

**Instituto de Biologia Molecular e Celular**

Group of Molecular Microbiology

**Tyrosine phosphorylation profile  
of *Listeria monocytogenes*  
infected cells:**

**Identification of new host factors  
hijacked to promote infection**

**Maria Teresa Pinto de Almeida**

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**Supervisor: Dr. Didier Cabanes**

**Co-Supervisor: Dra. Sandra Sousa**



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# Summary

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|                                                                                  |    |
|----------------------------------------------------------------------------------|----|
| Figure Index .....                                                               | 7  |
| Abstract .....                                                                   | 9  |
| Resumo .....                                                                     | 11 |
| Abbreviations.....                                                               | 13 |
| Introduction.....                                                                | 15 |
| I. <i>Listeria monocytogenes</i> .....                                           | 15 |
| General features .....                                                           | 15 |
| Listeriosis .....                                                                | 17 |
| Cycle of cellular infection.....                                                 | 19 |
| Major virulence factors .....                                                    | 21 |
| II. Phosphorylation events upon cellular invasion .....                          | 25 |
| InIA/E-cadherin-induced cellular invasion.....                                   | 28 |
| InIB/c-Met-induced cellular invasion .....                                       | 30 |
| Phosphorylation events triggered by other pathogens .....                        | 33 |
| III. Myosins: role in various cellular processes.....                            | 35 |
| Myosins and Phagocytosis.....                                                    | 38 |
| Myosins and Bacterial Infection.....                                             | 40 |
| IV. Intermediate Filaments .....                                                 | 42 |
| Keratins .....                                                                   | 45 |
| Keratins and Bacterial Infection.....                                            | 46 |
| Project Presentation .....                                                       | 47 |
| Material and Methods.....                                                        | 49 |
| Results .....                                                                    | 55 |
| I. Analysis of tyrosine phosphorylation patterns in infected cells .....         | 55 |
| II. Identification of proteins differentially phosphorylated during infection .. | 57 |
| III. Myosin-9 tyrosine phosphorylation during <i>Listeria</i> infection.....     | 61 |

|                                  |                                                                         |    |
|----------------------------------|-------------------------------------------------------------------------|----|
| IV.                              | Localization of Myosin-9 in <i>Listeria</i> -infected cells .....       | 65 |
| V.                               | Role of Myosin-9 in <i>Listeria</i> entry .....                         | 67 |
| VI.                              | Identification of Myosin-9 phosphorylation sites .....                  | 71 |
| VII.                             | Identification of Myosin-9 interacting proteins .....                   | 75 |
| VIII.                            | Cytokeratin 18 tyrosine phosphorylation during infection .....          | 77 |
| IX.                              | Localization of Cytokeratin 18 in <i>Listeria</i> -infected cells ..... | 79 |
| X.                               | Role of Cytokeratin 18 in <i>Listeria</i> entry .....                   | 79 |
| XI.                              | Identification of Cytokeratin 18 tyrosine phosphorylation sites .....   | 82 |
| Discussion and Perspectives..... |                                                                         | 85 |
|                                  | Myosin-9.....                                                           | 86 |
|                                  | Cytokeratin 18 .....                                                    | 91 |
| References .....                 |                                                                         | 95 |

# Figure Index

---

|                                                                                                                  |    |
|------------------------------------------------------------------------------------------------------------------|----|
| <b>Figure 1</b> - Sucessive steps of the <i>L. monocytogenes in vivo</i> infection.....                          | 16 |
| <b>Figure 2</b> - The cycle of cellular infection by <i>L. monocytogenes</i> .....                               | 18 |
| <b>Figure 3</b> - Schematic representation of InlA. ....                                                         | 22 |
| <b>Figure 4</b> - Schematic representation of InlB. ....                                                         | 22 |
| <b>Figure 5</b> - Intracellular movement of <i>L. monocytogenes</i> . Formation of actin tails through ActA..... | 24 |
| <b>Figure 6</b> - Major virulence genes of <i>L. monocytogenes</i> regulated by PrfA.....                        | 26 |
| <b>Figure 7</b> - Schematic model representing the host factors that mediate the InlA-dependent uptake.....      | 29 |
| <b>Figure 8</b> - Cell signaling pathways triggered by the interaction InlB-c-Met.....                           | 32 |
| <b>Figure 9</b> - Schematic representation of a Myosin.....                                                      | 36 |
| <b>Figure 10</b> - Secondary structure of human Cytokeratins.....                                                | 44 |
| <b>Figure 11</b> - Protein tyrosine phosphorylation patterns of <i>L. monocytogenes</i> infected cells.....      | 56 |
| <b>Figure 12</b> - Protein identification by Peptide Mass Fingerprinting (PMF).....                              | 58 |
| <b>Figure 13</b> - Myh9 expression in different cell lines.....                                                  | 60 |
| <b>Figure 14</b> - Myh9 tyrosine phosphorylation in response to <i>L. monocytogenes</i> infection.....           | 62 |
| <b>Figure 15</b> - Localization of Myh9 in <i>L. monocytogenes</i> infected Caco-2 cells.....                    | 64 |
| <b>Figure 16</b> - Effect of ML-7 and ML-9 inhibitors on <i>L. monocytogenes</i> internalization.....            | 66 |
| <b>Figure 17</b> - Effect of blebbistatin on <i>L. monocytogenes</i> internalization.....                        | 68 |
| <b>Figure 18</b> - Knock down expression of Myh9 in HeLa cells.....                                              | 70 |
| <b>Figure 19</b> - Effect of Myh9 knock down expression on <i>L. monocytogenes</i> internalization.....          | 70 |
| <b>Figure 20</b> - Computational prediction of Myh9 phosphorylated tyrosines.....                                | 72 |
| <b>Figure 21</b> - Silver stained gels of total Immunoprecipitated proteins using anti-Myh9.....                 | 74 |
| <b>Figure 22</b> - Green fluorescent protein (GFP) purification.....                                             | 76 |

|                                                                                                                  |    |
|------------------------------------------------------------------------------------------------------------------|----|
| <b>Figure 23</b> - CK18 expression in different cell lines.....                                                  | 78 |
| <b>Figure 24</b> - CK18 tyrosine phosphorylation in response to <i>L. monocytogenes</i> infection of Caco-2..... | 78 |
| <b>Figure 25</b> - Localization of CK18 in <i>L. monocytogenes</i> infected Caco-2 cells..                       | 80 |
| <b>Figure 26</b> - Effect of CK18 knock down expression on <i>L. monocytogenes</i> internalization.....          | 81 |
| <b>Figure 27</b> - Computational prediction of CK18 phosphorylated tyrosines.....                                | 83 |

# Abstract

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*Listeria monocytogenes* is a human food borne pathogen that may lead, in particular in immunocompromised individuals, to a severe disease characterized by septicemias, meningitis, meningo-encephalitis and abortions. The study of the cell biology of *Listeria* 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, different pathogens often hijacking 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 two cytoskeletal proteins, Myosin 9 (Myh9) and Cytokeratin 18 (CK18), differentially tyrosine-phosphorylated in response to *Listeria* uptake. We demonstrated that Myh9 was not only tyrosine-phosphorylated over the time of infection, but was also recruited with actin at the bacteria entry site. In addition, we were able to show that the inhibition of Myh9 activity blocked *Listeria* entry into non-phagocytic cells. Surprisingly, the reduction of Myh9 expression using RNAi techniques resulted in an increased *Listeria* uptake.

Together these results point to the role of a novel myosin class in the internalization of *Listeria*, correlating for the first time myosin post-translational modifications and *Listeria* infection. We also show here that CK18 is enriched in the vicinity of entering *Listeria* into epithelial Caco-2 cells. Moreover, our preliminary results indicate a reduced entry of *Listeria* in cells showing low levels of CK18 expression. The identification of Myh9 and CK18 tyrosine-phosphorylated residues during infection and their direct mutagenesis would help to unravel the direct role of these specific tyrosine phosphorylations on bacteria internalization. This work identified two novel host proteins involved in

the *Listeria* infectious process and should contribute to the identification of new signaling cascades hijacked by pathogens to promote their own internalization.

# Resumo

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A *Listeria monocytogenes* é um patógeno humano de origem alimentar. A ingestão de alimentos contaminados pode causar uma doença grave, principalmente em indivíduos imunocomprometidos. A listeriose é caracterizada por septicemias, meningites, meningo-encefalites e abortos. O estudo ao nível da biologia celular da infecção por *Listeria* tem proporcionado uma melhor compreensão dos mecanismos pelos quais a bactéria manipula o hospedeiro e tem revelado funções desconhecidas de determinadas proteínas celulares. Para causar infecção, os patógenos intrometem-se com vias de sinalização intracelulares essenciais das células do hospedeiro, diferentes patógenos muitas vezes sequestram as mesmas vias intracelulares. As cascatas de fosforilação das células do hospedeiro são um dos alvos preferenciais das bactérias infecciosas.

Neste estudo, usando *L. monocytogenes* como patógeno modelo, mostrámos que diferentes linhas celulares eucariotas apresentam um padrão de fosforilação de proteínas variável durante a infecção. Direcção-nos essencialmente para proteínas que apresentam níveis de fosforilação variáveis em resíduos de tirosina despoletados pela infecção por *Listeria*. Identificámos duas proteínas do citosqueleto, a miosina 9 (Myh9) e a citoqueratina 18 (CK18), diferencialmente fosforiladas em resposta à entrada de *Listeria* nas células do hospedeiro. Demonstrámos que a Myh9 não só é fosforilada em tirosina durante o tempo de infecção mas também é recrutada em conjunto com a actina ao local de entrada da bactéria. Conseguímos ainda mostrar que a entrada da *Listeria* em células não-fagocíticas é bloqueada por inibição química da actividade da Myh9. Surpreendentemente, a redução da expressão da Myh9, através de técnicas de RNAi, levou a um aumento da entrada de *Listeria* nas células testadas. Estes resultados apontam assim para o papel de uma nova classe de miosina na internalização de *Listeria*, correlacionando pela primeira vez uma modificação pós-transducional em miosina e o seu papel na infecção por *Listeria*. Neste estudo, mostramos ainda que há um enriquecimento de CK18 na proximidade dos locais de entrada de *Listeria* em

celulas epiteliais Caco-2. Os nossos resultados preliminares indicam que em células com baixa expressão de CK18 o nível de entrada de *Listeria* é reduzido.

A identificação dos resíduos fosforilados de tirosina da Myh9 e CK18 e a sua mutagénese directa irá ajudar a descrever o papel directo das tirosinas fosforiladas na entrada de bactérias.

Este trabalho identificou duas novas proteínas do hospedeiro envolvidas no processo infeccioso de *Listeria* e poderá contribuir para a identificação de novas cascatas de sinalização sequestradas por patógenos de modo a promover a sua internalização.

# Abbreviations

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5'-UTR – 5'-Untranslated region  
ATP – Adenosine tri-phosphate  
BHI – Brain heart infusion  
CK18 – Cytokeratin 18  
CK8 – Cytokeratin 8  
c-Met - Hepatocyte growth factor receptor  
DMEM - Dulbecco's modified Eagle medium  
EMEM - Eagle's Minimal Essential Medium  
EPEC - *Enteropathogenic Escherichia coli*  
FBS – Fetal bovine serum  
GAP – GTPase activating protein  
GFP – Green fluorescence protein  
HGF – Hepatocyte growth factor  
IFs – Intermediate filaments  
InIA – Internalin A  
InIB – Internalin B  
IP – Immunoprecipitation  
IQ motif - Isoleucine-Glutamine motif  
IR – Inter repeat region  
K – Keratin  
LAP – Localization and affinity purification  
LLO- Listeriolysin O  
LRR – Leucine rich repeats  
MAP – Mitogen activating protein  
MAPK - Mitogen-activated protein kinase  
MBD – Met binding domains  
MHC – Myosin heavy chain  
MLC – Myosin light chain  
MLCK – Myosin light chain kinase  
MOI – Multiplicity of infection

Mpl- Metalloprotease  
mRNA – Messenger RNA  
MS – Mass spectrometry  
Myh9 – Myosin 9  
NF-κB – Nuclear factor-κB  
NMHCIIA – Non-muscle myosin heavy chain isoform IIA  
NMHCIIIB – Non-muscle myosin heavy chain isoform IIB  
NMHCIIIC – Non-muscle myosin heavy chain isoform IIC  
PBS - Phosphate-buffered saline  
PC- Phosphatidylcholine  
PC-PLC/PLC-B – Phosphatidylcholine-specific phospholipase C  
PFA – Paraformaldehyde  
PI 3 kinase - Phosphatidylinositol 3- kinase  
PI- Phosphoinositol  
PIP - Phosphatidylinositol 1-*phosphate*  
PIP<sub>2</sub> – Phosphatidylinositol 2-*phosphate*  
PI-PLC/PLC-A - Phosphatidylinositol-specific phospholipase C  
PKA – Protein kinase A  
PKC – Protein kinase C  
PMF – Peptide mass fingerprinting  
PTB – Phosphotyrosine binding domains  
PTK - Protein tyrosine kinase  
RLC – Regulatory light chain  
RNAi – RNA interference technique  
RTK – Receptor tyrosine kinase  
SDS-PAGE - SDS-polyacrylamide gel electrophoresis  
SH2 – Src homology-2 domain  
siRNA – Small interference RNA  
T4SS – Type IV secretion system  
TTSS – Type III secretion system  
WB – Western Blot

# Introduction

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Bacterial pathogens became a common topic of interest due not only to economical reasons but also due to obvious health impact in our societies.

These microorganisms are considered one of the major threats in both undeveloped and developed countries. A big effort has to be undertaken not only in the creation of new drugs but also to better understand the basis of the molecular and cellular mechanisms of the infection. Even though different pathogens tend to develop different infectious diseases the way they establish infections resides in several common strategies.

It is known that bacterial pathogens make use of host cellular components in their own profit and by mimicking normal cell physiological processes enable invasion and establishment of infection.

## ***I. Listeria monocytogenes***

### **General features**

*Listeria monocytogenes* was first discovered in 1926 by Murray and his colleagues after an outbreak of infection in animal care house in Cambridge (Murray et al., 1926; Pirie, 1927). Human cases were reported in 1929 and listeriosis was long considered as a zoonosis. The first human listeriosis outbreak and the direct association to the consumption of *Listeria*-contaminated food was reported in 1983 (Schlech et al., 1983). *L. monocytogenes* is now recognized as a food-borne pathogen affecting industrialized countries. Foods most frequently implicated in listeriosis outbreaks are soft cheeses and dairy products, pâtés and sausages, smoked fish, salads, “delicatessen”, and in general industrially produced, refrigerated ready-to-eat products that are consumed without cooking or reheating.

*L. monocytogenes* is a facultative anaerobic rod-shaped Gram-positive bacterium and is one of the six species of the genus *Listeria*. This genus comprises pathogenic and non-pathogenic species, *L. monocytogenes* being one of the two pathogenic species. *L. monocytogenes* is ubiquitously distributed

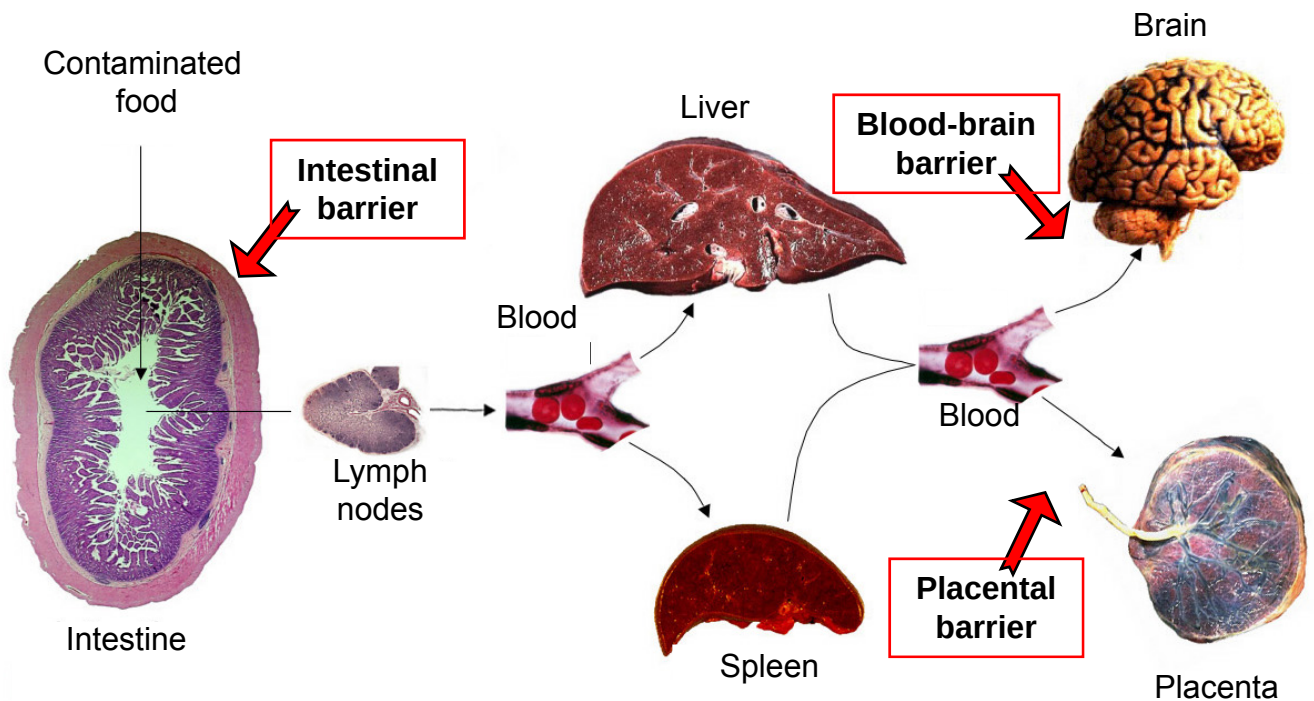


**Figure 1: Successive steps of the *L. monocytogenes* in vivo infection.**

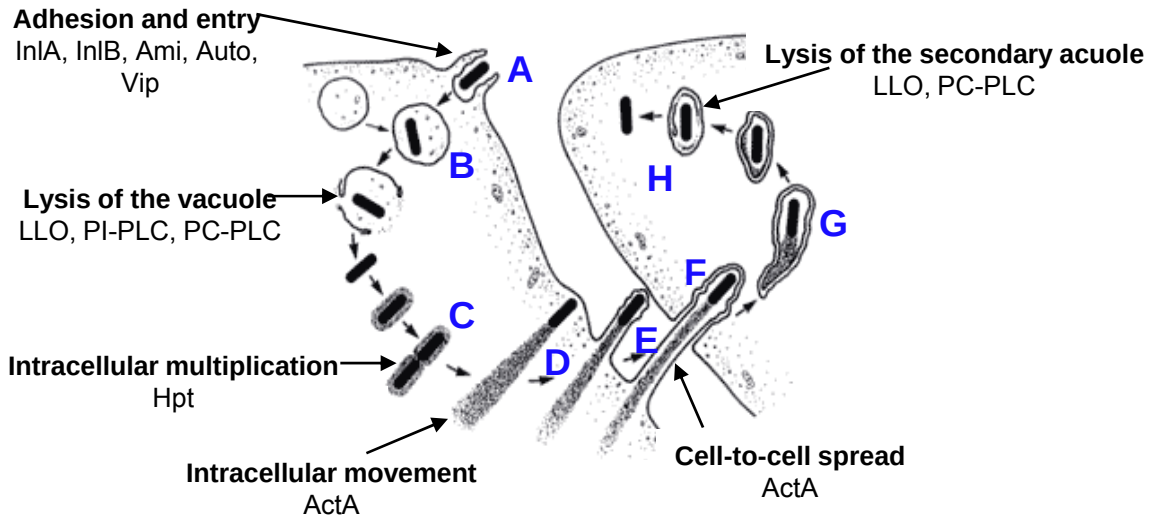
(adapted from Lecuit *et al.*, 2004)

in nature, and can be found in many different niches such as soil, water, plants, and food processing plants. It has great survival capacity to harsh conditions. Indeed, it can survive and multiply in high NaCl concentration (20%), pH values ranging from 4,5 to 9 and vast range of temperature values (1°C-45°C). These remarkable properties allow *L. monocytogenes* to be present in different environments increasing the risk of infection in humans and animals by a routinely exposure to the pathogen. This makes *Listeria* a serious threat to food safety and ranks it among the microorganisms that most concern the food industry {Khelef, 2004 #27; Vazquez-Boland, 2001 #16}.

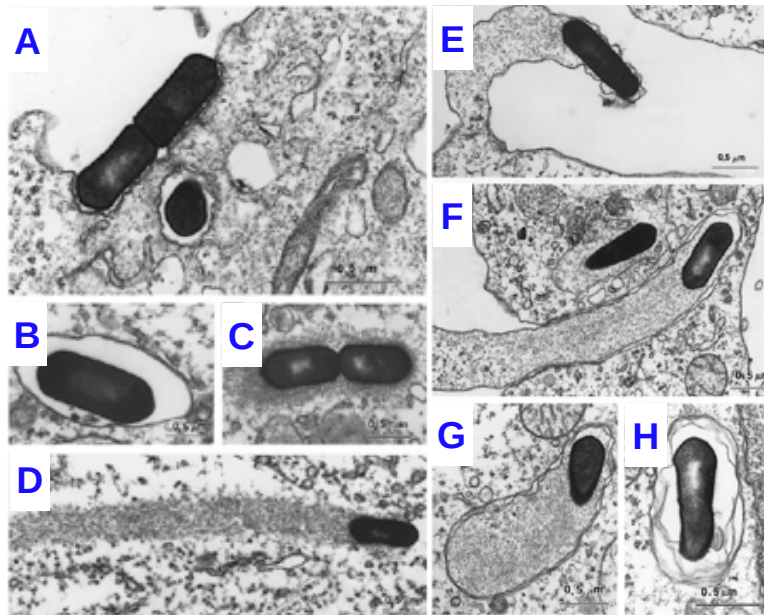
### **Listeriosis**

*L. monocytogenes* can be asymptotically present in the gastrointestinal tract of healthy humans. Although the incubation time is relatively long (30 days), in healthy individuals listeriosis can occur in less than 24 hours as a severe gastroenteritis. This is related to a non-invasive form of the disease and manifests after ingestion of highly contaminated food (Vazquez-Boland et al., 2001; Wing and Gregory, 2002). *L. monocytogenes* may also cause a serious invasive disease that primarily affects immune-compromised individuals, newborn babies, elderly or pregnant women (Cossart, 2007).

The incidence of listeriosis ranges from 0.1 and 11.3 / 1,000,000. The majority of invasive listeriosis cases appear as life-threatening in one of three clinical syndromes: maternofetal listeriosis or neonatal listeriosis, blood stream infection, and meningoencephalitis. Listeriosis has an average case-fatality rate of 20-30%, and besides the invasive forms, listeriosis might be present as febrile gastroenteritis and as cutaneous listeriosis. *L. monocytogenes* is naturally susceptible to a variety of antibiotics, such as penicilins, aminoglycosides, trimethoprim, tetracycline, macrolides, and vancomycin. Treatment of invasive listeriosis requires the use of antibiotics combination, in general high doses of intravenous penicillin in association with an aminoglycoside during at least 3 weeks. The non-invasive form of the disease usually resolves spontaneously. The major line of defense against listeriosis is cell-mediated immunity explaining the fact that individuals with T-cell



**A. Schematic representation of the different steps of the *L. monocytogenes* cellular infection.**  
 The major virulence factors of *L. monocytogenes* implicated in each step are indicated.  
 (adapted from Tilney et Portnoy 1989)



**B. Electron microscope photographs showing the different steps of *L. monocytogenes* cellular infection cycle.**

(adapted from Kocks *et al.*, 1992)

**A.** Adhesion and entry; **B.** Bacteria inside the first vacuole; **C.** Intracellular multiplication; **D.** Intracellular movement; **E.** Protrusion formation; **F.** Cell-to-cell spread; **G.** Bacteria inside the double membrane vacuole; **H.** Lysis of the secondary vacuole

dysfunction are prone to contracting the disease (Swaminathan and Gerner-Smidt, 2007).

Human listeriosis develops through successive steps (Fig.1). Infection occurs through the ingestion of contaminated food. Bacteria resist to the stomach acidity and reach the intestinal lumen. *Listeria* invade the enterocytes, cross the host intestinal-barrier and it is thought to disseminate from mesenteric lymph nodes to the liver and the spleen via the blood circulation. In the liver and spleen *L. monocytogenes* can replicate. A more severe state of the disease can be achieved in a second stage of the infection if *Listeria* reach the brain or the placenta, by crossing the blood-brain barrier or placental barrier respectively. These last steps of infection result in meningitis or encephalitis in immunocompromised patients, abortions in pregnant women, and septicemia in infected neonates (Lecuit, 2007).

### **Cycle of cellular infection**

*L. monocytogenes* is a facultative intracellular pathogen, and the cell biology of its infectious process has been widely studied. The key feature of *Listeria* is its capacity to enter 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. In addition, these intracellular bacteria exploit the host cytoskeleton to move intracellularly through an actin-dependent process. They form protrusions that invade adjacent cells, allowing dissemination by direct cell-to-cell spread, without returning to the extracellular environment, therefore escaping the extracellular defenses, such as antibodies and complement {Hamon, 2006 #184; Khelef, 2004 #27}.

Infection at the cell level proceeds in a step-wise mode: (i) adhesion to host cells, (ii) bacterial-induced internalization, (iii) lysis of the primary vacuole, (iv) multiplication in the host cytosol, (v) actin-based intracellular movement and (vi) intercellular spread (Fig. 2). *Listeria* evolved a variety of molecular weapons in order to exploit and turn in its own profit important cellular processes. Indeed, the *Listeria* virulence factors already identified interact with particular cellular components and are involved in specific steps of the cellular infection.

(i) *adhesion to host cells*

The first step of cellular infection corresponds to the adhesion of bacteria to the host cell surface. Several bacterial proteins expressed at the surface of *Listeria* (Fig.2) interact with specific proteins expressed at the surface of the host cell. Bacteria are therefore closely associated to the host cell membrane and induce host signaling cascades in order to promote their internalization.

(ii) *bacterial- induced internalization*

*In vivo*, as well as in *in vitro* cultured cells, *L. monocytogenes* can enter and survive in professional phagocytic cells such as macrophages, neutrophils and dendritic cells. Importantly, as described above, this bacterium has also the capacity to induce its own internalization in non-phagocytic cells.

*L. monocytogenes* invades host cells by the so-called zipper mechanism characterized by intimate interactions established between the bacteria and the host cell. This process is followed by the progressive invagination of host plasma membrane leading to the consequent bacterial engulfment (Fig.2). The entry is a crucial step in the infection process controlled by different bacterial factors (Fig.2), including the *Listeria* surface proteins InlA and InlB (described below).

(iii) *lysis of the primary vacuole*

Once inside the cell, *L. monocytogenes* resides inside a vacuole surrounded by a single membrane. Rapidly after internalization the vacuole acidifies and is disrupted by *Listeria*. The bacteria persist free in the cytosol (Fig.2). The disruption of the vacuole is controlled by three *Listeria*-secreted proteins described below (LLO, PI-PLC and PC-PLC).

(iv) *multiplication in the host cytosol*

By lysing the vacuole *Listeria* becomes free in the cytosol and starts multiplying, acquiring nutrients from the cytosol. Several genes encoding virulence or metabolic determinants are induced during *L. monocytogenes* intracellular life, including those involved in vacuole lysis, actin-based motility, and cell-to-cell spreading.

(v) *actin-based intracellular movement*

*Listeria* is able to move inside host cytoplasm exploiting the cellular machinery. Indeed, the bacterium is able to polymerize host actin and form filaments at one pole, creating a structure resembling a comet tail (Fig.2). The constant polymerization and depolymerization of actin in the comet generates the driven force used for bacterial movement inside the host cytoplasm.

(vi) *intercellular spread*

This actin comet-like tail propels bacteria in random directions and occasionally to the cell periphery, generating a protrusion which contains the bacteria (Fig.2). If the moving bacterium encounters the membrane of a neighboring cell, the protrusion invaginates in this cell, generating a double membrane vacuole. The vacuole of the secondarily infected cell is then lysed liberating bacteria into the cytosol and starting a new infectious cycle. The process of cell-to-cell spread without contacting the extracellular milieu allows *L. monocytogenes* to escape to host immune response.

