CHARACTERIZING THE ROLE OF THE SPECTRIN CYTOSKELETON DURING ADHERENT AND INVASIVE BACTERIAL PATHOGENESIS

by

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Abstract

The enteric pathogens, enteropathogenic *Escherichia coli* (EPEC), *Salmonella* Typhimurium and *Listeria monocytogenes* cause millions of infections worldwide each year. A shared host cell target of these microbes is the cytoskeleton, which is reorganized as part of the infectious process. Because the hijacking of the cytoskeleton is a pre-requisite for disease, I hypothesized that the spectrin cytoskeleton would be targeted by these pathogens. I investigated three major components of the spectrin cytoskeleton: spectrin, adducin and protein 4.1. Using immunolocalization techniques, I identified spectrin cytoskeletal components recruited to EPEC pedestals, *S.* Typhimurium membrane invasion ruffles and *Salmonella* containing vacuoles (SCVs), as well as *L. monocytogenes* actin phagocytic cups and sites of initial comet tail formation. When any of the spectrin cytoskeletal proteins were knocked down, pathogenesis of each organism was severely attenuated. These findings reveal a novel host cell cytoskeletal network that is crucial for both adherent and invasive bacterial disease.

Keywords: spectrin; cytoskeleton; Enteropathogenic *E. coli;* Attaching and Effacing bacteria; *Listeria monocytogenes; Salmonella* Typhimurium; siRNA; invasive triggering; invasive zippering

Dedication

This work is dedicated to my parents who have guided and inspired me throughout life. Without their support and inspiration I surely would not be accomplishing my dreams. I further dedicate this to my grandparents, who endured far more adversity in their youth than I, maintaining optimism and happiness throughout their life.

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1: Introduction

1.1 *Escherichia coli*

Escherichia coli (*E. coli*) are the most abundant commensal facultative anaerobes inhabiting the colons of mammals [1]. However, certain strains of *E. coli* have acquired virulence genes through horizontal gene transfer, deletion, or modification, which benefit the organism but can have detrimental affects on the host. Large clusters of virulence genes can be found throughout the genome of pathogenic *E. coli* strains; within distinct regions termed pathogenicity islands (PAIs) as well as on plasmids and prophages [2]. Acquisition of such traits imparts a fitness advantage to these bacteria as they evolve within the host [3]. Presently, 7 distinct intestinal pathovars have been identified in *E. coli,* based on specific virulence factors and varying manifestations of disease in their hosts [4]. The first identified pathogenic *E.coli* strain was Enteropathogenic *E. coli* (EPEC), which was attributed to an outbreak of human diarrhea in 1945 [5].

1.1.1 Enteropathogenic *E.coli*

Enteropathogenic *E.coli* (EPEC) are Gram-negative bacteria that are part of a family of pathogens infecting millions of people each year [6]. Despite their identification as a cause of disease in 1945, it was not until 1978 when EPEC was isolated from infants with diarrhea and then administered to adult volunteers, confirming EPEC's pathogenic abilities [5,7]. Presently, EPEC remains a public

health concern largely because it is the leading cause of infantile diarrhea worldwide. Infantile diarrhea claims the lives of 2 million children each year [8].

1.1.2 EPEC pathogenesis overview

EPEC is a member of the attaching and effacing (A/E) group of bacteria. Another family members is Enterohaemorrhagic *E*. coli (EHEC), sharing similar pathogenic strategies to EPEC, but exhibiting more severe symptoms of infection associated with production of a shiga-like toxin [9]. Because EPEC and EHEC are human pathogens and do not cause disease in mice, the closely related murine pathogen *Citrobacter rodentium* is used to model A/E bacterial infections *in vivo* [10]. Upon ingestion, these microbes occupy the lumen of host intestines and use pili, rope-like structures, to form their initial attachment to host intestinal epithelial cells [11,12]. This attachment brings the bacteria within close proximity to the host cell, which is followed by formation of a needle-like apparatus known as a type-three secretion system (T3SS) that allows for the translocation of bacterial proteins (effectors) into the host cell cytoplasm [13]. Upon effector delivery, these proteins function by controlling the sub-cellular machinery of the host cell. A common characteristic of these infections is the production of A/E lesions. These lesions are identified by the localized effacement of epithelial microvilli and formation of an actin-rich membrane protrusion or "pedestal" that forms beneath the bacteria [2,5]. EPEC remain on their pedestals throughout the course of pathogenesis, remaining mostly extracellular during infection, thus these bacteria are limited to the intestinal tract [14,15].

1.1.3 Symptoms of A/E infections

EPEC infections induce a number of pathophysiological changes within the intestine. The resulting symptoms include intestinal inflammation, fever, vomiting and persistent diarrhea, which can ultimately lead to death [6,16]. Diarrhea associated water loss is not completely understood, but is thought to be a result of a combination of complications. As part of A/E lesion formation, these pathogens cause dramatic changes to many of the water channels, ion channels and exchangers that regulate nutrient and water uptake across the intestinal barrier [17,18,19]. These changes result in an imbalance of the essential exchanges of Na^{+}, Ca^{2+}, Cl^{-}, across the intestinal epithelium, reducing water absorption and contributing to diarrhea [20,21].

Water transport in the intestinal epithelium is tightly regulated, and can be disrupted by A/E bacterial infection leading to symptoms of diarrhea. Aquoporin (AQP) channels are expressed in the intestine and are involved in water transport across cellular membranes [22]. They allow water passage into intestinal epithelial cells while maintaining ion gradients by selective exclusion of ion passage through the channel [22]. Water exchange imbalance during A/E infections is altered through aquoporin redistribution from the plasma membrane to the host cell cytoplasm during infection. Aquoporin redistribution during infection is a phenotype initiated in part by the *C. rodentium* effectors EspF and EspG [17].

A/E bacterial pathogens also target gap junctions of the intestinal epithelium. Gap junctions are composed by end-to-end linkage of 2 hemichannels, normally found to connect the cytoplasm of neighbouring epithelial cells. The linked

hemichannels allow signals, fluids, and ions to be exchanged between cells [23]. Further exacerbating diarrhea, gap junction hemichannels are localized to the apical membrane of cells during infection [24]. Hemichannels can maintain their function when expressed on the plasma membrane in the absence of a pairing partner [25], thus providing an additional mechanism of water release during A/E infections [24].

In addition to water channels, ion channels and exchanger dysregulation, the pathophysiology of A/E bacterial infections are a result of a number of other intestinal epithelial malfunctions. Overall changes in epithelial barrier structure and function, as well as inflammatory responses further exacerbate symptoms of infection. The intestinal barrier is maintained by tight junctions (TJs) found within the apical junction complex (AJC) [26]. TJs provide a selective barrier, and together with the epithelial cells, determine the net epithelial absorption and secretion [26]. AJC's are found in association with a circumferential belt of actin microfilaments, in association with myosin light chain (MLC) 2, forming a contractile actomyosin ring around the apical perimeter of the intestinal epithelium [27]. Phosphorylation of MLC initiates contraction of the actomyosin ring, which extends the tight junctions and has been implicated in increased paracellular permeability [28]. EPEC infections activate a number of kinases that phosphorylate the myosin light chain II (MLC), suggesting a possible mechanism of increased junction permeability [29]. Additionally, specific tight junction proteins such as ZO1 and claudins 1, 3 and 5 are redistributed during A/E bacterial infections contributing to the associated water loss [30,31]. The re-distribution of these key junctional proteins results in a disruption of

epithelial polarity, intensifying the electrochemical gradient imbalance and water loss at the intestinal epithelium [30].

Other symptoms of A/E infection are fueled by the host immune response. EPEC flagellin stimulates interleukin-8 production, a protein key in the recruitment of inflammatory cells to the intestinal mucosa [32,33]. Further immune response occurs upon bacterial attachment and induction of major host cell cytoskeleton rearrangements. During EPEC pedestal formation, interleukin-8 and other proinflammatory cytokines are expressed, intensifying the inflammatory response. Even mild inflammation can elicit changes in gastrointestinal nerve and smooth muscle function. Inflammation directly affects the enteric nervous system, causing hypersensitivity of gastrointestinal neurons, which can result in cramping, pain, and diarrhea [34,35]. Thus A/E infection induced inflammation is likely to contribute to symptoms of these infections.

In addition to the inflammatory response effects on the gastrointestinal nervous system, *in vivo* A/E infections with *C. rodentium* also have more direct affects on neuronal signalling within the intestine. Two key modulators of gastrointestinal motility are the secretion of serotonin by enterochromaffin cells and serotonin removal by serotonin reuptake transporters (SERTs) [36]. Serotonin is a paracrine messenger that exudes its effects on primary afferent neurons within the lumen of the intestine [36]. The excessive release of serotonin, or the inability of SERT to remove serotonin from the intestinal lumen, results in increased gastrointestinal motility and diarrhea [37,38]. *C. rodentium* infections, through unidentified mechanisms, induce the release of serotonin from enteroendocrine cells

and cause a drastic reduction in SERT expression on the plasma membrane of epithelial cells [39]. Although direct correlations to disease are not confirmed, it is expected that the excess serotonin in the intestinal lumen during A/E infection may contribute to symptoms of disease.

1.1.4 A/E lesions: the Tir:Intimin interaction

Within the lumen of the intestine, prior to forming the characteristic A/E lesions, EPEC attaches to the intestinal epithelial cells. Initial attachment is mediated in part by a type IV bundle forming pilus (BFP) encoded on the EPEC adherence factor (EAF) plasmid [12,40]. These pili interact with *N*-acetyl-lactosamine-containing receptors lining the host epithelial surface to bring EPEC within close proximity of the epithelial cells [41]. In addition to mediating initial attachment to host cells, pili enable attachments to neighbouring EPEC that are simultaneously anchored to the host cell. The presence of BFP initiates formation of microcolonies of bacteria on the surface of the host cell [41]. Infection of human volunteers with an EAF plasmid cured strain revealed a drastic reduction in disease symptoms, suggesting a crucial role for this plasmid in EPEC pathogenesis [11] . In addition to BFP, there are a number of other investigated, and yet unidentified, adhesins that are thought to contribute to adherence to epithelial cells [42].

Many of the phenotypes associated with EPEC and other A/E diseases are encoded by genes located on a 35 kb pathogenicity island termed the locus of enterocyte effacement (LEE) [43]. The LEE encodes proteins that execute many key processes during EPEC infection, including the formation of the T3SS [13]. This syringe-like structure spans the bacterial membranes forming contact with the host

cell plasma membrane allowing the translocation of bacterial effectors into the host cell [44]. A crucial effector for A/E bacterial pathogenesis is the translocated intimin receptor (Tir), which upon delivery into the host cell, embeds in the host plasma membrane. Tir inserts in a specific orientation forming an extracellular hairpin loop domain, with both the N- and C-terminal tails located within the host cytoplasm [45]. The extracellular domain binds the bacterial outer membrane protein intimin, forming a firm attachment between EPEC and the intestinal epithelial cells [46,47]. Subsequently, the C-terminus of Tir is phosphorylated at a variety of tyrosine residues, most importantly at tyrosine 474 (Y474) [47]. This occurs through the actions of the host cell kinases c-fyn, Abl1, Abl2, and the Tec family [48,49,50]. The host cell protein nck directly binds to the phosphorylated Y474 of tir and is crucial for the efficient formation of EPEC pedestals [47,51]. Nck recruitment to Tir initiates a cascade of events involving the recruitment of actin-associated proteins N-WASP and Arp2/3, culminating in the polymerization of actin at the site of bacterial attachment (Figure 1-1)[52]. Actin polymerization occurs with the barbed ends orientated toward the pedestal membrane (Appendix 3). In addition to Nck, N-WASP, and actin, a number of other host cell proteins are recruited to pedestals that serve various functions in the formation and maintenance of these structures [53]. Yet, the binding of Nck to Tir is sufficient to generate actin polymerization, making this process the minimum requirements for pedestal formation [51]. The function of the pedestal is unclear; however, it has been well established that the Tir:intimin interaction which leads to pedestal formation is an essential event for A/E disease [51,54].

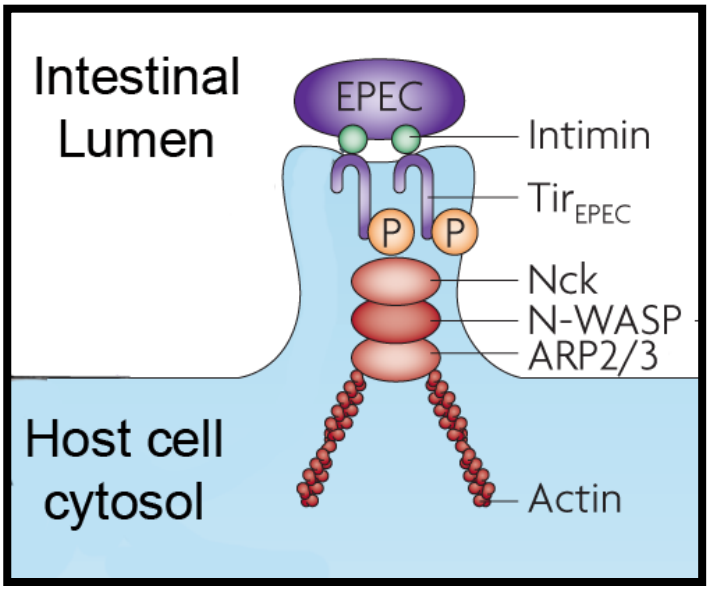

Figure 1-1 EPEC pedestal.

Upon BFP mediated initial attachment to the host intestinal epithelial cell, EPEC utilizes a T3SS to translocate bacterial effectors into the host cell cytosol. One such effector, Tir inserts into the host cell plasma membrane, forming an extracellular binding domain for the bacterial membrane protein intimin. Tir:intimin interaction is followed by the clustering of Tir beneath EPEC and subsequent phoshporylation of Tir Y474. Upon TirY474 phosphorylation, Tir recruits the host cell actin associated protein nck, which then recruits N-WASP leading to Arp2/3 recruitment and eventual actin polymerization beneath attached EPEC. Actin polymerization at the tail of Tir produces a force that culminates in EPEC rising off the natural surface of the host cell, residing on the actin-rich pedestal throughout the course of pathogenesis (Image modified from [2]).

1.1.5 A/E lesion accessory EPEC effectors

In addition to Tir interactions with intimin, there are a number of bacterial proteins involved in A/E lesion formation. A number of LEE encoded bacterial effectors such as espF, Map, espH, espG, and espZ (formerly sepZ), are translocated into the host cytosol [53]. The most versatile of EPEC effectors is espF. This effector has been shown to trigger mitochondrial dysfunction, inhibit phagocytosis, aid in membrane remodelling, modulate cytoskeletal dynamics, disrupt epithelial

tight junctions and induce host cell apoptosis [55,56,57,58]. Another multifunctional effector, the mitochondrial-associated protein (Map), acts to disrupt mitochondrial structure and respiratory function, in addition to disrupting epithelial barrier permeability [59]. Map belongs to a group of proteins characterized by a WxxxE motif [60], which enables map to have additional functions as a mimic of host cell guanine nucleotide exchange factors (GEF's) for cdc42, an established actin remodelling Rho-GTPase. Thus Map aids in modulating actin dynamics by encouraging filopodial formation around EPEC microcolonies [60,61]. Contrasting the functions of Map, the espH effector was identified to inhibit filopodial dynamics, and additionally enhance actin pedestal formation [62]. Specifically, espH directly targets Rho-GEFs inhibiting Rho-GTPase activation, which in addition to inhibiting filopodia, is implicated in preventing phagocytosis [63]. Further targeting the host cell cytoskeleton, espG targets tubulin and has been proposed to disassemble microtubules at sites of EPEC pedestals [64]. EspG also contributes to tight junction disruption, as infections with espG mutated EPEC strains showed decreased affects on Caco-2 monolayer permeability [64,65]. Finally, another LEE encoded effector espZ, localizes to the pedestal and aids in pathogenesis by interacting with the host cell protein CD98, facilitating host cell survival [66]. Several other effector proteins are translocated into the host cell and have been implicated in various aspects of EPEC pathogenesis, while many others have been identified with undiscovered function (reviewed in [2,53]).

1.2 Salmonella

Salmonellae bacteria are responsible for approximately 1.3 billion cases of human disease each year; resulting in 3 million deaths [67,68]. *Salmonella* species are rod shaped, gram negative, intracellular pathogens capable of infecting a wide range of hosts [68]. *Salmonella* infections are manifested as a broad range of clinical illnesses, depending on the host species and serovar causing the infection [69]. The genus *Salmonella* is divided into two species: *Salmonella bongori* and *Salmonella enterica*. *S. bongori* are mostly commensals of cold-blooded animals [68]. *S. enterica* are divided into 6 subspecies (I-VI), which incorporate over 50 serogroups (based on lipopolysaccharide antigens) and greater than 2500 serovars (based on flagellar antigens). *S. enterica* ssp.I is the primary source of two human diseases: typhoid fever and gastroenteritis [70]. Typhoid fever results from infection with *Salmonella enterica* serovars Typhi or Paratyphi [68]. *S. enterica* serovar Enteriditis (*S.* Enteriditis) and Typhimurium (*S.* Typhimurium) are the pathogens responsible for gastroenteritis; a human disease characterized by diarrhea, nausea, vomiting and fever, which can cause death [71].

1.2.1 *Salmonella* Typhimurium

S. Typhimurium is a major cause of food poisoning in the world and is responsible for the majority of *Salmonella*-based infections (Salmonellosis) [68]. These infections often remain localized in the gastrointestinal system causing acute enterocolitis, characterized by mucosal edema and intestinal inflammation. Enterocolitis is a result of bacterial invasion of intestinal cells as well as the robust immune response elicited by these pathogens [68,72,73]. Unlike *S.* Typhi, which

only infects humans, *S.* Typhimurium can infect a broad range of hosts and can result in drastically different disease progression depending on the host and route of infection [69]. This allows *S.* Typhimurium infections to act as a model for both types of human salmonellosis; *S.* Typhimurium infections of mice model human typhoid fever, while infections of a calf or cow strikingly resembles human gasteroenteritis [68]. Researchers have further developed *S.* Typhimurium gastroenteritis models through the use of direct infections of rabbit ileal loops [74]. Other gastroenteritis models developed involve infections of mice with *S.* Typhimurium strains attenuated for systemic infection or mice pre-treated with streptomycin to encourage colon colonization [75,76]. *S.* Typhimurium's prevalence and lethality worldwide, and ability to model both forms of salmonellosis, make it the most studied serovar of *Salmonella*.

