adaptins), one medium (μ) and one small (σ) subunit.

AP complexes play an important role in the formation of clathrin-coated vesicles by recruiting clathrin, as well as in the incorporation of cargo membrane proteins into nascent clathrin-coated vesicles by recognizing sorting signals such

as dileucine- and tyrosine-based sorting motifs (Ohno et al. 1995, Heilker et al. 1996).

The β-adaptin subunit of AP1 is commonly found inside the cell on the cytoplasmic face of clathrin-coated vesicles located at the GC. There, it mediates the recruitment of clathrin and the recognition of sorting signals that are located on the cytosolic tails of transmembrane cargo molecules. (β) and medium (μ) subunits AP1 recognize both tyrosine-based (YXXφ) (Carvajal-Gonzalez et al. 2012) and dileucine-based ([DE]XXXLL) (Rapoport et al. 1998) sorting signals. AP-1 is involved in the basolateral recycling of transmembrane proteins controlling viral infection (Diaz et al. 2009) and is required for *Lm* cell invasion (Pizarro-Cerda et al. 2007). Remarkably, NMHC-IIA contains both a dileucine-based sorting motif within the tail domain used in our Y2H screen and a tyrosine-based sorting motif exactly including its tyrosine-158 that we showed to be phosphorylated upon infection. The sorting signal of the YxxΦ type (where Y is tyrosine, x is any amino acid, and Φ is any hydrophobic amino acid) mediates protein targeting and internalization through tyrosine phosphorylation (Marks et al. 1996), so we hypothesize that Y158 phosphorylation might regulate functional properties of NM-IIA in the interaction with other adaptor or effector proteins namely β-adaptin, that also recognizes YxxΦ motifs (Carvajal-Gonzalez et al. 2012). Noticeably, regulation of other tyrosine-based sorting motifs occurs through phosphorylation (Bonifacino and Traub 2003).

In addition, β-adaptin, was shown to be recruited at focal adhesions, at the leading edges of migrating cells (invadipodia) (Pignatelli et al. 2012). Therefore, it is important to continue the study of β-adaptin function and its interaction with NM-IIA.

Hsp56

Hsp56 is composed of an N-terminal peptidylprolyl cis-trans isomerase (PPIase) domain responsible for the chaperoning function in protein folding processes (Schiene and Fischer 2000), and a C-terminal tetratricopeptide repeat (TPR) domain, which is responsible for mediating protein-protein interactions (Goebl and Yanagida 1991). It is considered a high-molecular weight immunophilin, a known target of the immunosuppressive drug FK506, and is

therefore also named FKBP4 (FK506-binding protein 4). Hsp56 is known to associate with other heat shock proteins (Hsp90) and to play a role in the intracellular trafficking of hetero-oligomeric protein complexes of steroid receptors, between the cytoplasmic and nuclear compartments (Tai et al. 1986). To this purpose, Hsp56 binds a motor protein, dynein, allowing the translocation of the hormone complex from the cytoplasm to the nucleus (Czar et al. 1994, Pratt and Welsh 1994, Galigniana et al. 2001). Importantly, Hsp90 is a Gp96 paralogue, which in turn is a known *Lm* receptor (Cabanes et al. 2005) and whose subcellular localization was shown to be affected during infection (Martins et al. 2012). Moreover, we could envision that *Lm*-induced NMHC-IIA-Gp96 interaction may be stabilized by protein complex formation with the chaperone Hsp56.

One can assume that bacteria by sequestering NM-IIA could interfere with either inward or outward vesicular trafficking of any cargo, and facilitate *Lm* infection.

Moreover, in studies of recurrent spontaneous abortions in women, Hsp56 gene expression appears decreased in placentas of terminated pregnancies. Abortions caused by *Lm* infection are still not well understood, in particular the tropism of the bacteria to this organ (Chen et al. 2014). Therefore, Hsp56 function and interaction with NM-IIA are important to evaluate.

Gp96

Gp96 is a chaperone glycoprotein that belongs to the Hsp90 family, and is localized within the lumen of the endoplasmic reticulum (ER) (Yang and Li 2005). The expression of Gp96 is highly increased in stressful conditions leading to the accumulation of misfolded proteins. Gp96 has multiple protein substrates and has been implicated in both innate and adaptive immunity. It chaperones antigenic peptides, delivers them to antigen-presenting cells (APC), and activates dendritic cells (DCs). Importantly, Gp96 is required for the proper folding and cell surface distribution of TLRs and integrins (Yang et al. 2007, Wu et al. 2012).

In particular conditions such as cell activation, necrotic cell death or infection, Gp96 is exposed at the cell surface and is often hijacked as a membrane protein serving as receptor for bacterial virulence factors. Besides its role as *Lm* receptor, Gp96 is a key mediator in the establishment of various human infections.