*L. monocytogenes* infection was first an useful model for immunologists in the study of the host T-cell response. In the last decades, cell biologists have also extensively studied *Listeria* to unravel the mechanisms underlying general cell biology processes such as actin-based motility.

### **Major virulence factors**

Internalins are key bacterial effectors of *Listeria* invasion (Bierne et al., 2007). The internalization of *L. monocytogenes* in non-phagocytic cells is mainly promoted by two proteins from the internalin multigenic protein family, **InlA** and **InlB**. The genes *inlA* and *inlB* are encoded by an operon and their expression is under the control of PrfA, the major transcriptional regulator of *Listeria* virulence genes (Fig.6). These genes can be co-transcribed or transcribed separately. Recent studies showed that other molecules are also necessary for internalization, revealing a complex interaction between *Listeria* and eukaryotic

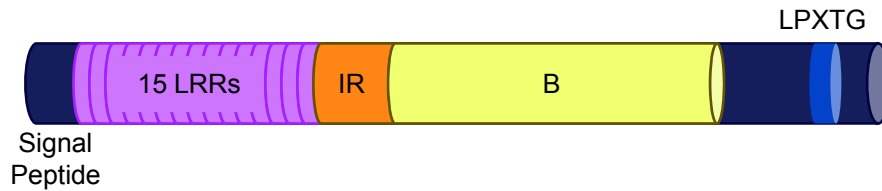


**Figure 3: Schematic representation of InIA.**

LRRs: Region of leucine rich repeats; IR: Region of inter-repeats; B: Region of B repeat; LPXTG: Cell-wall sorting motif allowing the covalent anchorage.

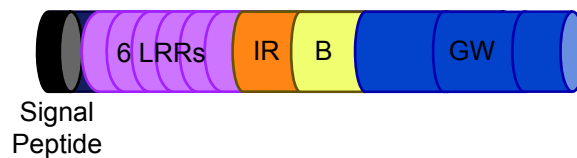


**Figure 4: Schematic representation of InIB.**

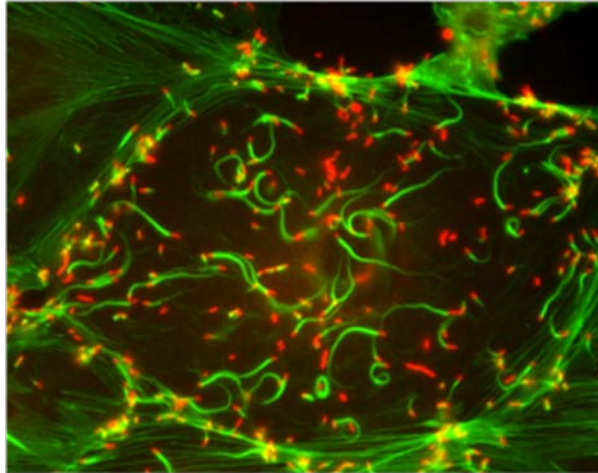
LRRs: Region of leucine rich repeats; IR: Region of inter-repeats; B: Region of B repeat; GW: GW Modules allowing the labile association to the surface.

cells during the early steps of the infectious cycle {Cabanes, 2004 #210; Cabanes, 2005 #8; Dussurget, 2004 #49}.

InlA is composed of 800 amino acids and is required for the invasion of epithelial cells expressing its specific receptor, the E-cadherin (see below), such as human Caco-2 cells. At the N-terminus, InlA has a peptide signal followed by a region of 15 leucine rich repeats (LRRs) consisting of tandem repeats of 20-22 amino acids {Bierne, 2007 #173; Schubert, 2001 #38}. 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 allowing a covalent linkage of InlA to the bacterial peptidoglycan (Fig.3). The LRR and IR regions are both necessary and sufficient to promote *Listeria* entry into epithelial cells (Dussurget et al., 2004; Lecuit et al., 1997).

InlB is a 630-amino acid protein involved in the entry of *L. monocytogenes* into a broad range of cell types (Dramsi et al., 1995; Gaillard et al., 1991). Its amino-terminal domain presents a signal peptide sequence that is followed by seven LRR repeats, one IR region, and one B repeat. The carboxyl-terminal domain has three tandem repeats of 80 amino acids that begin with the sequence GW (named GW modules) and mediate the loosely attachment of InlB to the *Listeria* cell wall *via* a non-covalent interaction with the lipoteichoic acids (Fig.4). Due to its labile association to the bacterial membrane, InlB can be released in the extracellular medium and can act as a soluble factor interfering with host cell signaling. GW modules and the B repeat are also involved in interactions with target cells. The LRR region of InlB is sufficient to confer invasiveness to non-invasive *Listeria innocua* (Braun et al., 1999; Jonquieres et al., 1999).

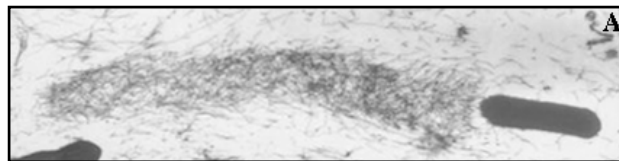
Listeriolysin O (**LLO**) is a cholesterol-dependent cytolysin encoded by *L. monocytogenes* and plays a key role in the disruption of the phagocytic vacuole. LLO is a major virulence factor of *L. monocytogenes*; mutants for LLO are non-hemolytic and unable to escape from the vacuole. LLO is a pore-forming toxin. Rapidly after the internalization, the *Listeria*-containing vacuole acidifies allowing the maximum activity of LLO. As a consequence of pore formation, the pH of the vacuole rises and LLO is progressively inactivated. LLO also appears



**A. Immunofluorescence photograph showing an infected cell and the *L. monocytogenes* actin tails.**

Green: Actin; Red: *L. monocytogenes*

( Khelef *et al.*, 2004)



**B. Electron microscope photograph of *L. monocytogenes* actin tails labelled with myosin S1 fragment.**

( Gouin *et al.*, 1999)

**Figure 5: Intracellular movement of *L. monocytogenes*.  
Formation of actin tails through ActA.**

to trigger histone modifications prior to bacterial entry and, as a consequence, down regulates a subset of genes, including immunity genes (Hamon et al., 2007). In addition to LLO, the lysis of primary and secondary vacuole also involves two secreted phospholipases. PI-PLC (or **PLC-A**) is specific for phosphoinositol (PI) and is implicated in the disruption of primary vacuole. PC-PLC (or **PLC-B**) is specific for phosphatidylcholine (PC) and plays an important role in the disruption of the double membrane vacuole. The activation of PC-PLC requires the proteolytic cleavage of its immature form by the metalloprotease **Mpl**.

The intracellular motility of *L. monocytogenes* requires the bacterial protein **ActA** (Domann et al., 1992; Kocks et al., 1992). ActA is a 639-amino-acid protein that presents a signal peptide and a transmembrane motif that anchors this molecule to the bacterial surface. ActA localizes at one pole of the bacteria and recruits cellular actin, as well as other host cell proteins involved in the polymerization and depolymerization of actin filaments (Fig.5).

Other well-characterized virulence factors of *L. monocytogenes* are involved in different steps of the infectious cycle mentioned above and as indicated in figure 2.

It is important to mention that the expression of key *Listeria* virulence factors is controlled by the transcriptional activator **PrfA** (Fig.6), a protein of 233 amino acids that belongs to the Crp/Fnr family (Khelef et al., 2004; Sheehan et al., 1996). Interestingly, the PrfA protein itself is only expressed at 37°C (Renzoni et al., 1997). The 5'UTR of the PrfA mRNA can adopt a hairpin structure which at low temperatures prevents access to the ribosomes. At high temperatures, the mRNA secondary structure is destabilized, the ribosome-binding site becomes accessible and PrfA is expressed (Johansson et al., 2002). This regulatory mechanism allows the expression of virulence factors in the context of the host at 37°C.

## II. Phosphorylation events upon cellular invasion

In the past decades, a big effort has been done in order to understand the mechanisms underlying interactions between intracellular bacteria and their host cells. Bacterial pathogens have evolved a panoply of virulence factors that

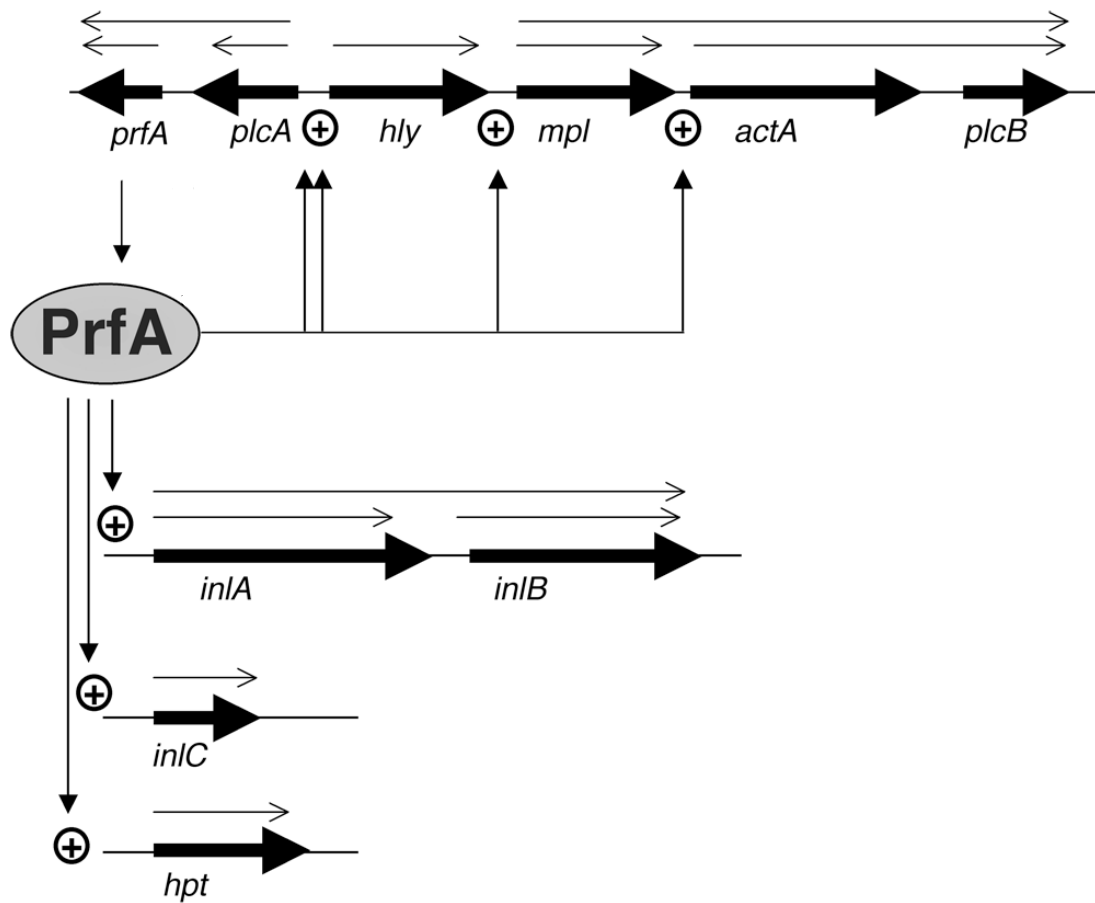


**Figure 6: Major virulence genes of *L. monocytogenes* regulated by PrfA.**

(Khelef *et al.*, 2004)

hijack the host cell machinery to create a suitable niche for their survival and proliferation. Despite their evolutionary divergence, different bacterial effectors often target the same host pathways. A beneficial strategy used by many pathogens is to interfere with the phosphorylation cascades of intracellular-signaling pathways of the host cell. Phosphorylation states are usually controlled by protein kinases and phosphatases, and the functions of these enzymes are mimicked by bacterial effector proteins. Indeed, phosphorylation cascades have been described as preferential targets for several human pathogens (Alto et al., 2006; Bhavsar et al., 2007).

Bacterial surface proteins mediate the attachment to host cell surface receptors. This interaction is usually followed by stimulation of signaling events that induce plasma membrane remodeling and cytoskeletal rearrangement and promote close bacteria-host association and/or bacterial internalization (Mattoo et al., 2007). A combination of genetic tools with *in vitro* and *in vivo* studies leads to the identification of some cellular effectors as well as internalization routes subverted by virulence factors.

The facultative intracellular bacterial pathogen *L. monocytogenes* has evolved multiple strategies to invade different cell types. Host cell invasion is critical for listeriosis pathology due to the initial crossing of the host intestinal barrier and further colonization of diverse target organs. It is therefore crucial for the comprehension of listeriosis to understand how *Listeria* induces its own uptake by the host cells. Previous studies indicate a complexity of interactions between multiple bacterial and specific host factors that cooperate during the entry process, and help to understand the molecular basis of the tissular tropism of *L. monocytogenes* (Seveau et al., 2007).

The cellular receptors for InlA and InlB have been identified as **E-cadherin** and hepatocyte growth factor receptor (**c-Met**), respectively (Mengaud et al., 1996; Shen et al., 2000). The molecular signaling cascades triggered downstream the engagement of these receptors during *Listeria* entry into host cells are being characterized in detail {Bierne, 2002 #45; Bonazzi, 2008 #66; Cossart, 2003 #48; Hamon, 2006 #184; Pizarro-Cerda, 2006 #7; Seveau, 2007 #5; Sousa, 2005 #43; Sousa, 2007 #6; Sousa, 2004 #2}.

**InlA/E-cadherin-induced cellular invasion**

E-cadherin is a 120 kDa transmembrane glycoprotein that belongs to the cadherin superfamily of calcium-dependent cell adhesion molecules and mediates adherens junction formation (Halbleib and Nelson, 2006). Homophilic interactions mediated by the first two extracellular domains of E-cadherin establish direct contact between adjacent cells, while the intracellular domain, by interacting with catenins, anchors the adhesive complexes to the cortical actin cytoskeleton (Pokutta and Weis, 2007). E-cadherin proteins are not stably exposed at the cell surface; instead they cycle on and off the plasma membrane in a dynamic fashion by exo- and endocytic events (Akhtar and Hotchin, 2001; Xiao et al., 2005; Yap et al., 2007).

Internalization of E-cadherin from adherens junctions is initiated by the Src-mediated tyrosine phosphorylation of E-cadherin and its ubiquitination by the cbl-like ubiquitin-ligase Hakai and further internalization within clathrin-coated endosomes (Bonazzi et al., 2008; Fujita et al., 2002; McLachlan et al., 2007; Papkoff, 1997).

$\beta$ -Catenin binds to the carboxyl terminus of E-cadherin and connects E-cadherin with  $\alpha$ -catenin that tightly controls the actin remodeling necessary to adherens junctions dynamics (Drees et al., 2005; Gates and Peifer, 2005; Yamada et al., 2005). The extracellular domain, contrarily to the cytoplasmic domain has so far only few interactors: E-cadherin itself,  $\alpha E\beta 7$  integrins, InlA expressed at the surface of *L. monocytogenes* and Als3 an invasin of *Candida albicans* (Higgins et al., 1998; Karecla et al., 1996; Mengaud et al., 1996; Phan et al., 2007). The interaction of InlA/E-cadherin has been shown to be critical in the crossing of intestinal and placental barriers (Lecuit et al., 2004; Lecuit et al., 2001). Intriguingly, E-cadherin molecules are usually restricted to basolateral membranes in polarized epithelial layers and thus they are not easily reached by pathogens found in the intestinal lumen. Only when E-cadherin is transiently exposed to the luminal surface during junctional remodeling, a specific entry portal for *Listeria* is provided (Pentecost et al., 2006).

When InlA interacts with E-cadherin, it triggers a series of events that ultimately stimulate actin polymerization and bacterial entry (Fig.7). InlA interaction with E-cadherin activates both  $\beta$ - and  $\alpha$ -catenin-mediated signaling

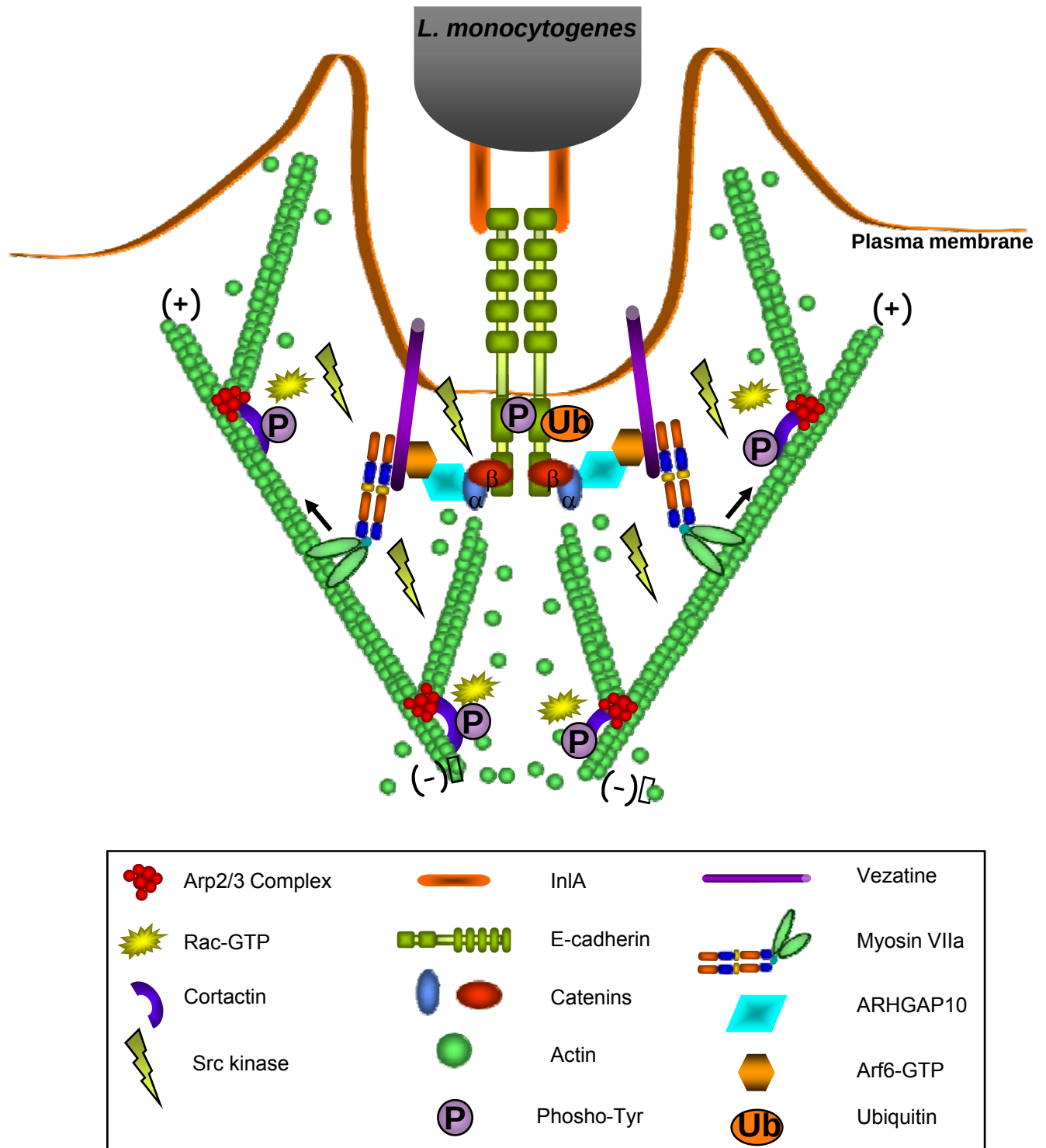


**Figure 7. Schematic model representing the host factors that mediate the InlA-dependent uptake.**

(adapted from Sousa *et al.*, 2007)

pathways involved in the formation of adherens junctions, suggesting that *Listeria* exploits the same molecular scaffold for inducing its entry into host cells (Lecuit et al., 2000). Other proteins, such as the unconventional myosin VIIa, vezatin and ARHGAP10 have been implicated in InlA-mediated invasion (Sousa et al., 2005; Sousa et al., 2004). These proteins could also be involved in the actin-dependent remodeling of the plasma membrane during adherens junctions formation. Upon infection, Src, a signaling non-receptor tyrosine kinase is also activated. The Src substrate, cortactin, is recruited and Arp2/3 complex is in turn activated. In addition, the small GTPase Rac1 is also required for *Listeria* internalization (Sousa et al., 2007). These data suggest a model in which activated Src-tyrosine kinase, together with Rac1, promotes recruitment and phosphorylation of cortactin and activation of Arp2/3 complex at the *Listeria* entry site. Actin polymerization in the InlA-dependent pathway relies on Rac1, cortactin and Arp2/3 complex however how this whole process is orchestrated is poorly understood.

A very recent study showed that InlA triggers two successive E-cadherin post-translational modifications, the Src-mediated tyrosine phosphorylation of E-cadherin followed by its ubiquitination by the ubiquitin-ligase Hakai (Bonazzi et al., 2008). E-cadherin ubiquitination induces the recruitment of clathrin that is required for optimal bacterial internalization (Bonazzi et al., 2008).

### **InlB/c-Met-induced cellular invasion**

InlB has been reported to interact with several host molecules such as gc1qR, glycosaminoglycans and c-Met (Braun et al., 2000; Jonquieres et al., 2001; Shen et al., 2000). c-Met is considered the major host ligand for InlB promoting *Listeria* entry into non-phagocytic cells. In addition to promote uptake, the interaction InlB/c-Met induces cell scattering and membrane ruffling (Shen et al., 2000).

c-Met belongs to the family of receptor tyrosine kinases (RTKs), one of the most important families of transmembrane signaling receptors expressed by a variety of cells. It is important in organ morphogenesis, cell proliferation, migration and differentiation, and also controls growth and invasion during the process of metastasis (Birchmeier et al., 2003).

c-Met is synthesized as a large protein that is cleaved into  $\alpha$  and  $\beta$  subunits. The mature form expressed at the cell surface corresponds to a disulfide-linked heterodimer comprising the extracellular  $\alpha$  subunit and the transmembrane  $\beta$  subunit that contains the tyrosine kinase catalytic domain. Once activated by its endogenous ligand HGF, c-Met undergoes an autophosphorylation on specific tyrosine residues in its cytoplasmic domain increasing c-Met kinase activity. A multifunctional signal transducer docking site is formed by phosphorylation of other tyrosines that serve to recruit signaling molecules and intracellular adaptors (Shc and Gab1) *via* Src homology-2 domains (SH2), phosphotyrosine binding domains (PTB) and Met binding domains (MBD) (Ferracini et al., 1991; Maulik et al., 2002).

Briefly, Gab1 is recruited to activated c-Met by direct binding to the tyrosine phosphate groups of the receptor (Weidner et al., 1996). The c-Met/Gab1 complex binds to downstream molecules, including SHP2, phosphatidylinositol 3-kinase (PI 3-kinase) and Grb2 (Franke et al., 2008). c-Cbl is a substrate of the activated tyrosine kinase receptor c-Met (Garcia-Guzman et al., 2000) and can also activate PI 3-kinase (Fournier et al., 2000). Induction of the signaling cascades by HGF can therefore be very broad, leading to different biological outcomes.

InIB only functionally mimics HGF: these molecules are dissimilar in sequence and structure and do not compete for binding to c-Met, suggesting that they associate with different sites of the receptor (Shen et al., 2000). Comparative studies demonstrated that InIB activates c-Met in different way as compared to HGF. InIB induces a less sustained phosphorylation of c-Met and a more intense activation of the Ras-MAPK (mitogen-activated protein kinase) pathway (Copp et al., 2003).

Upon stimulation by InIB, phosphorylation of c-Met, Cbl, Shc and Gab1 occurs in host cells (Fig.8) (Ireton et al., 1996; Shen et al., 2000). The recruitment and activation of Gab1 by InIB can take place by two redundant pathways that require either phosphorylation of c-Met tyrosines and Gab1-binding to the c-Met multidocking site or activation of the PI 3-kinase that generates PIP<sub>3</sub> and Gab1 recruitment. Gab1, Shc and Cbl are also involved in the activation of PI 3-kinase (Ireton et al., 1996). PI 3-kinase then is involved in activation of small GTP-binding protein Rac1 and induction of actin

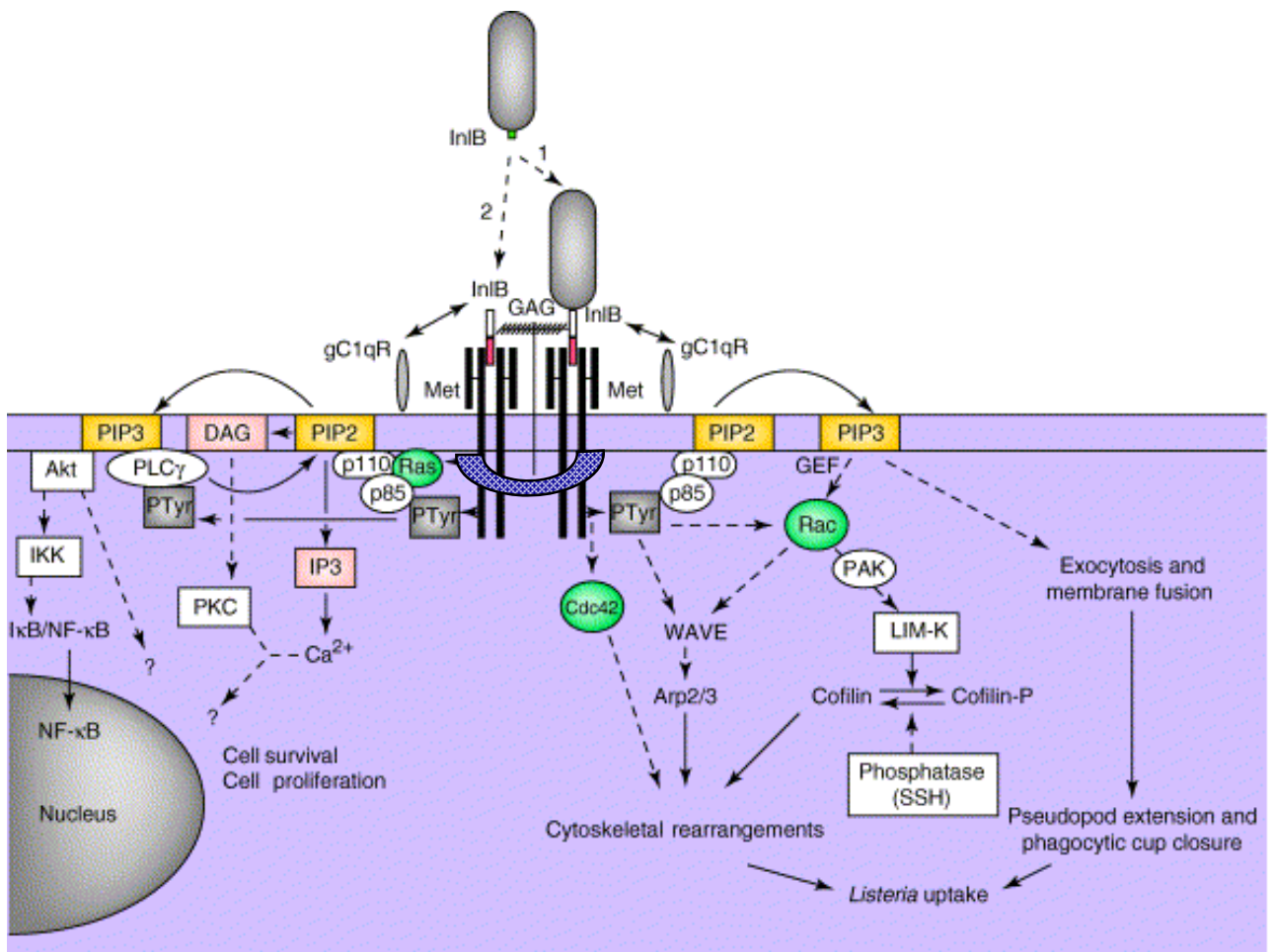


**Figure 8:Cell signaling pathways triggered by the interaction InlB-c-Met.**

(adapted from Cossart *et al.*, 2003)

InlB can interact with its cellular receptors (c-Met, gC1qR et GAGs) in association with the bacteria (1) or in its soluble form (2). The interaction InlB-c-Met induces the recruitment of adaptor proteins that are tyrosine phosphorylated, as well as the recruitment of PI-3 kinase. Downstream the activation of PI-3 kinase, a series of signaling events lead to the actin polymerization at the entry site and membrane remodeling that allows *L. monocytogenes* internalization. Another signaling pathway involving PLC $\gamma$  and Akt is also activated. This internalization process is also dependent on the endocytosis machinery involved in the normal trafficking of c-Met.

polymerization through the activity of WAVE and the Arp2/3 complex (Bierne et al., 2005). Both PI 3-kinase and Rac1 are necessary for bacterial engulfment.