1.2.2 *S.* Typhimurium Pathogenesis overview

Upon ingestion of *S.* Typhimurium, the bacteria remain inactive until reaching the small intestine, where environmental cues such as reduced bile content, high osmolarity and low oxygen induce expression of key virulence factors associated with pathogenesis [77,78]. At this point the bacteria can 1) be passively taken up by M-cells within distinct regions of the intestine known as Peyer's patches, 2) be captured in the lumen by macrophages and dendritic cells, or 3) actively invade epithelial cells [79,80,81,82,83]. The ability to invade epithelial cells as well as survive within macrophages is a key pathogenic processes required for full virulence. Life within host cells provides niches for *S.* Typhimurium throughout the course of infection [79,84,85,86]. Yet, life in the macrophage may

be short lived, as *S.* Typhimurium presence in human macrophages *in vitro* induces cell death within 4 hours of infection [87]. When *S.* Typhimurium induced macrophage death is elicited, a substantial immune response is generated, recruiting neutrophils to uptake and kill *S.* Typhimurium [88]. Consequently, when neutrophil deficient mice are challenged with *Salmonella* during the enterocolitis infection model, the disease is severely exacerbated and systemic infection is established [89,90]. These findings may explain why *S.* Typhimurium infections are effectively localized to the intestinal tract of immune competent humans, because they are limited to the epithelial cells and short existence within macrophages. Hence, many studies have investigated mechanisms by which *S.* Typhimurium invade intestinal epithelial cells *in vitro* [81,91,92]. Corresponding *in vivo* studies have shown a significant reduction in virulence of *S.* Typhimurium strains that lack the ability to invade epithelial cells [76,84,85,93,94,95]. Thus, epithelial cell invasion is a key determinant of *S.* Typhimurium virulence.

The process of active uptake into non-phagocytic cells is tightly regulated by a series of bacterial proteins (effectors) that are translocated into the host cell cytoplasm where they initiate massive cytoskeletal rearrangements [96], culminating in bacterial uptake into the cell (reviewed in [82]). *S.* Typhimurium effector translocation is achieved via a T3SS with striking structural similarities to the EPEC T3SS [13]. The T3SS and most of its effectors are encoded within a distinct region of the genome known as a Salmonella pathogenicity island (SPI-1) [71]. Upon translocation, effectors act to commandeer host cell machinery to mediate uptake of the bacteria into the host cell [97]. After entry into the host cell, *S.* Typhimurium are

contained within a phagosomal compartment, termed the Salmonella containing vacuole (SCV). Life within the SCV is primarily regulated by a second T3SS, encoded by the SPI-2 region of the *Salmonella* genome [98]. The effectors direct the SCV to the perinuclear region of the Golgi apparatus. Bacterial replication occurs within SCVs docked near the golgi. At this point, unique tubulovesicular structures, termed Salmonella induced filaments (Sifs), form around the SCVs (Extensively reviewed in [67,98].

It has not been established how *Salmonella* proceed to disseminate to neighbouring epithelial cells *in vivo*. Proposed mechanisms involve *Salmonella* induced necrosis or apoptosis of host intestinal epithelial cells, which upon shedding and membrane degradation, would release bacteria into the lumen to infect neighbouring cells [99,100]. Others have suggested the bacteria remain within the epithelial cell until it is shed into the intestinal lumen as part of normal epithelial cell turnover, at which point cell death occurs and the bacteria would be free to infect surrounding epithelial cells [72]. Other factors involve the ability to briefly survive within the host macrophages, induce their death, and disseminate to neighbouring cells [101]. Despite uncharacterized mechanisms of dissemination, it is clear that *S.* Typhimurium efficiently invades host epithelial cells and macrophages *in vivo*, generating a robust infection, which culminates in gastroenteritis.

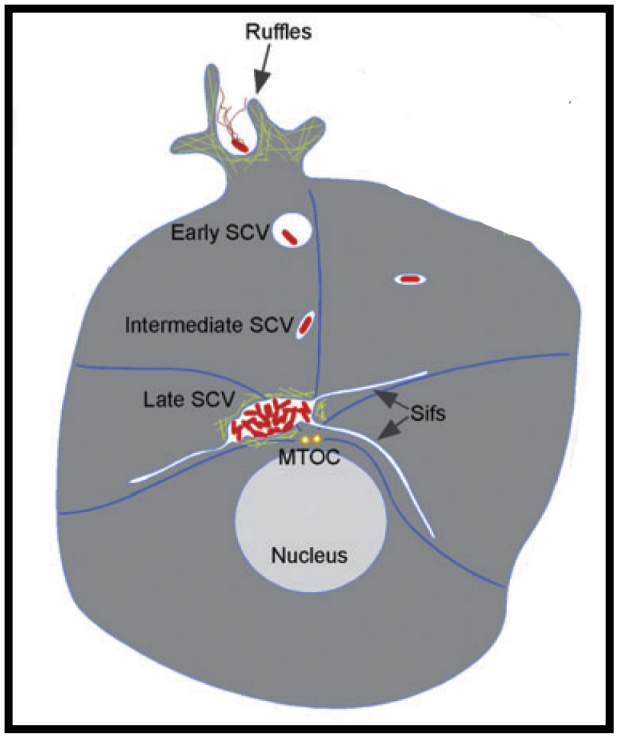

Figure 1-2 *S.* Typhimurium pathogenesis overview.

S. Typhimurium uses the SPI-1 encoded T3SS to translocate bacterial effectors into the host cell. Effectors act to commandeer host cell actin machinery to produce actin-rich membrane ruffles at sites of bacterial attachment. Membrane ruffles engulf the bacteria into a vesicle within the host cell termed the *Salmonella* Containing Vacuole (SCV). SPI-2 effectors work to direct trafficking of the SCV to the perinuclear region of the cell, at which point bacterial replication within the mature SCV occurs. During replication, characteristic *Salmonella* induced filaments (Sifs) form around the SCV. (Image modified from Ibarra [82]).

1.2.3 *S.* Typhimurium symptoms of disease

S. Typhimurium infection causes acute intestinal inflammation. During their pathogenesis they cause the migration of neutrophils (polymorphonuclear leukocytes, PMNs) across the epithelial barrier into the lumen of the intestine, resulting in the release of cytotoxic granules, damaging the mucosal cells and

causing inflammation [75,102]. *S.* Typhimurium strains that do not induce neutrophil migration are attenuated for diarrheal symptoms [102]. PMN migration is initiated in part by the *S.* Typhimurium secreted protein SipA. SipA secretion into the lumen of the intestine initiates PMN recruitment via the N-terminal domain of SipA though unidentified mechanisms. Infections *in vitro* demonstrated SipA mutants had dramatically reduced ability to elicit PMN migration across polarized human colonic cells [103]. Additionally it was found that purified SipA protein alone is sufficient to elicit migration [103,104]. PMN transepithelial passage is intensified by the recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) of the host innate immune system. Examples of PAMPs include the *Salmonella* proteins composing fimbriae, flagella, or LPS, which are all recognized by specific host cell Toll-like receptors (TLRs, a class of PRRs), expressed on the surface of intestinal epithelial cells [105,106,107,108,109]. Binding of bacterial PAMPs with TLRs initates TLR signaling; a process well characterized to elicit inflammatory responses and diarrhea (reviewed in [110]). The intestinal inflammation associated with infection disrupts epithelial barrier function and consequently contributes to intestinal cramping and diarrhea [34,35].

Not unlike EPEC infections, *S.* Typhimurium infections are associated with epithelial barrier disruption leading to the associated symptoms of disease [76,111,112]. The robust migration of neutrophils across the intestinal barrier not only helps localize *S.* Typhimurium infection, but also is associated with damage to the intestinal mucosa, contributing to leakage of fluids across the barrier [85].

Neutrophil migration induces Cl^- secretion into the intestinal lumen, which also is associated with water efflux and corresponds with the underlying symptoms of diarrhea [113]. Similarly to EPEC infection, *S.* Typhimurium infection is associated with actinomyosin ring contraction at the apical junction complex of epithelial cells. Actinomysin contraction is thought to increase permeability of tight junctions, thus increasing the escape of water across the barrier [114]. Tight junctions are also altered as a result of the invasion of intestinal epithelial cells. Throughout epithelial cell invasion, host cell cytoskeletal changes occur, which have been implicated in dismantling of tight junctions, thus increasing their permeability [115]. The *S.* Typhimurium translocated effectors SopE, SopE2, SopB, and SipA are all associated with the redistribution of tight junction proteins ZO-1 and occludin, to the cytosol of the host cell [116].

The associated intestinal pathophysiology that results from the inflammatory response and pathogenic strategies of *S.* Typhimurium not only contribute to symptoms of diarrhea, but also are required for full virulence *in vivo* [76,84,85,93,94,95]. Ultimately, the two key processes that elicit the inflammatory response and symptoms of *S.* Typhimurium disease are the ability to invade intestinal epithelial cells and live within macrophages; functions encoded by various genomic regions termed pathogenicity islands.

1.2.4 Salmonella pathogenicity Islands

S. Typhimurium (and other *Salmonella* serovars) has gained a unique arsenal of virulence proteins through the acquisition of gene elements in distinct regions within its genome termed *Salmonella* pathogenicity islands (SPI's) [71]. Many of

these SPI's are found next to tRNA genes (sequences containing repeats often targeted during insertion events), and their G + C content is lower than the rest of the genome, suggesting the SPI's have arrived in the genome via horizontal gene transfer [71,117,118]. Virulence genes within these islands code for proteins that commandeer the host cell machinery during many essential stages of pathogenesis. Proteins from different SPI's and from elsewhere in the genome often act in concert during the various stages of epithelial cell invasion and intracellular life [119,120]. Cell invasion and intracellular survival are tightly regulated events and rely upon the correct timing of bacterial protein activity at key stages of pathogenesis. For example, during invasion of intestinal epithelial cells the first proteins to be deployed must be involved in forming the T3SS, followed by effectors involved in forming pores in the host cell membrane, at last followed by effectors involved in the invasion event. Hence, certain bacterial proteins are involved in tightly regulating the timing of translocation of effectors involved in pathogenesis [120,121,122]. The various virulence proteins are largely encoded within 21 SPI's that have been identified and characterized to varying degrees (as reviewed in [71]). Two extensively characterized regions are SPI-1 and SPI-2; essential for invasion and intracellular survival respectively [71].

1.2.5 SPI-1: encoding invasion of intestinal epithelial cells

SPI-1 is a 40kb region of the genome crucial for the process of intestinal epithelial cell invasion [123]. It is hypothesized that SPI-1 acquisition occurred around the time *Escherichia* and *Salmonella* genera separated from a common ancestor, enabling *Salmonella* to become an invasive pathogen [118,124,125]. *S.*

Typhimurium SPI-1 mutated strains are severely attenuated during *in vivo* infections [126,127]. SPI-1 supports three key roles in pathogenesis by encoding: the components of a T3SS (T3SS-1), effectors crucial for host cell cytoskeletal remodelling and the sitABCD iron acquisition system. The SPI-1 encoded proteins PrgH, PrgK, InvG, PrgI, and PrgJ constitute the base units that compose the T3SS-1 needle complex [128,129]. The T3SS-1 enables the translocation of bacterial effectors into the host cytosol; a process essential for invasion of intestinal epithelial cells. The SPI-1 encoded proteins SipB, SipC, and SipD are instrumental in forming a translocation complex in the host cell plasma membrane that enables delivery of bacterial effectors into the host cell cytosol [130]. Subsequently, the SPI-1 effectors SopB/E/E2 are translocated into the host cytosol where they dramatically rearrange the actin cytoskeleton to initiate *S.* Typhimurium uptake into the host cell [93,131]. Other translocated SPI-1 effectors include SipA, AvrA, and SptP. SipA plays a crucial role in bundling actin during intestinal cell invasion [132], while AvrA and SptP are both involved in minimizing the host inflammatory responses and in enhancing bacterial virulence within host cells, albeit through different mechanisms [133,134]. Finally, the sitABCD transporter is thought to be crucial for iron acquisition within the host, and is necessary for full virulence of *S.* Typhimurium *in vivo* [135].

1.2.6 SPI-2: regulating life within the SCV

SPI-2 is a 40kb locus that is essential for *S.* Typhimurium replication and survival within the SCV of host cells and therefore necessary for full virulence *in vivo* [101,136,137]. SPI-2 encodes a second T3SS (denoted T3SS-2) that enables

bacterial proteins to be translocated from within the SCV into the host cell [138,139]. SPI-2 encoded proteins compose the T3SS-2 needle apparatus and proteins that form the translocon, enabling the various T3SS-2 effectors to be translocated across the SCV membrane. Acting upstream of most SPI-2 proteins, SipC appears to be a key component of SPI-2 function, as strains mutated in this protein are unable to survive within macrophages and have diminished virulence in mice [140]. The phenotype of SipC mutant infection, nearly recapitulates a SPI-2 null mutant infection [141]. The SipC mutant phenotype may be largely attributed to the inability to translocate three key SPI-2 encoded proteins: SseB, SseC, and SseD [142,143]. The SseB, SseC, and SseD proteins are essential for effector translocation, forming the translocon complex of the T3SS2 [139]. In addition to SipC, the needle apparatus and translocon complex, SPI-2 encodes at least three chaperone proteins, SscA, SscB and SseA. These chaperones are all involved in coordinating the translocation of various T3SS-2 effectors into the host cell [144,145]. T3SS-2 secreted proteins (most of which are not SPI-2 encoded) have many functions during the intracellular stage of infection ranging from directing the endocytic trafficking of the SCV [140], maintaining SCV membrane integrity [146,147], and avoiding macrophage killing thus enabling bacterial replication within the SCV [148,149].

1.2.7 Invasion of intestinal epithelial cells

a. Initial attachment

Although SPI-1 is necessary for invasion of non-phagocytic cells, it is not sufficient. Prior to the SPI-1 mediated invasion, *S.* Typhimurium must come into

close contact with the intestinal epithelial cell. This process is mediated by a type-one secretion system (T1SS) encoded within SPI-4. SPI-4 encodes the giant non-fimbrial adhesin SiiE, which enables intimate contact between the bacteria and the host intestinal epithelial cell (see figure 1-3) [119]. SiiE mediates the initial attachment and subsequent destruction of the surrounding microvilli [119].

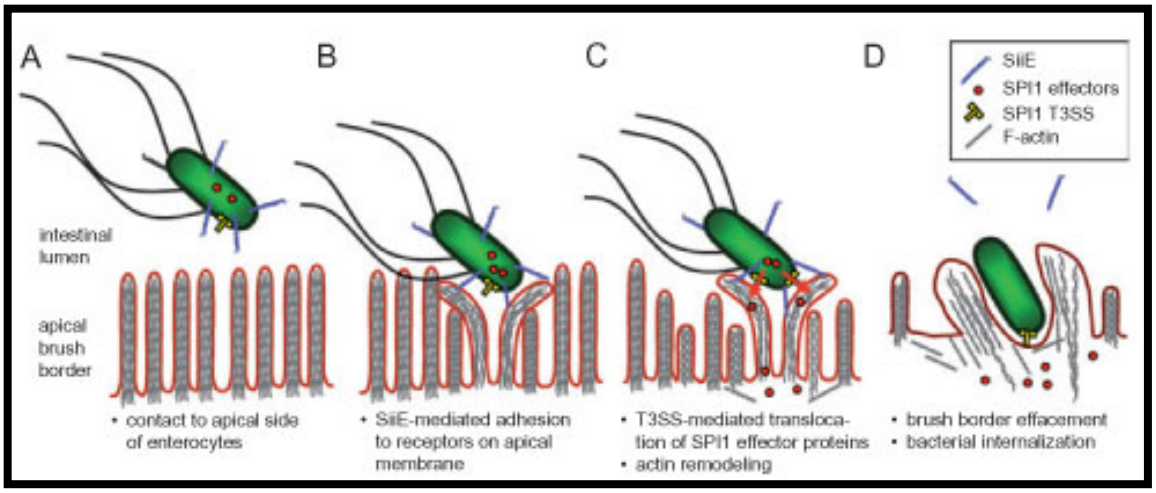

Figure 1-3 SiiE mediated attachment.

The SiiE non-fimbrial adhesin aids S. Typhimurium in creating a firm attachment to the intestinal epithelial microvilli, allowing subsequent translocation of effectors into the host cell. SiiE knockouts lack the ability to invade the apical surface of polar epithelial cells (Image modified from [119]).

b. Inducing membrane ruffling and uptake

During *S.* Typhimurium pathogenesis, actin rearrangements at the cell surface are essential to bacterial uptake into the host cell. Instrumental to actin manipulation, is the commandeering of host cell Rho-GTPases by *S.* Typhimurium effector proteins [97]. Rho-GTPases are a family of highly conserved proteins expressed in all eukaryotic cells that act as switches, cycling between active GTP-bound and inactive GDP-bound states [150]. Cycling of Rho GTP/GDP-bound

states is tightly regulated by guanine nucleotide exchange factors (GEF's) and GTPase activating proteins (GAP's). GEF's catalyze the exchange of GDP for GTP (activating the switch), while GAP's stimulate the GTPase to hydrolyze GTP (inactivating the switch) (See figure 1-4**a**) [150]. The downstream effects of RhoGTPase activities are centralized around remodelling the actin cytoskeleton but can also contribute to a wide array of cellular activities involving cell polarization, microtubule dynamics and membrane transport [151]. The three most well characterized RhoGTPases are Rho, Rac and CDC42, which are all involved in regulating actin; as part of myosin contractile filaments, lamellipodia dynamics, and filopodial dynamics respectively [151]. Upon GTP-activation, the three GTPases recruit actin-associated proteins, culminating in the formation and organization of actin filaments. Actin dynamics of branched actin networks are mediated through recruitment of WASP or structurally related proteins (WAVE) that subsequently sequester the Arp2/3 complex to initiate actin assembly into filaments (Figure 1-4**b**) [152]. These actin rearrangements can mediate migration of cells across a substrate (Fig 1-4**c**) [150].