Neisseria gonorrhoeae surface protein PorBIA interacts with Gp96 allowing an increase in adherence (Rechner et al. 2007). Gp96 also serves as receptor for enterotoxin A from *Clostridium difficile* (Na et al. 2008), OmpA expressed at the surface of *E. coli* K1 (Prasadarao et al. 2002, Prasadarao et al. 2003, Maruvada et al. 2008) and Als3 invasin of *Candida albicans* (Liu et al. 2011). In addition Bap, a biofilm matrix protein of *Staphylococcus aureus*, has been shown to prevents bacterial entry into epithelial cells through binding to Gp96 receptor (Valle et al. 2012)

Some intracellular pathogens use mechanisms that trigger interactions of the pathogen-containing compartments with secretory pathway components culminating in either pathogen resilience or replication (Canton and Kima 2012). Moreover, TTSS bacterial effectors directly manipulate components of the secretory pathway that are responsible for intracellular vesicle trafficking (Hilbi and Haas 2012). *Chlamydia* is an obligate intracellular pathogen whose release from cells has been described to occur by either cellular lysis or extrusion of the bacterial containing vacuoles. Inhibition or depletion of NM-IIA reduced chlamydial extrusion (Hybiske and Stephens 2007, Lutter et al. 2013). Gp96-NMHC-A interaction in *Lm* infection was LLO-dependent. Considering that NMHC-IIA, described to facilitate vesicle trafficking in membrane repair (Lin et al. 2012), and *Chlamydia* infection findings, one can assume that *Lm* through secretion of LLO manipulates cell membrane integrity and hijack a similar system. It is not known if NMHC-IIA is tyrosine-phosphorylated in membrane repair or which are the responsible *Lm* virulence factors for this PTM. Although we have recently observed that LLO toxicity induces NMHC-IIA tyrosine phosphorylation (Mesquita, F.S. unpublished results). Moreover, increased *Shigella* infection might correlate with *Lm*-LLO toxicity through the insertion of Ipa proteins into host cell membranes (Blocker et al. 1999, Mostowy et al. 2010). How LLO and NM-IIA-Gp96 interaction influences membrane extrusion mechanisms in *Lm* infection will be important to address (Koster et al. 2014, Seveau 2014).

Despite the progress in understanding the roles of Gp96 during infection much remains to be learned concerning the mechanisms inducing Gp96-cell membrane association and triggering of signaling pathways downstream Gp96 engagement.

All the proposed NMHC-IIA-interacting proteins discussed above are of extreme importance for dissecting the intracellular pathways hijacked during infection. Moreover, understanding the cellular processes where these proteins are involved will allow us to track the respective signaling cascades and define the exact role of NMHC-IIA in bacterial infection.

III. KERATINS

In our search to identify novel host proteins that would be tyrosine-phosphorylated in response to *Lm*, we followed an immunoprecipitation approach coupled to protein identification by mass spectrometry. We identified NMHC-IIA (described in section I of the results chapter) and an intermediate filament protein, keratin 18 (K18).

Intermediate filaments (IFs) are cytoskeletal polymers whose protein properties and intracellular organization provides crucial structural support in the cytoplasm and nucleus. Besides functions primarily related to structural integrity of cells and tissues, non-mechanical roles for IFs have been ascribed in a highly diverse range of cellular functions (Leduc and Etienne-Manneville 2015). These include cell adhesion and migration (Ivaska et al. 2007), organelle shaping and positioning (Toivola et al. 2005), in modification of various cellular processes, such as stress response and tissue growth, through their ability to regulate signaling molecules (Pallari and Eriksson 2006, Kim and Coulombe 2007). IFs are regulated by post-translational modifications, being phosphorylation the major regulatory event reported for these proteins. This regulation leads to the recruitment and sequestration of signaling molecules that participate in several cellular functions (Hyder et al. 2008).

Considering IF localization, there are four major types of IF proteins in vertebrate cells: epithelial (acidic-type I keratins and basic-type II keratins), neuronal (nestin, neurofilament proteins NF-L, NF-M and NF-H), nuclear (lamins A, B, and C) and in mesenchymally-derived cells (vimentin, desmin, glial fibrillary acidic protein, peripherin) (Leduc and Etienne-Manneville 2015).

Keratins represent the typical IF category in epithelial cells, showing a high degree of molecular diversity. As part of the epithelial cytoskeleton, keratins are important for the mechanical stability and integrity of epithelial cells and tissues (Toivola et al. 2015). In contrast to the other IF proteins keiratins only can constitute their filamentous stage by heteropolymeric pair formation between a type I and type II keratin. Individual keratin proteins deviating from the equimolar type I: type II amounts are rapidly degraded (Lu and Lane 1990). There are 28 type I keratin-encoding genes (17 epithelial keratins and 11 hair keratins) and 26 type II keratin-encoding genes (20 epithelial keratins and 6 hair keratins)

(Schweizer et al. 2006, Haines and Lane 2012). K18 is typically co-expressed with K8, and constitute the primary keratin pair of simple epithelial cells.

As members of the IF protein family they share a common tripartite protein-structure, with a central rod domain of *α*-helical conformation flanked by non-helical head and tail domains (Figure 10). Heterodimer formation occurs by association of the corresponding rod domains in *α*-helical coiled-coil conformation. The resulting polymerization forms the basic building units of the keratin filaments (Omary et al. 2006).

Besides being static intracellular skeletal structures, some keratins modulate intracellular signaling pathways of different dynamic cell processes such as cell polarization, migration, protein synthesis and membrane trafficking (Toivola et al. 2015). Several keratin variants have been associated with different diseases, either caused by gene mutation or altered keratin PTM patterns (Omary et al. 2009, Pan et al. 2013, Snider and Omary 2014).