Actin rearrangements are also controlled downstream of Rac1 by the actin depolymerizing protein cofilin and by the cofilin-modulating enzyme LIM-kinase (Fig.8) (Bierne et al., 2001; Ireton et al., 1996). Interestingly, Cbl promotes the recruitment and activity of the clathrin-dependent endocytosis machinery at the site of bacterial entry and internalization (Veiga and Cossart, 2005). InlB, thus, seems to exploit the normal trafficking of its receptor to promote invasion (Li et al., 2005; Pizarro-Cerda and Cossart, 2006a, b).

### **Phosphorylation events triggered by other pathogens**

*Salmonella* and *Shigella* are both Gram-negative pathogens. *Salmonella* colonize a wide range of animal hosts, including humans, and are an aetiological agent of both gastroenteritis and typhoid fever. *Shigella flexneri* is the leading cause of dysentery (shigellosis), often provoking severe colitis and diarrhea that dehydrates affected patients (Izard et al., 2006; Ly and Casanova, 2007). Both *Salmonella* and *Shigella* interact with host cells by a type III secretion system (TTSS) that allows the delivery of bacterial components directly into the host cytoplasm (Cossart and Sansonetti, 2004). Host-pathogen interaction takes place in subdomains enriched in signaling molecules, such as tyrosine kinases of the Src family. The invasion involves localized but massive rearrangements of the cell surface and cytoskeleton, characterized by the formation of filopodial structures that appear similar in *Salmonella* and *Shigella* (Cossart and Sansonetti, 2004). SopB, a *Salmonella* TTSS effector, functions as a phosphoinositide phosphatase that catalyses the dephosphorylation of host phosphatidylinositols stimulating actin rearrangements (Norris et al., 1998; Terebiznik et al., 2002). SptP, another *Salmonella* TTSS-secreted protein, has two activities: a tyrosine-phosphatase activity that regulates activity of the MAPK induced by entry, and a GAP activity on Cdc42 and Rac resulting in the shrinking of the entry focus by blocking further actin polymerization (Stebbins and Galan, 2000). In *Shigella* infection, IpaC is central to the activation of Cdc42 and Rac-1, which is followed by activation of the tyrosine kinase c-Src, recruitment of cortactin to the membrane upon its tyrosine phosphorylation, and

massive actin polymerization in the vicinity of the entry site (Bougneres et al., 2004). This process is amplified by IpgD, a phosphatidylinositol phosphatase that hydrolyzes PI(4,5)P<sub>2</sub> into PI(5)P, thus disconnecting the actin subcortical cytoskeleton from the membrane and favoring actin dynamics at the entry site (Niebuhr et al., 2002). The Abl family of tyrosine kinases is also involved in *Shigella* entry through phosphorylation of the adaptor molecule Crk (Backert et al., 2008; Bougneres et al., 2004).

Enteropathogenic *Escherichia coli* (EPEC) cause infantile diarrhea and are characterized by their ability to produce attaching and effacing lesions on the surface of intestinal epithelial cells. EPEC make use of a filamentous TTSS for the delivery of effector molecules to subvert mammalian cell function to generate actin- and cytokeratin- rich pedestals beneath adherent bacteria (Patel et al., 2006). During infection by EPEC, the intimin receptor (Tir) translocates into host cell and inserts into the host cell membrane. Tir becomes phosphorylated on tyrosine residue by host kinases, Fyn and Abl, leading to the generation of actin filaments beneath the attached bacteria and the formation of pedestal structure (Backert et al., 2008; Gruenheid et al., 2001).

*Yersinia* spp. causes gastroenteritis and the plague, representing historically devastating enteropathogens that are currently an important biodefense and antibiotic resistance concern. A critical virulence determinant is the *Yersinia* protein kinase A, or YpkA, a multidomain protein that disrupts the eukaryotic actin cytoskeleton. 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 modulates the actin cytoskeleton (Prehna et al., 2006). YopM, another *Yersinia* effector, simultaneously binds to and activates host protein kinases (McDonald et al., 2003). YopP/YopJ binds to and neutralizes the activity of a MAPK kinase, thereby blocking the activation of NF- $\kappa$ B (Autenrieth et al., 2007). In addition, *Yersinia* invasion induces the activation of several host kinases: FAK, Src and PI3K (Alrutz and Isberg, 1998; Cossart and Sansonetti, 2004).

*Rickettsiae* are strictly intracellular Gram-negative pathogens, which are transmitted to humans *via* arthropod organisms (Hackstadt, 1996). *Rickettsia conorii*, the causative agent of Mediterranean spotted fever, is able to attach to and invade a variety of cell types, both *in vitro* and *in vivo*. Previous studies

show that entry of *R. conorii* into non-phagocytic cells relies on actin polymerization, however the molecular details governing *Rickettsia*-host cell interactions and actin rearrangements are not fully understood. *Rickettsia* recruits the Arp2/3 complex to the site of entry foci and its invasion is dependent on PI 3-kinase and on protein tyrosine kinase (PTK) activities, in particular Src-family kinases. c-Src and its downstream target, cortactin, colocalize at entry sites in the infection process. *R. conorii* internalization correlated with the tyrosine phosphorylation of several other host proteins, including FAK. *Rickettsia* entry into non-phagocytic cells is dependent on the Arp2/3 complex and the interplay of pathways involving Cdc42, PI 3-kinase, c-Src, cortactin and tyrosine-phosphorylated proteins that regulate the localized actin rearrangements observed during bacterial entry (Martinez and Cossart, 2004).

*Helicobacter pylori* is a micro-aerophilic spiral-shaped Gram-negative bacterium that has colonized the gastric epithelium of half of the human population (Covacci et al., 1999; Hu et al., 1992). *H. pylori* is a risk factor for several diseases, such as gastric cancer and MALT lymphoma (Crabtree and Naumann, 2006). It has evolved elaborate mechanisms to manipulate host cells during infection. Through type IV secretion system (T4SS), *H. pylori* translocates into the host cytoplasm one of its main virulence factors, CagA, where it modulates cellular functions. Injected CagA can interact with host tyrosine kinases, becoming tyrosine-phosphorylated. Both Src and Abl family kinases are responsible for CagA tyrosine-phosphorylation. After phosphorylation by c-Src and Fyn, CagA remains at the plasma membrane where it interacts with a number of proteins, triggering signals that resemble the activation of the receptor-tyrosine kinase growth factors such as c-Met (Amieva and El-Omar, 2008; Backert and Selbach, 2008; Franke et al., 2008).

### III. Myosins: role in various cellular processes

In order to cells exert their function properly some of their components must be anchored to provide frameworks or structures. Other cell components have to be transported over long distances so that asymmetries in cell morphology and composition can be achieved. For a long-range transport, cells

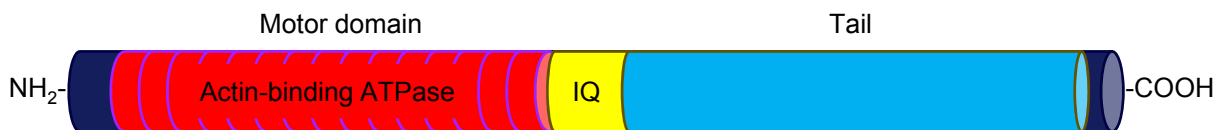


**Figure 9: Schematic representation of a Myosin.**

NH<sub>2</sub>-terminal **motor** or **head** domain (actin binding and ATP hydrolysis); neck region containing isoleucine-glutamine (**IQ**) motifs and a COOH-terminal **tail** domain

cannot rely solely on diffusion as many organelles and macromolecular complexes are immobilized in the cytosol. To overcome diffusion, cells exploit the free energy liberated by ATP hydrolysis through molecular motors.

The myosins are actin filament-based molecular motors and have evolved a variety of ways to contribute to cellular organization through numerous modifications to the manner they convert free energy into mechanical work (O'Connell et al., 2007). Myosins are typically composed of three domains with respective functions: (i) the N-terminal **motor** or **head** domain responsible for actin binding and ATP hydrolysis; (ii) a neck region containing one or more isoleucine-glutamine (**IQ**) motifs that bind light chains and calmodulin; and (iii) a C-terminal **tail** domain which directs the interaction of a given myosin with its cargo and is responsible for class-specific properties (Fig.9). This domain is also responsible for dimerization of heavy chains (Berg et al., 2001; Krendel and Mooseker, 2005; O'Connell et al., 2007; Sellers, 2000)

In addition, myosin 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. Several binding proteins have been identified which provided important insights for specific myosin function. Diversity in myosin function is also manifested by significant differences in the motor properties among members of the myosin family. The mechanochemical properties of motor domains have evolved unique characteristics and regulatory mechanisms to optimize function (Krendel and Mooseker, 2005; O'Connell et al., 2007). The neck regions are stabilized by the binding of light chains which allows them to swing like a lever arm (Howard, 1997). Besides providing certain rigidity to the neck regions, the light chains also participate in the regulation of the ATPase and motor activities. These light chains are either calmodulin or related to calmodulin and belong to the EF-hand family of proteins. Regulatory mechanisms involve direct binding of calcium ions to the light chains or the post-translational modification of the light chains by phosphorylation. The myosin head domain, together with the neck or regulatory domain, forms the motor region that is sufficient for mechanical force production along actin filaments (Bahler, 2000).

To date, the myosin superfamily consists of at least 25 different classes based on the sequence comparison of myosin head domain and tail domain

organization (Conti and Adelstein, 2008; Krendel and Mooseker, 2005). Many human pathologies have been associated with mutations within several myosin genes (Redowicz, 2002).

Myosins are fundamental in eukaryotic motility. They are important contributors in cytokinesis (e.g. class II myosins), organelle transport and organelle/particle trafficking (e.g. class I and V) (Thompson and Langford, 2002). Additionally, myosins are implicated in cell polarization (e.g. class V) (Yin et al., 2000), intracellular transport and signal transduction pathways (e.g. class I, III, VI, VII and IX), in which they are modulators of other protein activities and position signaling activities at the cytoskeleton-membrane interface (Bahler, 1996, 2000).

Myosins are even hypothesized to play important roles in cell crawling, phagocytosis (e.g. class II, IXb and X) (Groves et al., 2008), growth cone extension and maintenance of cell shape (e.g. class I and II) (Berg et al., 2001) and have been implicated in the polymerization of actin (e.g. class I), revealing the molecular basis of actin-based motility (Evangelista et al., 2000; Lechler et al., 2000; Lee et al., 2000). Cooperation between different domain functions, such as forces along actin filaments and modulation of signal transduction is ideally achieved to orchestrate initiation and flow of information.

Novel myosins are being identified in many eukaryotic cell types suggesting that each cell *per se* contains a variety of these proteins.

### **Myosins and Phagocytosis**

There is a constant exposure of eukaryotes to microbial pathogens. On the other hand, multicellular organisms need to get rid of cells when they become dangerous, because of apoptosis, necrosis or transformation, and debris generated during development, tissue turnover and repair. Eukaryotic cells have evolved a mechanism to overcome and destroy particles: phagocytosis. In its classical sense, phagocytosis is a receptor-mediated, actin-driven process and proceeds in a zipper-like manner (Griffin et al., 1975). However, particle internalization and bacterial invasion can also occur through macropinocytosis (Cardelli, 2001). Phagocytosis is a multi-step process that is initiated by particle recognition. This is mediated by a wide variety of cell

surface receptors that bind directly or indirectly to the particle. Apparently due to evolutionary pressure, ligands for phagocytic receptors tend to be common to classes of pathogens.

Upon interaction with surface receptors, a transient and localized actin polymerization is necessary for phagocytosis, as highlighted by the effects of drugs that inhibit actin filament assembly and block particle uptake (Axline and Reaven, 1974; Maniak et al., 1995; Zigmond and Hirsch, 1972). Actin dynamics is regulated by signaling pathways activated downstream of engaged phagocytic receptors. In macrophages, the pathways governing actin dynamics during phagocytosis originate from receptor-particle interaction in a rapid phosphorylation of tyrosine residues in the cytoplasmic tail of the receptor (Sanchez-Mejorada and Rosales, 1998). This is mediated by Src-family tyrosine kinases and is essential for local actin polymerization and phagocytosis (Ghazizadeh and Fleit, 1994). Not all phagocytic receptors rely on tyrosine phosphorylation to induce phagocytosis in macrophages, providing evidence for receptor-specific pathways in the initial phases of uptake.

In addition to actin polymerization, the myosin superfamily of motor proteins represents another driving force for phagosome formation through its contractile activities. A large number of myosin isoforms have been implicated in receptor-mediated phagocytosis. This suggests that different steps of phagocytosis are controlled by different myosins. Indeed, immunofluorescence analysis of myosin distribution in macrophages undergoing receptor-mediated phagocytosis has revealed that myosins II and IXb were recruited at early phagocytic cup structures, likely modulating pseudopod extension, while myosin IC was associated with the forming phagosome at later time points, in phagosome closure (Araki, 2006). During formation of the phagosome myosin V recruitment presented continuous increase and was significantly recruited after phagosome closure (Diakonova et al., 2002; Swanson et al., 1999).

Myosin II has been implicated in phagocytic cup squeezing; as treatment of macrophages with inhibitors that selectively target myosin II function does not affect pseudopod extension but prevents closure of phagocytic cups (Araki et al., 2003). Myosin II is also involved in receptor-mediated particle uptake, with a preponderant role in actin assembly (Olazabal et al., 2002).

Myosin IC is the only myosin isoform localized to the linking strings of the erythrocyte membrane shared by competing phagocytes, supporting a role for this particular myosin in the so-called 'purse-string-like' contraction accompanying phagosome closure (Swanson et al., 1999).

Myosin X is also recruited to macrophage phagocytic cups in a PI 3-kinase-dependent way. Its function within the phagocytic cup appears related to pseudopod extension, as phagocytosis of a large (6  $\mu\text{m}$ ) but not small (2  $\mu\text{m}$ ) particle is inhibited in cells expressing a truncated myosin X mutant (Cox et al., 2002). This size dependency was also exhibited by cells treated with PI 3-kinase inhibitors (Cox et al., 1999), suggesting that myosin X controls pseudopod extension as a downstream effector of PI 3-kinase.

Myosin V appears only after phagosome closure and it seems to regulate phagosomal movement in the cytosol. Myosin Va-null macrophages accumulate phagosomes in the perinuclear region suggesting that myosin Va plays a role in short-range movements at the cell periphery (Al-Haddad et al., 2001).

### **Myosins and Bacterial Infection**

Myosins have been demonstrated to be involved in the interactions between bacteria and the host cytoskeleton. For a number of bacteria, it has also been demonstrated that their movement in the cytosol depends on direct interaction with cytoskeletal network (Stevens et al., 2006).

*Neisseria gonorrhoeae* infection of mucosal epithelial tissues involves adherence of the bacteria to the host cell, followed by invasion, transepithelial trafficking across cells and finally, exocytosis. Host cell factors for gonococcal adherence, entry and transcytosis involve membrane receptors that interact with Opa neisserial proteins (Wang et al., 2008), however infection also relies in rearrangements of the host cytoskeleton (Pujol et al., 2000; Wang et al., 1998). Recently, it has been shown that myosin I is involved in *Neisseria* invasion and transcytosis but not in adherence. It is also suggested that Opa-expressing *gonococci* invasion might be driven by myosin I along actin filaments to the deeper side of the epithelium. However, it is not completely described which is the molecular basis of myosin I function in the alterations of the cytoskeleton upon *Neisseria* infection (Wang et al., 2008).

*Shigella flexneri*, the causative agent of bacillary dysentery, has been shown to disseminate through intestinal epithelial cells via protrusions that extend from infected cells and are engulfed by the neighboring cell (Rathman et al., 2000). Myosin has been shown to play a role in a variety of membrane phenomena. It has been shown that phosphorylation of myosin by its upstream regulator MLCK (myosin light chain kinase) leads to the formation of the bacteria-containing protrusion, and could also contribute to the protrusion engulfment by adjacent cells (Rathman et al., 2000). The class IX myosin Myr5 has been identified as a regulator of *Shigella* infection; it is recruited into *Shigella*-induced cellular projections essential for bacterial entry. Myr5 has a GAP subdomain in its tail domain that, together with its actin-binding site, links Rho-like GTPases to the cytoskeleton. Efficient entry is probably a result of tightly regulated Rho-dependent cytoskeletal rearrangements, demonstrating a sophisticated use of myosin and GAP activity of a class IX myosin by *Shigella*, during infection of epithelial cells (Graf et al., 2000).

*Salmonella enterica* serovar *typhimurium*, a facultative intracellular pathogen, causes gastroenteritis in humans and a lethal systemic disease in certain strains of mice (Tsolis et al., 1999). As described above, invasion of epithelial cells by *Salmonella* is directed by bacterial proteins that interact with host signaling proteins leading to rearrangements in actin cytoskeleton (Galan and Curtiss, 1989; Galan and Zhou, 2000). Recent studies show that upon bacteria uptake, driven by cell surface ruffling, *Salmonella* gets into vacuoles and myosins seem to have a role in the dynamics of these vacuoles within infected host cells. Furthermore, vacuole positioning is driven by specific bacterial effectors, through activation of myosin II (Wasylnka et al., 2008).

EPEC destroys intestinal microvilli and suppresses phagocytosis by injecting effectors into infected cells a TTSS. EspB, a component of the TTSS is injected into the cytoplasm of host cells and binds to myosins. Recent studies identified Myosin-Ic as an EspB-binding protein. EspB inhibits the interaction of myosin Ic with actin, suggesting that EspB inhibits myosin functions and thereby facilitates efficient infection by EPEC (Iizumi et al., 2007).

*L. monocytogenes* invasion involves coordinated membrane remodeling and actin cytoskeleton rearrangements. Cellular proteins required for *Listeria* entry are recruited at the entry site, acting as driving forces that favor

internalization. Previous studies identified two novel proteins, the unconventional myosin MyoVIIa and its ligand vezatin in *Listeria*-induced phagocytosis.

Myo VIIa and vezatin are recruited at adherens junctions and at the entry site of *Listeria*, where they localize with actin, suggesting that vezatin acts as the molecular link between myosin VIIa and the E-cadherin/catenins/actin complex. It was shown that myosin VIIa is required for the InlA/E-cadherin-mediated internalization of *Listeria* but not for entry mediated by InlB, another invasion protein of *Listeria* (Sousa et al., 2004). Vezatin is a transmembrane protein previously shown to bind myosin VIIa and to recruit it to adherens junctions (Kussel-Andermann et al., 2000).

#### IV. Intermediate Filaments

Among fibrous cytoskeletal polymers like F-actin and microtubules, intermediate filaments (IFs) are cytoskeletal polymers whose protein properties and intracellular organization provides crucial structural support in the cytoplasm and nucleus. Frequently the three cytoskeletal polymers work together to enhance structural integrity, cell shape, and cell and organelle motility. IFs range in diameter from 8-12 nm an intermediate in size as compared with actin filaments and microtubules (Hyder et al., 2008). Besides functions primarily related to structural integrity of cells and tissues, non-mechanical roles for IFs have been implicated in a highly diverse range of cellular functions. These include cell adhesion and migration (Ivaska et al., 2007), cell 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) and vectorial processes including protein targeting in polarized cellular settings (Kim and Coulombe, 2007). IF function in regulation of key signaling pathways that control cell survival and cell growth was also characterized (Pallari and Eriksson, 2006).

Each IF monomer consists of an  $\alpha$ -helical rod domain which connects the amino (head) and carboxyl (tail) terminals.

Structurally, IF proteins are characterized by their highly conserved  $\alpha$ -helical rod domain that acts as the major determinant of self-assembly, flanked by the N-terminal head and the C-terminal tail domain. The edges of the rod domain consist of highly conserved amino acid regions that are crucial for polymerization and are frequently mutated in human disease (Kim and Coulombe, 2007).

The nature of the IFs to dimerize and form coiled-coil structures is defined by the structural properties of the rod domain (Strelkov et al., 2003). Recent research has changed the view of interactions between IFs and other cellular molecules. Indeed, in addition to cytoskeletal linker proteins IFs also interact with signaling determinants, such as kinases (Ivaska et al., 2007; Pallari and Eriksson, 2006). The ability of IFs to sequester or act as a scaffold for signaling molecules renders them exceptional organizers of different physiological processes. IFs are dynamically regulated by their post-translational modifications. Initially, these modifications were found to regulate filament dynamics and organization. However, in the last few years, their roles have extended significantly to facilitating, for example, the recruitment and sequestration of signaling molecules that regulate a wide range of cellular functions. While phosphorylation has been established as the principal post-translational modification regulating IF function, other modifications with co-operative roles are emerging, adding new perspectives to IF-mediated signaling. O-linked glycosylation appears as an additional regulator of IF phosphorylation (Hyder et al., 2008).

The relatively high abundance of IFs in most cells makes them highly efficient as signaling scaffolds, even for high-abundance signaling molecules. It seems that post-translational modifications are the key to functional diversity, adding complexity to the functions of the cell.

Considering IF localization there are four major types of IF proteins in vertebrate cells: nuclear (lamins A, B, and C), vimentin-like (vimentin, desmin, glial fibrillary acidic protein, peripherin), epithelial (acidic-type I keratins and basic-type II keratins) and axonal (neurofilament proteins NF-L, NF-M, and NF-H) (Alberts et al., 2007).

Further identification of binding partners for IF proteins will help to better understand their roles in the cell.

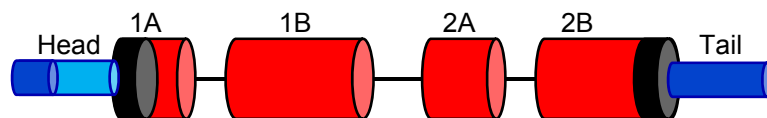


**Figure 10. Secondary structure of human Cytokeratins.**

The large red boxes indicate the four  $\alpha$ -helical subdomains (1A, 1B, 2A, and 2B), and the black boxes indicate the highly conserved end domains of the rod. Solid bars denote non-helical head and tail domains, and the head domain contains a highly conserved subdomain (light blue).

(adapted from Shiratsuchi *et al.*, 2002)

### **Keratins**

IF proteins are expressed in a highly cell type-specific manner, and herein keratins represent the typical IF category of epithelial cells. In some, but not all epithelia, keratin filaments are conspicuously bundled as tonofilaments. Inside the cell they braid the nucleus, span through the cytoplasm and are attached to the cytoplasmic plaques of the typical epithelial cell–cell junctions, the desmosomes (Waschke, 2008). This feature already suggests that keratins play a major functional role in the integrity and mechanical stability of both the single epithelial cells and, *via* cell–cell contacts, of that of the epithelial tissues.

Keratin (K) is the typical IF proteins of epithelia, showing an outstanding degree of molecular diversity. Another unique property of the keratins is that, in contrast to the other IF proteins, they only can constitute their filamentous stage by heteropolymeric pair formation. Formation of heterodimers is made between a type I (“acidic”) and type II (“basic to neutral”) keratin in an equimolar type of (1:1) molecules. 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).

All these keratins belong to the family of IF proteins and therefore share common protein-structural characteristics, such as the central rod domain of the  $\alpha$ -helical conformation flanked by non-helical head and tail domains with variable length (Fig.10). Heterodimer formation occurs by association of the corresponding rod domains in  $\alpha$ -helical coiled-coil conformation. The resulting heterodimers and tetramers form the basic building units of the keratin filaments (Moll et al., 2008). Single keratin proteins deviating from equimolar type I/type II amounts are rapidly degraded (Lu and Lane, 1990).

As part of the epithelial cytoskeleton, keratins are important for the mechanical stability and integrity of epithelial cells and tissues. Moreover, some keratins also have regulatory functions and are involved in intracellular signaling pathways (Moll et al., 2008). Since keratin filaments are important structural stabilizers of epithelial cells, there is unabatedly high interest in keratins in biology, embryology, pathology, and dermatology (McLean and Irvine, 2007; Uitto et al., 2007).

It has been recognized that keratins are not simply static intracellular skeletal structures but rather highly dynamic, therefore new functional roles of keratins have been defined (Magin et al., 2007; Oshima, 2007).

K8/K18/K19 are involved in protection of the placental and trophoblast barrier function (Hesse et al., 2000; Jaquemar et al., 2003), K8/K17 in protection from apoptosis (Caulin et al., 2000; Ku et al., 2003b; Tong and Coulombe, 2006), K8/K18 in the protection of the liver against stress and from injury (Ku et al., 2003a; Zatloukal et al., 2000), and K17 in the regulation of protein synthesis and cell size during wound healing involving intracellular signaling pathways (Kim et al., 2006). Keratins may also play a role in epithelial polarity and membrane traffic (Oriolo et al., 2007). Therefore, keratins obviously exert broad and variable signaling functions beyond their mechanical roles.

### **Keratins and Bacterial Infection**

In what concerns bacterial infection and recruitment/activation of keratins, only a few studies were able to associate keratins and infection.

*Salmonella typhimurium* initiates infection of a mammalian host by inducing its own uptake into intestinal cells. The secreted invasion-mediating proteins interact with host cell components inducing alterations in the actin cytoskeleton. SspC, a TTSS effector (so-called SipC), appears to bind IF proteins such as cytokeratin-8 (CK8) and cytokeratin-18 (CK18) during *Salmonella* invasion (Carlson et al., 2002; Scherer et al., 2000)

Recent studies identified cytokeratin-18 as a novel interacting protein of EPEC receptor Tir. CK18 is recruited to the EPEC-induced pedestal and has a direct role in actin accumulation and cytoskeleton reorganization. This study implicates, for the first time, IF in microfilament reorganization following EPEC infection (Batchelor et al., 2004). Furthermore, it has been additionally suggested that CK18 forms a complex with EPEC effector protein EspF, in infected intestinal epithelial cells (Viswanathan et al., 2004).

# Project Presentation

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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, *L. monocytogenes* is one of the most documented. *Listeria* enters into cells mainly *via* two bacterial proteins, InlA and InlB, which interact with cell surface receptors, E-cadherin and c-Met, respectively. These interactions initiate a series of signaling cascade events that leads to actin polymerization, membrane invagination and subsequent bacterial internalization. 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. *L. monocytogenes*, as other human pathogens, induces the phosphorylation of its cellular receptors and triggers a series of phosphorylation events that contribute to the bacterial internalization.

The main goal of the project I developed was the identification of new host intracellular pathways that are exploited by the pathogens in order to cause and establish infection, using *L. monocytogenes* as the pathogen model. 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.

# Material and Methods

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## **Bacterial strains, cell lines and culture media**

*L. monocytogenes* strain EGDe was grown in brain heart infusion (BHI) (Difco Laboratories, Becton, Dickinson and Company, USA) at 37°C with agitation. *L. monocytogenes* transformed with pNF8-GFP was grown in BHI supplemented with Erythromycin (5 µg.ml<sup>-1</sup>). 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). Jeg-3 cells were grown in EMEM supplemented with 2 mM non-essential aminoacids, 2 mM sodium pyruvate and 10% 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 37°C in a 7% CO<sub>2</sub> atmosphere. All the media and products for cell culture were purchased from Lonza.

## **Antibodies and other reagents**

Monoclonal antibody against phosphotyrosine (clone 4G10) was purchased from Upstate Biotechnology (UBI). Monoclonal antibody against actin (AC-15) was obtained from Sigma. Mouse monoclonal anti-InlA (L7.7) and anti-*Listeria monocytogenes* (R11) were gently provided by Pr Pascale Cossart, Institut Pasteur, Paris. Polyclonal antibody against Myh9 (M8064) was purchased from Sigma and mouse monoclonal antibody against Cytokeratin-18 (DC-10) was purchased from Santa Cruz Biotechnology. Appropriate Cy3- and Cy5-conjugated secondary antibodies were used following manufacturer's instructions (Jackson ImmunoResearch). HRP-conjugated secondary antibodies were from P.A.R.I.S (Cambridge Bioscience). Alexa-Phalloidin 488 (Molecular Probes) was used to label actin filaments.

Chemical inhibitors ML-9 and ML-7 were obtained from Sigma. Blebbistatin was purchased from Calbiochem.