Upon *S.* Typhimurium firm attachment to the host cell, SPI-1 effectors are translocated via the T3SS-1 into the host cell cytosol. Primarily 6 effectors (SipA, SipC, SopB, SopD, SopE, SopE2) are responsible for membrane and cytoskeletal reorganization culminating in bacterial uptake into the host cell [67]. SopE and SopE2 mimic host cell GEF's to activate Cdc42 and Rho (see figure 1-4**a**), resulting in substantial actin polymerization creating membranous ruffles at sites of bacterial attack (Figure 1-4**c**) [153,154]. The two effectors share 69% sequence similarity,

yet have different substrate specificities; SopE targets Rac-1 and Cdc42, while SopE2 preferentially activates Cdc42 [153,154]. The catalytic domain of SopE contains an entirely unique architecture from that of other host cell RhoGEF's, yet strikingly acts upon Cdc42 in a mechanistically identical fashion [155]. SopE2 also targets Cdc42 and contains a similar catalytic core motif as SopE [155]. NMR solution structures revealed SopE2 utilizes the same residues as SopE for Cdc42 interaction and similar mechanistic approaches to activation [156]. The cumulative SopE/E2 Cdc42 (and Rho) activation leads to the downstream formation of actin filaments at the site of bacterial invasion, promoting the engulfment of the bacterium into the host cell (Fig 1-4**d**) [97].

SopB (formerly called SigD) indirectly increases the intensity of actin ruffling at invasion sites through its phosphatase activities and activation of host cell GEF's. SopB dephosphorylates phosphoinositide phosphate (PIP) and inositol phosphate (IP), leading to downstream activation of host cell cdc42 Rho-GTPases through unidentified mechanisms [131,157]. SopB also activates the SH3-containing guanine nucleotide exchange factor (SGEF), an exchange factor for RhoG, which in turn further initiates actin polymerization [131]. The cumulative effects of SopE/SopE2/SopB activation of various host cell Rho GTPases, creates massive actin ruffles at the site of bacterial attachment, sufficient to bring bacteria into the host cytosol (see figure 1-4) [157].

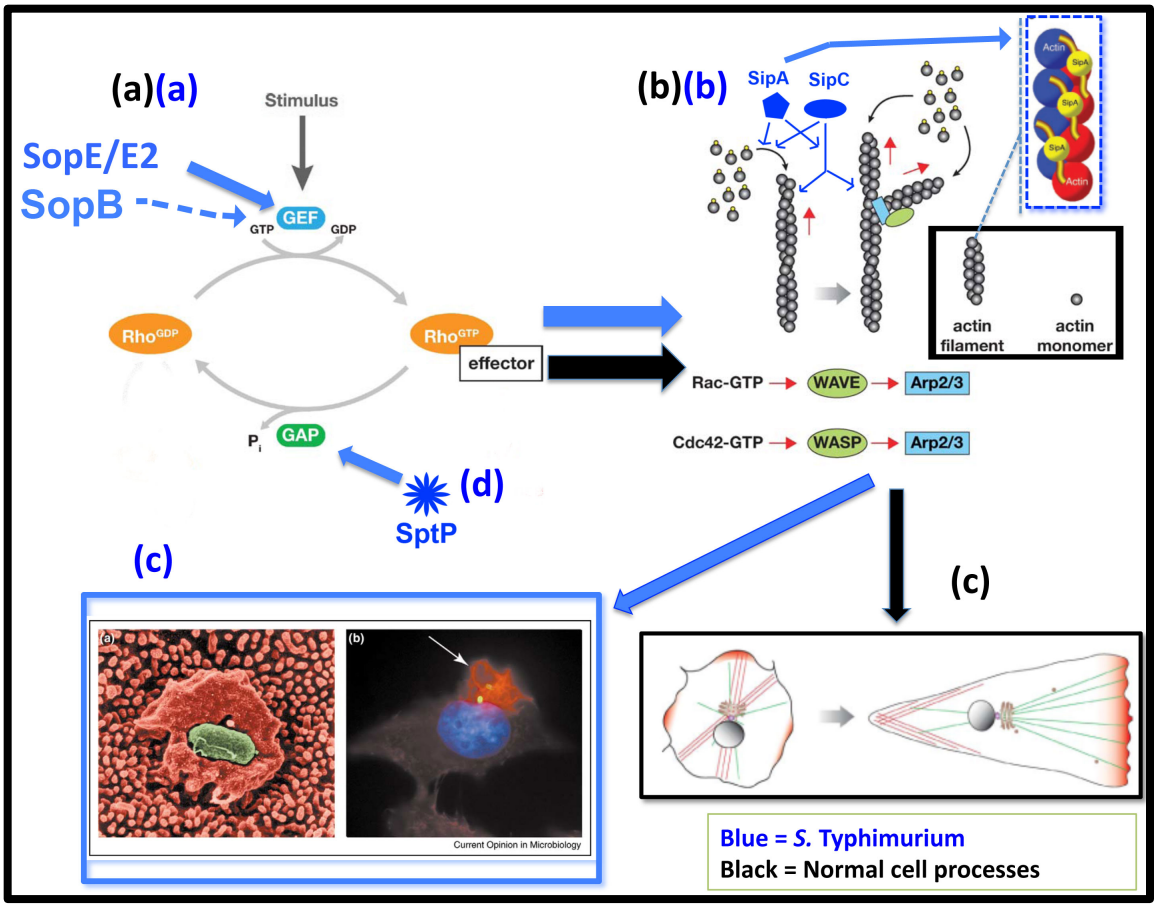

Figure 1-4 *S.* Typhimurium actin remodelling during invasion.

Black arrows represent pathways and proteins involved in normal processes such as cell migration, blue arrows and proteins represent *S.* Typhimurium processes enabling invasion of the host cell. Black Arrows (a) Stimulus event leads to GEF activation of RhoGTPases. (b) RhoGTPases (Rac and Cdc42) recruit actin-associated proteins, culminating in Arp2/3 activation, and subsequent actin polymerization. (c) Cell migration mediated by actin rearrangements at cells leading edge. Blue Arrows (a) SopE/SopE2/SopB *S.* Typhimurium effectors mimic host cell GEF's to exchange GDP for GTP in RhoGTPases to initiate their activity. (b) Upon activation Rac and cdc42 (RhoGTPases) recruit actin-associated proteins, leading to Arp2/3 recruitment and initiation of actin filament formation. SipA and SipC promote actin filament bundling and actin polymerization to enhance membrane ruffling. (c) *S.* Typhimurium invasion mediated by actin polymerization and subsequent ruffle formation at site of bacterial attachment. (d) The SptP effector of *S.* Typhimurium mimics host cell GAP's by initiating RhoGTPases to hydrolyze GTP to GDP, thereby inactivating them, returning the host cytoskeleton to a resting state. [Images modified from [150] (a,b,c), [97] (d), and [158] (b)]

c. Invasion accessory proteins

Although SopE, SopE2 and SopB are the minimum proteins required for

Salmonella invasion of non-phagocytic cells, a number of other effectors are required to enhance the efficiency of invasion. SipC and SipA are unique effectors that bind actin directly. Both effectors are capable of increasing the kinetics of actin monomer assembly in forming filaments, and also encourage bundling of actin filaments (Figure 1-4**b**) [157,159]. The SipC proline-rich N-terminal domain directly binds to and promotes actin filaments to form bundles *in vitro*, while the C-terminal domain is proposed to nucleate actin polymerization [159]. Similarily, SipA was identified to enhance filament bundling by directly binding actin, promoting filament formation and preventing de-polymerization *in vitro* [132]. The SipA protein is composed of a globular domain with 2 non-globular arms that extend out to tether and stabilize opposing actin strands, encouraging the bundling of filaments (Figure 1-4**b**) [158]. Through x-ray crystallography and electron microscopy, researchers propose a model for SipA-actin interactions whereby SipA acts as a 'molecular staple' tethering actin protomers in adjacent actin polymers (Figure 1-4**b**)[158]. SipA also prevents the actin filament severing activity of gelsolin [160]. Thus, SipA and SipC work collectively to stabilize actin filaments, producing more intense membrane ruffling, to encourage bacterial uptake into the host cell.

d. Vesicle fission and returning membrane to resting state

At the final stages of *S.* Typhimurium entry into the host cell, a fission event occurs releasing the SCV into the host cell cytoplasm [82]. The fission event is mediated by SopB, which eliminates host cell PtdIns(4,5)P2 in the membrane at the site of invasion, a process thought to reduce membrane rigidity, and promote the

fission event [161]. Following entry into the host cell, SopB remains active within the membrane of the SCV, however its activity is down-regulated at the host cell plasma membrane, helping return the host cell plasma membrane to a resting state [162]. In addition to SopB, the SptP effector is active after internalization, acting as a GAP to catalyze the hydrolysis of GTP and essentially inactivate Rac-1 and Cdc42 host cell RhoGTPases (Figure 1-4**d**) [163]. SptP returns the host cell plasma membrane to a resting state. The massive cytoskeletal rearrangements and membrane ruffling, which aid in bacterial uptake, can also trigger host immune responses [69]. As a by-product of SPI-1 cdc-42 activation, a number of host inflammatory cytokines are produced eliciting the production of the potent proinflammatory cytokine IL-8 in cultured epithelial cells. By SptP returning the membrane to resting state after internalization, *S.* Typhimurium may minimize this immune response. Hence, *Salmonella* can remain undetected in the host cell within the SCV, establishing a replicative niche.

1.3 Listeria

In 1926 *Listeria monocytogenes* was isolated from diseased rabbits becoming the first species of the genus *Listeria* to be identified. *L. monocytogenes* is the primary *Listeria* species that causes human disease worldwide [164,165]. They are facultative anaerobes, Gram-positive bacteria, capable of living in diverse environmental conditions such as diverse temperatures, pH ranges and high salt concentrations [166]. The ability to withstand various environments enables *L. monocytogenes* to occupy a myriad of habitats ranging from soil, living as saprophytes feeding on decomposing organic material, to the intestinal tract and cell

cytosol of animals, where they obtain nutrients and establish replicative niches. Because *L. monocytogenes* reside in drinking water, vegetation and food processing plants, this enables a multitude of sources for transition to human hosts where they cause the disease Listeriosis [166,167]. *L. monocytogenes* infection of humans is decreasing in North America, likely a result of aggressive testing at food processing plants and good manufacturing practices [167,168]. Yet *L. monocytogenes* remains a public health concern due to high mortality rates associated with infection of immune compromised individuals and pregnancy complications arising from infections.

1.3.1 *L. monocytogenes* pathogenesis overview

Upon entry into the mammalian host intestinal tract, *L. monocytogenes* deposits proteins on the surface of its membrane, which recognize and bind host cell receptors. After *Listeria* surface protein-receptor binding, a series of signalling events are initiated culminating in bacterial uptake into the host intestinal cell [169]. Bacterial uptake is identified by formation of an actin phagocytic cup in conjunction with recruitment of clathrin and calveolin endocytic machinery [170,171]. Within minutes post invasion, *Listeria* breaks free of the internalization vacuole releasing into the host cell cytoplasm, where they replicate [172,173]. Cytosolic bacteria then form the hallmark actin-rich "comet tail" at one pole of the bacterium, which produces a propulsive force enabling *L. monocytogenes* motility throughout the host cell cytoplasm. Comet tail motility also enables dissemination to neighbouring cells, which can eventually lead to invasion of macrophages, and subsequently the liver and spleen where they form replicative niches [166].

1.3.2 Symptoms of *L. monocytogenes* infections

Infections of immunocompetent individuals are usually restricted to the intestinal tract, causing inflammation and diarrhea, contributing to gastroenteritis. However when immunocompromised patients or pregnant women are infected with these microbes they are at an increased risk to develop systemic infections, which can be fatal. Systemic infection of immunocompromised individuals can lead to infections of the liver and kidneys, followed by hematological dissemination to the brain and other organs of the body [174,175]. Up to 70% of systemic infections result in infection of the central nervous system (CNS) [176]. CNS infection results in meningoencephalitis, which is associated with debilitating symptoms of headache, visual impairment and vomiting, followed by severe neurological shock that can lead to death [176]. In the case of pregnant women, fetal developmental complications often culminate in abortion [166].

During life within host cells, *L. monocytogenes* induces apoptosis of the host cell [177,178,179,180]. Research has identified the *Listeria* listeriolysin (LLO) virulence protein to be responsible for induction of apoptosis [177]. Infections with strains mutated in LLO are unable to elicit an immune response *in vitro* [177]. Apoptosis is associated with neutrophil recruitment, and subsequent neutralization of infection. Thus during infection of immunocompetent individuals, apoptosis of intestinal epithelial cells would generate the collective symptoms of gastroenteritis, while limiting infection to the intestinal tract [176]. Conversely, during systemic infection of immune compromised individuals, induced apoptosis of hepatocytes and other cells and tissues of the body contribute to the exacerbated symptoms of disease

that can lead to organ failure and death. Furthermore, it is suspected that apoptosis of dendritic cells, which are important antigen presenting cells, may impede their ability to elicit further immune response to clear the infection [174,177].

Despite decreased levels of infections in developed countries, *L. monocytogenes* remains a serious public health concern due to the high mortality rates, as 36% of established systemic infections result in death [167,168]. As recently as 2008, there was an outbreak across Canada resulting in 53 confirmed *Listeria* infections, resulting in 20 deaths [181]. Additionally, there is growing evidence that *Listeria*, and other invasive intestinal pathogens (including *Salmonella*), may contribute to the onset or exacerbation of inflammatory bowel diseases [182]. Hence, researchers continue to investigate the mechanisms of *Listeria* pathogenesis, to ensure appropriate treatments are available to infected individuals. Ultimately, *L. monocytogenes* infection of the host depends on the ability to cross a multitude of host cellular and tissue barriers, a process enabled by a number of bacterial virulence proteins.

1.3.3 *L. monocytogenes* invasion of host cells

L. monocytogenes ability to invade such a diverse group of host cells and tissues is mediated by a number of bacterial proteins expressed on the surface of the bacterial membrane. Internalization involves the binding of bacterial surface ligands to endogenous host cell receptors, creating a firm attachment to the host cell and initiating endocytic processes to internalize the bacteria. *L. monocytogenes* invasion has been termed the "zippering" mode of entry, highlighting the progressive binding

of bacterial surface proteins with their corresponding receptors during the entry event [174].

There are a number of surface proteins implicated in *L. monocytogenes* internalization into non-phagocytic cells including: InlA, InlB, Vip, Auto, and LapB [183]. InlA recognizes the host cell receptor E-cadherin, mostly expressed on cells of epithelial origin, allowing InlA to mediate passage across the intestinal barrier [184,185]. InlB recognizes the ubiquitously expressed hepatocyte receptor Met, enabling InlB mediated entry into a broad range of cell types [185,186]. Thus the InlB protein is necessary for systemic infection and colonization of many organs *in vivo* [187,188]. Auto is an autolysin necessary for invasion of a number of different cell types *in vitro* as well as for infection of guinea pigs and mice [189]. Similar to InlA, Vip is also important for invasion of human enterocytes as well as guinea pig epithelial cells *in vitro* [190]. Finally, LapB is also important for invasion of human epithelial cell lines *in vitro* [183]. Thus *L. monocytogenes* has developed multiple surface proteins with some redundant functions in mediating uptake into various cells and tissues of the body.

Internalization into cells and tissues is not always a result of exclusive use of one particular bacterial surface protein, rather it can be a result of multiple types of surface proteins; as is the case in crossing the placental barrier whereby InlA and InlB work synergistically [191,192]. To characterize the internalization pathways and avoid misinterpretations based on utilization of multiple surface proteins, researchers have manipulated non-invasive *Listeria* strains (i.e. *L. innocua*) to express just one of the internalization proteins, thereby allowing in-depth

examination of a specific mode of entry [193,194]. Others have expressed the various internalization proteins on ligand-coated beads to reveal host cell receptors and other proteins involved in invasion [195,196]. Alternatively, *in vitro* work with certain cell lines has elucidated mechanisms of specific *Listeria* internalization proteins, such as HeLa cells in which InlB is the sole surface protein utilized for entry [171]. Two of the most crucial and well-characterized internalization pathways involve the crossing of the intestinal epithelial barrier and entry into hepatocytes of the liver, mediated by the bacterial membrane surface proteins InlA and InlB respectively. Research has identified a shared strategy of these pathways, identifying clathrin endocytic machinery as important components mediating entry [171,188].

1.3.4 Clathrin mediated *L. monocytogenes* invasion

Involvement of clathrin endocytic machinery during *L. monocytogenes* invasion was first identified while investigating InlB mediated invasion of HeLa cells. In that system, the binding of InlB to the host cell receptor Met resulted in the rapid recruitment of the ubiquitin ligase Cbl to the Met receptor. During normal Met receptor regulation, Cbl ligase activity initiates endocytosis and subsequent lysosomal targeting and degradation of Met receptors [197]. The binding of InlB to Met initiates a strikingly similar series of events. InlB:Met interaction initiates monoubiquitination of Met by Cbl, commencing a cascade of events culminating in the recruitment of clathrin endocytic machinery (Figure 1-3). In addition to clathrin, the endocytosis associated proteins eps15, grb2, dynamin, CIN85, CD2AP, cortactin and hrs are all recruited and critical for efficient entry of *L. monocytogenes* [171].