Published data on the involvement of K18 in several bacterial infectious processes (Scherer et al. 2000, Carlson et al. 2002, Batchelor et al. 2004, Kumar and Valdivia 2008, Rodel et al. 2012, Saberi et al. 2012) supported our decision to further study K18 tyrosine phosphorylation and its role in *Lm* infection. Here I will present and discuss our first observations and results concerning K18.

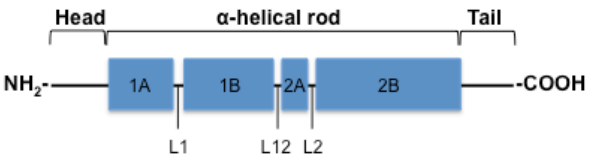


Figure 20. Schematic representation of keratin domain structure. Keratins share a common domain structure consisting of a conserved α-helical rod domain that is flanked by variable non-helical head and tail regions. Subdomains of the rod domain (1A/1B/2A and 2B) are interrupted by non-helical linkers (L1/L2 and L12). Adapted from (Eriksson et al. 2009).

III. EXPERIMENTAL PROCEDURES

Bacterial strains, cell lines and culture media*. Lm* strain EGDe (ATCC BAA-679) was grown in brain heart infusion (BHI) broth (Difco Laboratories, Becton, Dickinson and Company, USA) at 37ºC with agitation. Caco-2 cells were grown in Eagle's Minimal Essential Medium with L-Glutamine (EMEM) supplemented with 2 mM non- essential aminoacids, 2 mM sodium pyruvate and 20% fetal bovine serum FBS). HeLa cells were grown in Dulbecco's modified Eagle medium with 4.5 g/L Glucose and L-Glutamine (DMEM) supplemented with 10% FBS. The cells were grown without antibiotics at 37ºC in a 5% CO2 atmosphere. All media and products for cell culture were purchased from Lonza.

Antibodies. Monoclonal antibody raised against phosphotyrosine (clone 4G10) was purchased from Millipore (05-321). Antibodies to detect K8 (sc-8020), K18 (sc-6259) and GAPDH (sc-32233) were obtained from Santa Cruz. Rabbit K18 antibody was from Abcam (ab52948). α-Catenin antibody was purchased from BD Biosciences (610193). HRP-conjugated secondary antibodies were from P.A.R.I.S (Cambridge Bioscience).

Infection and immunoprecipitation assays. Caco-2 cells were seeded onto 75 cm2 flasks and incubated at 37°C in a 5% CO_2 atmosphere, until confluence. The day of the experiment, cells were washed twice with phosphate-buffered saline (PBS) and serum-starved during 5 h at 37°C and 5% CO_2. Then, cells were either left uninfected or infected with *Lm* EGDe at a MOI of 200 bacteria per cell, during different periods of time. After each time point, cells were washed twice with ice-cold PBS and lysed in 1 ml of lysis buffer (1% NP-40, 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM AEBSF, PhosSTOP (Roche Pharmaceuticals) and Complete Protease Inhibitor Cocktail (Roche Pharmaceuticals)). Lysates were centrifuged at 15 000 g for 10 min at 4°C and immunoprecipitated with 2 µg of anti-phosphotyrosine antibody (4G10) overnight at 4°C. Immune complexes were captured with 50 µl of PureProteome Protein A magnetic beads (Millipore) at 4°C and washed three times with wash buffer (0.2% NP-40, 50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM AEBSF, PhosSTOP, Complete Protease Inhibitor Cocktail). Immunoprecipitated proteins were eluted and boiled in Laemmli buffer containing 0.1 mM DTT and 5% β-mercaptoethanol.

Protein identification by mass spectrometry (MS). Protein identification was performed by MALDI TOF/TOF mass spectrometry as described (Osorio and Reis 2013). Protein bands were excised from SDS-PAGE gels, reduced with dithiothreitol, alkylated with iodacetamide and in gel digested with trypsin. Peptides were extracted, desalted, concentrated using ZipTips (Millipore), crystallized onto a MALDI sample plate and analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems). Peptidic mass spectra were acquired in reflector positive mode at a 700-4000 m/z mass window and proteins identified by Peptide Mass Fingerprint using the Mascot software (Matrix Science, UK) integrated in the GPS Explorer software (ABSCIEX, CA) and searched against the SwissProt/UniProt *Homo sapiens* protein sequence database. The maximum error tolerance was 35 ppm and up to two missed cleavages were allowed.

Immunoblotting. Proteins were resolved by SDS-PAGE and transferred onto Nitrocellulose membranes (Hybond ECL, GE Healthcare Life Sciences). Membranes were blocked with 5% skimmed milk in buffer A (150 mM NaCl, 20 mM Tris- HCl pH 7.4 and 0.1% Triton X-100) for 1 h at room temperature and incubated overnight with primary antibodies diluted in 2.5% skimmed milk in buffer. Membranes were then washed and incubated with mouse or rabbit HRP-conjugated secondary antibodies for 1 hour. The signal was developed with the ECL Chemiluminescent Detection System (Pierce).

Determination of intracellular bacteria. Infection experiments were performed as previously described (Reis et al., 2010). For invasion assays, cells were challenged with *Listeria* at MOI of 50 for one hour. After one hour infection the inoculum was removed and cells were treated with 20 µg/ml gentamicin for 90 min. Cells were further washed three times with PBS, lysed with 0.2% Triton X-100 and serial dilutions plated for CFU counting. Assays were performed in triplicate and repeated 3 times.