**Infection assays and Immunoprecipitation**

Cells were seeded onto 75 cm<sup>2</sup> flasks and incubated at 37°C in a 7% CO<sub>2</sub> atmosphere, until confluence. The day of the experiment, cells (approximately 5 x 10<sup>6</sup> cells) were washed twice with phosphate-buffered saline (PBS) and serum-starved during 5 h at 37°C and 7% CO<sub>2</sub>. Then, cells were either left uninfected or infected with *L. monocytogenes* EGDe at a multiplicity of infection (MOI) of 200, during different times. 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), Complete Protease Inhibitor Cocktail (Roche Pharmaceuticals)). Lysates were centrifuged at 15 000 g for 10 min at 4°C and the supernatants were clarified with 60 µl of a 20% (v/v) suspension of Protein G Sepharose beads (SIGMA) for 1 h at 4°C. Lysates were then immunoprecipitated with 2 µg of anti-phosphotyrosine antisera (4G10) overnight at 4°C. Immune complexes were captured with 60 µl of a 20% (v/v) suspension of Protein G Sepharose at 4°C, washed three times (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).

**Western Blot (WB) Analysis**

Immunoprecipitated proteins were eluted and boiled in Laemmli buffer containing 5% β-mercaptoethanol. Samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane blocked overnight at 4°C with Membrane Blocking Solution (ZYMED). The membrane was then immunoblotted overnight at 4°C with primary antibody diluted in Membrane Blocking Solution: 1:1000 antiphosphotyrosine antisera (4G10), 1:3000 anti-Myh9, 1:200 anti-CK18, 1:5000 anti-Actin or 1:500 anti-α-Catenin. Nitrocellulose membrane was washed 3 times at room temperature, during 10 minutes, and incubated 1h at room temperature with appropriate goat anti-mouse or anti-rabbit secondary antibodies HRP-conjugated (1:3000). Then washed again 3 times for 10 minutes at room temperature. The signal was developed with the ECL Chemiluminescent Detection System (Amersham).

**Silver staining**

Following SDS-PAGE, gels were silver-stained using the ProteoSilver™ Plus Silver Staining Kit (Sigma-Aldrich, USA) as described by the manufacturer.

**Protein identification by mass spectrometry (MS)**

Protein bands were extracted from the silver-stained SDS-polyacrylamide gel and digested with trypsin. The proteolysis was carried out overnight and the resulting peptides were extracted with acetonitrile and dried under vacuum. Proteolytic peptides were then dissolved in a small amount of distilled water and subjected to MS analysis followed by database searching. Using dedicated software algorithms, the proteins from which the mass-measured peptides were derived were identified. Procedure described above relies on peptide mass fingerprinting (PMF) and was performed by the Proteomics Unit at IPATIMUP, Universidade do Porto.

**Invasion assays**

Invasion assays were performed in 24-well plates using the gentamicin survival assay as previously described (Pizarro-Cerdà et al., 2002). For pre-treatment, 30 min prior to infection, cells were incubated at 37°C and 7% CO<sub>2</sub> with serum-free medium containing 80 µM ML-9 in DMSO or 30 µM ML-7 in DMSO or blebbistatin (10, 20, 50 and 100 µM) in DMSO 90%. As control, cells were incubated with serum-free medium containing DMSO (0.3-0.8%). Bacteria were added to cells at a MOI of 50. Cells were incubated with bacteria for 1 hour at 37°C and 7% CO<sub>2</sub> in the absence or in presence of inhibitors (ML-9, ML-7 or blebbistatin). The media was then discarded and the cells incubated for 90 min at 37°C and 7% CO<sub>2</sub> in presence of gentamicin (20 µg ml<sup>-1</sup>). The media was discarded and the cells washed 3 times with PBS. Cells were then lysed with 0.2% Triton X-100 in H<sub>2</sub>O and serial dilutions of the lysates were plated for bacterial counting.

**Immunofluorescence analysis**

For immunofluorescence cells were grown on coverslips and infected with *L. monocytogenes* EGDe or EGDe transformed with pNF8-GFP plasmid (MOI = 50). Cells were fixed in 3% paraformaldehyde (PFA) or in methanol at 4°C.

Cells were permeabilized in 0.1% Triton X-100 in PBS for 2 min, blocked with 1% BSA in PBS or 1% Gelatine in PBS for 30 min, and then incubated for 45 min with primary antibodies diluted in blocking buffer: anti-InlA (1:250), anti-Myh9 (1:100), anti-*Listeria*/R11 (1:250) and anti-CK18 (1:100). Cells were incubated with secondary antibodies or Phalloidin Alexa-488 diluted in blocking buffer (1:250). DNA was counterstained with DAPI (4,6-diamidino-2-phenylindole) in a 1:500 dilution. Cells were washed with PBS before and after incubation with primary and secondary antibodies. Slide preparations were mounted with 5 µl MOWIOL coverslip mounting solution. Images were collected by a Zeiss Axio Imager Z1 fluorescence microscope.

### **Green fluorescent protein (GFP) purification**

The plasmid encoding the GFP fused to an His tag (pET-GFP-His) was kindly provided by Dr Helder Maiato (IBMC, Porto, Portugal). *E. coli* BL21 (DE3) were transformed with pET-GFP-His and grown in Luria-Broth medium supplemented with Kanamycin (20 µg.ml<sup>-1</sup>) at 37°C until OD<sub>600nm</sub>=0,5. Overexpression of the GFP-His fusion protein was induced by addition of IPTG (0,15 mM). After 3 hours, bacterial culture (1 l) was harvested, centrifuged and the bacterial pellet was resuspended in 30 ml of Lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub> pH 8, 300 mM NaCl, 10 mM Imidazol, 0.5 mg/ml lysozyme) and stored at -20°C overnight. Bacteria were lysed by 6 cycles of 10 sec sonication with 15% of amplitude (Sonifier 150, Branson). GFP-His protein was then purified on HisTrap FF 1 ml Ni<sup>2+</sup> Sepharose column (GE Healthcare). The protein was eluted using a gradient of imidazol ranging from 10 to 500 mM. This method allowed us to purify approximately 20 mg of protein.

### **Transfection of siRNA duplexes**

Two different Myh9 siRNA duplexes were used: a commercially available Myosin IIa siRNA sc-61120 (Santa Cruz Biotechnology) and the Myh9 siRNA (5'-GGAGCGTTACTACTCAGGG-3') designed to match the 3' of human myosin9 mRNA and chemically synthesized by Qiagen. CK18 siRNA duplex was purchased from Sigma (5'-GAGAGGAGCUAGACAAGUA-3'). Control RNA (control siRNA-A: sc-37007) was purchased from Santa Cruz Biotechnology. HeLa cells were transfected using either the HiPerfect Reagent (Qiagen) or the

siRNA Transfection Reagent (Santa Cruz Biotechnology), following manufacturer's instructions. Cells were harvested 24, 48h and 72h after transfection and the efficiency of siRNA-treatment was assessed by western blot.



# Results

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## I. Analysis of tyrosine phosphorylation patterns in infected cells

Several studies have shown how different pathogens hijack signaling cascades and activate host kinases/phosphatases to promote phosphorylation or dephosphorylation of either bacterial or cellular proteins. These phosphorylation features are important in promoting infection. Phosphorylation in tyrosines, serines and threonines is commonly described. In order to identify novel host proteins involved in *Listeria* infection, we decided first to analyze the tyrosine phosphorylation patterns of infected as compared to non-infected cells. With this purpose, we performed infection assays with EGDe (*L. monocytogenes* wild-type strain) in Caco-2 cells.

The total cell lysates, including a non-infected control, were collected at different time-points of infection and subjected to an immunoprecipitation (IP) using anti-phosphotyrosine antibodies (4G10). This approach allowed us to recover the pool of proteins that were phosphorylated on tyrosine residues, for each infection time-point. The immunoprecipitates were solved in acrylamide gel and proceeded for WB detection using the 4G10 antibodies. Our results (Fig. 11A) showed that some bands disappeared during infection in Caco-2 cells. This suggests that, during *Listeria* infection, some tyrosine-phosphorylated proteins undergo dephosphorylation, probably following the activation of host phosphatases. We also performed the same experiments in HeLa cells. These experiments were repeated several times in both cell lines. In the case of HeLa cells we observed the appearance or the increase of intensity of bands in the course of the infection (data not shown). This reveals the increase of tyrosine phosphorylation events probably associated with the activation of host kinases. Together, these preliminary studies indicated that our technique could allow us to detect proteins that are differentially tyrosine-phosphorylated upon infection.

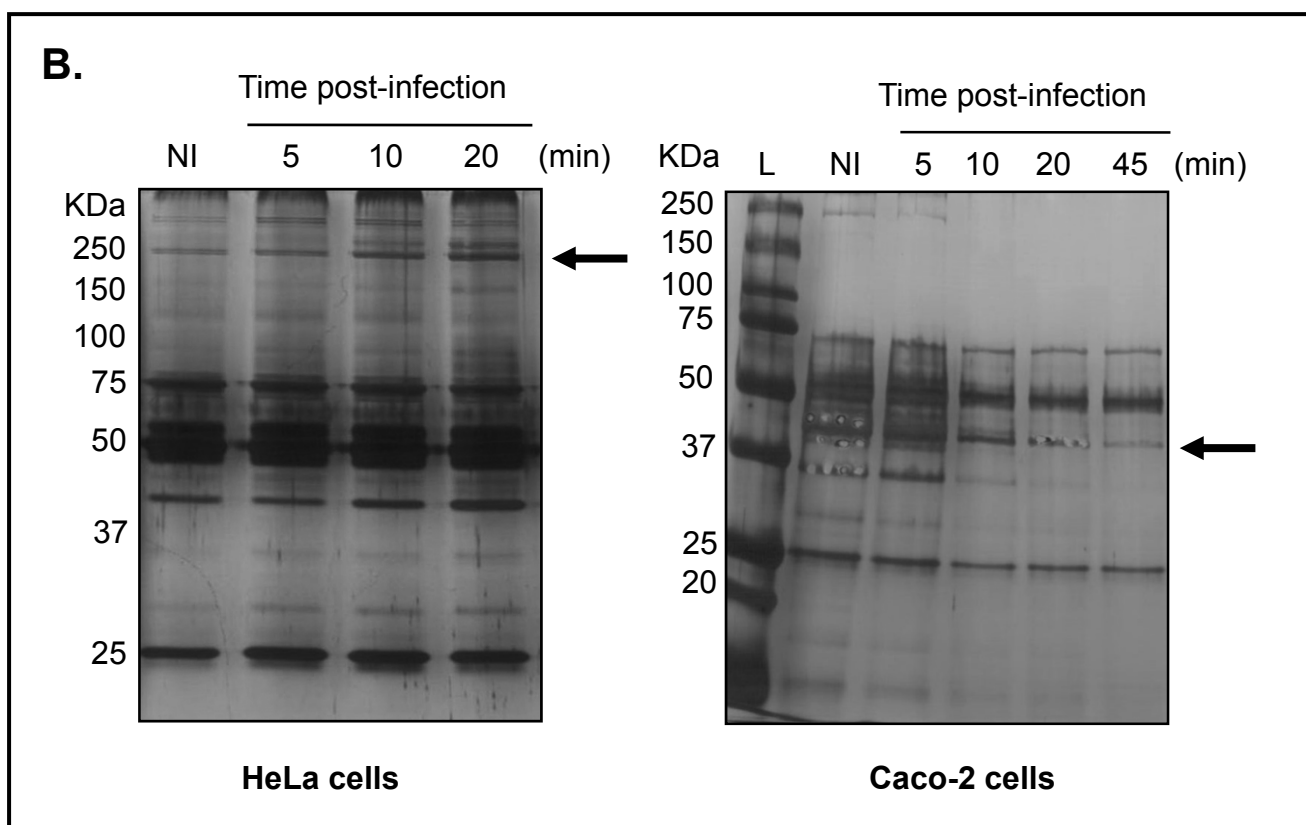
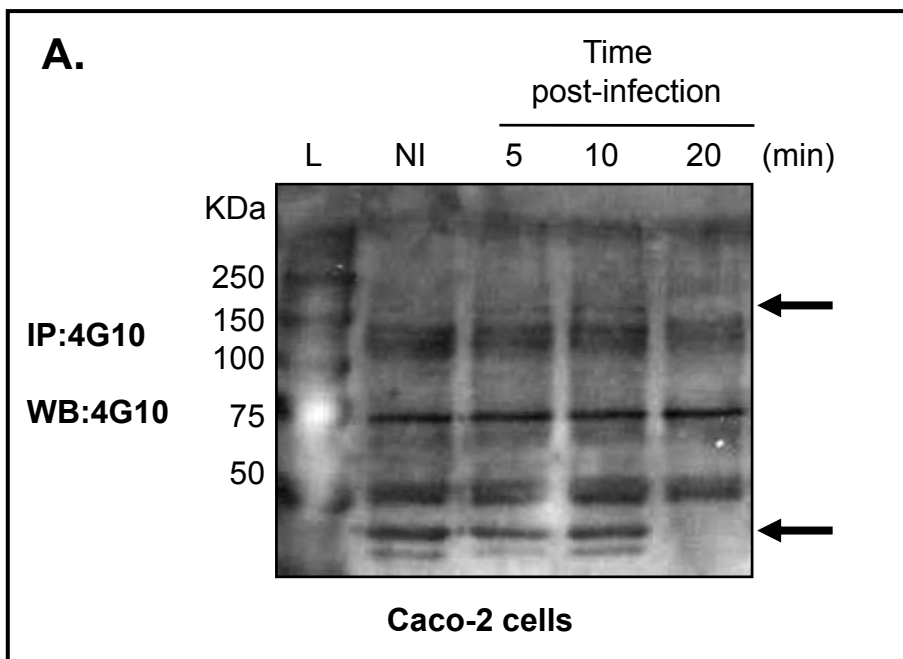


**Figure 11. Protein tyrosine phosphorylation patterns of *L. monocytogenes* infected cells.** (A) Caco-2 cells were left non-infected (NI) or infected with *L. monocytogenes* for different periods of time (5,10 and 20 minutes). Total cell extracts were immunoprecipitated using 4G10 antibody and the immunoprecipitates were revealed by WB using 4G10. Black arrows indicate bands disappearing during infection. (B) Silver staining detection of total tyrosine phosphorylated proteins immunoprecipitated with 4G10 in whole cell extracts from non-infected or infected HeLa (left) and Caco-2 (right) cell lines. Black arrows indicate bands with variable intensity over time of infection. Standard molecular weight ladder (L) is indicated.

Supported by these observations, we decided to move forward in trying to identify these differentially tyrosine-phosphorylated proteins. New infection assays were performed in Caco-2 and HeLa cells. Total cell lysates were collected at different time-points and immunoprecipitated with 4G10 antibodies as described above. Samples were solved by acrylamide gel followed by silver staining. Despite the lower detection sensitivity of silver staining, as compared to the WB, we still observe different tyrosine-phosphorylated patterns for the different time points of the infection (Fig.11B). The bands showing differences in silver staining do not correspond necessarily to the bands of interest observed in WB. The silver staining profiles were carefully analyzed and several bands that should correspond to proteins differentially phosphorylated during infection were selected for protein identification.

On HeLa cells, a band around 250 kDa became more intense over the time of infection. This suggested that a protein with a molecular weight around 250 kDa is being tyrosine-phosphorylated during *Listeria* infection. On Caco-2 cells experiment, we selected a band with a decreased intensity in the course of cellular infection, corresponding to a protein around 37-50 kDa that becomes dephosphorylated. This observation suggests that a phosphatase is activated during *Listeria* infection.

## **II. Identification of proteins differentially phosphorylated during infection**

SDS-acrylamide gels of both HeLa and Caco-2 infection experiments were sent to the Proteomics Unit at IPATIMUP, in Porto, for excision of the two bands described above. The corresponding proteins were identified by molecular weight determination and peptide mass fingerprinting (PMF). This method, also known as protein fingerprinting, is an analytical technique for protein identification. Proteins of interest were first cleaved by trypsin. The peptides were subjected to mass spectrometric analysis and peptides absolute masses were accurately measured. This analysis produced a list of molecular weights called peak list. The peptide masses obtained were then compared to protein databases, such as Swissprot and Genbank, which contain protein

| PMF Myosin-9 [MYH9_HUMAN]<br>226.4 kDa |             | PMF Cytokeratin 18 [gi 30311]<br>47.3 kDa |            |
|----------------------------------------|-------------|-------------------------------------------|------------|
| Peptide mass<br>(Da)                   | Sequence    | Peptide mass<br>(Da)                      | Sequence   |
| 917.4686                               | DLEGLSQR    | 718.3916                                  | IMADIR     |
| 924.4937                               | VVFQEFR     | 758.4406                                  | IIEDLR     |
| 961.485                                | NTNPNFVR    | 807.3995                                  | LAADDFR    |
| 975.4584                               | NWQWWR      | 837.4465                                  | LASYLDR    |
| 999.5621                               | GDLPFVVR    | 848.4108                                  | SLETENR    |
| 1039.6622                              | VKPLLQVSR   | 889.4737                                  | ASLENSLR   |
| 1045.571                               | LEVNLQAMK   | 924.5261                                  | IREHLEK    |
| 1155.6633                              | GDLPFVVR    | 965.4686                                  | AQYDELAR   |
| 1193.6161                              | ALELDSNLYR  | 975.453                                   | STFSTNYR   |
| 1205.6412                              | TDLLLEPYNK  | 982.4417                                  | DWSHYFK    |
| 1215.6692                              | ASREEILAAK  | 1041.6051                                 | IVLQIDNAR  |
| 1220.6521                              | KFDQLLAEEK  | 1046.5477                                 | VIDDTNITR  |
| 1223.6378                              | AGVLAHLEER  | 1065.5575                                 | LEAEIATYR  |
| 1242.5896                              | THEAQIQEMR  | 1093.4943                                 | ETMQSLNDR  |
| 1257.6797                              | KEEELQAALAR | 1239.6401                                 | VKYETELAMR |

**Figure 12. Protein identification by Peptide Mass Fingerprinting (PMF).** Tables show trypsin digested representative peptides obtained by the mass spectrometry analysis of the excised bands. Peptides map with Myh9 (left) and CK18 (right) trypsin digested. The respective molecular masses and aminoacid sequences are indicated. In brackets: accession numbers of the respective proteins.

sequence information. Software programs were design (Hufnagel and Rabus, 2006) to digest all the proteins into peptides with trypsin. The absolute mass of all these peptides was theoretically calculated. A comparison was made between the peak list we obtained and the databases of theoretically calculated masses. The results were statistically analyzed.

The spectrometric analysis reports allowed the identification of the two proteins, one for the HeLa cells and the other for Caco-2 cells infection experiments. The approximately 250 kDa band from HeLa cells, showing an increased intensity over infection, was identified as human Myosin-9 (Myh9); also called non-muscle myosin II isoform A (NMHC-IIA). Figure 12 summarizes the PMF identification report. These same reports further revealed a protein score C.I. of 100% corresponding to the perfect match between experimentally and theoretically calculated peptide masses. Myh9 calculated molecular weight is 226 kDa that is in accordance with the apparent molecular weight estimated from our silver-stained acrylamide gel (Fig. 11B). This identification succeeded not only by matching with silver stain results but also by identifying a protein prone to undergo tyrosine phosphorylation, as predicted by databases. Moreover, myosins have been long implicated in phagocytosis processes, including in the *Listeria*-induced phagocytosis in epithelial cells (Groves et al., 2008; Sousa et al., 2004). Taken together, these results reveal Myosin-9 as a promising candidate involved in *Listeria* infection.

The band extracted from the gel corresponding to the Caco-2 cells experiment resulted in the identification of human Cytokeratin-18 (CK18). This identification showed a protein score C.I. of 100%. Some of the trypsinized peptides we obtained, as well as their corresponding molecular masses are represented in figure 12. CK18 has a molecular weight of 48 kDa sustaining our prediction from silver-stained gel (37-50 kDa). Cytokeratins are known to be functionally regulated by phosphorylation and CK18 is known to be recruited at EPEC pedestals (Batchelor et al., 2004; Viswanathan et al., 2004), therefore we decided to study its role in *Listeria* infection. Together with the fact of being an epithelial and not a follicular keratin, this PMF results minimize the possibility of either immunoprecipitation or mass spectrometry contamination.

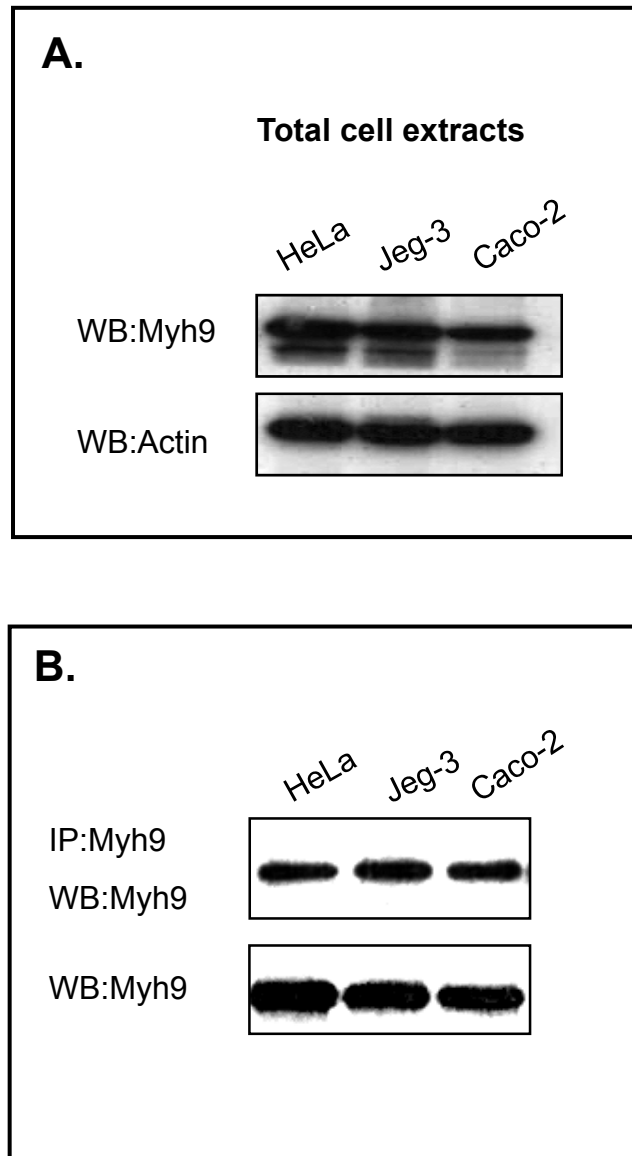


**Figure 13. Myh9 expression in different cell lines. (A)** Whole cell extracts of non-infected HeLa, Jeg-3 and Caco-2 cells were used for WB detection of Myh9, using an anti-Myh9 specific antibody. The same membrane was incubated with an anti-actin antibody for loading control. **(B)** Immunoprecipitation capacity of the anti-Myh9 antibody. Whole cell extracts of the same cell lines were subjected to immunoprecipitation assays, using the anti-Myh9 and 1 mg of total protein extracts. Immunoprecipitates were revealed by WB using the same anti-Myh9 (upper pannel). As control, Myh9 was detected by WB in 50  $\mu$ g of Jeg-3, HeLa and Caco-2 whole cell extracts (lower pannel).

### III. Myosin-9 tyrosine phosphorylation during *Listeria* infection

In order to investigate the expression pattern of Myh9, we first analyzed the expression of Myh9 in different cell lines relevant for *Listeria* infection. It was important to first ascertain if Myh9 was or not expressed in normal cellular conditions or if its expression was dependent on a stimulus or a signaling cascade. We used HeLa cells, in which we detected the tyrosine phosphorylation increase during *Listeria* infection, Jeg-3 cells that are human placenta cells used for mimicking the placental barrier, and Caco-2 cells that are human colon cells habitually used for mimicking intestinal barrier. Non-infected HeLa, Jeg-3 and Caco-2 cells were lysed and the total extracts were quantified. Total proteins were run in a gel and proceed for WB. Using specific antibodies, we detected Myh9 and also revealed actin as a loading control. As expected, we detected Myh9 in non-infected HeLa cells but also in Jeg-3 and Caco-2 cells. No variation in Myh9 expression was observed between the three cell lines (Fig.13A). The levels of actin were also similar in the three cell lines indicating that the quantity of total proteins loaded in each lane was the same. These results indicate a constitutive expression of Myh9 in the cell lines tested and highlight Myh9 as an important cell physiological component rather than a *Listeria* exclusive host cell effector.

In order to validate the results obtained following 4G10 immunoprecipitation, we analyzed the tyrosine phosphorylation of Myh9 upon *Listeria* infection, using Myh9-specific antibodies. We planned to use the antibody anti-Myh9 in immunoprecipitation of whole infected and non-infected cell lysates. This technique should allow the increase of Myh9 concentration and diminish the amount of other phosphotyrosine proteins in the immunoprecipitates. In addition, using this technique we would also be able to purify with the Myh9 its interacting proteins that would be later identified by mass spectrometry. We therefore determined the immunoprecipitation capacity of the antibody specific for Myh9. Anti-Myh9 immunoprecipitation was performed in 1 mg of total protein from non-infected cell lysates of HeLa, Jeg-3 and Caco-2 cells. Myh9 immunoprecipitates were solved in acrylamide gel and

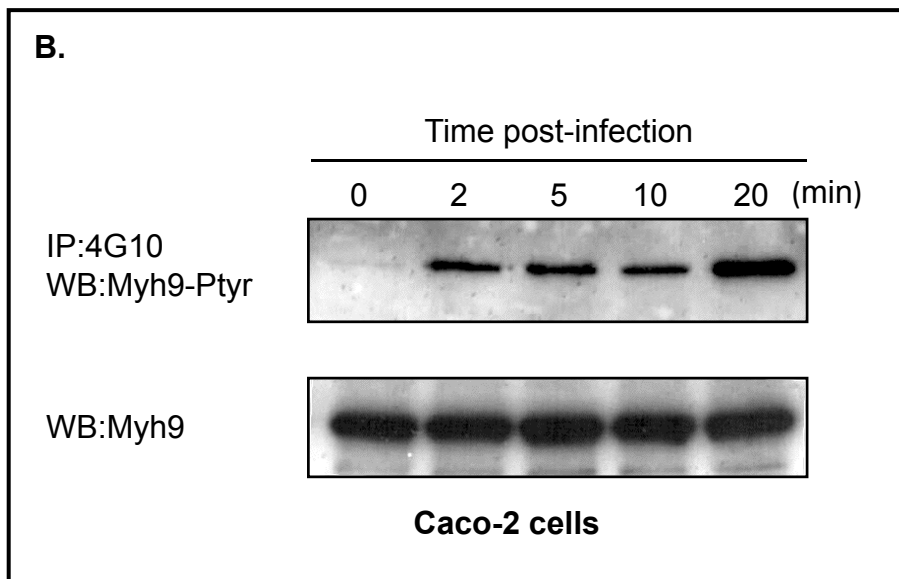
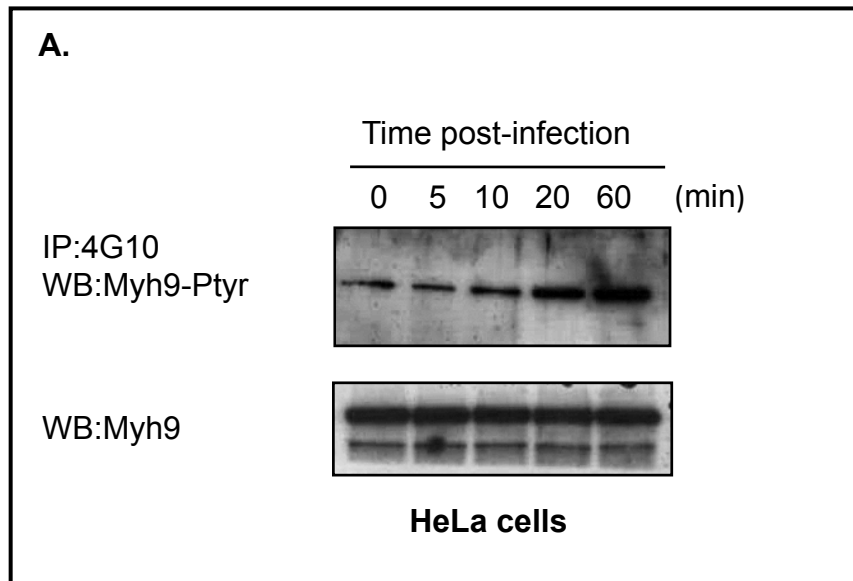


**Figure 14. Myh9 tyrosine phosphorylation in response to *L. monocytogenes* infection.** Hela cells (**A**) or Caco-2 cells (**B**) were left non-infected (lanes 0), or infected with *L. monocytogenes* during different periods of time. Cells were lysed and tyrosine phosphorylated proteins were immunoprecipitated with 4G10 antibody. Myh9 (Myh9-Ptyr) was detected using the anti-Myh9 in the 4G10 immunoprecipitates. As control, the total Myh9 (phosphorylated and non-phosphorylated) was revealed by WB in the total cell extracts.