Clathrin and associated proteins form a sub-membranous scaffold to produce membrane curvature and a substrate for endocytic associated proteins to bind, in a process necessary for endocytosis [198]. In addition to the endocytic machinery, the sub-membranous actin network is also commandeered and required for *Listeria* interalization [188]. As part of the Met receptor cascade, a downstream affect is the activation of the Rho-GTPases rac and/or cdc42, leading to recruitment of N-WASP/WAVE and Arp2/3 culminating in actin polymerization [199,200]. The precise role of actin in bacterial uptake is unclear, yet actin disruption by the fungal metabolite cytochalasin inhibits *L. monocytogenes* invasion, revealing the necessity for actin polymerization for the entry event [201]. Actin polymerization at the site of entry may provide force for the efficient internalization and subsequent vesicle fission, as has been suggested for other clathrin mediated endocytic events [198].

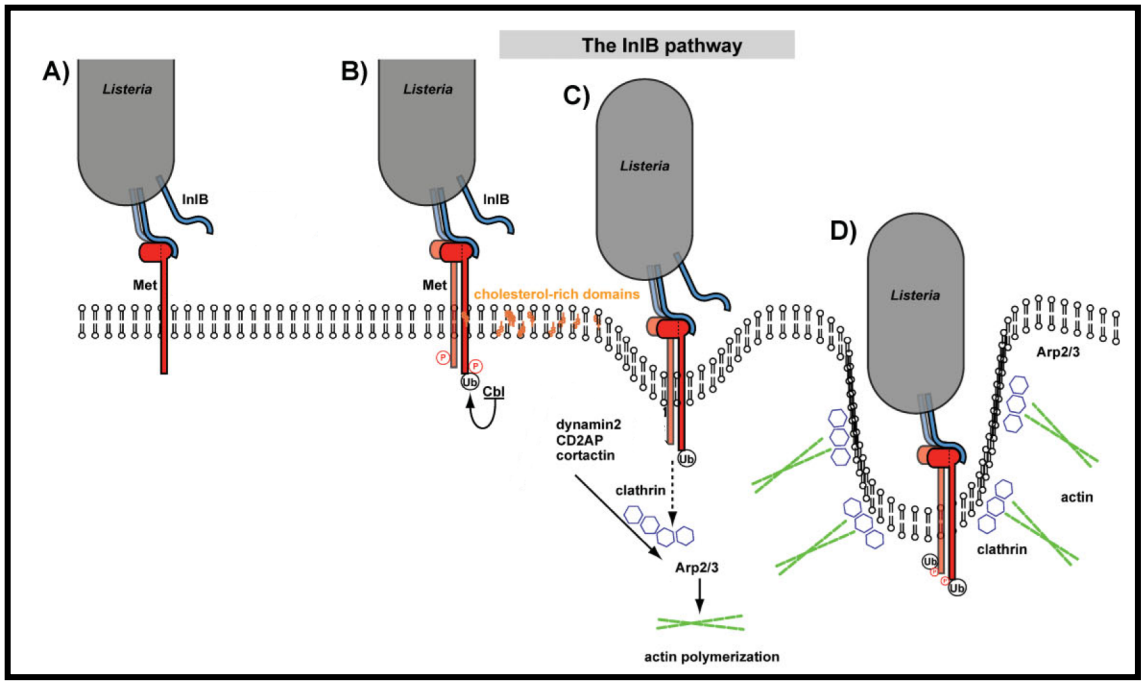

Figure 1-5 *L. monocytogenes* InlB mediated entry.

The bacterial surface protein InlB binds Met, leading to recruitment of the host cell ubiquitin ligase Cbl. Upon Met monoubiquitination by Cbl, downstream signalling leads to recruitment of the clathrin endocytic machinery. Additionally, Arp2/3 is recruited, leading to actin polymerization at the site of invasion. Clathrin and actin machinery work in concert to internalize the bacterium. (Image modified from [202])

1.3.5 Intracellular life

Upon internalization, *L. monocytogenes* are briefly retained within a vacuole. As early as 10 minutes post invasion, the bacterium secretes listeriolysin O (LLO); a pore-forming, cholesterol-dependant cytolysin which is responsible for vacuolar destruction and bacterial escape into the host cytosol [203,204]. Following vacuole escape, *L. monocytogenes* are able to efficiently replicate within the host cytosol, accumulating numbers as high as 200 bacteria in a single cell, without having any cytotoxic effect [173]. Intracellular bacteria express the 67 kDa ActA protein which

localizes asymmetrically at one pole on the surface of the bacterial membrane, which mimics WASP/WAVE proteins to recruit the Arp2/3 complex [205,206,207]. This complex initiates actin polymerization forming the comet tail that enables motility within the host cell [208,209].

1.3.6 Comet tail formation

There are two morphologically distinct stages of comet tail formation. The first is within an hour of infection in cell culture, when ActA, Arp2/3 and actin surround the entire membrane of the bacterium. Approximately 2 hours after entry, the bacteria begin to divide with unique differential cell wall growth rates, which contributes to the progressive localization of ActA and actin to one pole of the bacteria [207]. The second stage of tail formation is approximately 2.5 to 4 hours post invasion, when mature full-length comet tails are formed [207]. Mature comet tails are morphologically distinct, 10-40 μm long actin-dense tails extending from one pole of the bacteria, providing the propulsive forces necessary for motility [207,210]. As bacteria manoeuvre through the host cell and encounter the plasma membrane, the comet tail generated force creates a protrusion leading to eventual engulfment within a double-membranous vacuole in the new host. The bacteria again utilize LLO to break free of the vacuole and multiply within the new host cell [211] .

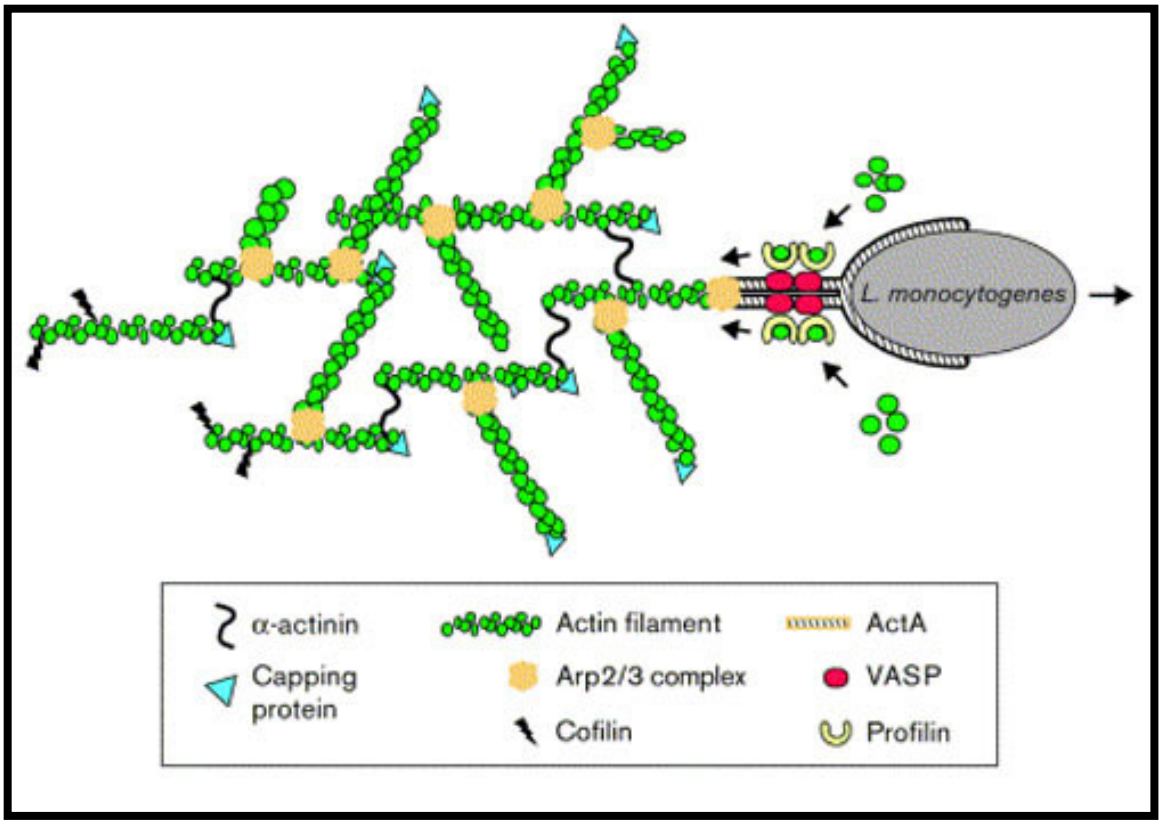

Figure 1-6 *Listeria* comet tail

Upon internalization into the host cell, *L. monocytogenes* expresses the surface protein ActA at one pole of the bacterium. ActA recruit's host cell actin associated proteins, including VASP and the Arp2/3 complex, which facilitate actin polymerization at the bacterial membrane in a polar manner. The host cell proteins profilin and cofilin help to either recruit actin monomers or displace them from the comet tail respectively. Other host cell proteins help stabilize actin filaments within the comet tail by cross-linking them, achieved by α-actinin, or by capping them with various capping proteins. Actin polymerization produces a propulsive force that drives *Listeria* throughout the host cell cytosol and into neighbouring cells (Image modified from the Pasteur Institute).

1.4 The spectrin cytoskeleton

The spectrin cytoskeleton was first identified in human erythrocytes in 1968 [212] and later visualized in 1973 [213]. This cytoskeletal system was thought to be

unique to erythrocytes for years after its discovery [214]. It has since been identified as a protein network ubiquitously expressed in cells and tissues across the animal kingdom (reviewed in [215]). This cytoskeletal meshwork resides beneath the plasma membrane and associates with select organelle membranes of cells, providing mechanical stability and acting as a protein-sorting machine to promote the formation of specialized membrane domains [216,217,218,219]. Similar to the clathrin lattice, the spectrin meshwork forms a lattice beneath the plasma membrane. Furthermore, the spectrin lattice enables the collection of specific membrane-associated proteins and restricts their distribution within discrete membrane domains. The classic example of spectrin dependant protein sorting is the localization of Na^+/K^+ ATPases to basolateral domains of polar epithelial cells [216,220]. SiRNA depletion of spectrin or associated proteins results in loss of Na^+/K^+ ATPase localization at the basolateral membrane [221]. Spectrin and associated proteins are also necessary for the correct localization of E-cadherin, a protein essential to establish lateral membrane domains of polar epithelia [222]. For both Na^+/K^+ ATPases and E-cadherin, spectrin mutations or knockdowns resulted in loss of the membrane localization of these proteins [220,222]. Other examples include Ca^{2+} regulating proteins, L1CAM's, glyophorin C, glutamate receptors, ionotropic receptors, rhesus proteins, and band 3 are all membrane associated proteins that also rely on the spectrin scaffold for correct localization to their respective membrane domains [221,223,224,225,226].

The core units of the spectrin cytoskeleton are the α- and β-spectrin subunits that align anti-parallel as dimers via interactions of triple helical repeat domains

[227,228]. A single dimer then forms head to head connections with another dimer, assembling the basic heterotetramer building blocks of the spectrin network (FIG 1-5) [228,229,230]. Spectrin tetramers can link to adjacent tetramers forming the well-characterized spectrin-actin junction complex (Fig 1-8) [231]. Actin binds the spectrin CH domain, allowing small actin filaments of 14 to 16 monomers to link adjacent spectrin tetramers together (Figure 1-8). Thus the basic units of the spectrin cytoskeleton are spectrin heterotetramers that are interconnected through spectrin-actin junction complexes.

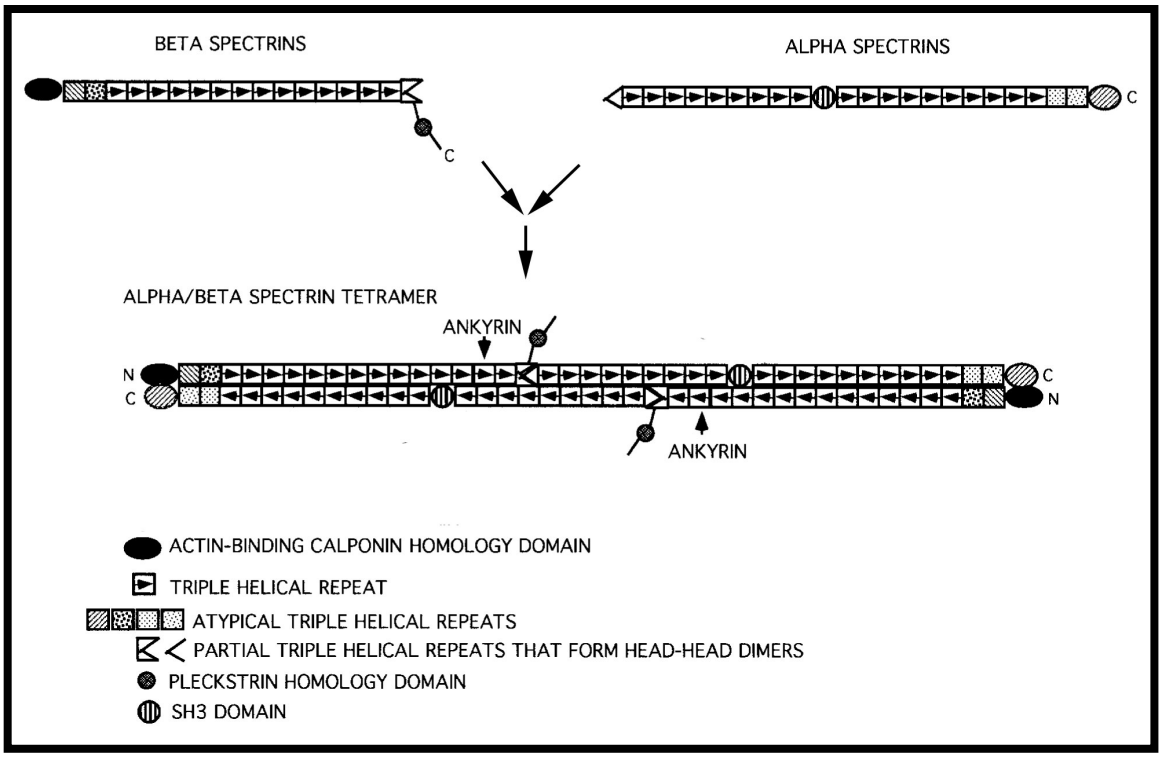

Figure 1-7 Spectrin protein interactions.

Spectrin proteins are largely composed of triple helical repeats. The NH2 terminal of β-spectrin contains a calponin homology (CH) domain that enables binding to actin. α- and β-spectrin dimmers are formed through interaction between terminal triple helical domains. Furthermore, the NH2 terminal of α-spectrin contains a helical repeat with only 1 helix, while the most C-terminal triple helical repeat of β-spectrin is comprised of two helices, which interacts with the single helix of α-spectrin forming head-to-head associations that allow the two dimers to form tetramers. The pleckstrin homology (PH) domains of β-spectrin mediate associations with membrane lipids and other proteins. The SH3 domains of α-spectrin are common in many cell-signalling proteins and mediate interactions with proline rich regions within other proteins. (Modified from [215]).

The spectrin cytoskeleton is able to interact with a diverse group of proteins, not only through distinct domains within the spectrin proteins, but also through spectrin interactions with adaptor proteins that link the spectrin scaffold to various membrane-associated proteins. In addition to spectrin and actin, there are three additional proteins that are key components of the spectrin cytoskeleton; adducin, protein 4.1 (p4.1) and ankyrin. Spectrin-actin associations *in vitro* are very weak, thus they utilize accessory proteins such as adducin and p4.1 to enhance the kinetics of binding [215]. Adducin recruits spectrin to fast growing ends of actin filaments [232,233]. While encouraging spectrin-actin interactions, adducin also associates with membrane-associated proteins, linking the complex to the plasma membrane (FIG 1-6) [234,235]. Additionally, adducin blocks elongation and depolymerization at the barbed end of actin filaments, making it an actin capping protein [236].

P4.1 also plays a key role in promoting spectrin-actin interactions, whereby its presence provides a 10^9 increase in the association constant of the spectrin-actin complex *in vitro* [237]. It not only promotes complex stability, but also provides links to a number of membrane-associated proteins via its FERM and C-terminal domains [238].

In addition to p4.1 and adducin, the spectrin associated protein ankyrin binds β-spectrin and links it to a number of membrane-associated proteins. The ankyrin protein contains ANK repeats that enable interactions with a number of membrane associated proteins including (but not limited to): anion exchangers, voltage gated sodium channels, various ion channels and a number of cell adhesion molecules including E-cadherin and L1-CAM [223,239,240,241,242,243,244]. Thus, amongst p4.1, adducin and ankyrin, the spectrin cytoskeletal lattice is capable of binding a large repertoire of membrane proteins. Furthermore, mutation or functional disruption of any spectrin cytoskeletal components often results in the loss of membrane localization and function of many of these associated membrane proteins [217,220,222].