Transfection of siRNA duplexes. HeLa cells seeded in 24 well plates were transfected with 60 nM of control siRNA-D (sc-44232, Santa Cruz Biotechnology) or specific siRNAs for K8 and/or K18 depletion, using HiPerfect Reagent (Qiagen) following manufacturer's instructions. Assays were performed 72 h later. K8 (CUGGGAAGGAGGCCGCUAU) and K18 (GAGAGGAGCUAGACAAGUA) siRNA duplexes (Sigma) were transfection into Caco-2 cells with the Nucleofector system (Amaxa).

III. RESULTS

III.A. K18 tyrosine phosphorylation during infection

Infection experiments were performed for different periods of time, and total cell lysates were subject to IPs using 4G10 antibody. Eluates were further resolved by SDS-PAGE and visualized by silver staining as described for NMHC-IIA identification.

As previously described we searched for bands showing variable intensities over the time of infection, indicating differential tyrosine phosphorylation profiles in response to bacterial interaction with host cells. Such bands were analyzed by mass spectrometry for protein identification. A band extracted from an infection experiment in Caco-2 cells was identified as being K18. The spectrum of trypsin-digested peptides obtained by MS, as well as their corresponding molecular masses are represented in Figure 21A. K18 has a molecular weight of 48 kDa, which corresponds to the molecular weight of the band extracted from the polyacrylamide gel (37-50 kDa).

We started by analyzing the expression levels of K18 by WB in different cell lines relevant for *Lm* infection studies, such as Caco-2, Jeg-3 and HeLa, and confirmed that K18 was expressed in all three cell lines (Figure 21B).

A

PMF Keratin 18 [gi|30311] 47.3 kDa	
Peptide mass (Da)	Sequence
718.3916	IMADIR
758.4406	IIEDLR
807.3995	LAADDFR
837.4465	LASYLDR
848.4108	SLETENR
889.4737	ASLENSLR
924.5261	IREHLEK
965.4686	AQYDELAR
975.453	STFSTNYR
982.4417	DWSHYFK
1041.6051	IVLQIDNAR
1046.5477	VIDDTNITR
1065.5575	LEAEIATYR
1093.4943	ETMQSLNDR
1239.6401	VKYETELAMR

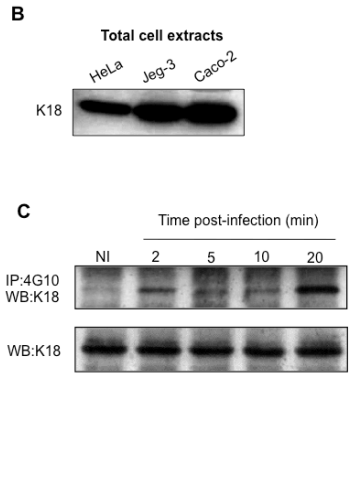


Figure 21. Identification and validation of K18 as a tyrosine-phosphorylated protein in *Lm* infection. (A) Trypsin-digested peptides of K18 identified by PMF. (B) Evaluation of the expression of endogenous K18 in human cell lines relevant for *Lm* infection. (C) K18 tyrosine phosphorylation in response to *Lm* infection of Caco-2 cells. IP of tyrosine-phosphorylated proteins in total cell extracts (infected and non-infected) with 4G10 and WB detection of K18 in the immunoprecipitates.

To further confirm that K18 is differentially tyrosine-phosphorylated in response to infection, experiments with Caco-2 cells incubated with *Lm* and further 4G10 IPs of the cell lysates were repeated, as described above, and 4G10 eluates were resolved by SDS-PAGE. WB analysis with anti-K18 showed that the amount of K18 in the 4G10 IP fraction increases with infection. This result shows that the amount of tyrosine-phosphorylated K18 is higher at 20 min post-infection, (Figure 21C). The total amount of K18 in the different whole cell lysates of non-immunoprecipitated extracts was unchanged, thus ensuring that the expression of total K18 remains the same in the course of the infection. Surprisingly, the enrichment of K18 fractions was not observed in phosphotyrosine-immunoprecipitated fractions of infected HeLa cells (data not shown).

These results show for the first time that *Lm* infection induces an increase of K18 tyrosine phosphorylation in Caco-2 cells.

III.B. Role of K8 and K18 during infection

The increase in tyrosine phosphorylation of K18 upon *Lm* infection suggests a role for K18 in *Lm* cellular infection. Therefore we decided to study K8/K18 role in *Lm* infection.

We first decided to evaluate bacterial entry in cells depleted for K18 and K8, through an RNAi approach. Experiments were performed using either K18 or K8 small interference RNA (siRNA) on Caco-2 and HeLa cells. Cells were harvested 72h hours after siRNA transfection, and protein depletion assessed by WB for K18 and K8, using GAPDH as a loading control (Figure 22A).

While the intracellular levels of *Lm* were not affected by the depletion of K18 and/or K8 in Caco-2 cells (Figure 22B), they were strongly reduced in depleted HeLa cells (Figure 22C).