Myh9 was detected by WB, using the same antibody. In the same gel were loaded 50 µg of the total protein lysates for further detection of Myh9. In figure 13B, we show that, indeed, anti-Myh9 immunoprecipitates the respective protein in either of the three cell lines. However, taking in account that only 50 µg of non-immunoprecipitated protein extracts show more Myh9 than Myh9-immunoprecipitates from 1 mg of total protein we conclude that our antibody had low immunoprecipitation capacity. We expected to concentrate Myh9 using immunoprecipitation and therefore to obtain a stronger signal in our immunoprecipitated samples as compared to whole cell lysates. In order to exclude the possibility that the antibody was used in limiting quantities we performed the same experiment using a large excess of anti-Myh9. The obtained results were the same, definitively showing that this antibody is not appropriate to use in immunoprecipitation studies (data not shown). For the validation of our Myh9 tyrosine phosphorylation data, we decided to follow another approach using 4G10 antibody for immunoprecipitation and then reveal the Myh9 with the anti-Myh9 antibody that showed satisfactory results in WB procedure.

Thus, infection experiments with EGDe were repeated on HeLa cells. The protein concentration in whole cell lysates for different infection time-points was quantified and 1 mg of total protein extracts was further used for immunoprecipitation with anti-phosphotyrosine (4G10). Immunoprecipitated tyrosine-phosphorylated proteins were solved by SDS-acrylamide gel proceeding for WB analysis using anti-Myh9. As control, 50 µg of total protein extracts (non-immunoprecipitated) from each infection lysates were run in a SDS-acrylamide gel and also blotted with anti-Myh9. Remarkably, in agreement with our previous observations, we could detect an increase of Myh9 phosphorylation in HeLa cells upon infection by *Listeria* (Fig.14A). The observed tyrosine phosphorylation increase was continuous in the course of the infection, reaching a maximum 60 minutes after infection, in our experiments. This increase was similar to the one previously observed in silver-stained gel (Fig.11B). In our control total protein extracts, we observed no variation in the amount of Myh9 upon infection, confirming that variations observed in the immunoprecipitate samples were indeed due to differences in tyrosine phosphorylation rather than in the Myh9 quantities. In addition, detection of

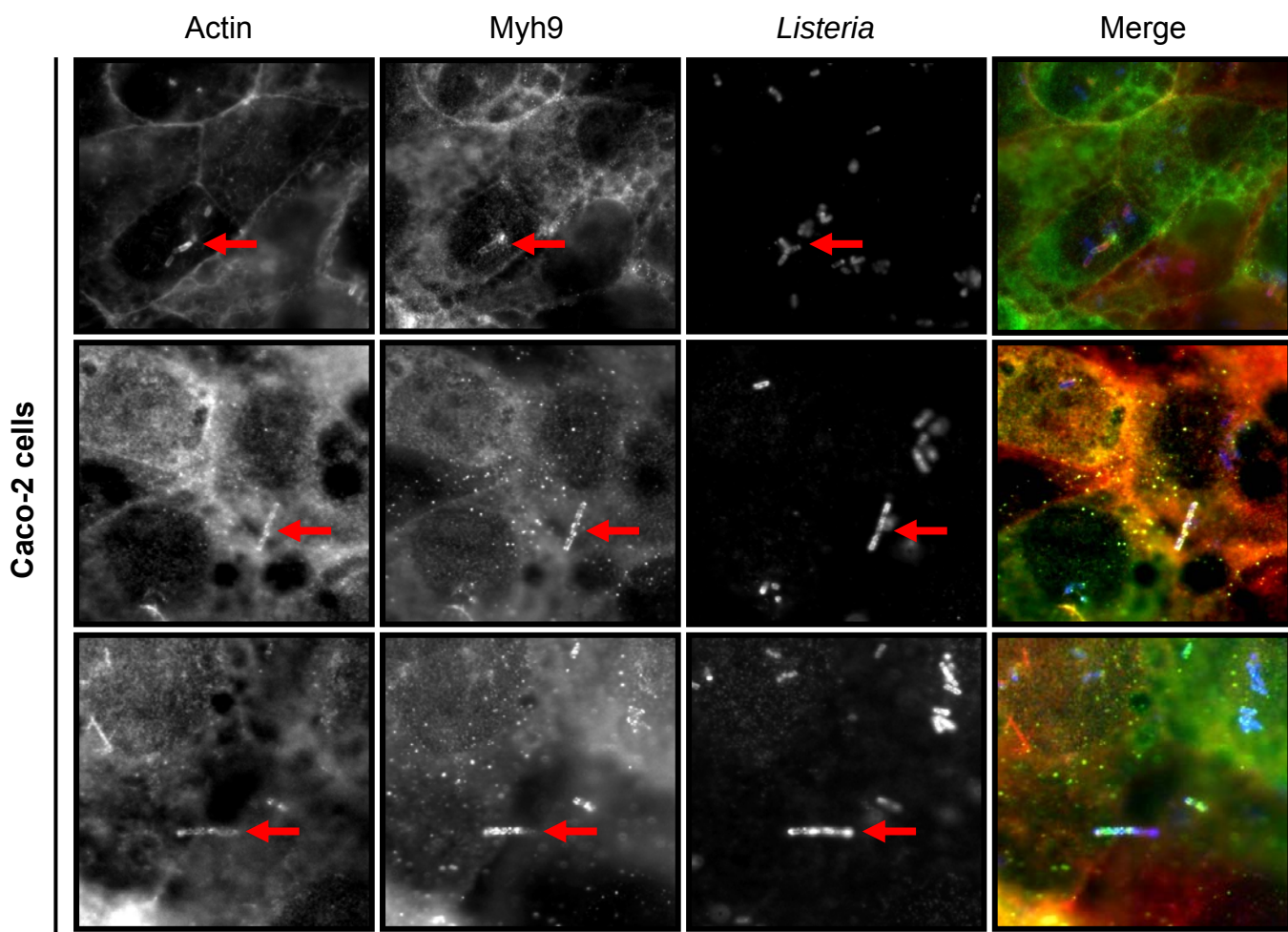


**Figure 15. Localization of Myh9 in *L. monocytogenes* infected Caco-2 cells.** Immunofluorescence staining for actin, Myh9 and *Listeria*. Red arrows show sites where endogenous Myh9 is recruited at the entry site of *L. monocytogenes* and colocalizes there with actin.

Myh9 in 4G10 immunoprecipitates certified that this protein is really tyrosine-phosphorylated and that this phosphorylation is triggered by *Listeria* infection. This is the first report describing the tyrosine phosphorylation of Myh9 in response to bacterial infection. Caco-2 cell line represents an important cell system in the study of *Listeria* infection, since it mimics the intestinal barriers as described before. Based on this, we decided to look at the Myh9 tyrosine phosphorylation behavior on *Listeria*-infected Caco-2 cells. The same procedure was used for infection assays. As in HeLa cells, we observed a differential tyrosine phosphorylation of Myh9 in response to infection. In Caco-2, the tyrosine phosphorylation of Myh9 increases immediately after infection and in our experiments reaches a maximum 20 minutes post transfection (Fig.14B). These results confirm the stimulation of Myh9 tyrosine phosphorylation in response to *Listeria* infection and strongly suggest a role for Myh9 in the cellular infection.

#### **IV. Localization of Myosin-9 in *Listeria*-infected cells**

Cellular proteins required for *Listeria* entry are generally recruited at the entry site. We thus investigated the localization of endogenous Myh9 during *Listeria* entry into cells. Caco-2 cells infected with *L. monocytogenes* EGDe (MOI = 50) were fixed and incubated with antibodies raised against Myh9 (anti-Myh9), InlA that stains *L. monocytogenes* and phalloidin to label actin which is known to be recruited at the entry site of *Listeria* (Lecuit et al., 2000). As shown in figure 15, endogenous Myh9 was recruited at the site of entry of *Listeria*, where it colocalized with actin. This recruitment indicates an enrichment of Myh9 at the bacteria entry site that probably corresponds to protein mobilization to a site where its activity is needed. Same results were observed in Jeg-3 cells (data not shown). These results are in favor of an important role of Myh9 in the entry process of *L. monocytogenes* into eukaryotic cells.

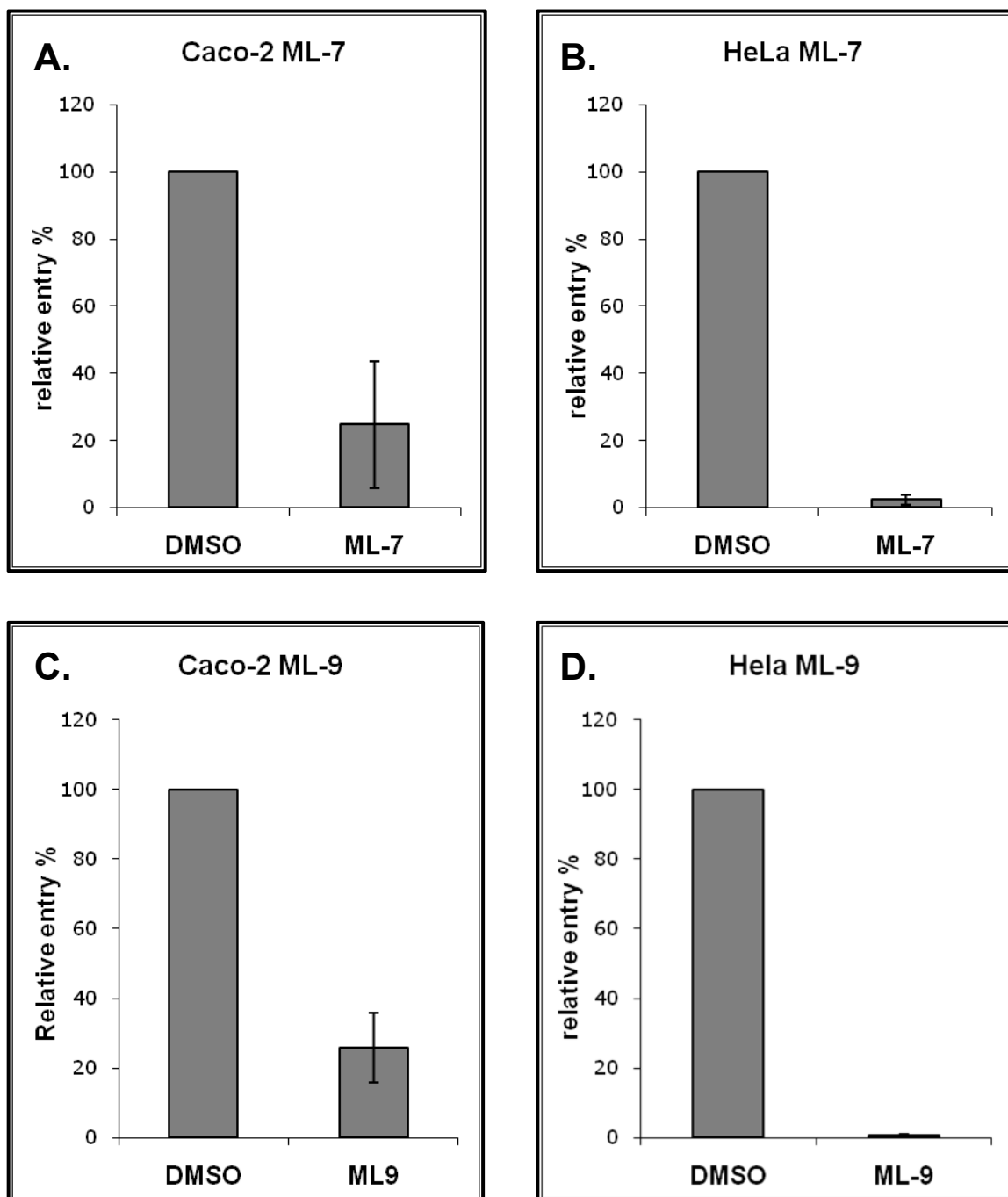


**Figure 16. Effect of ML-7 and ML-9 inhibitors on *L. monocytogenes* internalization.** Entry levels of *L. monocytogenes* in the presence of ML-7 and ML-9 was evaluated and compared to entry levels obtained in DMSO-treated cells. **(A)** and **(B)** ML-7 effect on *Listeria* entry into Caco-2 and HeLa cells respectively. **(C)** and **(D)** ML-9 effect on *Listeria* entry into Caco-2 and HeLa cells respectively. Entry in DMSO-treated cells has been normalized to 100 and the levels of entry in cells treated with inhibitors are expressed as relative values. Results are means  $\pm$ SD from four independent experiments, each done in triplicate.

## V. Role of Myosin-9 in *Listeria* entry

### A. Chemical Inhibition

Together the previous results strongly suggested that Myh9 plays a role in the infection. The recruitment of Myh9 at the entry site of the bacteria put forward two hypotheses. The protein is enriched at this specific site because: its activity is important for the bacterial invasion process or it is a structural protein that recruits itself other proteins important for the process. In the second hypothesis, we can speculate that the protein is necessary but its activity is dispensable for the infection. In order to investigate the exact role of Myh9 during infection, we decided first to evaluate how *Listeria* entry into Caco-2 and HeLa cells, was affected by the inhibition of Myh9 activity. Myh9 is activated upon phosphorylation of its regulatory light chain (RLC) by myosin light chain kinase (MLCK). This phosphorylation in serine residues is known to regulate interaction of Myh9 with actin. Therefore, we used for this study two specific MLCK inhibitors: ML-7 and ML-9. In the presence of either ML-7 or ML-9, MLCK is not able to phosphorylate Myh9 that, thus, remains inactivated. Because both inhibitors are dissolved in DMSO, as control we used cells treated with DMSO. HeLa and Caco-2 cells were first pre-treated with ML-7 or ML-9 for 30 min and then infected, in the presence of ML-7 or ML-9, with *L. monocytogenes* EGDe strain during one hour. The experiment proceeded as a classic gentamicin invasion assay and lysates were plated for further bacterial counting and calculation of *Listeria* relative entry values. In Caco-2 cells infection experiments, we observed a 4- to 5-fold decrease in bacterial relative entry following ML-7 and ML-9 inhibitions, corresponding to approximately 80% reduction of invasiveness (Fig.16A and C). Additionally, either ML-7 or ML-9 inhibition on HeLa cells resulted in a nearly 100% reduction of bacterial entry (Fig.16B and D). These values represent an effective reduction in *Listeria* entry in both cell types. We can actually assert that *Listeria* entry is blocked upon inhibition of the Myh9 activator. Nevertheless these experiments correspond to indirect inhibitions of Myh9 activity; altogether the results obtained not only indicate a role for Myh9 activity in the uptake of *Listeria* but also strongly

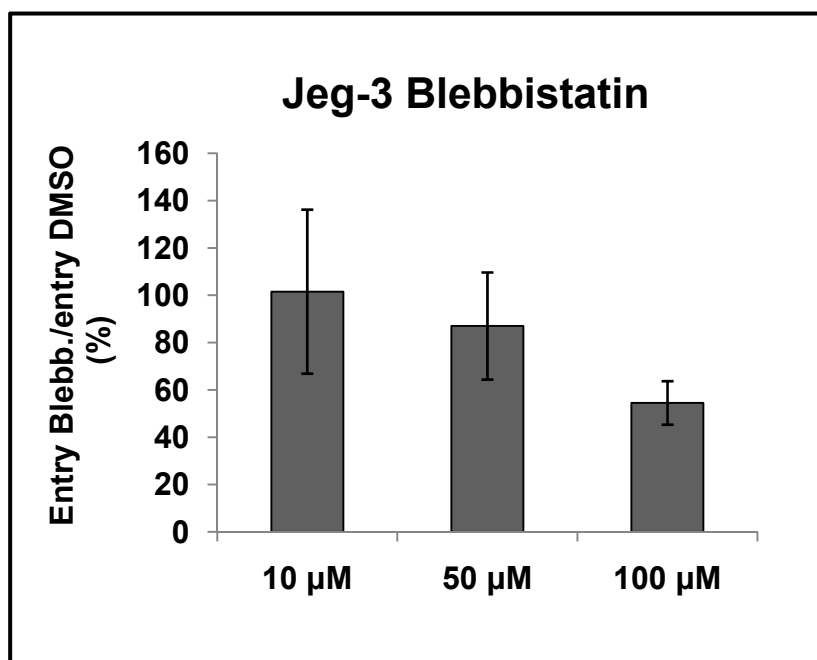


**Figure 17. Effect of Blebbistatin inhibitor on *L. monocytogenes* internalization.** Blebbistatin dose dependent effect on *Listeria* entry into Jeg-3 cells. Entry levels of *L. monocytogenes* in the presence of Blebbistatin were evaluated and compared to entry levels obtained in DMSO-treated cells. The results are presented as the ratio between entry levels in Blebbistatin-treated cells and DMSO-treated cells. Results are means  $\pm$ SD from two independent experiments.

suggest the requirement of Myh9 tyrosine phosphorylation in the course of infection.

Indirect inhibition, although promising, brought the necessity to evaluate the effect of a direct inhibition of Myh9. Blebbistatin is a small molecule inhibitor showing high affinity and selectivity toward myosin II isoforms {Kovacs, 2004 #205} (remember that Myh9 is also called Myo IIA). HeLa and Caco-2 cells were first pre-treated with blebbistatin 20  $\mu$ M for 30 min and then infected, in the presence of blebbistatin, with *L. monocytogenes* EGDe strain during one hour. The experiment proceeded as a classic gentamicin invasion assay and lysates were plated for further bacterial counting and calculation of *Listeria* relative entry values. Both on HeLa and Caco-2 cells series of experiments we observed different results between the cell lines and also when comparing different experiments (data not shown). This lack of consistency needs to be further evaluated. We then decide to evaluate the *Listeria* entry levels in presence of other blebbistatin concentrations values and other cell lines. For this we pre-treated Jeg-3 cell line with blebbistatin also for 30 min and then infected these cells, in the presence of blebbistatin, with *L. monocytogenes* EGDe strain during one hour. In this experiment we used blebbistatin at different concentrations, 10, 50 and 100  $\mu$ M. The highest concentration of blebbistatin is commonly used to block the activity of Myo II. Entry levels of *L. monocytogenes* in the presence of blebbistatin were evaluated and compared to entry levels obtained in DMSO-treated cells. In Jeg-3 cells, we observed a decrease in *Listeria* entry in a blebbistatin dose-dependent manner (Fig.17). This dose dependent decrease in bacterial entry was observed in two independent experiments and will be repeated not only in Jeg-3 but will be performed on HeLa and Caco-2 cells to identify blebbistatin effective concentration that appears to be cell line dependent. These results together with MLCK inhibition reinforce the fact that Myh9 activity is needed for *Listeria* internalization, since its direct inhibition also leads to a decrease in bacterial entry.

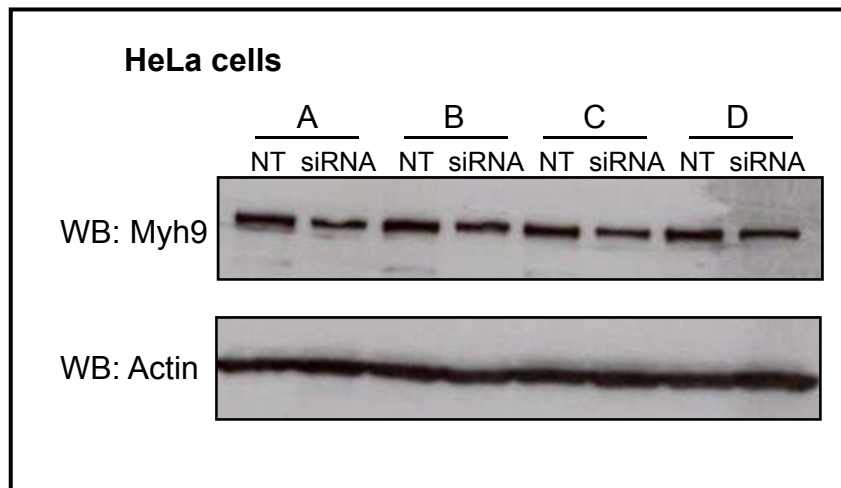


**Figure 18. Knock down expression of Myh9 in HeLa cells. (A)-(D)** Different siRNA protocols. For each protocol the efficiency of Myh9-siRNA was evaluated at the protein level. HeLa cells were left non-transfected (NT) or transfected with Myh9 siRNA duplexes (siRNA). The amount of Myh9 was checked in total protein extracts 48h post-transfection, using the anti-Myh9 antibody. The same membrane was incubated with an anti-actin antibody for loading control.

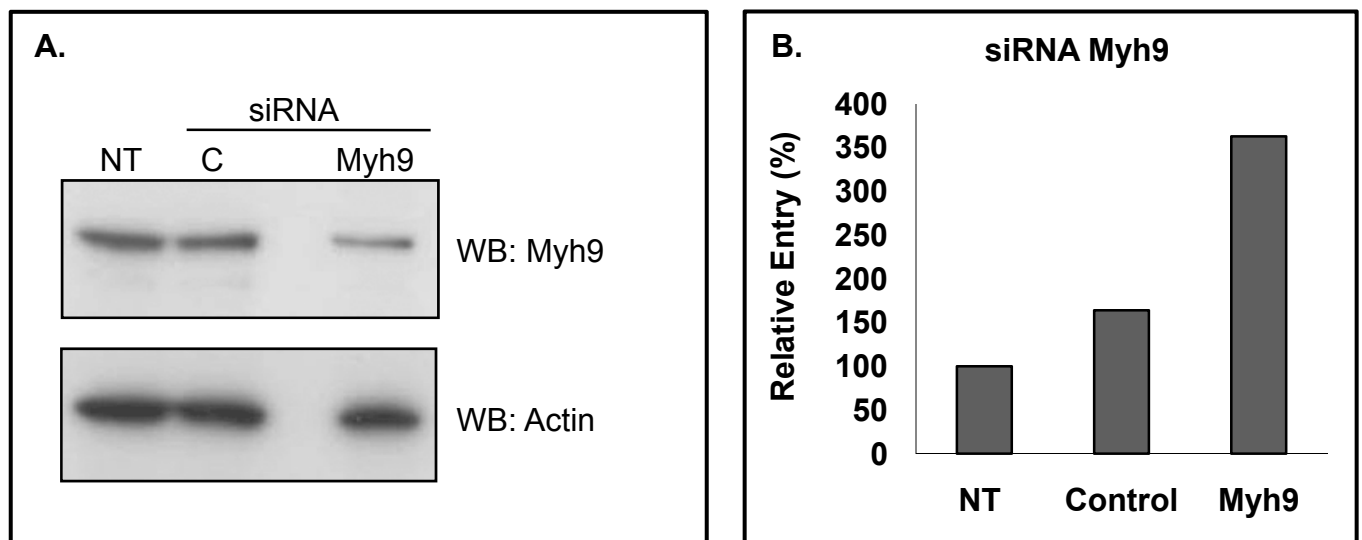


**Figure 19. Effect of Myh9 knock down expression on *L. monocytogenes* internalization .** HeLa cells were left non-transfected (NT) or transfected with either Control (C) or Myh9 siRNA duplexes. **(A)** The amount of Myh9 was checked in total protein extracts 48h post-transfection, using the anti-Myh9 antibody. The same membrane was incubated with an anti-actin antibody for loading control. **(B)** Entry levels of *L. monocytogenes* in the presence of control or Myh9 siRNAs were evaluated and compared to entry levels obtained in NT cells. Entry in NT cells has been normalized to 100 and the levels of entry in cells treated with siRNAs are expressed as relative values.

### **B. Myh9 gene silencing by interference RNA (RNAi) technique**

Chemical inhibition of Myh9 acts at protein activity level. As described above, chemical inhibition results already implied a dependency of *Listeria* entry on Myh9 activity. For further confirmation of Myh9 requirement during infection, we decided to evaluate bacterial entry in Myh9-RNAi treated cells. This approach is Myh9-specific when compared to MLCK inhibition. Optimization experiments were performed by using two different Myh9 small interference RNAs (siRNAs). siRNAs were either used independently or in combination trying to achieve the highest reduction on Myh9 expression. Cells were harvested in Laemmli buffer, 24 and 48 hours after siRNA transfection, and cell lysates solved by SDS-acrylamide gel and western blotted for Myh9 and actin. In four tested protocols (Fig.18 A-D), a decreased Myh9 expression was observed in Myh9-siRNA transfected HeLa cells. By a computational analysis of the intensity of the bands, an average reduction of 50% was obtained for our different siRNA approaches, 48 hours post-transfection. To assess the effect on bacterial entry of this two-fold reduction in Myh9 expression, conditions defined for experimental settings A (Fig.18) were used for new siRNA experiments and further invasion assay analysis. Cells were transfected with siRNA, both control and Myh9 specific according to protocol and 48h post-transfection proceeded for standard invasion assay. Surprisingly the two-fold reduction of Myh9 expression, confirmed by WB using actin as a load control, promoted an increase in *Listeria* entry on these transfected HeLa cells (Fig.19). Several experiments have been performed and this same result was observed, this consistency represents this way a strong evidence for the association of Myh9 gene silencing and the observed entry effect. These results indicate that in cells showing a reduced Myh9 expression, the entry of *Listeria* is promoted.

## **VI. Identification of Myosin-9 phosphorylation sites**

We have been able to show an increase in Myh9 tyrosine phosphorylation in response to *Listeria* infection. We planned to address the role of such tyrosine phosphorylation in the activity/role of Myh9 during bacterial

| Myh9 Tyrosine predictions |                    |       |
|---------------------------|--------------------|-------|
| Position (aa)             | Context            | Score |
| 9                         | AADK <b>Y</b> LYVD | 0.941 |
| 11                        | DKYL <b>Y</b> VDKN | 0.975 |
| 105                       | LKERYYSGL          | 0.378 |
| 106                       | KERYYSGLI          | 0.115 |
| 111                       | SGLI <b>Y</b> TYSG | 0.806 |
| 113                       | LIYTYSGLF          | 0.004 |
| 124                       | VINPYKNLP          | 0.312 |
| 130                       | NLPIYSEEI          | 0.081 |
| 138                       | IVEMYKGKK          | 0.053 |
| 151                       | PPHI <b>Y</b> AITD | 0.539 |
| 158                       | TDTA <b>Y</b> RSMM | 0.690 |
| 190                       | KVIQYLAYV          | 0.014 |
| 193                       | QYLAYVASS          | 0.126 |
| 248                       | DVNGYIVGA          | 0.457 |
| 257                       | NIETYLLEK          | 0.063 |
| 278                       | FHIFYLLS           | 0.009 |
| 279                       | HIFYYLLSG          | 0.023 |
| 297                       | LLEPYNKYR          | 0.023 |
| 300                       | PYNKYRFLS          | 0.043 |
| 400                       | VGRD <b>Y</b> VQKA | 0.800 |
| 422                       | AKAT <b>Y</b> ERMF | 0.707 |
| 471                       | LCINYTNEK          | 0.098 |
| 492                       | EQEE <b>Y</b> QREG | 0.963 |
| 573                       | CIIHYAGKV          | 0.023 |
| 579                       | GKVDYKADE          | 0.410 |
| 650                       | VGQLYKEQL          | 0.045 |
| 721                       | FRQRYEILT          | 0.458 |
| 754                       | DSNLYRIGQ          | 0.181 |
| 793                       | CCRGYLARK          | 0.103 |
| 819                       | NCAAYLKLR          | 0.155 |
| 1408                      | KVAAYDKLE          | 0.052 |
| 1460                      | ISAKYAEER          | 0.231 |
| 1805                      | VKSKYKASI          | 0.491 |
| 1861                      | NAEQ <b>Y</b> KDQA | 0.956 |

**Figure 20. Computational prediction of Myh9 phosphorylated tyrosines.** The table shows the phosphorylation potential (score) of all Myh9 tyrosine (Y) residues obtained in NetPhos 2.0 server. Highlighted in yellow are the tyrosines with high scores and identified by mass spectrometry from infected Caco-2 and HeLa cells.

infection. We first intended to identify the Myh9 tyrosine residues that were phosphorylated upon *Listeria* infection. We planned a directed mutagenesis approach in which each tyrosine residue would be mutated and the effect on infection of each single mutation would be evaluated. The analysis of the Myh9 amino acid sequence revealed more than 30 tyrosine residues, the majority of them located at the N-terminus where the myosin motor domain is located. The large number of tyrosine residues makes a straight mutational approach quite difficult and time-consuming. We then decided to investigate which tyrosine residues are preferential sites to be phosphorylated, for further mutation analysis experiments. Initially, we used a computational approach (NetPhos 2.0 Server). Based on the amino acid content we could predict which tyrosine residues are more prone to undergo phosphorylation (Fig.20). We also attempted to identify by mass spectrometry analysis the tyrosine residues phosphorylated on Myh9 expressed in *Listeria*-infected HeLa cells.