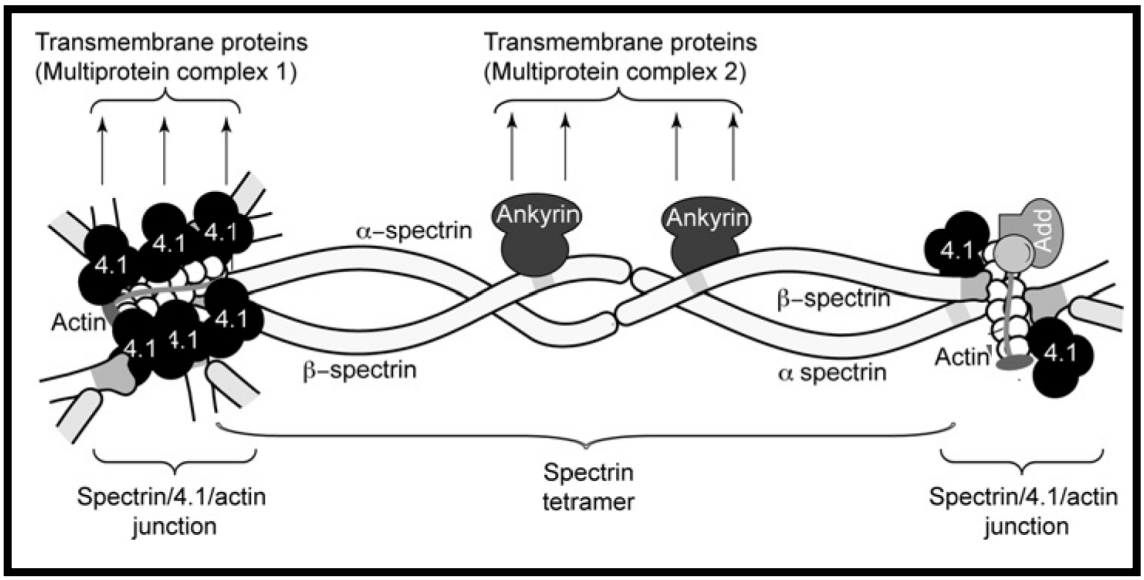

Figure 1-8 The spectrin-actin complex

Adjacent spectrin heterotetramers are linked through spectrin-actin junction complexes. Spectrin can directly bind actin via the β-spectrin CH domain and through actin interactions with triple helical repeats within β-spectrin. The spectrin-actin binding interactions are greatly increased in the presence of spectrin associated proteins adducin and p4.1. Adducin, p4.1 and ankyrin can each additionally interact with various membrane-associated proteins linking the spectrin-actin network to the plasma membrane. (Modified from [217])

Through interactions with various cytoskeletal and membrane associated proteins, the spectrin cytoskeleton is also involved in dynamic membrane reorganization events. During cell migration, spectrin interacts with the actin re-organizing protein, EVL, located at the leading edge of filopodia and lamellipodia [245,246]. Spectrin also associates with a number of motor proteins (Kinesin, dynein, and dynactin) involved in cellular transport and cell motility [247]. Additionally, the spectrin network is found at the terminal web of mirovilli, cross-linking the actin core bundles within the apical cytoplasm [248]. These associations and spectrin localization at the cell periphery and apical cytoplasm, implicate the importance of the spectin cytoskeleton in dynamic reorganization of the plasma membrane [245].

1.5 Research hypothesis

EPEC, *S.* Typhimurium and *L. monocytogenes* all commandeer the host cell cytoskeletal machinery as crucial events during their pathogenesis. Yet, the spectrin cytoskeleton has gone un-investigated throughout the manifestation of these diseases. The sub-membranous localization and known actin associations of the spectrin cytoskeleton, together with the dramatic reorganization of the host cell cytoskeletal networks, identify the spectrin cytoskeleton as a potential target of these

pathogens. Therefore, I hypothesized that the spectrin cytoskeleton would be an integral component for both adherent and invasive bacteterial pathogenesis. Accordingly, I hypothesized spectrin's involvement in EPEC pedestals, *S.* Typhimurium host cell invasion and life within the SCV, as well as *L. monocytogenes* invasion and motility within the host cell. Furthermore, I predicted that when spectrin or the associated proteins are knocked down, pathogenesis of each organism would be inhibited.

2: The Spectrin Cytoskeleton is Crucial for Adherent and Invasive Bacterial Pathogenesis

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Author contributions: TR was involved in experimental design, execution of experiments (excluding SC contributions), data analysis and writing the paper. SC was involved in execution of experiments for the majority of *Salmonella* studies and the *Listeria* initial invasion study. JAG was involved in experimental design and paper writing. All authors discussed results and commented on manuscripts.

2.1 Abstract

Various enteric bacterial pathogens target the host cell cytoskeletal machinery as a crucial event in their pathogenesis. Despite thorough studies detailing strategies microbes use to exploit these components of the host cell, the role of the spectrin-based cytoskeleton has been largely overlooked. Here we show that the spectrin cytoskeleton is a host system that is hijacked by adherent (Entropathogenic *Escherichia coli* [EPEC]), invasive triggering (*Salmonella enterica* serovar Typhimurium [*S.* Typhimurium]) and invasive zippering (*Listeria monocytogenes*) bacteria. We demonstrate that spectrin cytoskeletal proteins are recruited to EPEC pedestals, *S.* Typhimurium membrane ruffles and *Salmonella* containing vacuoles (SCVs), as well as sites of invasion and comet tail initiation by *L. monocytogenes*. Spectrin was often seen co-localizing with actin filaments at the cell periphery, however a disconnect between the actin and spectrin cytoskeletons was also observed. During infections with *S.*

Typhimurium Δ*sipA*, actin-rich membrane ruffles at characteristic sites of bacterial invasion often occurred in the absence of spectrin cytoskeletal proteins. Additionally, early in the formation of *L. monocytogenes* comet tails, spectrin cytoskeletal elements were recruited to the surface of the internalized bacteria independant of actin filaments. Further studies revealed the presence of the spectrin cytoskeleton during SCV and *Listeria* comet tail formation, highlighting novel cytoplasmic roles for the spectrin cytoskeleton. SiRNA targeted against spectrin and the spectrin-associated proteins severely diminished EPEC pedestal formation as well as *S.* Typhimurium and *L. monocytogenes* invasion. Ultimately, these findings identify the spectrin cytoskeleton as a ubiquitous target of enteric bacterial pathogens and indicate that this cytoskeletal system is critical for these infections to progress.

2.2 Introduction

The manipulation of the host cytoskeleton is a crucial step during infections caused by a variety of enteric bacterial pathogens including EPEC, *S.* Typhimurium and *L. monocytogenes*. EPEC attach to host intestinal epithelial cells and remain primarily extracellular during their infections [249]. These microbes utilize a type III secretion system (T3SS) to inject bacterially-derived effector proteins from the bacterial cytosol directly into the host cell cytoplasm [13]. One such effector, the translocated intimin receptor (Tir), is instrumental in anchoring EPEC to the host cell through its extracellular domains. Intracellularly, Tir recruits actin filaments through the binding of actin-related proteins to its cytosolic tail domains. The abundant polymerization of actin filaments beneath EPEC results in the bacteria

rising off the natural surface of the cell on actin-rich membrane protrusions called "pedestals", which are hallmarks of the disease [250,251].

S. Typhimurium also utilize T3SS's as part of their pathogenesis. These invasive pathogens inject a variety of effector proteins, including SopB (SigD), SopE, SopE2 and SipA which cause the host cells to generate intense actin-based membrane ruffles at sites of bacterial invasion [153,154,252,253]. The membrane ruffling engulfs the bacteria into the host cell resulting in their encasement in a vacuole called a *Salmonella* containing vacuole (SCV), providing these microbes a protective niche for replication [80,254].

L. monocytogenes, another invasive pathogen, does not utilize a T3SS but rather deposits its effector proteins on its surface. These bacteria primarily utilize 2 internalin proteins to efficiently enter non-phagocytic host cells; internalinA (InlA) and internalinB (InlB)[255]. Both proteins recruit clathrin and the clathrin associated endocytic machinery to sites of bacterial attachment [171,188]. This collection of proteins initially internalizes the bacterium into a vacuole within the host cytoplasm [169] [256]. Once within the host cell, *L. monocytogenes* quickly disrupts the vacuole that encapsulates it, then initiates the up-regulation and polarized distribution of the ActA effector on the bacterial plasma membrane [257]. ActA mimics N-WASp, thus recruiting the Arp2/3 complex, causing an actin-based comet tail to be generated at one end of the bacterium [257]. This comet tail propels the bacterium within the host cytosol and enables the microbe to disseminate to neighbouring cells [258].

The spectrin cytoskeleton is a well characterised, ubiquitously expressed sub-membranous cytoskeletal system that was first discovered in erythrocytes and has since been identified in a variety of epithelial cells [217,221,259]. The cornerstone of this cytoskeletal system is the filamentous polymer spectrin. Unlike other cytoskeletal systems, the spectrin cytoskeleton is thought to be restricted to membranous regions of the cell. Spectrin filaments provide stability and mechanical support to the plasma membrane as well as the Golgi, Golgi associated vesicles, ER and lysosomal membranes of the cell [216,235,260]. Spectrin interacts directly with actin filaments as well as the spectrin-associated proteins adducin, protein 4.1 (p4.1) and ankyrin, which provide a bridge between the spectrin-actin cytosketal network and the plasma membrane [215]. Additionally, the spectrin cytoskeleton co-localizes with actin accessory proteins, acting as a "membrane protein-sorting machine" [216] at specific sub-membranous regions of the cell during dynamic membrane remodelling events such as during cell migration [216,221,245]. The sub-membranous localization and known actin associations of the spectrin cytoskeleton, together with the dramatic reorganization of the host cell plasma membrane and related cytoskeletal networks during various enteric bacterial infections, suggest that the spectrin cytoskeletal system may also be a target of these pathogens. To examine this, we investigated the role of the spectrin cytoskeleton during EPEC, *S.* Typhimurium and *L. monocytogenes* infections. Our findings show that a set of spectrin cytoskeletal components are targeted by these pathogens and the involvement of this cytoskeletal system is crucial for their pathogenesis.

2.3 Results

2.3.1 The EPEC effector Tir recruits spectrin, p4.1 and adducin to pedestals

To examine the role of the spectrin cytoskeleton during bacterial infections, we initially infected cultured cells with EPEC and immunolocalized ß$_2$-spectrin. We found that spectrin was recruited to EPEC pedestals (Figures 2-1a HeLa cells and 2-6S Caco cell) showing distinctly different localization as compared to the non-specific staining of primary antibody controls (2-7S). To determine whether proteins that are known to interact with spectrin were also present at these sites we immunolocalized the spectrin associated proteins α-adducin and p4.1 and found that they were also present at EPEC pedestals (Figure 2-1a HeLa cells and 2-7S controls). When their organization within these structures was analyzed, a slight separation between the bacteria and the spectrin cytoskeleton was observed. Although spectrin-associated proteins co-localized with the actin filaments at certain parts of the pedestals, they were primarily positioned at the basal regions of these structures (Figure 2-2 HeLa cells and 2-8S Caco cells).

To determine whether bacterial contact or effector translocation was responsible for spectrin cytoskeletal proteins being concentrated beneath EPEC, we used an EPEC T3SS mutant (EPEC Δ*escN*), mutated in a crucial ATPase needed for effector translocation [261]. Host cells infected with EPEC Δ*escN* did not recruit any components of the spectrin cytoskeleton to sites of bacterial attachment, suggesting that an effector was required (Figures 2-1b spectrin, 2-9S adducin and 2-10S p4.1). Because the EPEC effector Tir is needed for pedestal formation, we examined whether Tir mutants of EPEC concentrated any spectrin-associated

proteins at sites of bacterial contact. Infections using EPEC Δ*tir*, did not recruit any components of the spectrin cytoskeleton beneath the bacteria, whereas complemented bacteria (EPEC Δ*tir:tir*) restored the wild-type phenotype (Figure 2-1b specrtrin, 2-9S adducin and 2-10S p4.1). Although there are a variety of phosphorylation sites on the EPEC Tir protein that are involved in pedestal formation to varying degrees, by far the most crucial is the tyrosine 474 (Y474) phosphorylation site [51,262]. To determine whether this site was needed for spectrin cytoskeletal recruitment we used an EPEC Δ*tir* strain complemented with *tir* containing a point mutation at that site (Y474F)(EPEC Δ*tir:tir*Y474F) and examined the localization of spectrin during those infections. Here we again observed a lack of spectrin/adducin/p4.1 recruitment, demonstrating that Tir Y474 phosphorylation is crucial for their positioning during these infections (Figure 2-1b spectrin, 2-9S adducin and 2-10S p4.1). As other EPEC effectors such as EspH, EspZ, Map, EspG and EspF are also proposed to be involved in pedestal formation [53], we examined the recruitment of spectrin/adducin/p4.1 beneath the bacteria during infections with EPEC mutated in each of those effectors and found that in all cases, all three spectrin cytoskeletal proteins were present at pedestals (Figures 2-11S spectrin, 2-12S adducin and 2-13S p4.1).

2.3.2 Depletion of spectrin cytoskeletal proteins severely impairs EPEC infections

Because the spectrin cytoskeleton appeared to be a significant component of EPEC pedestals, we sought to functionally perturb individual host components to examine their roles in pedestal generation. To accomplish this, we separately transfected HeLa cells with siRNA targeted against β_2-spectrin, α-adducin and p4.1.

Knockdowns were confirmed by western blot analysis (Figures 2-1c spectrin, 2-14Sa adducin and c p4.1). SiRNA pre-treated cells were then infected with wild-type EPEC to examine pedestal formation. In cells with undetectable levels of β_2-spectrin or p4.1, attached EPEC were unable to form pedestals (Figures 2-1c and d spectrin RNAi, and figures 2-14Sd and e p4.1 RNAi). Despite this, the ability of the bacteria to attach to the host cells was not significantly altered by these treatments (Figure 2-15S). Interestingly, adducin knockdowns resulted in an inability of EPEC to attach to the host cell, thus subsequent pedestal presence was not observed (Figure 2-14Sb). To ensure the siRNA treatments were not having adverse effects on the cell, we performed cell viability assays and found no difference in the viability of cells treated with control pool siRNA when compared to spectrin, adducin or p4.1 siRNA treated cells (Figure 2-16S). Furthermore, the actin cytoskeleton of spectrin knocked-down cells was morphologically similar to untreated cells with cortical actin and stress fibers present (Figure 2-17S).

2.3.3 *S.* Typhimurium userp the spectrin cytoskeleton during multiple stages of infection

Based on our findings with EPEC, we investigated a potential role for the spectrin cytoskeleton during the pathogenesis of another T3SS dependent microbe, *S.* Typhimurium. We found that spectrin was recruited to the actin-rich membranous ruffles at sites of *S.* Typhimurium invasion (Figures 2-3a HeLa cells, and 2-18S Caco cells), but only partially colocalized with actin when examined in detail (2-18S Caco cells and 2-19S HeLa cells). This lack of complete colocalization suggests that the presence of spectrin at these sites was not merely a byproduct of actin

recruitment (Figure 2-18S and 2-19S). The disconnect of actin and spectrin cytoskeletons was confirmed in uninfected cells which showed a lack of spectrin recruitment to stress fibers (Figure 2-20S). In addition to spectrin, the same spectrin associated proteins that were identified at EPEC pedestals (adducin, and p4.1) were also recruited to invasion sites (Figure 2-3a). To investigate the bacterial factors responsible for this recruitment, we utilized a *S.* Typhimurium Δ*sopE/sopE2/sopB* mutant, deficient in the effectors primarily responsible for membrane ruffling and bacterial invasion during these infections [263]. Infections with this mutant did not generate actin-mediated membrane ruffling and concomitantly the recruitment of the spectrin cytoskeleton to sites of bacterial contact was absent (Figure 2-21S).

S. Typhimurium contains the bacterial effector, SipA, which is known to bundle actin and increase efficiency of invasion [158]. To determine if this effector influenced spectrin cytoskeletal protein recruitment to sites of invasion, we immunolocalized spectrin, adducin and p4.1 together with actin during infections with a *S*. Typhimurium *sipA* mutant. Infections with *S*. Typhimurium Δ*sipA* showed that the spectrin and actin cytoskeletons were independently recruited; as actin-rich membrane ruffles remained present but often did not concentrate spectrin or adducin at sites of invasion (Figure 2-3b spectrin, 2-22S adducin, and 2-23S over-enhanced images of both spectrin and adducin). When compared to WT *S*. Typhimurium, *S*. Typhimurium Δ*sipA* invasion sites showed a significantly decreased ability to recruit spectrin and adducin to invasion sites [43% and 89 % reduced respectively] (Figure 2-24S). *S*. Typhimurium Δ*sipA* complimented with *sipA* restored the recruitment of spectrin and adducin to the membrane ruffles (Figures 2-3b spectrin and 2-22S

adducin). P4.1 remained at membrane ruffles irrespective of the presence or absence of SipA (Figures 2-25S).

To investigate the potential involvement of the spectrin cytoskeleton at later time points of infections, when *S.* Typhiurium reside within the SCVs [254], we immunolocalized the spectrin cytoskeletal proteins at 90 minutes post invasion. We found that spectrin, but not adducin or p4.1, was recruited to SCVs (Fig 2-3c spectrin and 2-26S adducin and p4.1). We observed distinct localization of spectrin surrounding multiple bacteria within the protective vacuole (Figure 2-3c). Spectrin, adducin and p4.1 were not observed localizing to bacteria at earlier time points during the intracellular stage of the infections (data not shown).

2.3.4 RNAi of spectrin, adducin, or p4.1 proteins abolishes *S.* Typhimurium invasion

To determine the role of spectrin cytoskeletal components during *S.* Typhimurium invasion, we knocked down individual components of this cytoskeletal system in cultured cells and studied the effects on invasion. Knockdown of spectrin, adducin, or p4.1 proteins in host cells resulted in the near complete cessation of *S.* Typhimurium invasion (Figures 2-3d spectrin results and 2-27S adducin and p4.1 results). Quantification of *S.* Typhimurium invasion was assessed by immunofluorescent imaging in which cells were first identified that had undetectable levels of the targeted protein, then the number of bacteria that had infected those cells was counted. Microscopy counts of cells with undetectable levels for each of the three proteins showed an average of 8% invasion compared to control treatments (Figure 2-3d spectrin results and 2-27S adducin and p4.1 results).

We then quantified invasion efficiencies using classical invasion assay methods. Invasion assays with siRNA pretreated cells resulted in a significant decrease in invasion with an average of 35%/65%/60% (spectrin/adducin/p4.1 RNAi treated) invasion as compared to controls (Figure 2-3d spectrin results and 2-27S adducin and p4.1 results). As expected, microscopic analysis showed that our siRNA transfection efficiencies were not %100, with some cells having incomplete knockdown of the targeted protein. The observed increase in invasion efficiencies using the classical invasion assay method as compared to the microscopy-based counts can be attributed to the invasion of unsuccessfully transfected cells and those with only partial knockdowns being present in these assays.