Given that the early events of *Lm* infection in HeLa cells are driven by c-Met and InlB interaction, these results show that K18/K8 are required during the *Lm* infectious process probably through an InlB-dependent mechanism. Although not required in a InlA-dependent pathway, as seen in siRNA experiments in Caco-2 cell, tyrosine phosphorylation occurs in this cell line suggesting a possible role of K8/K18 in processes unrelated with *Lm* entry.

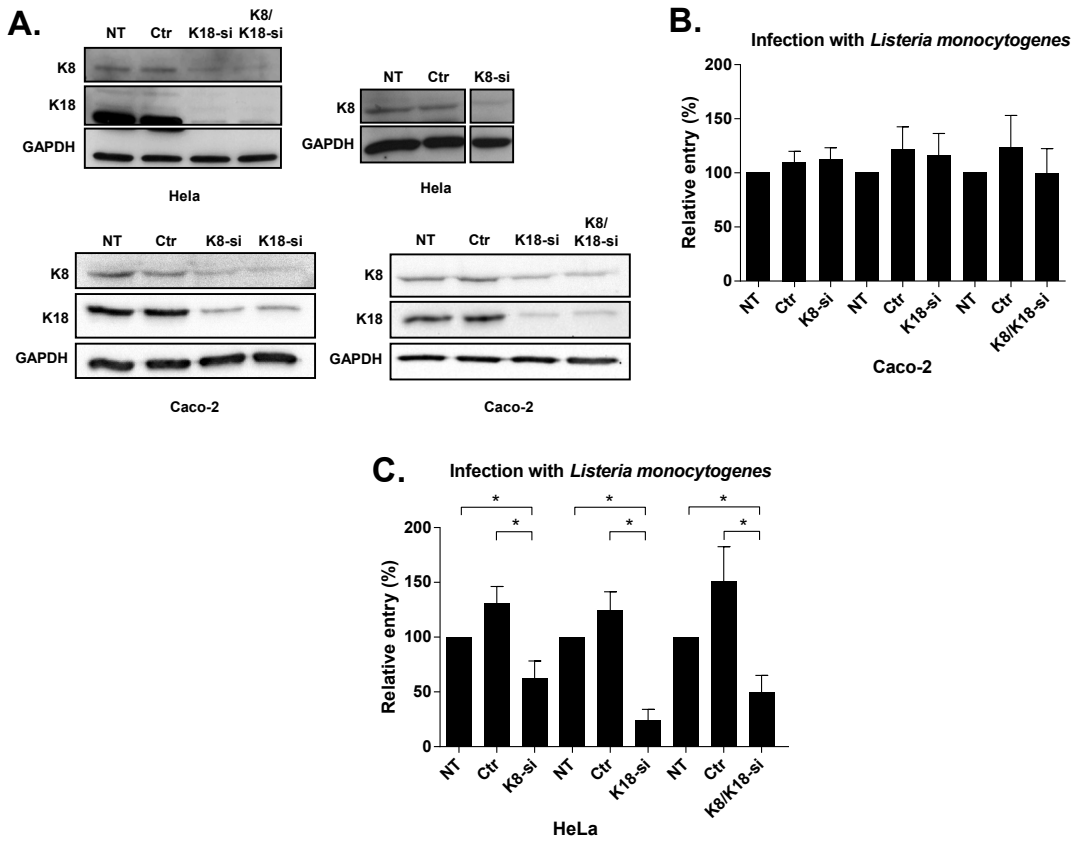


Figure 21. The requirement of K8 and K18 in *Lm* invasion is cell line-dependent. (A) WB detection of the expression levels of K8 and K18 on HeLa (top panels) and Caco-2 (bottom panels) cells left non-transfected (NT) or transfected with control (Ctr) or specific siRNAs for K8 (K8-si), K18 (K18-si) or both (K8/K18-si). GAPDH was used as loading control. (B and C) Caco-2 (B) and HeLa (C) cells were infected with *Lm* and levels of intracellular bacteria were determined. Bacterial counts in NT cells were normalized to 100 and bacterial counts in cells treated with siRNAs are expressed as relative values. Results shown are the mean of at least three independent experiments. *p<0,05.

III. DISCUSSION

We present here the first evidence of increased K18 tyrosine phosphorylation in response to *Lm* infection. Despite K19 and K8 tyrosine phosphorylation having already been characterized (Feng et al. 1999, Zhou et al. 2010, Snider et al. 2013), to our knowledge studies reporting K18 tyrosine phosphorylation have not yet been published.

Functionally, distinct keratins emerged as protein scaffolds, contributors to cell size determination, protein synthesis, cell proliferation, organelle transport or cell migration. All of these properties are controlled by highly complex patterns of phosphorylation and molecular associations (Magin et al. 2007). Keratin phosphorylation commonly occurs within the tail and/or head domains.