Infection assays on HeLa cells were performed as described above and total lysates of infected cells were immunoprecipitated with anti-Myh9. Immunoprecipitates were solved in acrylamide gel and further silver-stained (Fig.21). The bands corresponding to Myh9 were excised and sent to Proteomics Unit for identification of phosphorylated tyrosines. The Myh9 band from non-infected cells and the one from the longest infection time-point (60 min) were chosen for this experiment, being the less and more tyrosine-phosphorylated, respectively. From the computationally predicted phosphorylated tyrosines (Fig.20) four tyrosines were experimentally confirmed as being phosphorylated (tyrosine 9, 297, 650 and 1460). Interestingly, only tyrosine 9 corresponded to a high-scored phosphorylation prediction. The same experiments have been performed in Caco-2 cells. From the list of predicted phosphorylated tyrosines, we were able to confirm experimentally three as phosphorylated (tyrosine 400, 1460 and 1805), two of them different from those identified in HeLa cells. Tyrosine 400 matches a high-scored phosphorylation prediction. Importantly, tyrosines 9 and 400 are located at the motor domain known to be responsible for myosin binding to actin and ATP, and therefore participating in cytoskeletal rearrangements.

These two tyrosines are thus very good candidates for our directed mutagenesis analysis.

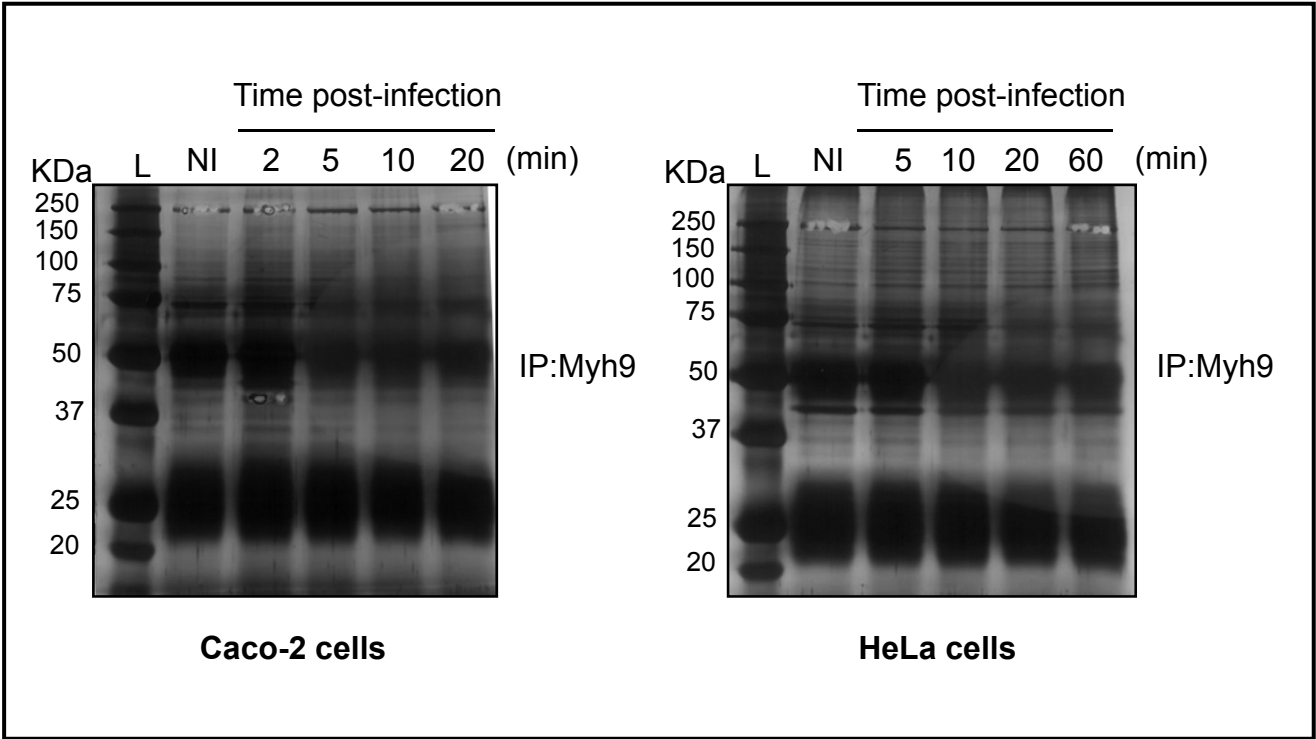


**Figure 21. Silver stained gels of total Immunoprecipitated proteins using anti-Myh9.** Caco-2 (left panel) and HeLa cells (right panel) were left non-infected (NI) or infected with *L. monocytogenes* during different time periods. Total cell extracts were subjected to immunoprecipitation using the anti-Myh9 antibody and immunoprecipitated proteins were silver stained. The bands corresponding to Myh9 were excised for mass spectrometry analysis. Standard molecular weights ladder (L) is indicated.

## VII. Identification of Myosin-9 interacting proteins

As described above, immunoprecipitation studies of Myh9 were also used to try to identify potential Myh9-interacting proteins. Unfortunately, the western blot analysis (Fig.13B), as well as silver staining gels (Fig.21) did not show a representative enrichment of Myh9. The poor immunoprecipitation capacity leads to the appearance of a multitude of protein contaminants that probably do not correspond to Myh9-interacting proteins (Fig.21). To overcome this technical problem, we decided to identify possible Myh9 interacting proteins by Localization and Affinity Purification – LAP. This purification technique will be based on the expression of a Myh9-LAP fusion protein with two distinct purification tags (GFP and S protein-binding tag).

For LAP procedure, a stable cell line expressing the Myh9-LAP fusion protein will be generated. The combination of two purification steps should result in a highly pure sample that can be analyzed by mass spectrometry, allowing the identification of Myh9-interacting proteins, as well as the confirmation of tyrosine phosphorylation sites.

The Myh9-LAP fusion is under construction. In order to accomplish the first purification step, we plan to use an antibody raised against GFP. Such antibody is presently being produced. We produced and purified GFP protein from *E. coli* BL21 (DE3) strain overexpressing a GFP-His tag protein. GFP-His protein was then purified on Ni<sup>2+</sup> Sepharose column. From a successful purification (Fig.22A), eluates from 12 to 18 were pooled together and further concentrated in PBS by successive steps of concentration (Fig.22B). This method allowed us to purify approximately 20 mg of GFP protein that have been injected in rabbits for anti-GFP production.

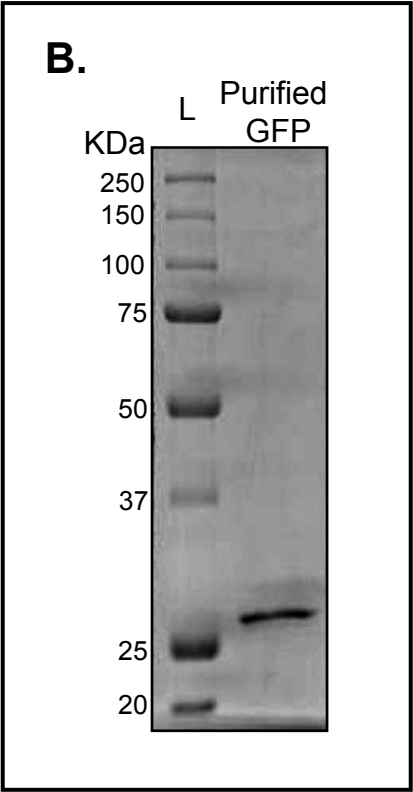
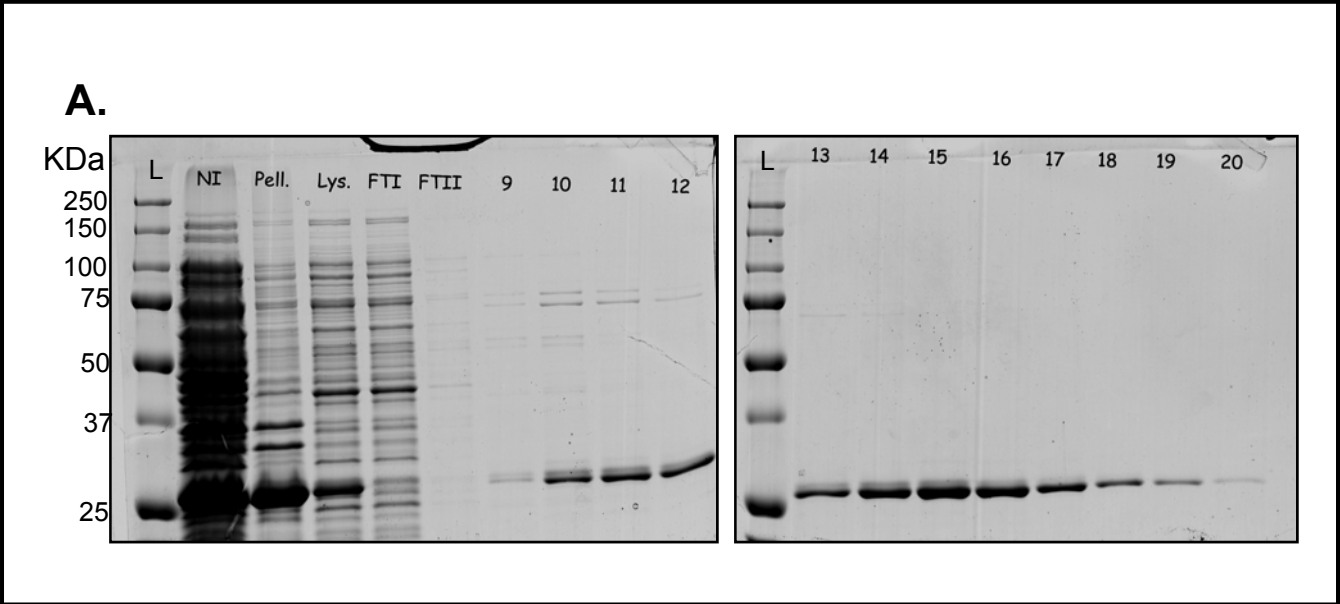


**Figure 22. Green fluorescent protein (GFP) purification. (A)** Coomassie Blue stained gels of collected samples from different steps of GFP purification process (L: ladder; NI: non-induced; Pell: pellet from IPTG induced cultures; Lys: supernatant from IPTG induced cultures; FT: flow-through and 9-20: 2  $\mu$ l of GFP purification eluates). **(B)** Coomassie Blue stained gel of 1  $\mu$ l of purified and concentrated GFP.

## VIII. Cytokeratin 18 tyrosine phosphorylation upon *L. monocytogenes* infection

CK18 was identified in a band extracted from a silver stained gel containing anti-4G10 immunoprecipitates from Caco-2 cells infected by *Listeria* (Fig.11B). To test the anti-CK18 antibody and study CK18 expression in different human cell lines, non-infected HeLa, Jeg-3 and Caco-2 cells were lysed; the total extracts were quantified and run in a gel and for WB analysis. Using specific antibodies, we detected CK18. As expected from previous Caco-2 mass spectrometry results, we detected CK18 in non-infected Caco-2 cells but also in Jeg-3 and HeLa cells. For the same amount of total protein extract expression of CK18 observed in the three cell lines showed differences. CK18 appear to be less expressed in HeLa cells (Fig.23) The levels of a control protein should be now addressed in the three cell lines to confirm that the quantity of total proteins loaded in each lane was the same. These results indicate a variable but constitutive presence of CK18 in the cell lines tested and highlights CK18 as an important cell physiological component.

To further confirm the differential phosphorylation of CK18 in response to infection, we decided to study tyrosine phosphorylation of CK18 in Caco-2 infected cells. Infection experiments with EGDe were repeated on Caco-2 cells. The concentration of total protein in the whole cell lysates, for each time-point, was quantified and 1 mg of total cell extracts was immunoprecipitated with anti-phosphotyrosine (4G10). Immunoprecipitates, containing tyrosine-phosphorylated proteins were solved by SDS-acrylamide gel proceeding for WB analysis with anti-CK18 immunoblotted membranes. As control, 50 µg of total protein extract (non-immunoprecipitated) from each time-point infection lysates were run in a SDS-acrylamide gel and also immunoblotted with anti-CK18. Remarkably, WB results showed that CK18 tyrosine phosphorylation increased during infection, being highly phosphorylated 20 min post-infection (Fig.24). The amount of CK18 in the different whole cell lysates of non-immunoprecipitated extracts was unchanged, thus ensuring that the expression of total CK18

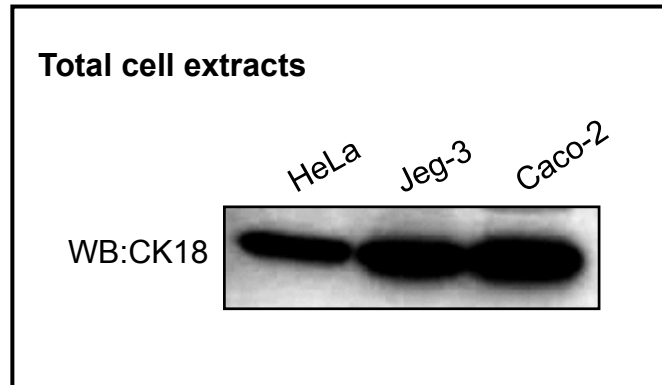


**Figure 23. CK18 expression in different cell lines.** Whole cell extracts of non-infected HeLa, Jeg-3 and Caco-2 cells were used for WB detection of CK18, using an anti-CK18 specific antibody.

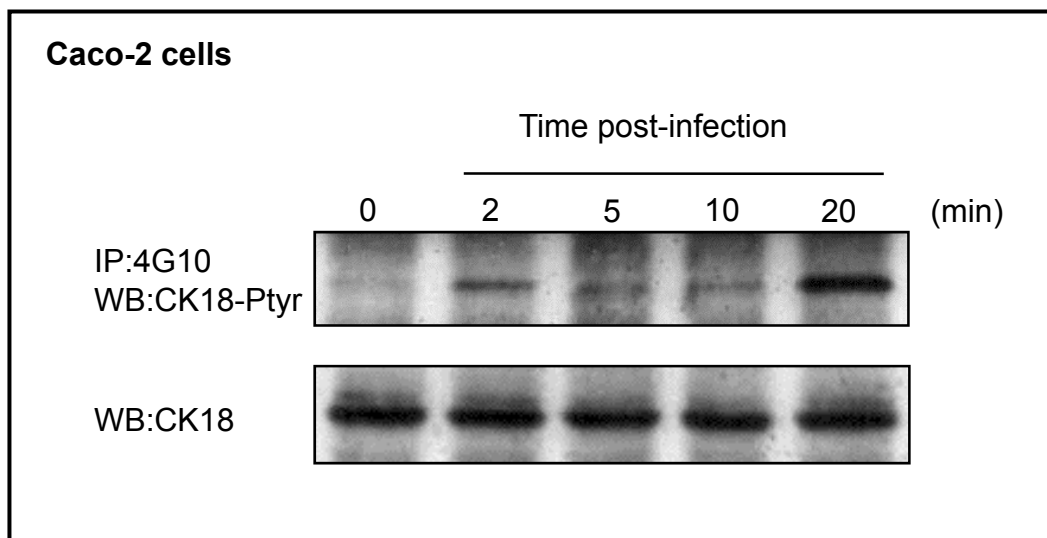


**Figure 24. CK18 tyrosine phosphorylation in response to *L. monocytogenes* infection of Caco-2.** Caco-2 cells were left non-infected (lane 0) or infected with *L. monocytogenes* during different periods of time as indicated. Cells were lysed and tyrosine phosphorylated proteins were immunoprecipitated with 4G10 antibody. CK18 (CK18-Ptyr) was detected using the anti-CK18 in the 4G10 immunoprecipitates. As control, the total CK18 (phosphorylated and non-phosphorylated) was revealed by WB in the total cell extracts.

remains the same in the course of the infection. These results associate for the first time CK18 and *Listeria* infection.

## **IX. Localization of Cytokeratin 18 in *Listeria*-infected cells**

Tyrosine phosphorylation of CK18 in response to infection suggests that this protein may play an important role in infection. As already mentioned cellular proteins required for *Listeria* entry are generally recruited at the entry site. We thus investigated the localization of endogenous CK18 during *Listeria* entry into cells. Caco-2 cells infected with *L. monocytogenes* EGDe (MOI = 50) expressing GFP were fixed in Methanol at 4°C and incubated with antibodies raised against CK18 (anti-CK18). As shown in figure 25, endogenous CK18 was recruited at the site of entry of *Listeria*. These results are in favor of an important role of CK18 in the entry process of *L. monocytogenes* into eukaryotic cells.

## **X. Role of Cytokeratin 18 in *Listeria* entry**

### **A. CK18 gene silencing by RNAi technique**

For further confirmation of CK18 requirement during infection, we decided to evaluate bacterial entry in CK18-RNAi treated cells. A single experiment was performed using CK18 small interference RNA (siRNA) on HeLa cells. siRNA was used individually in conditions to achieve the highest reduction on CK18 expression. Cells were harvested in Laemmli buffer, 72h hours after siRNA transfection, and cell lysates solved by SDS-acrylamide gel and western blotted for CK18 and  $\alpha$ -catenin. Also, at 72h post-transfection we performed an invasion assay in siRNA treated cells and evaluate the *Listeria* entry levels in order to establish a dependency on CK18. Although issue from a single experiment, these results show a significative reduction in CK18 expression, and this reduction seems to interfere with bacterial internalization.

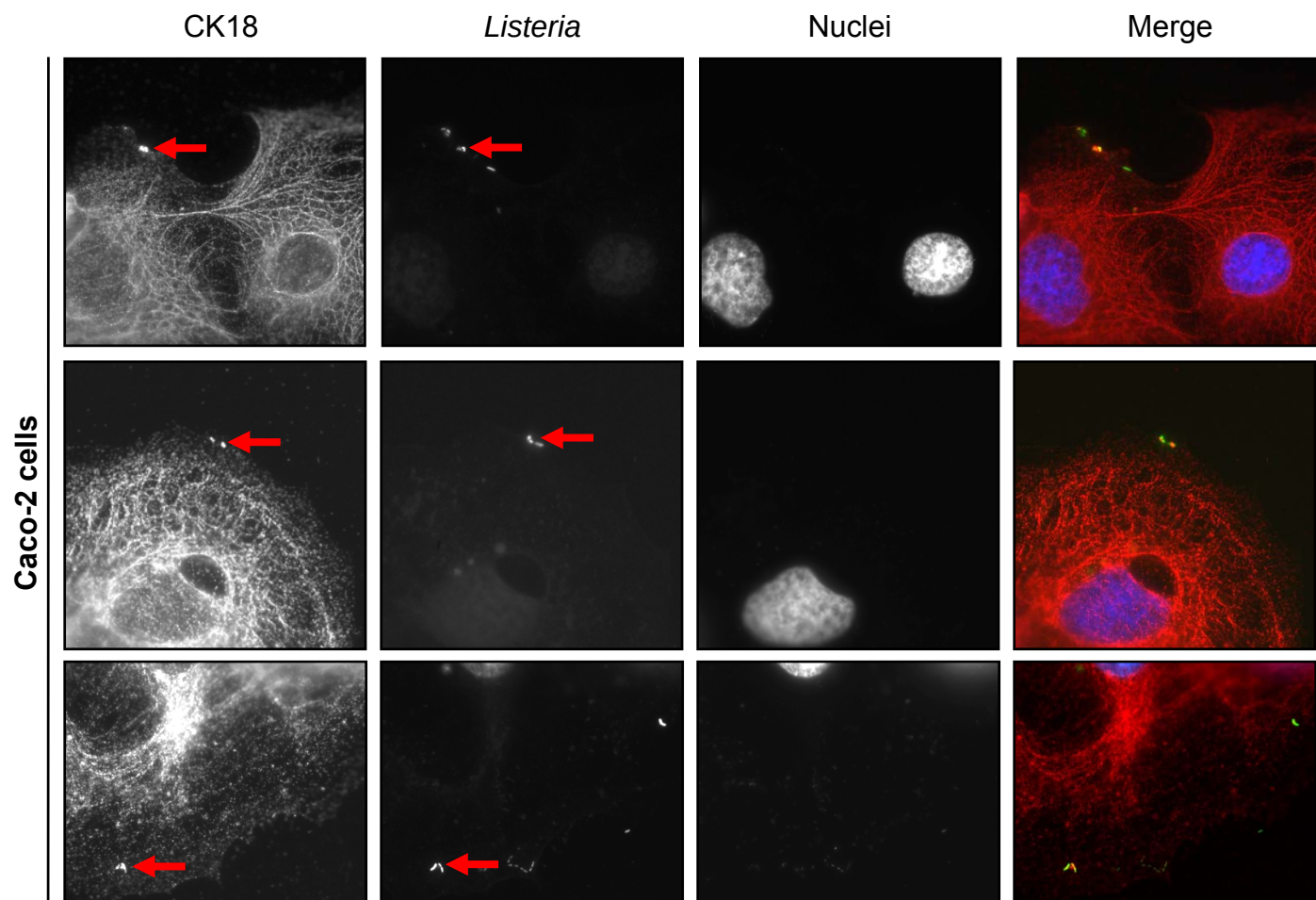


**Figure 25. Localization of CK18 in *L. monocytogenes* infected Caco-2 cells.** Immunofluorescence staining for Nuclei, CK18 and *Listeria*. Red arrows show sites where endogenous CK18 is recruited at the entry site of *L. monocytogenes* and colocalizes there with *Listeria*.

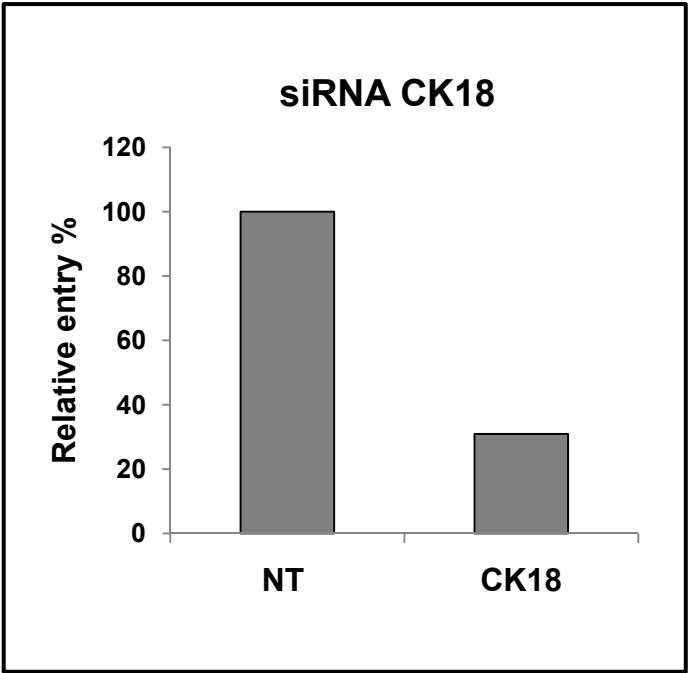
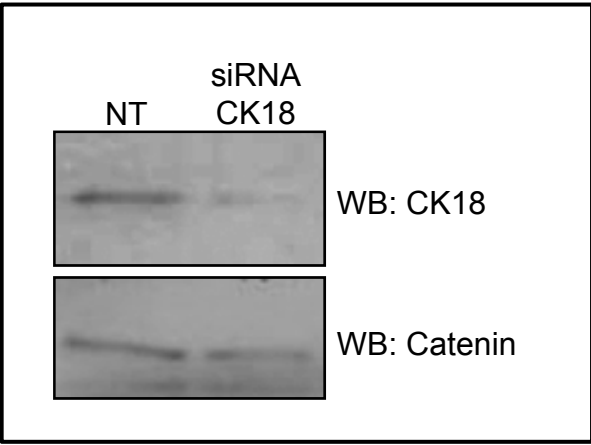


**Figure 26. Effect of CK18 knock down expression on *L. monocytogenes* internalization .** HeLa cells were left non-transfected (NT) or transfected with CK18 siRNA duplex. The amount of CK18 was checked in total protein extracts 72h post-transfection, using the anti-CK18 antibody. The same membrane was incubated with an anti- $\alpha$ -Catenin antibody for loading control. Entry levels of *L. monocytogenes* in the presence of CK18 siRNA were evaluated and compared to entry levels obtained in NT cells. Entry in NT cells has been normalized to 100 and the levels of entry in cells treated with siRNAs are expressed as relative values. Data resulted from one single experiment.

As shown in figure 26 when compared to non-transfected cells siRNA treated cells reveal a 70% reduction in bacteria internalization. This result brings considerable importance for CK18 in *Listeria* cellular infectious process.

## **XI. Identification of Cytokeratin 18 tyrosine phosphorylation sites**

As described for Myh9, we decided to study how important is the tyrosine phosphorylation of CK18 for bacterial internalization. By computational prediction (NetPhos 2.0 Server), CK18 phosphorylated tyrosine residues were listed depending on their phosphorylation potential (Fig.27). Eleven tyrosine residues compose the CK18 amino acid content and, from these, two show a high phosphorylation potential (tyrosine 13 and tyrosine 270). Further independent mutation of these two residues should be addressed to determine the implication of these phosphorylations during *Listeria* infection.

| CK18 Tyrosine predictions |                    |       |
|---------------------------|--------------------|-------|
| Position (aa)             | Context            | Score |
| 13                        | FSTN <b>Y</b> RSLG | 0.706 |
| 24                        | QAPSYGARP          | 0.434 |
| 36                        | AASVYAGAG          | 0.326 |
| 94                        | RLASYLDRV          | 0.053 |
| 129                       | DWSHYFKII          | 0.110 |
| 168                       | FRVKYETEL          | 0.390 |
| 256                       | IRAQYDELA          | 0.106 |
| 270                       | ELDK <b>Y</b> WSQQ | 0.860 |
| 331                       | VEARYALQM          | 0.092 |
| 363                       | QAQYEYALL          | 0.319 |
| 380                       | EIAT <b>Y</b> RRLI | 0.024 |

**Figure 27. Computational prediction of CK18 phosphorylated tyrosines.** The table shows the phosphorylation potential (score) of all CK18 tyrosine (Y) residues obtained in NetPhos 2.0 server. Highlighted in yellow are the tyrosines with higher scores.



# Discussion and Perspectives

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Host cell signaling involving phosphorylation cascades have long been described as preferential targets for microbial pathogens. Indeed, pathogens evolved a multitude of virulence factors that interfere with host signaling pathways in order to promote infection. In this study, we search for new host proteins/signaling pathways explored by pathogens. We used *L. monocytogenes* as a model pathogen and analyzed the tyrosine phosphorylation profiles of infected as compared to non-infected cells. We identified two differentially tyrosine-phosphorylated cytoskeletal proteins, Myh9 and CK18, whose phosphorylation was triggered in the course of the infection. In addition, we showed that both Myh9 and CK18 are recruited at the entry site of *Listeria* in infected cells and their activity appear to be required for cellular infection.

Even though the technique we used allowed the identification of two novel proteins involved in the *L. monocytogenes* infection process, we are aware that we missed certainly a great number of other differentially phosphorylated proteins.

Phosphorylation is one of the post-translational modifications that allow protein function regulation. Phosphorylations occur in serine, threonine and tyrosine residues. Tyrosine phosphorylation events occurring downstream of the interaction of a pathogen with its host are commonly described, whereas serine and threonine phosphorylations are not often reported in response to microbes. Our laboratory recently described the activation of the tyrosine kinase Src during *Listeria* invasion (Sousa et al., 2007). We thus hypothesized and investigated first the existence of uncharacterized tyrosine phosphorylation events downstream the activation of Src. In previous studies (Sousa et al., 2007), our group already used, with success, the anti-phosphotyrosine 4G10 in immunoprecipitation studies. Immunoprecipitation using 4G10 allows the concentration of the sample in tyrosine-phosphorylated proteins thus rendering their detection possible by simple silver staining. The same type of approach

using anti-phosphoserine and anti-phosphothreonine antibodies is currently ongoing in the laboratory.