2.3.5 *Listeria monocytogenes* requires the spectrin cytoskeleton for efficient invasion

We further characterized the role of the spectrin cytoskeleton during bacterial invasion by studying *L. monocytogenes* infections. Infections of cultured cells, which allow only the InlB invasion pathway to ensue [171], showed spectrin/adducin/p4.1 lining the characteristic actin-rich sites of *L. monocytogenes* internalization (Figure 2-4a) [199]. Individual siRNA-based depletion of spectrin/adducin/p4.1 nearly abolished the ability of *L. monocytogenes* to invade the host cell (Figure 2-4b spectrin results and 2-29S adducin and p4.1 results). Microscopy counts of cells with undetectable levels of spectrin/adducin/p4.1 showed 17%/15%/6% invasion compared to control treatments (Fig 2-4c spectrin results and 2-29S adducin and p4.1 results). Classical invasion assays performed on these samples (which include unsuccessfully transfected cells) resulted in significantly

different levels of invasion when RNAi treated cells were compared to controls [21%/52%/60% spectrin/adducin/p4.1 respectively] (Figures 2-4c spectrin results and 2-29S adducin and p4.1 results).

2.3.6 *L. monocytogenes* recruits spectrin and p4.1 to initial stages of comet tail formation using the ActA effector

Following entry into host cells, *L. monocytogenes* up-regulate the ActA effector to initiate the formation of the characteristic actin-rich comet tails [264]. We found that spectrin was recruited to the initial stages of comet tail formation (Figure 2-4d HeLa cells and 2-28S Caco cells). Additionally, p4.1 was recruited to the bacteria at the initial stages of comet tail formation, whereas adducin was not (Figure 2-30S). Detailed analysis revealed that in some instances spectrin was localized to the bacteria independent of actin (Figure 2-5 HeLa cells and 2-28S Caco cells). At 30 minutes post infection, 70% of the bacteria had spectrin lining the membrane in the absence of actin, whereas after 90 minutes of infection only 7% of internalized bacteria were associated with spectrin alone (Figure 2-31S). Infections with *L. monocytogenes* ActA mutants (*L. monocytogenes* Δ*actA*) resulted in the absence of spectrin and p4.1 with the internalized bacteria, suggesting that ActA is needed for their recruitment (Figures 4d spectrin results and S25 adducin and p4.1 results). Upon mature, full-length comet tail formation, spectrin as well as adducin and p4.1 were absent (Figure 2-32S). Consequently, the presence or absence of functional ActA had no effect on their recruitment to comet tails (Figure 2-30S).

2.3.7 Discussion

In this study we have shown that a set of spectrin cytoskeletal proteins are co-opted during a variety of enteric bacterial infections. We have demonstrated that spectrin, adducin and p4.1 are crucial proteins involved in EPEC pedestal formation, *S.* Typhimurium and *L. monocytogenes* epithelial cell invasion and subsequent stages of their intracellular life cycles. By functionally perturbing these host proteins, infections were efficiently halted demonstrating that this cytoskeletal system is integral to the pathogenesis of these bacteria.

During our examination of EPEC infections we showed that spectrin was specifically concentrated at the base of pedestals, partially colocalizing with actin. This basal localization resembles that of other membrane protruding structures, namely microvilli and filopodia. Such structures contain a spectrin-based scaffold that provides a secure foundation for protein machinery localization, thus enabling the remodeling of the plasma membrane [245,248]. Consequently, spectrin may be providing a similar function during EPEC pedestal formation, by providing a substratum for membrane protrusion and pedestal formation. This is supported by evidence demonstrating that attached EPEC were unable to recruit actin beneath the bacteria when any of the spectrin cytoskeletal components were knocked-down.

S. Typhimurium internalization is heavily dependant on actin-based membrane ruffles, however evidence presented here demonstrates that spectrin cytoskeletal components are also needed for maximal invasion. When any of the three spectrin cytoskeletal proteins were knocked-down, we observed ~8% invasion efficiencies when invaded bacteria were counted by microscopy in cells with undetectable levels of those cytoskeletal components. *S.* Typhimurium use a

multitude of effector proteins to efficiently invade non-phagocytic cells. During infections with *S.* Typhimurium mutated in SipA, an effector involved in actin bundling that is known to aid in invasion [253], we found that actin-rich membrane ruffles remained present but often lacked spectrin or adducin. Those results suggested that the presence of SipA was required for the efficient targeting of those 2 components to the ruffles. Others have shown that infections using *S.* Typhimurium Δ*sipA* resulted in ~60% invasion efficiency compared to wild-type infections [253]. Our classical invasion assay results demonstrated similar invasion efficiencies when spectrin cytoskeletal components were knocked-down. Taken together these results support an important role for the SipA effector in spectrin/adducin recruitment and suggest that *S.* Typhimurium posses strategies to control the spectrin cytoskeleton independently of the actin cytoskeleton.

L. monocytogenes utilize clathrin-mediated endocytosis (CME) to gain entry into non-phagocytic cells [171,188]. The involvement of the spectrin cytoskeleton during CME has been examined by others and was shown to be excluded from clathrin-coated pits to encourage budding from the plasma membrane [260,265,266]. Based on this, we expected that spectrin would be absent from *L. monocytogenes* invasion sites in a similar fashion to classical CME. However, we found that spectrin was recruited to sites of *L. monocytogenes* invasion. Furthermore, when we knocked down spectrin using siRNA, infections were inhibited; demonstrating that spectrin is needed for clathrin mediated *L. monocytogenes* uptake. Although entry of *L. monocytogenes* into epithelial cells involves the internalization of a structure that is large in comparison to a classically formed endocytic particle [171,188], our

results contradict the traditional views of spectrin's role in CME and require further scrutiny.

The spectrin cytoskeleton has been extensively characterized as a network restricted to the eukaryotic plasma membrane and membrane domains of the Golgi, Golgi associated vesicles, ER and lysosomes [221,267] Michaely et al., 1999). Accordingly, we anticipated that internalized bacteria found within the host cell cytosol would not associate with the spectrin cytoskeleton. However, we observed that after internalization, *L. monocytogenes* were able to recruit spectrin and p4.1 to sites of initial comet tail formation suggesting that this cytoskeletal system is not restricted to membranous regions of eukaryotic cells as previously thought.

Clues to understanding the function of spectrin during *L. monocytogenes* comet tail formation may lie in other systems. During cell migration, spectrin associates with actin machinery to facilitate actin polymerization for subsequent motility [216] [245]. Although this potential function provides a likely role for spectrin during *L. monocytogenes* infections, we were unable to directly investigate bacterial motility in the absence of spectrin cytoskeletal components due to the severe defects of *L. monocytogenes* invasion in cells knocked-down in any of the spectrin cytoskeletal proteins. Despite this we were able to determine whether spectrin cytoskeletal components required any bacterial surface protein for their recruitment to the bacteria. During, infections with *L. monocytogenes* Δ*actA*, the bacteria were able to invade cells but were unable to recruit spectrin and p4.1 to internalized bacteria, suggesting that the spectrin cytoskeleton was not simply

recruited to the bacterial membrane, but required the presence of the ActA effector to initiate its recruitment at the bacteria for subsequent comet tail formation.

Although our findings have demonstrated an integral role for the spectrin cytoskeleton during a variety of pathogenic infections our findings have opened the door to many important questions that will require future examination. First will be to investigate the crucial domains of spectrin, adducin and p4.1 that are responsible for their recruitment to sites of infection. Also, further exploration into the dynamics of spectrin cytoskeletal protein recruitment in relation to actin cytoskeletal components during these infections is required. Finally, understanding how the depression of adducin expression interferes with EPEC binding to the host cells and mechanistically elucidating the precise influence that SipA has on the spectrin cytoskeleton during *S.* Typhimurium infections will require further scrutiny.

Ultimately, our identification of the spectrin cytoskeleton as a target during key stages of adherent, triggering and zippering enteric bacterial pathogenesis, demonstrates that this previously overlooked cytoskeletal system is integral to a variety of infections. This recruitment, coupled with the demonstration that the depletion of spectrin cytoskeletal proteins from host cells during these infections results in the inhibition of bacterial attachment and invasion, highlights the importance of this cytoskeletal system in disease progression. Accordingly, the broad involvement of this cytoskeletal system with enteric microbial pathogenesis reveals a new potential target for therapeutic treatments of these infections.

2.4 Materials and Methods

2.4.1 Cells, Bacteria, and Growth Conditions

Cells were (ATCC) grown in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) supplemented with 10% (20% for Caco cells) Fetal Bovine Serum (FBS) (Sigma). HeLa cells are a commonly used cell line for invasion and pedestal research [171,188,268,269,270]. We opted to use them due to their flat morphology and ease of imaging. The bacterial strains used in this study included wild-type Enteropathogenic *E.coli* (EPEC) strain E2348/69, EPEC Δ*escN*, EPEC (strain JPN15) and mutants from the same strain including Δ*tir*, Δ*tir* complimented with EPEC *tir*, and Δ*tir* complimented with EPEC *tir* Y474F (JPN15 Δ*tir* + *tir*ΔY474), wild-type *Salmonella* Typhimurium (strain SL1344) S. Typhimurium SL1344 mutants (Δ*sipA,* Δ*sopB(sigD)/sopE/sopE2,* and Δ*sipA:sipA*), S. Typhimurium SL1344 GFP , wild type *L. monocytogenes* (strain EGD600) and *L. monocytogenes* mutants Δ*actA* (strain 2140) and a hyperinvasive strain, expressing an *inlB* derivative containing an NH2 terminal region (reference the paper where this was used). All EPEC and S. Typhimurium strains were grown using standard luria broth (LB), and *L. monocytogenes* was grown using brain heart infusion (BHI) agar (BD Biosciences), including antibiotics where appropriate.

Caco-2 human colon epithelial cells were polarized using the BIOCOAT® HTS Caco-2 Assay System as per manufacturers instructions (BD Biosciences). Briefly, cells were grown to 100% confluency and maintained for 2 days prior to seeding on 1.0μm fibrillar collagen coated PET membranes. Seeding was performed in the seeding basal medium provided, then replaced 24 hours later by the Entero-STIM

Medium provided. All media was supplemented with MITO+ serum extender. After 48 hours the cells established a polarized monolayer [271]. At this point, the media was replaced with DMEM (with 10% FBS), and the cells were used for experiments.

2.4.2 Infections

For HeLa cell infections, cells were grown to approximately 70% confluency, whereas Caco cells were fully confluent. Following overnight cultures, EPEC was used to infect host cells at a multiplicity of infection (MOI) of 10:1 for 6 hours and followed procedures previously described [272]. For S. Typhimurium studies of initial invasion, subcultures of overnight bacteria were back-diluted 30X in fresh LB and grown at 37ºC (shaking) for 3 hours to activate the *Salmonella*, cells were infected at an MOI of 100:1 and the infections were carried out for 15 minutes. For *L. monocytogenes* studies, overnight bacterial cultures were diluted 10X, then cultured until $A_{600\ nm} = 0.8$. The cells were then infected at an MOI of 50:1. For initial invasion studies, we infected the cells for 15 minutes prior to fixation, whereas for comet tail studies infections persisted for 30 minutes at which point the media was swapped with warm media containing gentamicin for 1 hour (initial comet tail formation) or 4 hours (established comet tail studies).

2.4.3 Invasion Assays

To perform invasion assays *L. monocytogenes* or *S.* Typhimurium were incubated on host cells for 30 minutes. This was followed by a 1-hour incubation in media containing 50 mg/ml gentamicin (to kill external bacteria). Cells were then washed 5 times in PBS (Supplemented with magnesium and calcium; Hyclone), and

then permeabilized with 1% triton for 5 minutes. Serial dilutions were then prepared, spread on LB plates and incubated for 24 hours at 37 ºC prior to enumeration.

2.4.4 Antibodies and Reagents

Antibodies used in this study included a mouse monoclonal anti-β-Spectrin II antibody (used at 2.5µg/ml for immunofluorescence and 0.25µg/ml for western blots) (Becton Dickinson), rabbit anti-α-adducin (used at 2µg/ml for immunofluorescence and 0.2µg/ml for western blots) (Santa Cruz), rabbit anti-EPB41 (protein 4.1) (used at 1.7µg/ml for immunofluorescence and 0.17µg/ml for western blots) (Sigma), rabbit anti-calnexin (Becton Dickinson) (used at 1:2000). Secondary antibodies included a goat anti-mouse (or rabbit) antibody conjugated to AlexaFluor 568/594 (use at 0.02 µg/ml) (or HRP used at 1µg/ml for western blotting) (Invitrogen). For F-actin staining AlexaFluor 488 conjugated phalloidin (Invitrogen) was used according to the manufacturers instructions.

2.4.5 Immunofluorescent Localizations

Cells were fixed on cover slips with 3% paraformaldehyde for 15 minutes at room temperature, permeabilized using 0.1% Triton for 5 minutes at room temperature, then washed 3 times (10 minutes each) with PBS -/- (Hyclone). Samples were blocked in 5% normal goat serum in TPBS/0.1% BSA (0.05% Tween-20 and 0.1% BSA in PBS) for 20 minutes. Antibodies were then incubated on the cover slips overnight at 4ºC. The next day the cover slips were washed three times (10 minutes each) with TPBS/0.1% BSA. After the final wash, secondary antibodies

were applied for 1 hr at 37ºC. This was followed by three additional washes (10 minutes each) with TPBS/0.1% BSA. The cover slips were then mounted on slides using Prolong Gold with DAPI (Invitrogen).

2.4.6 Transfection of siRNA

β-Spectrin II, protein 4.1, α-adducin and a control pool of siRNA (Dharmacon) were transfected using the InterferIN transfection reagent (PolyPlus Transfection) according to the manufactures instructions. Transfections were incubated for 48 hours. The media was changed prior to the infections.

2.4.7 Western Blots for RNAi confirmation

Infections were performed as described above. Following the infections, the samples were placed on ice and 120 µl of ice-cold RIPA lysis buffer (150mM NaCl, 1M Tris pH7.4, 0.5M EDTA, 1% Nonidet P-40, 1% Deoxychloric acid, 0.1% SDS) with EDTA Free COMPLETE protease inhibitors (Roche). Protein lysate concentrations were determined using a bicinchoninic acid assay. The samples were processed and loaded into 6% (or 10% for Adducin and protein 4.1) poly-acrylamide gels and were run at 100V. The proteins were then transferred to nitrocellulose membranes (Trans-Blot transfer medium, Bio-Rad). Membranes were blocked with 5% Blotto (Santa Cruz Biotechnology) for 20 minutes prior to incubation with primary antibodies (for concentrations see 'Antibodies and Reagents' section) overnight at 4^0C. Blots were then washed three times with TPBS-BSA (1% Tween-20 in PBS, with 0.1% BSA) then incubated with HRP (at 1 µg/ml) for five minutes and visualized using chemiluminescence BioMax film

(Kodak). Blots were then stripped (with 2%SDS, 12.5% Tris pH 6.8, 0.8% b-mercaptoethanol) for 45 minutes at 50^0C, re-probed with antibodies used for loading controls and visualized by chemiluminescence.

2.4.8 Quantifying bacterial pathogenic events during siRNA knockdowns using microscopy

Quantitation of EPEC, *S.* Typhimurium and *L. monocytogenes* experiments in which specific proteins were knocked down by siRNA in host cells were performed by initially identifying cells with undetectable levels of the knocked down proteins (spectrin, adducin or p4.1). After identifying these cells, we manually counted the number of bacteria that had successfully generated pedestals (EPEC) or invaded (S. Typhimurium and *L. monocytogenes*) those cells.

2.4.9 Cell viability assays for siRNA treated cells

Cell viability assays performed on siRNA treated (or untreated) cells were performed using the LIVE/DEAD® Cell Viability Assay kit (Invitrogen), as per manufacturers instructions.

2.4.10 Controls

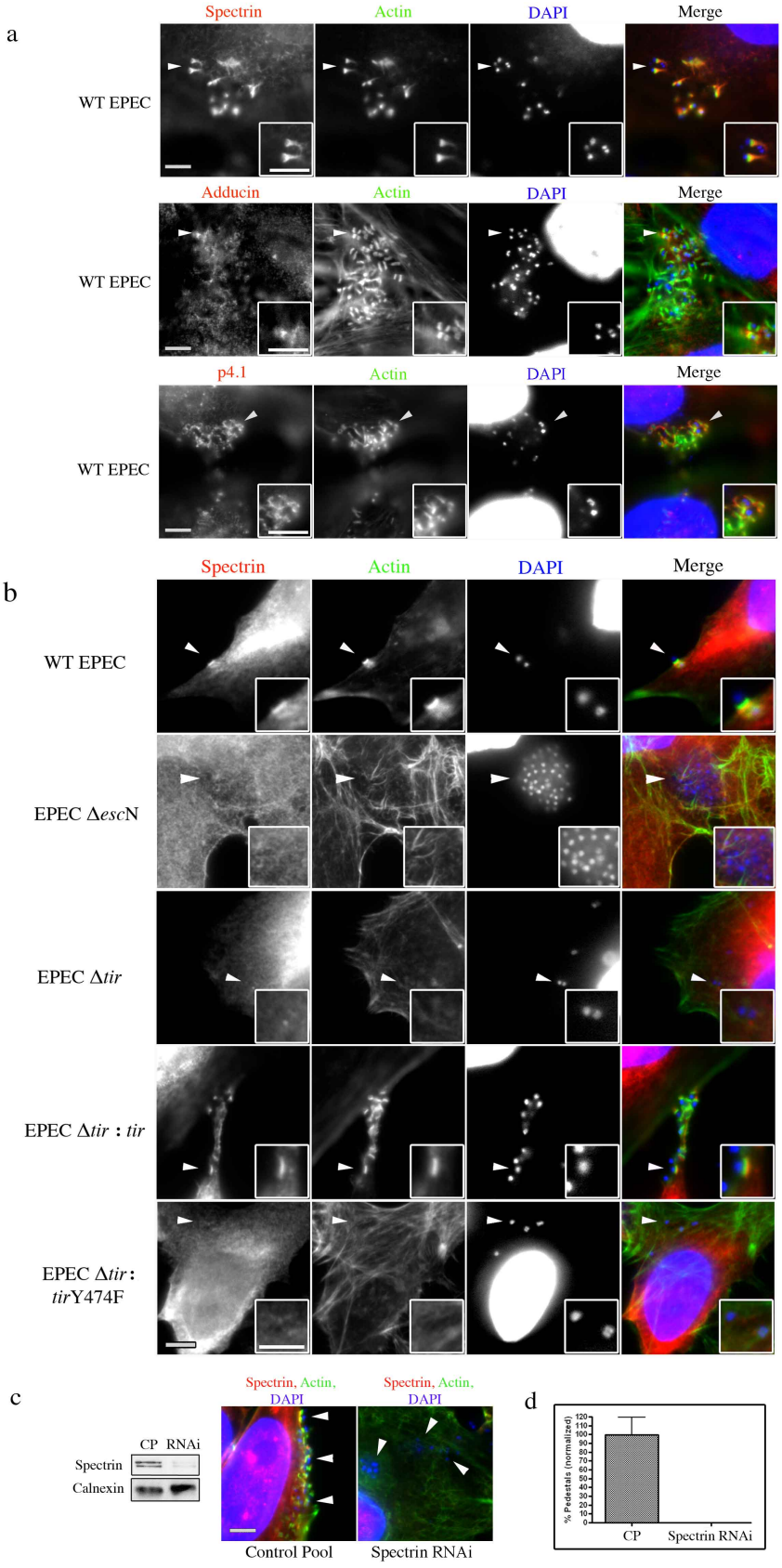
Primary antibody controls were performed by replacing the primary antibody with normal mouse IgG (Jackson ImmunoResearch) at the identical concentration to what the primary antibody was used at (Appendix 8). Secondary antibody controls were performed by replacing the primary antibody with TPBS/0.1%BSA (the carrier buffer for the primary antibodies), while all other procedures remained unchanged. We tested for autofluorecence in cells and bacteria by replacing the primary and

secondary antibodies with buffer and then mounting the cover slips with Prolong Gold (with Dapi).