Several serine phosphorylation sites and the respective kinases have been characterized in K8 and K18, supporting a role in filament solubility and reorganization and in regulating keratin interaction to other proteins (Snider and Omary 2014). Previous studies suggested a role for K8/K18 in cell spreading and motility. In terms of molecular regulation, the head and tail domains of K8 and K18 contain phosphorylation motifs at serine sites that are recognized by mitogen-activated protein (MAP) kinase cascades, protein kinase A (PKA) and protein kinase C (PKC) (Bordeleau et al. 2010, Busch et al. 2012, Fois et al. 2013). The dynamics of K8/K18 phosphorylation is controlled by kinases that are themselves involved in signaling pathways. It is suggested that K8/K18 may act as modulators of signaling pathways involving integrins at focal adhesions. In fact, it appears that K8/K18 depletion interferes with mechano-transduction signaling triggered by integrin β1 (Bordeleau et al. 2008). By a functional parallelism between cell adhesion molecules, such as cadherin and integrin, it sounds reasonable to believe that *Lm* hijacks cell adhesions through activation of K18. The physiological role of K8/K18 in signal transduction, involved in cell motility, might suggest that *Lm* uses these mechanisms in order to promote infection. In the analysis of the role of K18/K8 in infection our results show that K18/K8 are required in HeLa infection by *Lm*, pointing to an InlB-dependent mechanism since c-Met is the *Lm* receptor expressed in this cell line. Although not required in a InlA-E-cadherin dependent pathway, as seen in siRNA experiments in Caco-2 cells, tyrosine

phosphorylation results in this cell line suggest a possible role of K8/K18 in processes unrelated with *Lm* entry (Rotty and Coulombe 2012).

In what concerns bacterial infection and recruitment/activation of keratins, only a few studies were able to show a link between them. Previous studies have shown that both *Salmonella* and EPEC infections require K18. A yeast two-hybrid assay revealed K18 as an interacting partner of EPEC virulence factor EspF. Furthermore, it was shown that the adaptor protein 14-3-3 co-immunoprecipitated with EspF, suggesting that K18, 14-3-3 and EspF may form a complex in EPEC infected cells (Viswanathan et al. 2004). Others have identified K18 as a novel Tir-interacting protein that is recruited to EPEC-induced pedestals (Batchelor et al. 2004). Moreover, it has been demonstrated that 14-3-3 is recruited to the pedestal and can bind specifically to Tir (Patel et al. 2006). *Salmonella* invasion was inhibited in cells expressing dominant negative derivatives of K18. Moreover, SspC/SipC effector protein presents K8 and K18 as potential interactors (Scherer et al. 2000, Carlson et al. 2002). Furthermore, K18 protects *Chlamydia* inclusion contents from cytoplasmic innate immune system (Kumar and Valdivia 2008, Rodel et al. 2012). Contrarily, *Helicobacter pylori,* was shown to induce apoptosis by increasing a CagA-dependent K18 cleavage (Saberi et al. 2012). More recently, liver disease caused by hepatitis B viral infections demonstrated that viral replication requires K8 (Zhong et al. 2014). Nevertheless, the precise role of K18 and its partner K8 in infection are still unknown.

Currently, another PhD student in the laboratory is responsible to investigate at the molecular level the involvement of keratins in bacterial infection (Cruz, R.; unpublished results).

GENERAL DISCUSSION

Together with myosin VIIa (Sousa et al. 2004) and myosin VI (Bonazzi et al. 2011), NM-IIA emerges as a novel class of myosins participating in *Lm* infection. In particular, with this study we found that Src phosphorylates the NMHC-IIA Y158 residue upon *Lm* infection and that this PTM impairs *Lm* cell invasion. We also detected NMHC-IIA-pTyr in response to other human bacterial pathogens such as EPEC, EHEC and *Yersinia*, suggesting a central role for NMHC-IIA-pTyr in cellular infection. In addition, β-adaptin, Hsp56 and Gp96 were identified as novel NMHC-IIA-interacting proteins unveiling new paths to further explore NM-IIA function.

Considering that NMHC-IIA-pTyr occurs during bacterial infection, we hypothesized that this PTM was required for the establishment of infection. In the case of *Lm* infection, NMHC-IIA-pTyr kinetics were different in HeLa as compared to Caco-2 cells, possibly due to different signaling pathways being engaged by *Lm*. Indeed, in Caco-2 cells, *Lm* entry occurs mainly through a mechanism dependent on InlA/E-cadherin interaction, while *Lm* is internalized in HeLa cells by InlB-mediated c-Met activation. Moreover, invasion of Caco-2 cells by *Lm* is more efficient, which could explain the peak of NMHC-IIA-pTyr occurring at earlier time points in this cell line.

NMHC-IIA-pTyr was also detected in the context of *Yersinia*, EPEC and EHEC cellular infection. When we employed an artificial system (K12-inv) used in studies of invasin-mediated entry and invasin-triggered signaling pathways, we also observed strong NMHC-IIA-pTyr levels, demonstrating that invasin-mediated host cell infection also exploits NHMC-IIA-pTyr. Although to a lesser extent, NMHC-IIA-pTyr was also observed in infection by pathogenic *E. coli*. EPEC intimin-induced Tir clustering stimulates Tir_{Y474} phosphorylation by the Src-family kinase (SFK) c-Fyn, triggering actin polymerization and pedestal formation (Hayward et al. 2009) Others demonstrated that cortactin was also required for the formation of EPEC pedestals (Cantarelli et al. 2006), while experiments with Src and non-phosphorylatable mutants of cortactin showed an impairment in this process. This indicates that a dynamic tyrosine phosphorylation of cortactin by Src is important for pedestal formation (Huang et al. 1998). It is possible that Src or other SFKs could tyrosine-phosphorylate NMHC-IIA in distinct residues to differentiate the anti-phagocytic mechanisms triggered by extracellular pathogens from the internalization-promoting mechanisms used by intracellular bacteria. Our results show that NMHC-IIA-pTyr does not occur exclusively by an InlA- or an InlB-

activated signaling pathway, as it is also observed in other pathogen infections. Considering Src activation by other pathogens it would be interesting to investigate which tyrosine residue is phosphorylated in NMHC-IIA and how Y158F mutation could compromise *Yersinia*, EPEC and EHEC infections.