In this study, the immunoprecipitated proteins have been separated in 1D acrylamide gel. A more precise method for protein separation could be made in 2D gels in which proteins not only migrate according to their molecular weight but also to their isoelectric point. The 1D gels offer poor protein resolution; indeed proteins with similar molecular weight migrate very closely and appear as a single band in the gel. In these cases, protein identification by mass spectrometry is very difficult if not impossible. To overcome this limitation, in the future our samples will be separated in 2D gels. As an alternative to the immunoprecipitation approach, we also tested phosphoprotein enrichment kits that enable efficient isolation of phosphoproteins from complex cellular extracts. Using this strategy, we got concentrated phosphoprotein pools of phosphotyrosine, phosphoserine and phosphothreonine proteins, corresponding to more complex samples and difficult to analyze as compared to the ones obtained in specific immunoprecipitation.

### **Myosin-9**

Although with technique limitations, we showed here evidence that tyrosine phosphorylation of Myh9 was triggered during *Listeria* infection, and hypothesize that this post-translational modification could be necessary for bacterial invasion. Intriguingly, Myh9 phosphorylation pattern behaved differently in HeLa and Caco-2 cells. We can argue that different cell lines use different signaling pathways and that Myh9 could be participating through different mechanisms in *Listeria* infection. Identification of common but also distinct Myh9 phosphorylated tyrosines in these two cell lines could raise the point that tyrosine phosphorylation regulation of Myh9 is achieved by different residues in infected HeLa and Caco-2 cells. In the near future, we plan to assess the role of Myh9 tyrosine phosphorylation in infection. Together with Myosin VIIa (Sousa et al., 2004), Myh9 appears as a novel class of myosins participating in the *L. monocytogenes* infectious process.

We used in this study three pharmacological inhibitors, ML-7 and ML-9 and blebbistatin which block the activity of Myh9 and showed that, in presence

of these inhibitors the *Listeria* invasiveness is highly reduced. MLCK activates Myh9 by phosphorylation. Both ML-7 and ML-9 inhibitors used are indirect inhibitors of Myh9 activity that act on MLCK. To definitively confirm the role of Myh9 activity in *Listeria* uptake, the cell invasion capacity of *Listeria* is currently being tested using a specific Myh9 inhibitor, blebbistatin. Blebbistatin is a small molecule inhibitor discovered in a screen for inhibitors of nonmuscle myosin IIA (Myh9). This molecule potently inhibits vertebrate nonmuscle myosin IIA and IIB with IC<sub>50</sub> values ranging from 0.5 to 5  $\mu$ M. Blebbistatin inhibits the actin-activated MgATPase activity and *in vitro* motility of both isoforms equivalently since it directly binds ADP-bound myosin II, locking it in the low actin affinity rate {Kovacs, 2004 #205; Straight, 2003 #208}. In cells, has been shown to have an inhibitory effect on cytokinesis but did not inhibit representative myosin superfamily members from classes I, V, and X. Blebbistatin has some photochemical properties that may affect its behavior in cells {Limouze, 2004 #207; Sakamoto, 2005 #206}. This strong susceptibility of blebbistatin adds new surveillance need during the experiments especially due to its light inactivation. Our results on 20  $\mu$ M treated cells (data not shown) are not yet conclusive since *Listeria* entry levels on HeLa and Caco-2 treated cells are not consistent from one experiment to another. New experiments using a range of blebbistatin concentrations, special handling and experimental conditions, in particular to avoid blebbistatin photoinactivation, need to be performed to obtain confident results. Jeg-3 results, although preliminary, emerged very promising, we observed a decrease in *Listeria* entry in a dose dependent manner, consistent in both experiments, and we should now use the same approach for different cell lines. Using a range of concentration values for HeLa and Caco-2 cells we aim to determine the exact effect of this inhibitor in these cell lines. The role of Myh9 activity in the uptake of *Listeria* was also addressed by small interference RNA (siRNA) experiments. As we show, using a siRNA approach we obtained a 50% reduction in the expression of Myh9. The complete silencing of a gene is some times very difficult to obtain using siRNA; it depends on protein stability and varies from one cell type to another. Our laboratory has recently shown that a reduced expression of cortactin, even when not completely, contributes to a two-fold reduction in *Listeria* entry (Sousa et al., 2007). Taking this in account, we started evaluating the internalization of *Listeria* in cells showing a reduction

of 50% in Myh9 expression. Intriguingly our invasion assay results for this reduction consistently lead to an increase of *Listeria* entry into the cells. These results are in contradiction with results obtained with chemical inhibition. However one should consider that while in chemical inhibition we are inactivating both IIA and IIB myosin isoforms in siRNA we are specifically depleting isoform IIA. This is crucial in the understanding of a possible compensatory effect of the isoform IIB in the absence of IIA. Inhibiting both isoforms in a blebbistatin scenario we are effectively inactivating myosin function and therefore restrain *Listeria* internalization. In siRNA experiments we are specifically down regulating the expression of isoform IIA, and we could imagine that isoform IIB functionally replaces the absence of IIA. On the other hand depletion of isoform IIA has been shown to result in increases in protrusive activity {Sandquist, 2008 #209}. Together with a possible compensation of the still expressed isoform IIB we might be favoring bacteria internalization. We will further deplete isoform IIB or both IIA and IIB and evaluate *Listeria* infection in these conditions. Nevertheless, our results emphasize Myh9 importance in host cell machinery during *Listeria* infection.

Myosin is a mechanochemical transducer and serves as a motor for various motile activities, such as cell migration, cytokinesis, phagocytosis, maintenance of cell shape, and morphogenesis (Yumura and Uyeda, 2003).

Myosin 9 belongs to the myosin II subfamily of myosins that also includes skeletal, cardiac and smooth muscle myosins. All the members of the myosin II subfamily are formed by myosin light chains (MLC), which are very tightly but non-covalently bound to myosin heavy chains (MHC). MLC are stabilizers and regulators of myosin structure and activity. Regulation of myosin function is driven by MLC phosphorylation. Similarly to other myosins, myosins II bind to actin, hydrolyze ATP converting this way chemical energy into mechanical force and movement. Besides this motor activity, myosins II are also involved in filament formation through their tail domain and both myosin functions are dependent on actin-binding (Conti and Adelstein, 2008). There are three isoforms of nonmuscle myosins IIA (Myh9), IIB and IIC, being IIA the most abundant in cells, and each of them have specific cell localization and function (Conti et al., 2008; Krendel and Mooseker, 2005; Sellers, 2000). Myh9 has been widely studied, appearing now as an active participant in different but related

cell processes. In phagocytosis, Myh9 has been implicated in the phagocytic cup squeezing. Inhibition of Myh9 activation driven by MLCK does not affect pseudopod formation but prevents closure of phagocytic cups (Araki, 2006; Araki et al., 2003). Other studies have shown that Myh9 is one of the major regulators of cell migration. They showed that Myh9-deficient cells present broader lamellipodia and did not retract their trailing edges. These results associate Myh9 to cell protrusions, controlling their speed, stepwise pattern of extension and retraction (Vicente-Manzanares et al., 2007).

Cellular actomyosin and microtubule systems maintain a dynamic balance essential for cell contractility, polarization and migration. Recent studies indicate that Myh9 restrains random cell migration by a crosstalk with the microtubule system. A recent report showed that Myh9-deficient cells display pronounced contractile defects, increased non-directional migration and non-polarized membrane ruffling associated with stabilization of microtubules in lamellae (Even-Ram et al., 2007). *Listeria*, as already mentioned, infects cell in a stepwise mode in which a parallelism can be done with classical phagocytosis and its preceding cytoskeleton rearrangements. Pseudopod extension and phagocytic cup formation around the target predictably requires extensive remodeling of the actin cytoskeleton. *Listeria*, as many other pathogens, take advantage of host cellular processes in benefit of infection. We here identify Myh9 as a participant in *Listeria* infection. We can thus postulate that Myh9, by participating in membrane ruffling, protrusion formation and phagocytic uptake, plays a role in bacterial engulfment.

So far, regulation of Myh9 activity has only been associated with phosphorylation in serine and threonine residues at its regulatory light chain. Only a few reports have identified Myh9 phosphorylated tyrosine residues. The identification of Myh9 phosphotyrosine residues appears as a result of comparison between phosphorylation patterns in normal cell lines and cancer cell lines and does not make any association with regulatory roles of Myh9 (Guo et al., 2008; Rikova et al., 2007). The correlation between tyrosine phosphorylation and regulation of Myh9 activity has been briefly covered. A single report suggested that Myh9 is a target for protein tyrosine phosphatase SHP-1 during B-cell activation (Baba et al., 2003). Interesting studies on phosphotyrosine activation of Myh9 have recently pointed the role for phospho-

regulation of Myh9 in phagocytosis. Point mutations in two putative phosphosites of Myh9, the motor domain tyrosine 278 and the tail domain tyrosine 1805, lead to no significant localization of Myh9 in phagocytic synapse. This Myh9 regulation is driven by MHC and differs from the usually reported regulation occurring through the light chain (Conti and Adelstein, 2008; Tsai and Discher, 2008). Interestingly, tyrosine 1805 was one of the phosphorylated residues that we identified experimentally in Caco-2 infected cells, however a theoretical phosphorylation potential below the 0.5 threshold excluded this residue from our potential phosphorylation list. It seems now pertinent to re-evaluate the phosphorylation pattern concerning this specific residue. This residue will be included in a Myh9 direct mutagenesis approach.

Recently, it has been suggested that *Salmonella* effector SopB phosphorylates serine and threonine residues at myosin light chain (MLC) of Myh9. Surprisingly, the authors were unable to co-localize Myh9 with *Salmonella* inside the cells. However, the activated Myh9 was reported to play a role in controlling dynamics of *Salmonella*-containing vacuoles during infection (Wasylnka et al., 2008). Here we demonstrate by immunofluorescence that Myh9 colocalize with *Listeria* at the entry site and postulate that by being recruited Myh9 might play a role in the dynamics of *Listeria* infection.

We will in the near future concentrate our efforts in two different topics: (1) identification of the Myh9 tyrosine residues that are phosphorylated during infection and (2) identification of Myh9-interacting proteins important in *Listeria* invasion. We performed a first identification of tyrosine-phosphorylated sites that has to be further confirmed. Identification of Myh9 phosphorylated tyrosine residues was achieved from the immunoprecipitated Myh9 that includes both phosphorylated and non-phosphorylated states of the protein. This limitation, together with the low IP capacity of the anti-Myh9 antibody, could interfere with a consistent identification of tyrosine-phosphorylated residues. To overcome this difficulty, the 4G10 antibody even though not Myh9-specific, could be used in order to select only the Myh9 tyrosine-phosphorylated form. Otherwise, Myh9 could be expressed in cells as a protein fusion with GFP, and after infection the Myh9-GFP could be immunoprecipitated from total cell extracts, using an anti-GFP antibody. The Myh9-GFP would then be analyzed by mass spectrometry to identify the phosphorylated residues. This technique (LAP technique) will be

used in order to identify the proteins interacting with Myh9. We expect that the proteins interacting with Myh9 will co-purify with Myh9 in immunoprecipitation experiments. The immunoprecipitates will then be separated in acrylamide gels and silver-stained. Using mass spectrometry analysis, we aim to identify all the proteins that co-immunoprecipitate with Myh9.

### **Cytokeratin 18**

We present here the first evidence for tyrosine phosphorylation of CK18. We observed not only a tyrosine phosphorylation of CK18, but we also identified a variable CK18 tyrosine phosphorylation pattern dependent on *Listeria* infection. In our first experiments using 4G10 immunoprecipitates (Fig.11B), we observed the disappearance of the band supposed to correspond to CK18 during infection. Intriguingly, WB confirmation with anti-CK18 (Fig.24) showed a different CK18 phosphorylation behavior. Indeed, figure 24 shows an increase in CK18 tyrosine phosphorylation over time of infection. Considering the higher specificity of the WB strategy, we consider that CK18 appeared clearly tyrosine-phosphorylated upon *Listeria* uptake and predict a role of CK18 phosphorylation/activation in *Listeria* infection.

IF 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). The diversity of epithelial functions is reflected by the expression of distinct keratin pairs that are responsible to protect epithelial cells against mechanical stress and to act as signaling effectors. In addition to these structural aspects of keratin function, experimental evidence has led to a regulatory view of the keratin cytoskeleton. Distinct keratins emerge as protein scaffolds in different settings and contribute to cell size determination, translation control, proliferation, cell type-specific organelle transport, malignant transformation and various stress responses. All of these properties are controlled by highly complex patterns of phosphorylation and molecular associations (Magin et al., 2007).

Keratin phosphorylation occurs within the tail and/or head domains of all keratins that have been examined. Several serine phosphorylation sites and

some of the relevant kinases have been characterized in CK8 and CK18. Functions of keratin phosphorylation that have significant experimental support include a role in filament solubility and reorganization and a role in regulating keratin binding with other cytoplasmic proteins. Other associations with keratin phosphorylation include protection against cell stress, cell signaling, apoptosis, and cell compartment-specific roles (Omary et al., 2006).

CK18 is typically co-expressed with keratin CK8, constituting the primary keratin pair of simple epithelial cells. Interestingly, CK8 and CK18 may play a role in the regulation of the cell cycle, whereby serine phosphorylation of these keratins promotes binding of 14-3-3 adaptor proteins affecting CK18 organization and distribution (Hyder et al., 2008; Moll et al., 2008). 14-3-3 proteins are a family of regulatory molecules that bind to functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors. To date, more than 300 binding partners have been identified, of which most are phosphoproteins. It has become clear that 14-3-3 proteins are involved in the regulation of most cellular processes, including several metabolic pathways, redox-regulation, transcription, RNA processing, protein synthesis, protein folding and degradation, cell cycle, cytoskeletal organization and cellular trafficking (Kjarland et al., 2006; Mhawech, 2005).

Previous studies have shown that both *Salmonella* and EPEC infections require CK18. A yeast two-hybrid assay revealed CK18 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 CK18, 14-3-3 and EspF may form a complex in EPEC infected cells (Viswanathan et al., 2004). Others identified CK18 as a novel Tir partner protein which is recruited to EPEC-induced pedestals (Batchelor et al., 2004). Moreover, it has been demonstrated that 14-3-3 is recruited to the site of pedestal and can bind specifically to Tir (Patel et al., 2006). *Salmonella* invasion was inhibited in cells expressing dominant negative derivatives of CK18. Moreover, SspC/SipC effector protein presents CK8 and CK18 as potential interactors (Carlson et al., 2002; Scherer et al., 2000).

Recent studies show that CK8/CK18 provides a cytoskeletal network in simple epithelial cells as a response to cellular mechanical stress applied on integrins at focal adhesions. Based on knock-down expression studies it was

proposed that CK8/CK18 loss of function affects the mechano-transduction triggered at integrins (Bordeleau et al., 2008).

Others have previously linked CK8/CK18 in cell spreading and motility. In terms of molecular regulation, the head and tail domains of CK8 and CK18 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). The dynamics of CK8/CK18 is controlled by kinases that are themselves involved in signaling pathways. It is suggested that CK8/CK18 may act as modulators of signaling pathways. Knock-down experiments lead to a decreased cell spread. This effect is believed to be due to alterations in the bidirectional link between signal transducers and integrins at focal adhesions. In fact, it appears that in CK8/CK18-free cells integrin  $\beta 1$  responds to “inside-out” signals with an increase affinity for a fibronectin substrate (Galarneau et al., 2007).

By a functional parallelism between cadherin and integrins, it sounds reasonable to believe that CK18 is activated by a *Listeria* stimulus at the cell surface. Considering CK8/CK18 participation in normal cell processes, such as signal transduction and cell motility, *Listeria* might use these physiological mechanisms in order to promote its internalization. Here we complement this idea with the fact that CK18 is tyrosine-phosphorylated upon infection. In the near future, we aim to decipher how this phosphorylation in tyrosines is directly linked to the infection. For this, we are now in the process of identification of CK18 tyrosine residues that are phosphorylated in infected cells.

The variability of the physiological amounts of CK18 shown here in non-infected cells, appearing less expressed in HeLa cells and in more representative amounts on Caco-2 cells reinforces the fact that the mechanisms by which *Listeria* enters the cells is completely cell type dependent. Pathogen internalization relies this way in the availability of proteins in the infected cell. The role of CK18 in the *Listeria* infection is now being addressed. Endogenous CK18 localization studies present here for the first time evidence that in *Listeria* infected cells CK18 is recruited at the bacteria entry sites co-localizing with the pathogen. We were also able to show that the entry levels of *Listeria* in CK18-siRNA-transfected cells appear highly reduced and altogether these preliminary

results support a dependency of *Listeria* internalization on CK18 recruitment and phosphorylation.

As mentioned above, keratins work in pairs and CK18 partner is CK8. CK8 will therefore be a target of study, CK8-siRNA experiments will be performed and the entry level of *Listeria* in cells showing reduced CK8 expression will be evaluated. These experiments should give insights in the possible cooperative function of CK18 and CK8 during *Listeria* infection. As described for Myh9, the same approach will be followed with CK18 in order to identify the CK18 protein partners that play critical roles in response to *Listeria* infection.

Our long term goal is to integrate both Myh9 and CK18 in signaling cascades and try to decipher which cell receptors are upstream of the triggering host-pathogen interaction. In this context we will study the effect of HGF treatment in the tyrosine phosphorylation pattern of Myh9 and CK18. Upon HGF-treatment, c-Met present at the surface of cells is activated and triggers a phosphorylation signaling cascade. If a similar Myh9 or CK18 tyrosine phosphorylation pattern is observed, it will be a clue for the signaling pathway activated in our infection experiments.

We also plan to test whether or not the same newly identified host signaling pathways are exploited by other bacterial pathogens, such as *Salmonella*, *Shigella*, *Yersinia* and pathogenic *E. coli*. Cells will be infected with wild-type strains of the different pathogens and we will investigate the tyrosine phosphorylation pattern of Myh9 and CK18 in response to those infections.

Importantly, in this study we identified two novel host proteins tyrosine-phosphorylated in response to *Listeria* infection, Myh9 and CK18. Their activity was shown necessary for the entry process. Whether the tyrosine phosphorylation is implicated in this process remains to be clarified.

Further exploitation of the results presented here should contribute to the identification of new signaling cascades hijacked by pathogens to promote host colonization.

# References

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- Akhtar, N., and Hotchin, N.A. (2001). RAC1 regulates adherens junctions through endocytosis of E-cadherin. *Mol Biol Cell* 12, 847-862.
- Al-Haddad, A., Shonn, M.A., Redlich, B., Blocker, A., Burkhardt, J.K., Yu, H., Hammer, J.A., 3rd, Weiss, D.G., Steffen, W., Griffiths, G., *et al.* (2001). Myosin Va bound to phagosomes binds to F-actin and delays microtubule-dependent motility. *Mol Biol Cell* 12, 2742-2755.
- Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walte, r.P. (2007). The Cytoskeleton. *Molecular Biology of the Cell - Fifth Edition*, 983-985.
- Alrutz, M.A., and Isberg, R.R. (1998). Involvement of focal adhesion kinase in invasin-mediated uptake. *Proc Natl Acad Sci U S A* 95, 13658-13663.
- Alto, N.M., Shao, F., Lazar, C.S., Brost, R.L., Chua, G., Mattoo, S., McMahon, S.A., Ghosh, P., Hughes, T.R., Boone, C., *et al.* (2006). Identification of a bacterial type III effector family with G protein mimicry functions. *Cell* 124, 133-145.
- Amieva, M.R., and El-Omar, E.M. (2008). Host-bacterial interactions in *Helicobacter pylori* infection. *Gastroenterology* 134, 306-323.
- Araki, N. (2006). Role of microtubules and myosins in Fc gamma receptor-mediated phagocytosis. *Front Biosci* 11, 1479-1490.
- Araki, N., Hatae, T., Furukawa, A., and Swanson, J.A. (2003). Phosphoinositide-3-kinase-independent contractile activities associated with Fcgamma-receptor-mediated phagocytosis and macropinocytosis in macrophages. *J Cell Sci* 116, 247-257.
- Autenrieth, S.E., Soldanova, I., Rosemann, R., Gunst, D., Zahir, N., Kracht, M., Ruckdeschel, K., Wagner, H., Borgmann, S., and Autenrieth, I.B. (2007). *Yersinia enterocolitica* YopP inhibits MAP kinase-mediated antigen uptake in dendritic cells. *Cell Microbiol* 9, 425-437.
- Axline, S.G., and Reaven, E.P. (1974). Inhibition of phagocytosis and plasma membrane mobility of the cultivated macrophage by cytochalasin B. Role of subplasmalemmal microfilaments. *J Cell Biol* 62, 647-659.
- Baba, T., Fusaki, N., Shinya, N., Iwamatsu, A., and Hozumi, N. (2003). Myosin is an in vivo substrate of the protein tyrosine phosphatase (SHP-1) after mIgM cross-linking. *Biochem Biophys Res Commun* 304, 67-72.
- Backert, S., Feller, S.M., and Wessler, S. (2008). Emerging roles of Abl family tyrosine kinases in microbial pathogenesis. *Trends Biochem Sci* 33, 80-90.
- Backert, S., and Selbach, M. (2008). Role of type IV secretion in *Helicobacter pylori* pathogenesis. *Cell Microbiol* 10, 1573-1581.
- Bahler, M. (1996). Myosins on the move to signal transduction. *Curr Opin Cell Biol* 8, 18-22.

- Bahler, M. (2000). Are class III and class IX myosins motorized signalling molecules? *Biochim Biophys Acta* 1496, 52-59.
- Batchelor, M., Guignot, J., Patel, A., Cummings, N., Cleary, J., Knutton, S., Holden, D.W., Connerton, I., and Frankel, G. (2004). Involvement of the intermediate filament protein cytokeratin-18 in actin pedestal formation during EPEC infection. *EMBO Rep* 5, 104-110.
- Berg, J.S., Powell, B.C., and Cheney, R.E. (2001). A millennial myosin census. *Mol Biol Cell* 12, 780-794.
- Bhavsar, A.P., Guttman, J.A., and Finlay, B.B. (2007). Manipulation of host-cell pathways by bacterial pathogens. *Nature* 449, 827-834.
- Bierne, H., Gouin, E., Roux, P., Caroni, P., Yin, H.L., and Cossart, P. (2001). A role for cofilin and LIM kinase in *Listeria*-induced phagocytosis. *J Cell Biol* 155, 101-112.
- Bierne, H., Miki, H., Innocenti, M., Scita, G., Gertler, F.B., Takenawa, T., and Cossart, P. (2005). WASP-related proteins, *Abi1* and *Ena/VASP* are required for *Listeria* invasion induced by the *Met* receptor. *J Cell Sci* 118, 1537-1547.
- Bierne, H., Sabet, C., Personnic, N., and Cossart, P. (2007). Internalins: a complex family of leucine-rich repeat-containing proteins in *Listeria monocytogenes*. *Microbes Infect* 9, 1156-1166.
- Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G.F. (2003). *Met*, metastasis, motility and more. *Nat Rev Mol Cell Biol* 4, 915-925.
- Bonazzi, M., Veiga, E., Cerda, J.P., and Cossart, P. (2008). Successive post-translational modifications of E-cadherin are required for *InlA*-mediated internalisation of *Listeria monocytogenes*. *Cell Microbiol*.
- Bordeleau, F., Bessard, J., Sheng, Y., and Marceau, N. (2008). Keratin contribution to cellular mechanical stress response at focal adhesions as assayed by laser tweezers. *Biochem Cell Biol* 86, 352-359.
- Bougneres, L., Girardin, S.E., Weed, S.A., Karginov, A.V., Olivo-Marin, J.C., Parsons, J.T., Sansonetti, P.J., and Van Nhieu, G.T. (2004). Cortactin and *Crk* cooperate to trigger actin polymerization during *Shigella* invasion of epithelial cells. *J Cell Biol* 166, 225-235.
- Braun, L., Ghebrehiwet, B., and Cossart, P. (2000). *gC1q-R/p32*, a C1q-binding protein, is a receptor for the *InlB* invasion protein of *Listeria monocytogenes*. *EMBO J* 19, 1458-1466.
- Braun, L., Nato, F., Payrastre, B., Mazie, J.C., and Cossart, P. (1999). The 213-amino-acid leucine-rich repeat region of the *Listeria monocytogenes* *InlB* protein is sufficient for entry into mammalian cells, stimulation of PI 3-kinase and membrane ruffling. *Mol Microbiol* 34, 10-23.
- Cabanes, D., Dehoux, P., Dussurget, O., Frangeul, L., and Cossart, P. (2002). Surface proteins and the pathogenic potential of *Listeria monocytogenes*. *Trends Microbiol* 10, 238-245.
- Cardelli, J. (2001). Phagocytosis and macropinocytosis in *Dictyostelium*: phosphoinositide-based processes, biochemically distinct. *Traffic* 2, 311-320.

Carlson, S.A., Omary, M.B., and Jones, B.D. (2002). Identification of cytokeratins as accessory mediators of Salmonella entry into eukaryotic cells. *Life Sci* 70, 1415-1426.

Caulin, C., Ware, C.F., Magin, T.M., and Oshima, R.G. (2000). Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. *J Cell Biol* 149, 17-22.

Conti, M.A., and Adelstein, R.S. (2008). Nonmuscle myosin II moves in new directions. *J Cell Sci* 121, 11-18.

Conti, M.A., Kawamoto, S., and Adelstein, R.S. (2008). Nonmuscle myosin II. In *Myosins: A superfamily of Molecular Motors, Vol.7, Proteins and Cell Regulation* (ed. L. M. Coluccio). pp. 223-264. Dordrecht: Springer.

Copp, J., Marino, M., Banerjee, M., Ghosh, P., and van der Geer, P. (2003). Multiple regions of internalin B contribute to its ability to turn on the Ras-mitogen-activated protein kinase pathway. *J Biol Chem* 278, 7783-7789.

Cossart, P. (2007). Listeriology (1926-2007): the rise of a model pathogen. *Microbes Infect* 9, 1143-1146.

Cossart, P., and Sansonetti, P.J. (2004). Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science* 304, 242-248.

Covacci, A., Telford, J.L., Del Giudice, G., Parsonnet, J., and Rappuoli, R. (1999). Helicobacter pylori virulence and genetic geography. *Science* 284, 1328-1333.

Cox, D., Berg, J.S., Cammer, M., Chingwundoh, J.O., Dale, B.M., Cheney, R.E., and Greenberg, S. (2002). Myosin X is a downstream effector of PI(3)K during phagocytosis. *Nat Cell Biol* 4, 469-477.

Cox, D., Tseng, C.C., Bjekic, G., and Greenberg, S. (1999). A requirement for phosphatidylinositol 3-kinase in pseudopod extension. *J Biol Chem* 274, 1240-1247.

Crabtree, J.E., and Naumann, M. (2006). Epithelial Cell Signaling in Helicobacter pylori infection. *Current Signal Transduction Therapy* 1, 53-65.

Diakonova, M., Bokoch, G., and Swanson, J.A. (2002). Dynamics of cytoskeletal proteins during Fcγ receptor-mediated phagocytosis in macrophages. *Mol Biol Cell* 13, 402-411.

Domann, E., Wehland, J., Rohde, M., Pistor, S., Hartl, M., Goebel, W., Leimeister-Wachter, M., Wuenscher, M., and Chakraborty, T. (1992). A novel bacterial virulence gene in Listeria monocytogenes required for host cell microfilament interaction with homology to the proline-rich region of vinculin. *EMBO J* 11, 1981-1990.

Dramsi, S., Biswas, I., Maguin, E., Braun, L., Mastroeni, P., and Cossart, P. (1995). Entry of Listeria monocytogenes into hepatocytes requires expression of InlB, a surface protein of the internalin multigene family. *Mol Microbiol* 16, 251-261.

Drees, F., Pokutta, S., Yamada, S., Nelson, W.J., and Weis, W.I. (2005). Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* 123, 903-915.

Dussurget, O., Pizarro-Cerda, J., and Cossart, P. (2004). Molecular determinants of *Listeria monocytogenes* virulence. *Annu Rev Microbiol* 58, 587-610.