2.4.11 Statistics

Statistical analysis to compare the means of two samples, comprised an un-paired, single tailed, student t-tests, with *P* values as indicated.

2.5 Figures


a
Spectrin
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p4.1
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Spectrin
Actin
DAPI
Merge
WT EPEC
EPEC ΔescN
EPEC Δtir
EPEC Δtir : tir
EPEC Δtir : tirY474F
c
CP RNAi
Spectrin
Calnexin
Spectrin, Actin, DAPI
Spectrin, Actin, DAPI
Control Pool
Spectrin RNAi
d
% Pedestals (normalized)
CP
Spectrin RNAi

Figure 2-1 Characterizing the role of the spectrin cytoskeleton during EPEC infections.

(**a**) HeLa cells were infected with EPEC and immunolocalized with antibodies to spectrin, adducin and p4.1 together with probes to actin and DAPI. Each spectrin cytoskeletal protein is distinctly recruited to EPEC pedestals. (**b)** Immunolocalization of spectrin to sites of wild-type (WT) EPEC attachment and to EPEC effector mutants: Δ*escN*, Δ*tir*, Δ*tir*:*tir*, Δ*tir*:*tir*Y474F. (**c)** Western blot of siRNA treated HeLa cells targeted against spectrin (Spectrin RNAi) and non-targeting control pool siRNA (CP). Calnexin was used as a loading control. Immunofluorescent image of spectrin RNAi, attached bacteria show they are unable to form pedestals. (**d**) Quantification of the number of bacteria forming pedestals. For each treatment, 3 independent experiments were run; for microscopy counts n = 3 for each experiment, error bars show s.e.m. Statistics were not run due to a complete absence of pedestals in infected RNAi samples. Arrowheads indicate areas of interest that are found in the insets. Scale bars are 5 μm.

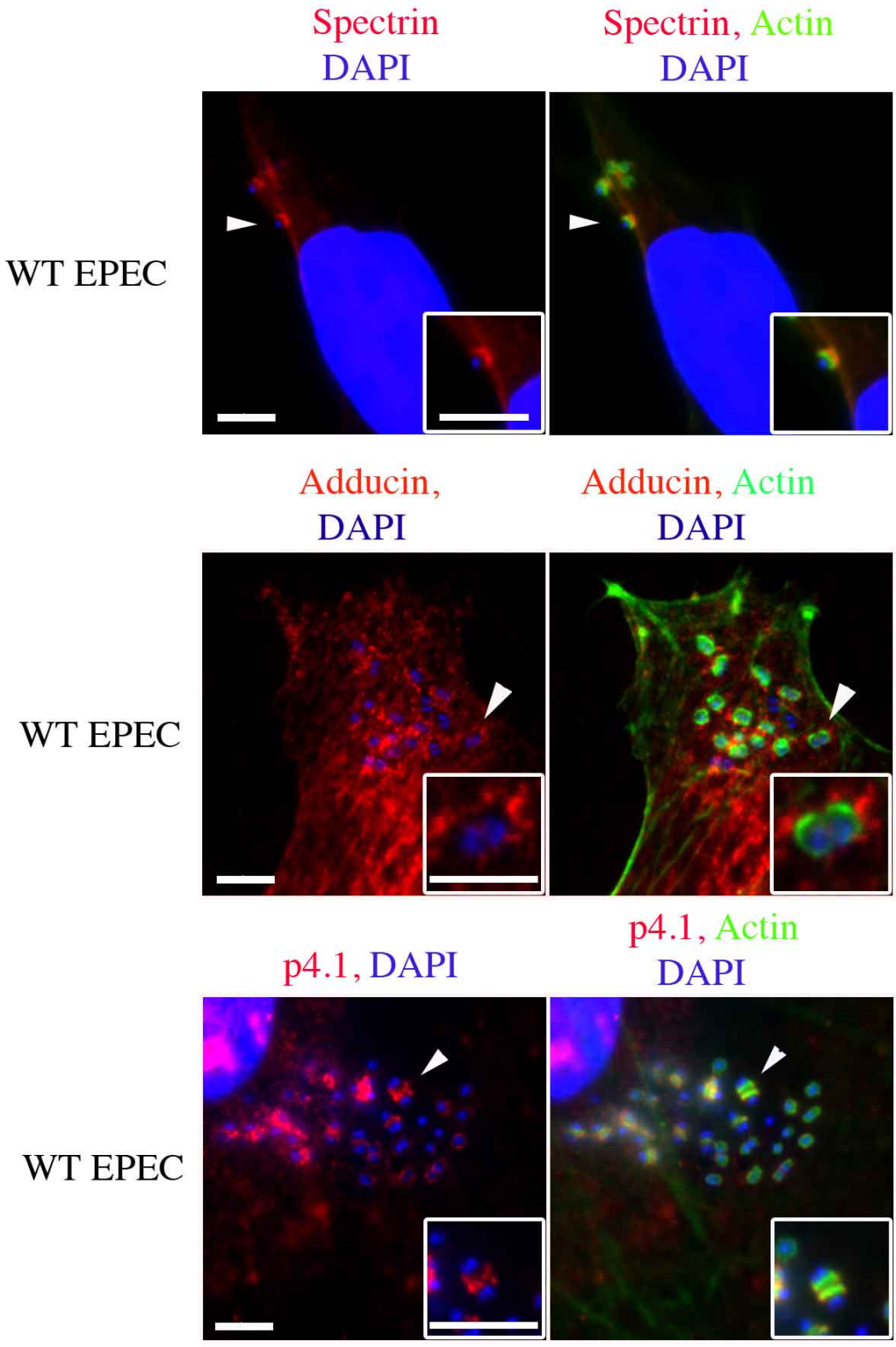

Spectrin
DAPI
Spectrin, Actin
DAPI
WT EPEC
Adducin,
DAPI
Adducin, Actin
DAPI
WT EPEC
p4.1, DAPI
p4.1, Actin
DAPI
WT EPEC

Figure 2-2 Investigating the precise localization of spectrin cytoskeletal proteins at EPEC pedestals.

HeLa cells were infected with EPEC and were immunolocalized with antibodies against spectrin, adducin and p4.1 as well as actin and DAPI. A distinct gap is observed between bacteria (stained with DAPI) and the spectrin cytoskeletal components. Actin fills the gap between spectrin cytoskeletal components and EPEC. Arrows indicate areas of interest that are found in the insets. Scale bars are 5 μm.

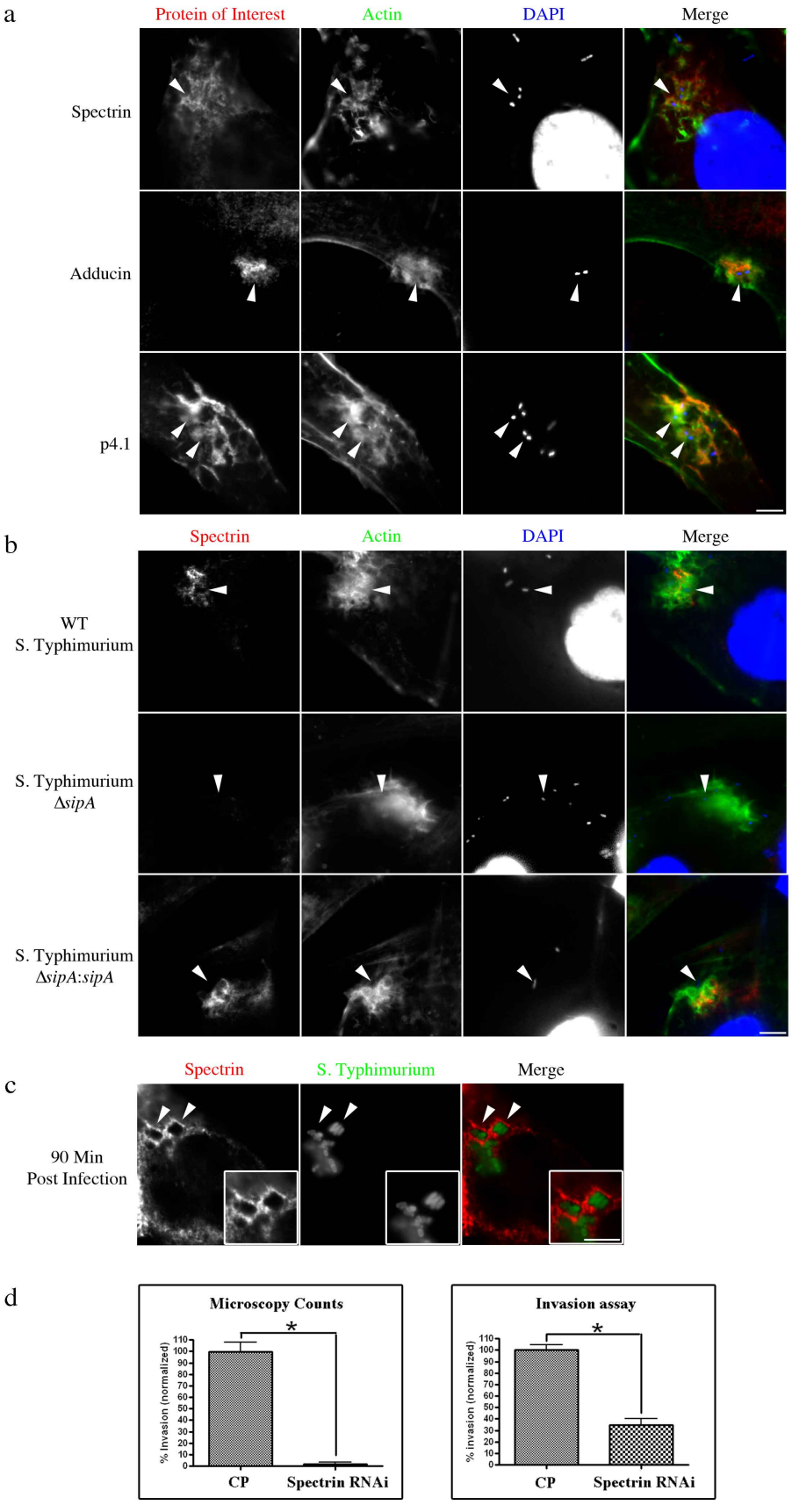

a
Protein of Interest
Actin
DAPI
Merge
Spectrin
Adducin
p4.1
b
Spectrin
Actin
DAPI
Merge
WT
S. Typhimurium
S. Typhimurium
ΔsipA
S. Typhimurium
ΔsipA:sipA
c
Spectrin
S. Typhimurium
Merge
90 Min
Post Infection
d
Microscopy Counts
% Invasion (normalized)
CP
Spectrin RNAi
Invasion assay
% invasion (normalized)
CP
Spectrin RNAi

Figure 2-3 Spectrin cytoskeleton distribution during *S*. Typhimurium infections.

(**a**) Immunolocalization of spectrin cytoskeletal elements, spectrin, adducin and p4.1 with actin and DAPI during *S*. Typhimurium SL1344 invasion in HeLa cells. Actin labeling identified invasion sites with actin-rich membrane ruffles. All three spectrin cytoskeletal components are recruited to invasion sites. (**b**) Images illustrating the inability of a Δ*sipA* mutant to recruit spectrin to sites of invasion. Δ*sipA*:*sipA* restored spectrin recruitment to invasion sites. (**c**) Spectrin shows incresed localization to sites of internalized *S*. Typhimurium SL1344-GFP. Areas of interest are indicated by arrowheads and highlighted in insets. (**d**) Quantifying invasion efficiency after depletion of spectrin with siRNA as compared to a non-targeting control pool (CP). For each treatment, 3 independent experiments were run; for microscopy counts n = 3, error bars show s.e.m, *P<0.0001 for all statistics. Scale bars are 5μm.

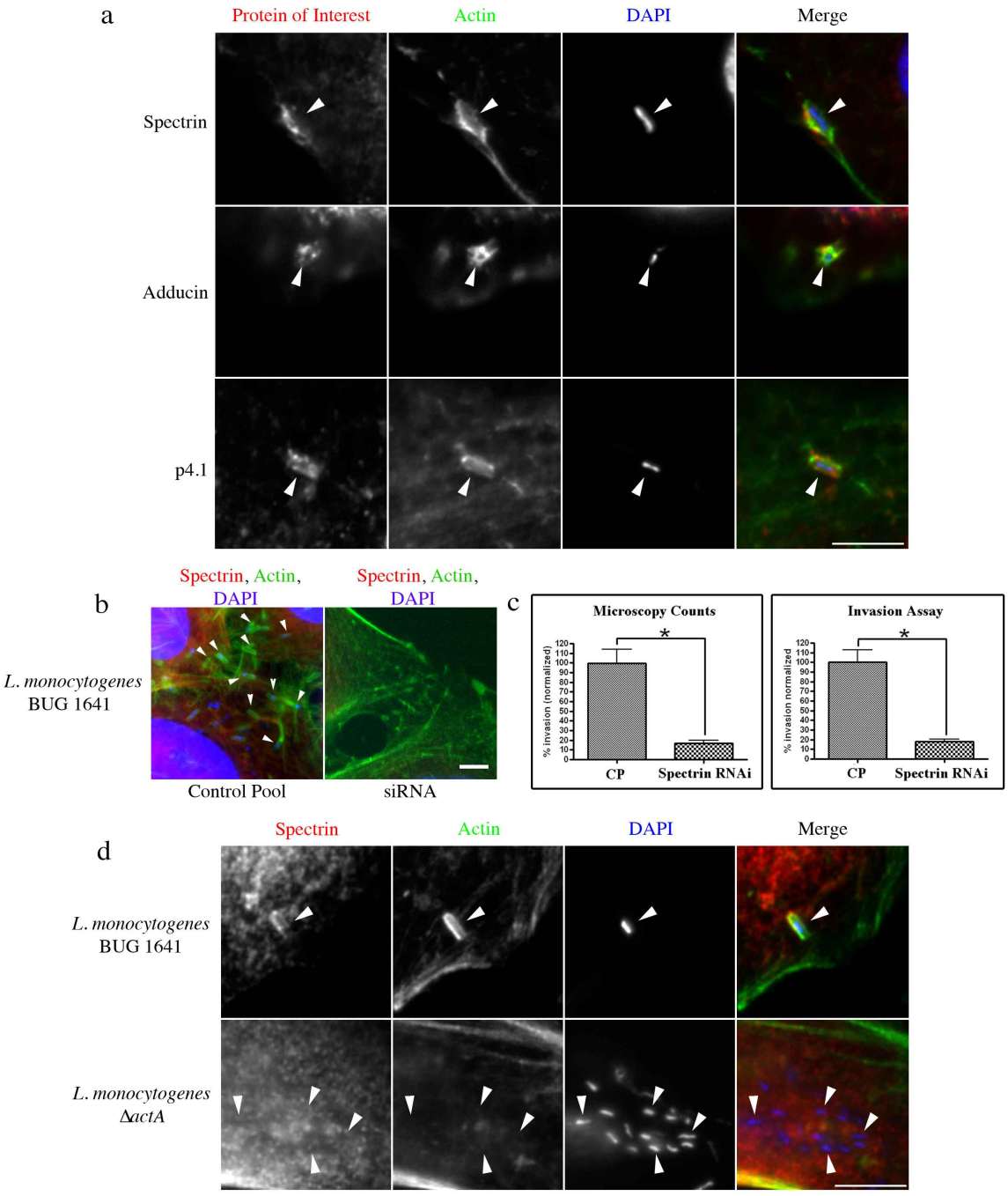

a
Protein of Interest
Actin
DAPI
Merge
Spectrin
Adducin
p4.1
b
Spectrin, Actin, DAPI
Spectrin, Actin, DAPI
L. monocytogenes BUG 1641
Control Pool
siRNA
c
Microscopy Counts
Invasion Assay
% invasion (normalized)
CP
Spectrin RNAi
d
Spectrin
Actin
DAPI
Merge
L. monocytogenes BUG 1641
L. monocytogenes ΔactA

Figure 2-4 The importance of the spectrin cytoskeleton during *L. monocytogenes* infections.

(**a**) Infections showing spectrin/adducin/p4.1 recruitment to the characteristic actin cups at sites of *L. monocytogenes* invasion. (**b**) Immunofluorescent image of spectrin RNAi showing inability of *L. monocytogenes* to invade cells with no detectable spectrin. Arrows indicate internalized bacteria. (**c**) Graphs quantifying invasion efficiency after depletion of spectrin with siRNA as compared to control pool (CP) siRNA treatment. For each treatment, 3 independent experiments were run, for microscopy counts n = 3, error bars show s.e.m, *P<0.0001 for all statistics. (**d**) Initial stages of *L. monocytogenes* comet tail formation, showing polar spectrin recruitment together with actin. Infection with *Listeria* Δ*actA* did not recruit spectrin. Scale bars are 5μm.