NMHC-IIA Y158 was predicted *in silico* to be a Src substrate and we showed that Y158 phosphorylation is indeed mediated by this tyrosine kinase. Src activation is known to be induced by the InlA/E-cadherin interaction, but it was never associated with the InlB/cMet internalization pathway. This suggests that interactions between other *Lm* virulence factors and their respective host receptors can potentially activate Src and induce NMHC-IIA Y158 phosphorylation. It would thus be important to identify and address the effect of other virulence factors in this response.

Our results do not provide a clear correlation between NMHC-IIA-pTyr and RLC activation and therefore we might be unraveling a novel functional property of NM-IIA, which is dependent on heavy chain tyrosine phosphorylation but independent of RLC phosphorylation. It is therefore important to determine how the NMHC-IIA-pTyr status relates with the motor activity levels of NM-IIA in the context of infection. For this, we should further characterize the NMHC-IIA Y158F mutant in relation to NM-IIA motor activation in different cell processes to confirm if Y158 phosphorylation functionally diverges from RLC phosphorylation. The construction and analysis of structural models of both non-phosphorylated and Y158-phosphorylated forms of NM-IIA should improve our knowledge of how the dynamic phosphorylation of this residue, located in an α-helix close to the ATP-binding pocket, would influence the Mg^{2+}-ATPase and actin-binding activity required for NM-IIA motor function (Figure 22). Interestingly, blebbistatin, which is known to bind NM-IIA close to the ATP-binding pocket, impairs the actin-activated Mg^{2+}-ATPase activity and blocks NM-II in a low actin affinity state (Straight et al. 2003, Kovacs et al. 2004). We postulate that phosphorylation dynamics of Y158 could have a similar impact.

We showed that NMHC-IIA is recruited at *Lm* entry sites. Intriguingly, the Y158F mutant is also recruited, suggesting that NM-IIA localization is either independent of the fine-tuned tyrosine phosphorylation mechanism or probably phosphorylation might happen downstream of its recruitment.

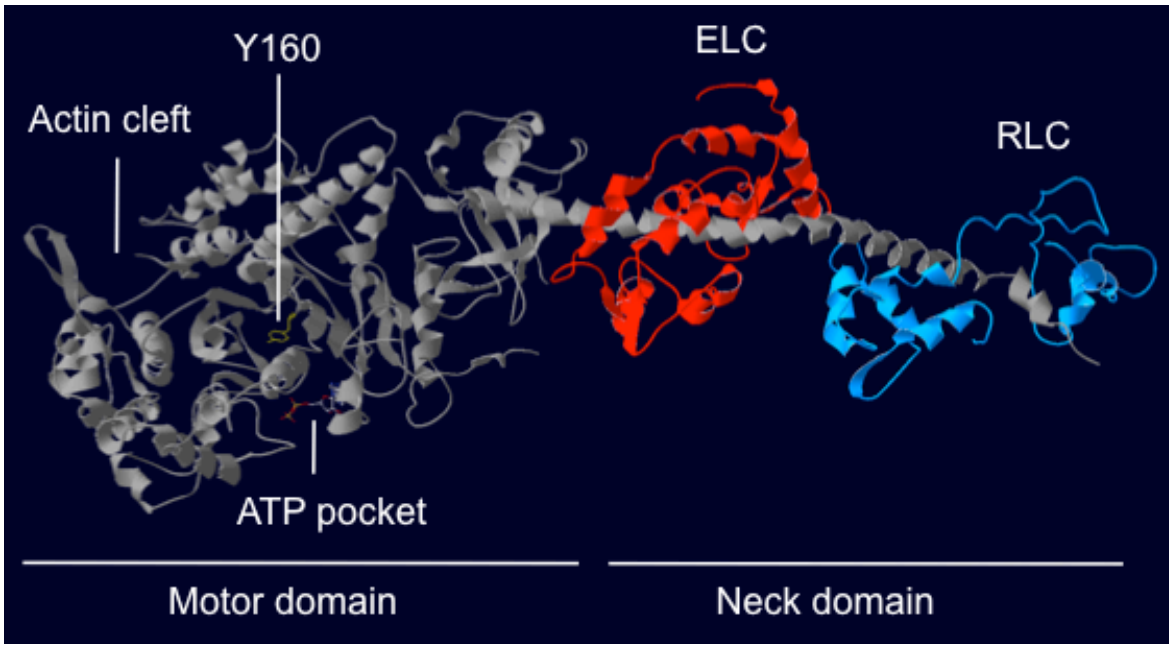


Figure 22. Structure of the *Argopecten irradians* (scallop) myosin heavy chain (motor and neck domains) and essential (ELC) and regulatory (RLC) light chains. *Schematic representation (adapted from RCSB Protein Data Bank file 1DFL) showing actin- and ATP-binding pockets (with bound ATP) as well as the Y160 residue, equivalent to human NMHC-IIA Y158 residue* (Houdusse et al. 2000).