Evangelista, M., Klebl, B.M., Tong, A.H., Webb, B.A., Leeuw, T., Leberer, E., Whiteway, M., Thomas, D.Y., and Boone, C. (2000). A role for myosin-I in actin assembly through interactions with Vrp1p, Bee1p, and the Arp2/3 complex. *J Cell Biol* 148, 353-362.

Even-Ram, S., Doyle, A.D., Conti, M.A., Matsumoto, K., Adelstein, R.S., and Yamada, K.M. (2007). Myosin IIA regulates cell motility and actomyosin-microtubule crosstalk. *Nat Cell Biol* 9, 299-309.

Ferracini, R., Longati, P., Naldini, L., Vigna, E., and Comoglio, P.M. (1991). Identification of the major autophosphorylation site of the Met/hepatocyte growth factor receptor tyrosine kinase. *J Biol Chem* 266, 19558-19564.

Fournier, T.M., Lamorte, L., Maroun, C.R., Lupher, M., Band, H., Langdon, W., and Park, M. (2000). Cbl-transforming variants trigger a cascade of molecular alterations that lead to epithelial mesenchymal conversion. *Mol Biol Cell* 11, 3397-3410.

Franke, R., Muller, M., Wundrack, N., Gilles, E.D., Klamt, S., Kahne, T., and Naumann, M. (2008). Host-pathogen systems biology: logical modelling of hepatocyte growth factor and *Helicobacter pylori* induced c-Met signal transduction. *BMC Syst Biol* 2, 4.

Fujita, Y., Krause, G., Scheffner, M., Zechner, D., Leddy, H.E., Behrens, J., Sommer, T., and Birchmeier, W. (2002). Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat Cell Biol* 4, 222-231.

Gaillard, J.L., Berche, P., Frehel, C., Gouin, E., and Cossart, P. (1991). Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell* 65, 1127-1141.

Galan, J.E., and Curtiss, R., 3rd (1989). Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. *Proc Natl Acad Sci U S A* 86, 6383-6387.

Galan, J.E., and Zhou, D. (2000). Striking a balance: modulation of the actin cytoskeleton by *Salmonella*. *Proc Natl Acad Sci U S A* 97, 8754-8761.

Galarneau, L., Loranger, A., Gilbert, S., and Marceau, N. (2007). Keratins modulate hepatic cell adhesion, size and G1/S transition. *Exp Cell Res* 313, 179-194.

Garcia-Guzman, M., Larsen, E., and Vuori, K. (2000). The proto-oncogene c-Cbl is a positive regulator of Met-induced MAP kinase activation: a role for the adaptor protein Crk. *Oncogene* 19, 4058-4065.

Gates, J., and Peifer, M. (2005). Can 1000 reviews be wrong? Actin, alpha-Catenin, and adherens junctions. *Cell* 123, 769-772.

Ghazizadeh, S., and Fleit, H.B. (1994). Tyrosine phosphorylation provides an obligatory early signal for Fc gamma RII-mediated endocytosis in the monocytic cell line THP-1. *J Immunol* 152, 30-41.

Graf, B., Bahler, M., Hilpela, P., Bowe, C., and Adam, T. (2000). Functional role for the class IX myosin myr5 in epithelial cell infection by *Shigella flexneri*. *Cell Microbiol* 2, 601-616.

Griffin, F.M., Jr., Griffin, J.A., Leider, J.E., and Silverstein, S.C. (1975). Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. *J Exp Med* 142, 1263-1282.

Groves, E., Dart, A.E., Covarelli, V., and Caron, E. (2008). Molecular mechanisms of phagocytic uptake in mammalian cells. *Cell Mol Life Sci* 65, 1957-1976.

Gruenheid, S., DeVinney, R., Blatt, F., Goosney, D., Gelkop, S., Gish, G.D., Pawson, T., and Finlay, B.B. (2001). Enteropathogenic *E. coli* Tir binds Nck to initiate actin pedestal formation in host cells. *Nat Cell Biol* 3, 856-859.

Guo, A., Villen, J., Kornhauser, J., Lee, K.A., Stokes, M.P., Rikova, K., Possemato, A., Nardone, J., Innocenti, G., Wetzel, R., *et al.* (2008). Signaling networks assembled by oncogenic EGFR and c-Met. *Proc Natl Acad Sci U S A* 105, 692-697.

Hackstadt, T. (1996). The biology of rickettsiae. *Infect Agents Dis* 5, 127-143.

Halbleib, J.M., and Nelson, W.J. (2006). Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev* 20, 3199-3214.

Hamon, M.A., Batsche, E., Regnault, B., Tham, T.N., Seveau, S., Muchardt, C., and Cossart, P. (2007). Histone modifications induced by a family of bacterial toxins. *Proc Natl Acad Sci U S A* 104, 13467-13472.

Hesse, M., Franz, T., Tamai, Y., Taketo, M.M., and Magin, T.M. (2000). Targeted deletion of keratins 18 and 19 leads to trophoblast fragility and early embryonic lethality. *EMBO J* 19, 5060-5070.

Higgins, J.M., Mandlebrot, D.A., Shaw, S.K., Russell, G.J., Murphy, E.A., Chen, Y.T., Nelson, W.J., Parker, C.M., and Brenner, M.B. (1998). Direct and regulated interaction of integrin alphaEbeta7 with E-cadherin. *J Cell Biol* 140, 197-210.

Howard, J. (1997). Molecular motors: structural adaptations to cellular functions. *Nature* 389, 561-567.

Hu, L.T., Foxall, P.A., Russell, R., and Mobley, H.L. (1992). Purification of recombinant *Helicobacter pylori* urease apoenzyme encoded by ureA and ureB. *Infect Immun* 60, 2657-2666.

Hufnagel, P., and Rabus, R. (2006). Mass spectrometric identification of proteins in complex post-genomic projects. Soluble proteins of the metabolically versatile, denitrifying '*Aromatoleum*' sp. strain EbN1. *J Mol Microbiol Biotechnol* 11, 53-81.

Hyder, C.L., Pallari, H.M., Kochin, V., and Eriksson, J.E. (2008). Providing cellular signposts--post-translational modifications of intermediate filaments. *FEBS Lett* 582, 2140-2148.

Iizumi, Y., Sagara, H., Kabe, Y., Azuma, M., Kume, K., Ogawa, M., Nagai, T., Gillespie, P.G., Sasakawa, C., and Handa, H. (2007). The enteropathogenic *E. coli* effector EspB facilitates microvillus effacing and antiphagocytosis by inhibiting myosin function. *Cell Host Microbe* 2, 383-392.

Ireton, K., Payrastre, B., Chap, H., Ogawa, W., Sakaue, H., Kasuga, M., and Cossart, P. (1996). A role for phosphoinositide 3-kinase in bacterial invasion. *Science* 274, 780-782.

Ivaska, J., Pallari, H.M., Nevo, J., and Eriksson, J.E. (2007). Novel functions of vimentin in cell adhesion, migration, and signaling. *Exp Cell Res* 313, 2050-2062.

Izard, T., Tran Van Nhieu, G., and Bois, P.R. (2006). *Shigella* applies molecular mimicry to subvert vinculin and invade host cells. *J Cell Biol* 175, 465-475.

Jaquemar, D., Kupriyanov, S., Wankell, M., Avis, J., Benirschke, K., Baribault, H., and Oshima, R.G. (2003). Keratin 8 protection of placental barrier function. *J Cell Biol* 161, 749-756.

Johansson, J., Mandin, P., Renzoni, A., Chiaruttini, C., Springer, M., and Cossart, P. (2002). An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* 110, 551-561.

Jonquieres, R., Bierne, H., Fiedler, F., Gounon, P., and Cossart, P. (1999). Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of gram-positive bacteria. *Mol Microbiol* 34, 902-914.

Jonquieres, R., Pizarro-Cerda, J., and Cossart, P. (2001). Synergy between the N- and C-terminal domains of InlB for efficient invasion of non-phagocytic cells by *Listeria monocytogenes*. *Mol Microbiol* 42, 955-965.

Karecla, P.I., Green, S.J., Bowden, S.J., Coadwell, J., and Kilshaw, P.J. (1996). Identification of a binding site for integrin  $\alpha$ 5 $\beta$ 1 in the N-terminal domain of E-cadherin. *J Biol Chem* 271, 30909-30915.

Khelef, N., Lecuit, M., Buchrieser, C., Cabanes, D., Dussurget, O., and Cossart, P. (2004). *Listeria monocytogenes* and the Genus *Listeria*. In *The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community*, M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, eds. (New York, Springer), p. <http://141.150.157.117:8080/prokPUB.index.htm>.

Kim, S., and Coulombe, P.A. (2007). Intermediate filament scaffolds fulfill mechanical, organizational, and signaling functions in the cytoplasm. *Genes Dev* 21, 1581-1597.

Kim, S., Wong, P., and Coulombe, P.A. (2006). A keratin cytoskeletal protein regulates protein synthesis and epithelial cell growth. *Nature* 441, 362-365.

Kjarland, E., Keen, T.J., and Kleppe, R. (2006). Does isoform diversity explain functional differences in the 14-3-3 protein family? *Curr Pharm Biotechnol* 7, 217-223.

Kocks, C., Gouin, E., Tabouret, M., Berche, P., Ohayon, H., and Cossart, P. (1992). *L. monocytogenes*-induced actin assembly requires the actA gene product, a surface protein. *Cell* 68, 521-531.

Krendel, M., and Mooseker, M.S. (2005). Myosins: tails (and heads) of functional diversity. *Physiology (Bethesda)* 20, 239-251.

Ku, N.O., Darling, J.M., Krams, S.M., Esquivel, C.O., Keeffe, E.B., Sibley, R.K., Lee, Y.M., Wright, T.L., and Omary, M.B. (2003a). Keratin 8 and 18 mutations are risk factors for developing liver disease of multiple etiologies. *Proc Natl Acad Sci U S A* 100, 6063-6068.

Ku, N.O., Soetikno, R.M., and Omary, M.B. (2003b). Keratin mutation in transgenic mice predisposes to Fas but not TNF-induced apoptosis and massive liver injury. *Hepatology* 37, 1006-1014.

Kussel-Andermann, P., El-Amraoui, A., Safieddine, S., Nouaille, S., Perfettini, I., Lecuit, M., Cossart, P., Wolfrum, U., and Petit, C. (2000). Vezatin, a novel transmembrane protein, bridges myosin VIIA to the cadherin-catenins complex. *EMBO J* 19, 6020-6029.

Lechler, T., Shevchenko, A., and Li, R. (2000). Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization. *J Cell Biol* 148, 363-373.

Lecuit, M. (2007). Human listeriosis and animal models. *Microbes Infect* 9, 1216-1225.

Lecuit, M., Hurme, R., Pizarro-Cerda, J., Ohayon, H., Geiger, B., and Cossart, P. (2000). A role for alpha-and beta-catenins in bacterial uptake. *Proc Natl Acad Sci U S A* 97, 10008-10013.

Lecuit, M., Nelson, D.M., Smith, S.D., Khun, H., Huerre, M., Vacher-Lavenu, M.C., Gordon, J.I., and Cossart, P. (2004). Targeting and crossing of the human maternofetal barrier by *Listeria monocytogenes*: role of internalin interaction with trophoblast E-cadherin. *Proc Natl Acad Sci U S A* 101, 6152-6157.

Lecuit, M., Ohayon, H., Braun, L., Mengaud, J., and Cossart, P. (1997). Internalin of *Listeria monocytogenes* with an intact leucine-rich repeat region is sufficient to promote internalization. *Infect Immun* 65, 5309-5319.

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., Babinet, C., and Cossart, P. (2001). A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science* 292, 1722-1725.

Lee, W.L., Bezanilla, M., and Pollard, T.D. (2000). Fission yeast myosin-I, Myo1p, stimulates actin assembly by Arp2/3 complex and shares functions with WASp. *J Cell Biol* 151, 789-800.

Li, N., Xiang, G.S., Dokainish, H., Ireton, K., and Elferink, L.A. (2005). The *Listeria* protein internalin B mimics hepatocyte growth factor-induced receptor trafficking. *Traffic* 6, 459-473.

Lu, X., and Lane, E.B. (1990). Retrovirus-mediated transgenic keratin expression in cultured fibroblasts: specific domain functions in keratin stabilization and filament formation. *Cell* 62, 681-696.

- Ly, K.T., and Casanova, J.E. (2007). Mechanisms of Salmonella entry into host cells. *Cell Microbiol* 9, 2103-2111.
- Magin, T.M., Vijayaraj, P., and Leube, R.E. (2007). Structural and regulatory functions of keratins. *Exp Cell Res* 313, 2021-2032.
- Maniak, M., Rauchenberger, R., Albrecht, R., Murphy, J., and Gerisch, G. (1995). Coronin involved in phagocytosis: dynamics of particle-induced relocation visualized by a green fluorescent protein Tag. *Cell* 83, 915-924.
- Martinez, J.J., and Cossart, P. (2004). Early signaling events involved in the entry of *Rickettsia conorii* into mammalian cells. *J Cell Sci* 117, 5097-5106.
- Mattoo, S., Lee, Y.M., and Dixon, J.E. (2007). Interactions of bacterial effector proteins with host proteins. *Curr Opin Immunol* 19, 392-401.
- Maulik, G., Madhiwala, P., Brooks, S., Ma, P.C., Kijima, T., Tibaldi, E.V., Schaefer, E., Parmar, K., and Salgia, R. (2002). Activated c-Met signals through PI3K with dramatic effects on cytoskeletal functions in small cell lung cancer. *J Cell Mol Med* 6, 539-553.
- McDonald, C., Vacratsis, P.O., Bliska, J.B., and Dixon, J.E. (2003). The yersinia virulence factor YopM forms a novel protein complex with two cellular kinases. *J Biol Chem* 278, 18514-18523.
- McLachlan, R.W., Kraemer, A., Helwani, F.M., Kovacs, E.M., and Yap, A.S. (2007). E-cadherin adhesion activates c-Src signaling at cell-cell contacts. *Mol Biol Cell* 18, 3214-3223.
- McLean, W.H., and Irvine, A.D. (2007). Disorders of keratinisation: from rare to common genetic diseases of skin and other epithelial tissues. *Ulster Med J* 76, 72-82.
- Mengaud, J., Ohayon, H., Gounon, P., Mege, R.M., and Cossart, P. (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell* 84, 923-932.
- Mhaweche, P. (2005). 14-3-3 proteins--an update. *Cell Res* 15, 228-236.
- Moll, R., Divo, M., and Langbein, L. (2008). The human keratins: biology and pathology. *Histochem Cell Biol* 129, 705-733.
- Murray, E.G.D., Webb, R.A., and Swann, H.B.R. (1926). A disease of rabbits characterized by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes* (n.sp.). *J Pathol Bacteriol* 29, 407-439.
- Niebuhr, K., Giuriato, S., Pedron, T., Philpott, D.J., Gaits, F., Sable, J., Sheetz, M.P., Parsot, C., Sansonetti, P.J., and Payrastre, B. (2002). Conversion of PtdIns(4,5)P(2) into PtdIns(5)P by the *S.flexneri* effector IpgD reorganizes host cell morphology. *EMBO J* 21, 5069-5078.
- Norris, F.A., Wilson, M.P., Wallis, T.S., Galyov, E.E., and Majerus, P.W. (1998). SopB, a protein required for virulence of *Salmonella dublin*, is an inositol phosphate phosphatase. *Proc Natl Acad Sci U S A* 95, 14057-14059.
- O'Connell, C.B., Tyska, M.J., and Mooseker, M.S. (2007). Myosin at work: motor adaptations for a variety of cellular functions. *Biochim Biophys Acta* 1773, 615-630.

Olazabal, I.M., Caron, E., May, R.C., Schilling, K., Knecht, D.A., and Machesky, L.M. (2002). Rho-kinase and myosin-II control phagocytic cup formation during CR, but not FcγR, phagocytosis. *Curr Biol* 12, 1413-1418.

Omary, M.B., Ku, N.O., Tao, G.Z., Toivola, D.M., and Liao, J. (2006). "Heads and tails" of intermediate filament phosphorylation: multiple sites and functional insights. *Trends Biochem Sci* 31, 383-394.

Oriolo, A.S., Wald, F.A., Ramsauer, V.P., and Salas, P.J. (2007). Intermediate filaments: a role in epithelial polarity. *Exp Cell Res* 313, 2255-2264.

Oshima, R.G. (2007). Intermediate filaments: a historical perspective. *Exp Cell Res* 313, 1981-1994.

Pallari, H.M., and Eriksson, J.E. (2006). Intermediate filaments as signaling platforms. *Sci STKE* 2006, pe53.

Papkoff, J. (1997). Regulation of complexed and free catenin pools by distinct mechanisms. Differential effects of Wnt-1 and v-Src. *J Biol Chem* 272, 4536-4543.

Patel, A., Cummings, N., Batchelor, M., Hill, P.J., Dubois, T., Mellits, K.H., Frankel, G., and Connerton, I. (2006). Host protein interactions with enteropathogenic *Escherichia coli* (EPEC): 14-3-3tau binds Tir and has a role in EPEC-induced actin polymerization. *Cell Microbiol* 8, 55-71.

Pentecost, M., Otto, G., Theriot, J.A., and Amieva, M.R. (2006). *Listeria monocytogenes* invades the epithelial junctions at sites of cell extrusion. *PLoS Pathog* 2, e3.

Phan, Q.T., Myers, C.L., Fu, Y., Sheppard, D.C., Yeaman, M.R., Welch, W.H., Ibrahim, A.S., Edwards, J.E., Jr., and Filler, S.G. (2007). Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol* 5, e64.

Pirie, J.H.H. (1927). A new disease in veld rodents, "Tiger river disease". *Publ S Afr Inst Med Res* 3, 163-186.

Pizarro-Cerda, J., and Cossart, P. (2006a). Bacterial adhesion and entry into host cells. *Cell* 124, 715-727.

Pizarro-Cerda, J., and Cossart, P. (2006b). Subversion of cellular functions by *Listeria monocytogenes*. *J Pathol* 208, 215-223.

Pizarro-Cerdà, J., Lecuit, M., and Cossart, P. (2002). Measuring and analysing invasion of mammalian cells by bacterial pathogens: the *Listeria monocytogenes* system. *Methods Microbiol* 31, 161-177.

Pokutta, S., and Weis, W.I. (2007). Structure and mechanism of cadherins and catenins in cell-cell contacts. *Annu Rev Cell Dev Biol* 23, 237-261.

Prehna, G., Ivanov, M.I., Bliska, J.B., and Stebbins, C.E. (2006). *Yersinia* virulence depends on mimicry of host Rho-family nucleotide dissociation inhibitors. *Cell* 126, 869-880.

Pujol, C., Eugene, E., Morand, P., and Nassif, X. (2000). Do pathogenic neisseriae need several ways to modify the host cell cytoskeleton? *Microbes Infect* 2, 821-827.

Rathman, M., de Lanerolle, P., Ohayon, H., Gounon, P., and Sansonetti, P. (2000). Myosin light chain kinase plays an essential role in *S. flexneri* dissemination. *J Cell Sci* 113 Pt 19, 3375-3386.

Redowicz, M.J. (2002). Myosins and pathology: genetics and biology. *Acta Biochim Pol* 49, 789-804.

Renzoni, A., Klarsfeld, A., Dramsi, S., and Cossart, P. (1997). Evidence that PrfA, the pleiotropic activator of virulence genes in *Listeria monocytogenes*, can be present but inactive. *Infect Immun* 65, 1515-1518.

Rikova, K., Guo, A., Zeng, Q., Possemato, A., Yu, J., Haack, H., Nardone, J., Lee, K., Reeves, C., Li, Y., *et al.* (2007). Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell* 131, 1190-1203.

Sanchez-Mejorada, G., and Rosales, C. (1998). Signal transduction by immunoglobulin Fc receptors. *J Leukoc Biol* 63, 521-533.

Scherer, C.A., Cooper, E., and Miller, S.I. (2000). The *Salmonella* type III secretion translocon protein SspC is inserted into the epithelial cell plasma membrane upon infection. *Mol Microbiol* 37, 1133-1145.

Schlech, W.F., 3rd, Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., *et al.* (1983). Epidemic listeriosis--evidence for transmission by food. *N Engl J Med* 308, 203-206.

Sellers, J.R. (2000). Myosins: a diverse superfamily. *Biochim Biophys Acta* 1496, 3-22.

Seveau, S., Pizarro-Cerda, J., and Cossart, P. (2007). Molecular mechanisms exploited by *Listeria monocytogenes* during host cell invasion. *Microbes Infect* 9, 1167-1175.

Sheehan, B., Klarsfeld, A., Ebright, R., and Cossart, P. (1996). A single substitution in the putative helix-turn-helix motif of the pleiotropic activator PrfA attenuates *Listeria monocytogenes* virulence. *Mol Microbiol* 20, 785-797.

Shen, Y., Naujokas, M., Park, M., and Ireton, K. (2000). InlB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. *Cell* 103, 501-510.

Shiratsuchi, H., Saito, T., Sakamoto, A., Itakura, E., Tamiya, S., Oshiro, Y., Oda, Y., Toh, S., Komiyama, S., and Tsuneyoshi, M. (2002). Mutation analysis of human cytokeratin 8 gene in malignant rhabdoid tumor: a possible association with intracytoplasmic inclusion body formation. *Mod Pathol* 15, 146-153.

Sousa, S., Cabanes, D., Archambaud, C., Colland, F., Lemichez, E., Popoff, M., Boisson-Dupuis, S., Gouin, E., Lecuit, M., Legrain, P., *et al.* (2005). ARHGAP10 is necessary for alpha-catenin recruitment at adherens junctions and for *Listeria* invasion. *Nat Cell Biol* 7, 954-960.

- Sousa, S., Cabanes, D., Bougneres, L., Lecuit, M., Sansonetti, P., Tran-Van-Nhieu, G., and Cossart, P. (2007). Src, cortactin and Arp2/3 complex are required for E-cadherin-mediated internalization of *Listeria* into cells. *Cell Microbiol* 9, 2629-2643.
- Sousa, S., Cabanes, D., El-Amraoui, A., Petit, C., Lecuit, M., and Cossart, P. (2004). Unconventional myosin VIIa and vezatin, two proteins crucial for *Listeria* entry into epithelial cells. *J Cell Sci* 117, 2121-2130.
- Stebbins, C.E., and Galan, J.E. (2000). Modulation of host signaling by a bacterial mimic: structure of the *Salmonella* effector SptP bound to Rac1. *Mol Cell* 6, 1449-1460.
- Stevens, J.M., Galyov, E.E., and Stevens, M.P. (2006). Actin-dependent movement of bacterial pathogens. *Nat Rev Microbiol* 4, 91-101.
- Strelkov, S.V., Herrmann, H., and Aepli, U. (2003). Molecular architecture of intermediate filaments. *Bioessays* 25, 243-251.
- Swaminathan, B., and Gerner-Smidt, P. (2007). The epidemiology of human listeriosis. *Microbes Infect* 9, 1236-1243.
- Swanson, J.A., Johnson, M.T., Beningo, K., Post, P., Mooseker, M., and Araki, N. (1999). A contractile activity that closes phagosomes in macrophages. *J Cell Sci* 112 ( Pt 3), 307-316.
- Terebiznik, M.R., Vieira, O.V., Marcus, S.L., Slade, A., Yip, C.M., Trimble, W.S., Meyer, T., Finlay, B.B., and Grinstein, S. (2002). Elimination of host cell PtdIns(4,5)P(2) by bacterial SigD promotes membrane fission during invasion by *Salmonella*. *Nat Cell Biol* 4, 766-773.
- Thompson, R.F., and Langford, G.M. (2002). Myosin superfamily evolutionary history. *Anat Rec* 268, 276-289.
- Toivola, D.M., Tao, G.Z., Habtezion, A., Liao, J., and Omary, M.B. (2005). Cellular integrity plus: organelle-related and protein-targeting functions of intermediate filaments. *Trends Cell Biol* 15, 608-617.
- Tong, X., and Coulombe, P.A. (2006). Keratin 17 modulates hair follicle cycling in a TNFalpha-dependent fashion. *Genes Dev* 20, 1353-1364.
- Tsai, R.K., and Discher, D.E. (2008). Inhibition of "self" engulfment through deactivation of myosin-II at the phagocytic synapse between human cells. *J Cell Biol* 180, 989-1003.
- Tsolis, R.M., Kingsley, R.A., Townsend, S.M., Ficht, T.A., Adams, L.G., and Baumler, A.J. (1999). Of mice, calves, and men. Comparison of the mouse typhoid model with other *Salmonella* infections. *Adv Exp Med Biol* 473, 261-274.
- Uitto, J., Richard, G., and McGrath, J.A. (2007). Diseases of epidermal keratins and their linker proteins. *Exp Cell Res* 313, 1995-2009.
- Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., and Kreft, J. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev* 14, 584-640.

- Veiga, E., and Cossart, P. (2005). *Listeria* hijacks the clathrin-dependent endocytic machinery to invade mammalian cells. *Nat Cell Biol* 7, 894-900.
- Vicente-Manzanares, M., Zareno, J., Whitmore, L., Choi, C.K., and Horwitz, A.F. (2007). Regulation of protrusion, adhesion dynamics, and polarity by myosins IIA and IIB in migrating cells. *J Cell Biol* 176, 573-580.
- Viswanathan, V.K., Lukic, S., Koutsouris, A., Miao, R., Muza, M.M., and Hecht, G. (2004). Cytokeratin 18 interacts with the enteropathogenic *Escherichia coli* secreted protein F (EspF) and is redistributed after infection. *Cell Microbiol* 6, 987-997.
- Wang, J., Gray-Owen, S.D., Knorre, A., Meyer, T.F., and Dehio, C. (1998). Opa binding to cellular CD66 receptors mediates the transcellular traversal of *Neisseria gonorrhoeae* across polarized T84 epithelial cell monolayers. *Mol Microbiol* 30, 657-671.
- Wang, J.A., Meyer, T.F., and Rudel, T. (2008). Cytoskeleton and motor proteins are required for the transcytosis of *Neisseria gonorrhoeae* through polarized epithelial cells. *Int J Med Microbiol* 298, 209-221.
- Waschke, J. (2008). The desmosome and pemphigus. *Histochem Cell Biol* 130, 21-54.
- Wasylnka, J.A., Bakowski, M.A., Szeto, J., Ohlson, M.B., Trimble, W.S., Miller, S.I., and Brumell, J.H. (2008). Role for myosin II in regulating positioning of *Salmonella*-containing vacuoles and intracellular replication. *Infect Immun* 76, 2722-2735.
- Weidner, K.M., Di Cesare, S., Sachs, M., Brinkmann, V., Behrens, J., and Birchmeier, W. (1996). Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature* 384, 173-176.
- Wing, E.J., and Gregory, S.H. (2002). *Listeria monocytogenes*: clinical and experimental update. *J Infect Dis* 185 Suppl 1, S18-24.
- Xiao, K., Garner, J., Buckley, K.M., Vincent, P.A., Chiasson, C.M., Dejana, E., Faundez, V., and Kowalczyk, A.P. (2005). p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin. *Mol Biol Cell* 16, 5141-5151.
- Yamada, S., Pokutta, S., Drees, F., Weis, W.I., and Nelson, W.J. (2005). Deconstructing the cadherin-catenin-actin complex. *Cell* 123, 889-901.
- Yap, A.S., Crampton, M.S., and Hardin, J. (2007). Making and breaking contacts: the cellular biology of cadherin regulation. *Curr Opin Cell Biol* 19, 508-514.
- Yin, H., Pruyne, D., Huffaker, T.C., and Bretscher, A. (2000). Myosin V orientates the mitotic spindle in yeast. *Nature* 406, 1013-1015.
- Yumura, S., and Uyeda, T.Q. (2003). Myosins and cell dynamics in cellular slime molds. *Int Rev Cytol* 224, 173-225.
- Zatloukal, K., Stumppner, C., Lehner, M., Denk, H., Baribault, H., Eshkind, L.G., and Franke, W.W. (2000). Cytokeratin 8 protects from hepatotoxicity, and its ratio to cytokeratin 18 determines the ability of hepatocytes to form Mallory bodies. *Am J Pathol* 156, 1263-1274.

Zigmond, S.H., and Hirsch, J.G. (1972). Effects of cytochalasin B on polymorphonuclear leucocyte locomotion, phagocytosis and glycolysis. *Exp Cell Res* 73, 383-393.