NM-IIA has crucial roles in cellular processes requiring force generation and membrane reshaping, namely cell membrane tension and cytoplasm cohesion (Cai and Sheetz 2009, Cai et al. 2010, Houk et al. 2012). Therefore, *Lm* locally manipulates NM-IIA-dependent membrane integrity and actin foci rearrangements to promote infection, while NMHC-IIA activity restricts infection most probably by delaying cellular invasion.

NMHC-IIA has been controversially proposed as an entry receptor for Herpes simplex virus-1 (HSV-1) (Arii et al. 2010) and promote viral infection via MLCK activation (Antoine and Shukla 2014). In addition, recent findings for the same viral family (Kaposi's sarcoma-associated herpesvirus) support a role for NM-IIA in viral signaling at the peri-membrane location through interaction with ubiquitin ligase c-Cbl and EphrinA2 (EPHA2) (Valiya Veettil et al. 2010, Chakraborty et al. 2012). EPHA2 is tyrosine kinase receptor also shown to interact with PI3K and adaptor proteins involved in bacterial infection such as Shc and Grb2 (Pandey et al. 1994, Pratt and Kinch 2002). Although our results do not depend on InlB expression, c-Cbl can be also recruited trough an alternative *Lm*-activated signaling machinery, which would depend on NM-IIA and EPHA2.

Cell migration is regulated by NMHC-IIA through interaction with the cytoplasmic tail of β1-integrin. Integrin receptors also have a role in InlB-dependent *Lm* invasion and both β1- and β3-integrin participate in bacterial internalization (Auriemma et al. 2010). In addition, LapB, which has been shown to be required for *Lm* adhesion and entry, contains an RGD (Arg-Gly-Asp) motif responsible for LapB-mediated invasiveness (Reis et al. 2010), which is also a core recognition sequence for potential integrin interaction (Reis, O. and Cabanes, D. unpublished results). Thus it would be interesting for this study to further characterize LapB-integrin interaction and relate it with NM-IIA function and tyrosine phosphorylation.

Increased *Lm* infection observed in conditions of absence or inactivation of NMHC-IIA, can be related with alterations in myosin–actin structures that can contribute to surface exposure of a new putative host cell receptor as it was described for integrins (Carragher et al. 2006) and also affect receptor conformation and/or membrane localization (Rodriguez-Fernandez et al. 2001, Shewan et al. 2005, Charrasse et al. 2006, Lawler et al. 2006, Wloka et al. 2013). Consequently, a putative receptor could be more accessible leading to increased bacterial adhesion and entry.

GENERAL PERSPECTIVES

In this project, the use of relevant infection models have deepened the molecular understanding of NM-IIA function and regulation. Since cellular response to pathogens provided the first evidence showing NMHC-IIA-pTyr and given that pathogens produce exacerbated signals, facilitating the dissection of signaling pathways, it would be important in the future to characterize in-depth the role of NMHC-IIA-pTyr with regard to the NM-IIA canonical functions as well as in the context of bacterial infection. NM-IIA-dependent cellular processes triggered by physiological stimuli should also be investigated, namely the engagement of pathogen-targeted host cell receptors by their cognate ligands (e.g. β1-integrins and c-Met. The NM-IIA Y158F mutant will be also an important tool to further determine how phosphorylation triggered during infection translates into the complex network of NMHC-IIA protein interactions and whether it is the molecular basis of the responsible mechanisms.

NMHC-IIA also presents an immunoreceptor tyrosine-based inhibitory motif (ITIM), which is a phosphorylation motif found in a large number of receptors and adaptor proteins. The ITIM motif of NMHC-IIA (IFY^{278}YLL) is described to be tyrosine-phosphorylated and consequently recruit SHP-1 phosphatase required for B cell activation (Baba et al. 2003). Characterization of a possible infection-driven Y278 phosphorylation in NMHC-IIA will be a valuable tool to improve our knowledge in the differential phosphotyrosine signaling triggered by NM-IIA.

To further explore NM-IIA functioning, we identified two novel NMHC-IIA partners by Y2H assay: β-adaptin and Hsp56. The central role of NM-IIA in cellular functions and the growing evidence pointing to the involvement of NM-IIA in bacterial infection, together with the cellular functions attributed to β-adaptin and Hsp56, render the NM-IIA-β-adaptin and NM-IIA-Hsp56 interactions significantly worthy of being studied in the context of bacterial infection, intracellular trafficking and cellular homeostasis.

Our results provide novel and significant insights to the molecular understanding of *Lm* pathogenesis, NM-IIA function and reveal new protein interactions important for intracellular trafficking, protein sorting and proper protein cellular localization. Our work contributes to both infection and cell biology fields, reiterating the importance of pathogens as tools to uncover novel cell biology mechanisms and regulatory pathways.

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ANNEX

This annex includes the following publication:

Martins M., Custódio R., Camejo A., **Almeida M.T.**, Cabanes D. and Sousa S. *Listeria monocytogenes* Triggers the Cell Surface Expression of Gp96 Protein and Interacts with Its N-Terminus to Support Cellular Infection. *J. Biol. Chem.* 2012, 287(51): 43083-43093.