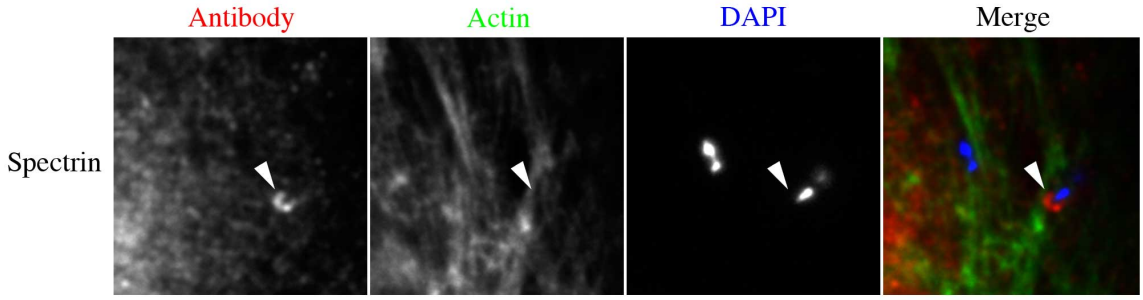

Antibody
Actin
DAPI
Merge
Spectrin

Figure 2-5 Spectrin is recruited to initial stages of *L. monocytogenes* tail formation, independent of actin.

Immunolocalization of spectrin identifying recruitment to internalized *L. monocytogenes*, in the absence of actin, at 30 minutes post infection. Scale bars are 5 μm.

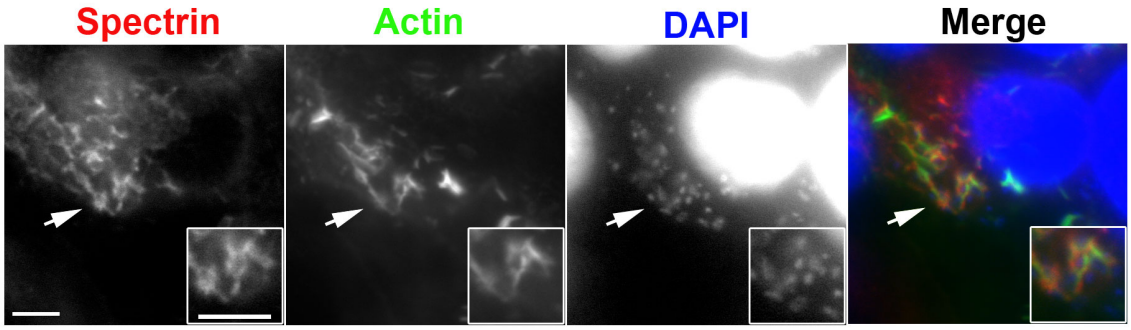

Spectrin
Actin
DAPI
Merge

Figure 2-6S Spectrin is recruited to EPEC pedestals on polarized Caco-2 cells.

Polar Caco-2 monolayers were infected with EPEC and stained for spectrin, actin and DAPI. Arrow points to area of actin and spectrin recruitment to pedestals, which is magnified within the inset. Scale bars are 5μm.

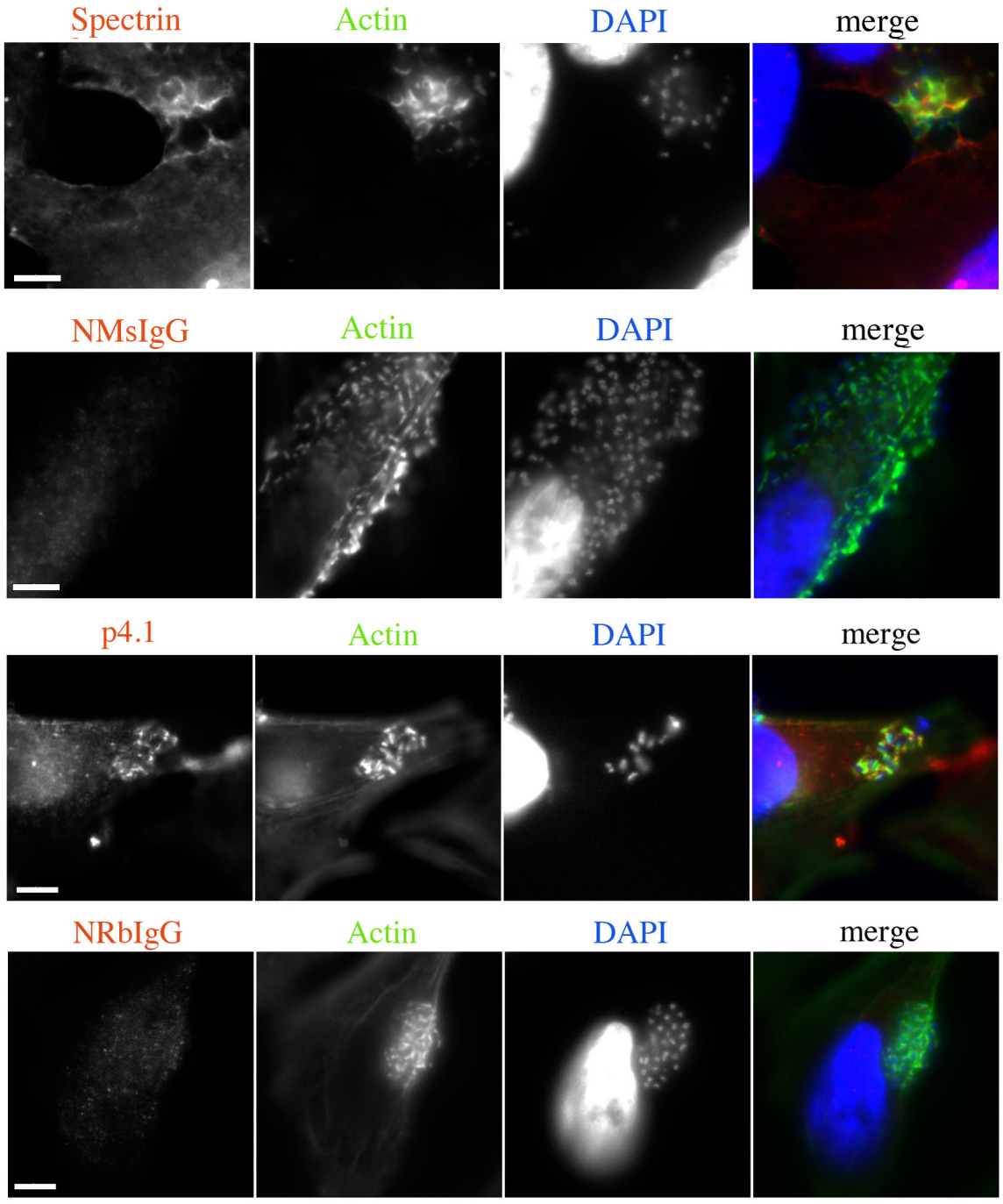

Spectrin
Actin
DAPI
merge
NMsIgG
Actin
DAPI
merge
p4.1
Actin
DAPI
merge
NRbIgG
Actin
DAPI
merge

Figure 2-7S Primary antibody controls show no specific staining at EPEC pedestals.

HeLa cells were infected with EPEC for 6 hours. Cells were treated with antibodies specific to spectrin or p4.1 and compared to cells stained with normal mouse IgG (NMsIgG) or normal rabbit IgG (NRbIgG), at identical concentrations to the spectrin and p4.1 antibodies respectively. Primary antibodies or non-specific IgG were co-localized with probes for DAPI and actin to identify attached EPEC and their pedestals. Primary antibodies non-specifically label the entire cell, with no increased localization at sites of EPEC pedestals. Scale bars are 5 μm.

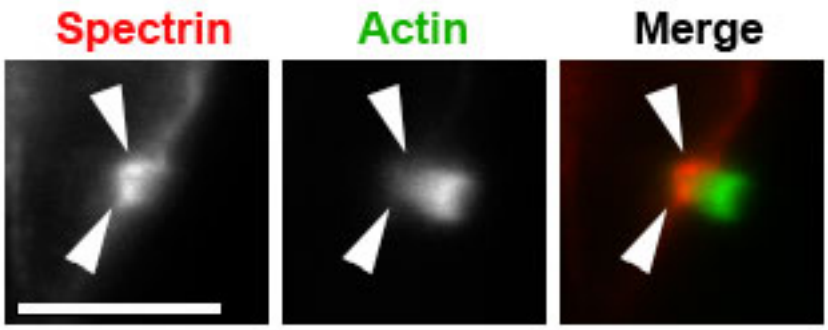

Spectrin
Actin
Merge

Figure 2-8S Spectrin localizes to the basal region of EPEC pedestals.

HeLa cells were infected with EPEC and stained for spectrin and actin. Arrows indicate a concentration of spectrin at the pedestal but it is not recruited to areas of actin filament concentration.

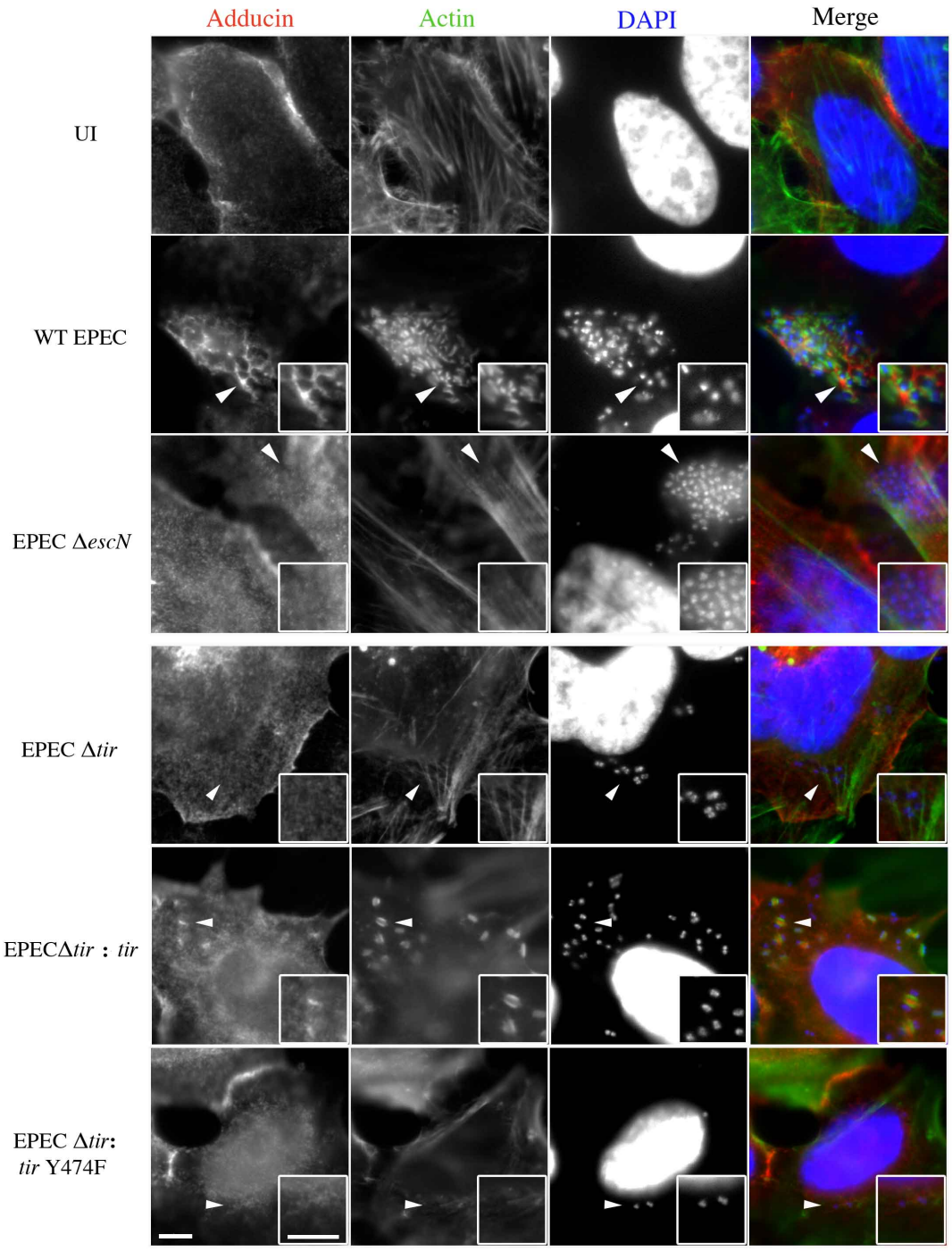

Adducin
Actin
DAPI
Merge
UI
WT EPEC
EPEC ΔescN
EPEC Δtir
EPECΔtir : tir
EPEC Δtir:
tir Y474F

Figure 2-9S The role of EPEC effectors in adducin recruitment to pedestals.

HeLa cells were infected with EPEC or EPEC effector mutants, and immunolocalized with adducin antibodies, as well as actin and DAPI. Images identify the Tir Y474 phosphorylation event as an essential step in adducin recruitment to the pedestal. Arrows indicate areas of interest that are found in the insets. Images examining adducin localization in uninfected (UI) or infections with WT EPEC, EPEC Δ*esc*N, EPEC Δ*tir*, EPEC Δ*tir*:*tir*, and EPEC Δ*tir*:*tir*Y474F. Scale bars are 5 μm.

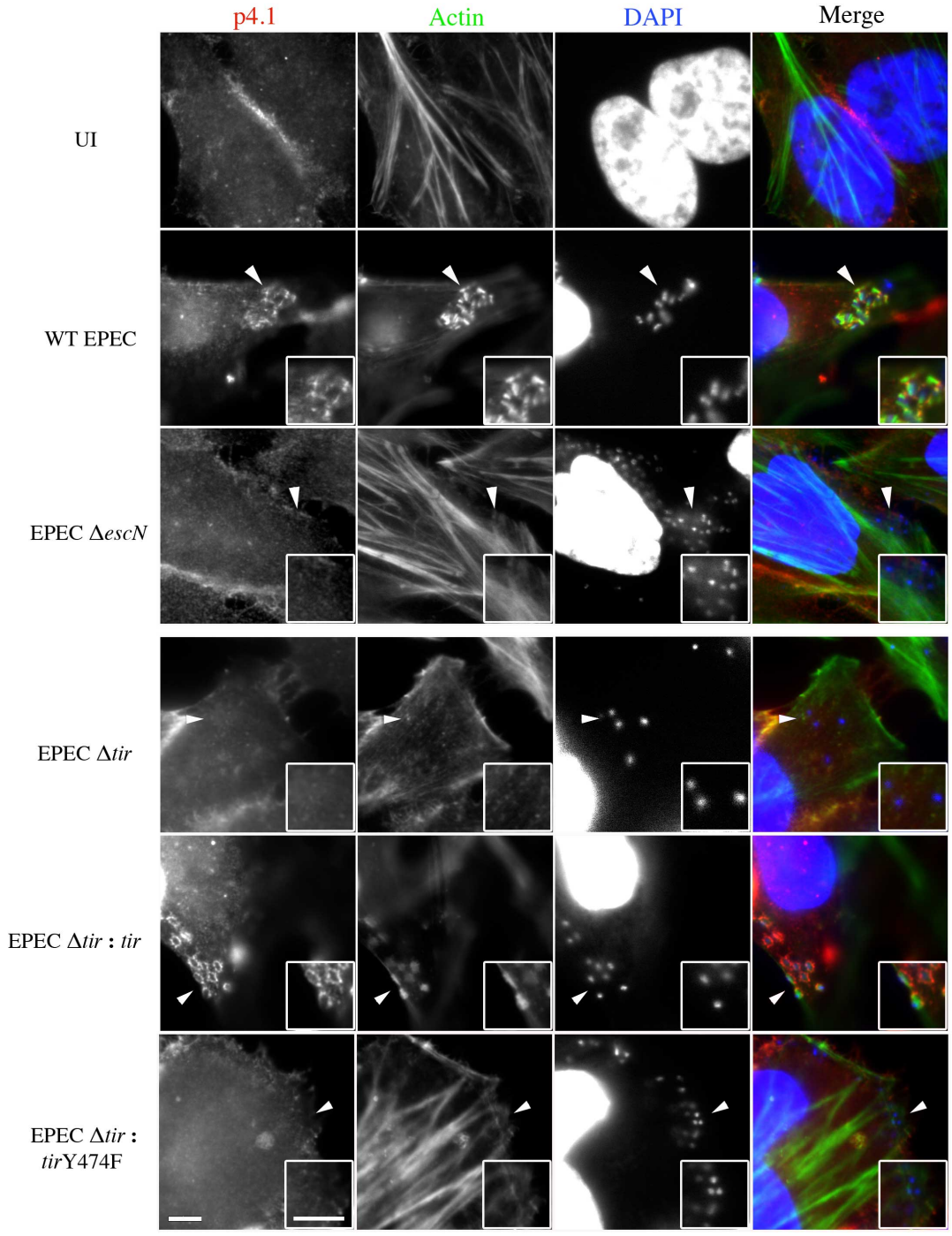

p4.1
Actin
DAPI
Merge
UI
WT EPEC
EPEC ΔescN
EPEC Δtir
EPEC Δtir : tir
EPEC Δtir : tirY474F

Figure 2-10S P4.1 recruitment in host cells during EPEC infections.

HeLa cells were infected with various EPEC effector mutants and immunolocalized with an antibody targeted against p4.1, as well as probes to actin and DAPI. TirY474 phosphorylation is shown to be necessary for p4.1 recruitment to the pedestal. Arrows indicate areas of interest that are found in the insets. Figure shows immunolocalization of p4.1 during infections with WT EPEC, EPEC Δ*esc*N, EPEC Δ*tir*, EPEC Δ*tir*:*tir*, EPEC Δ*tir*:*tir*Y474F. Scale bars are 5 μm.

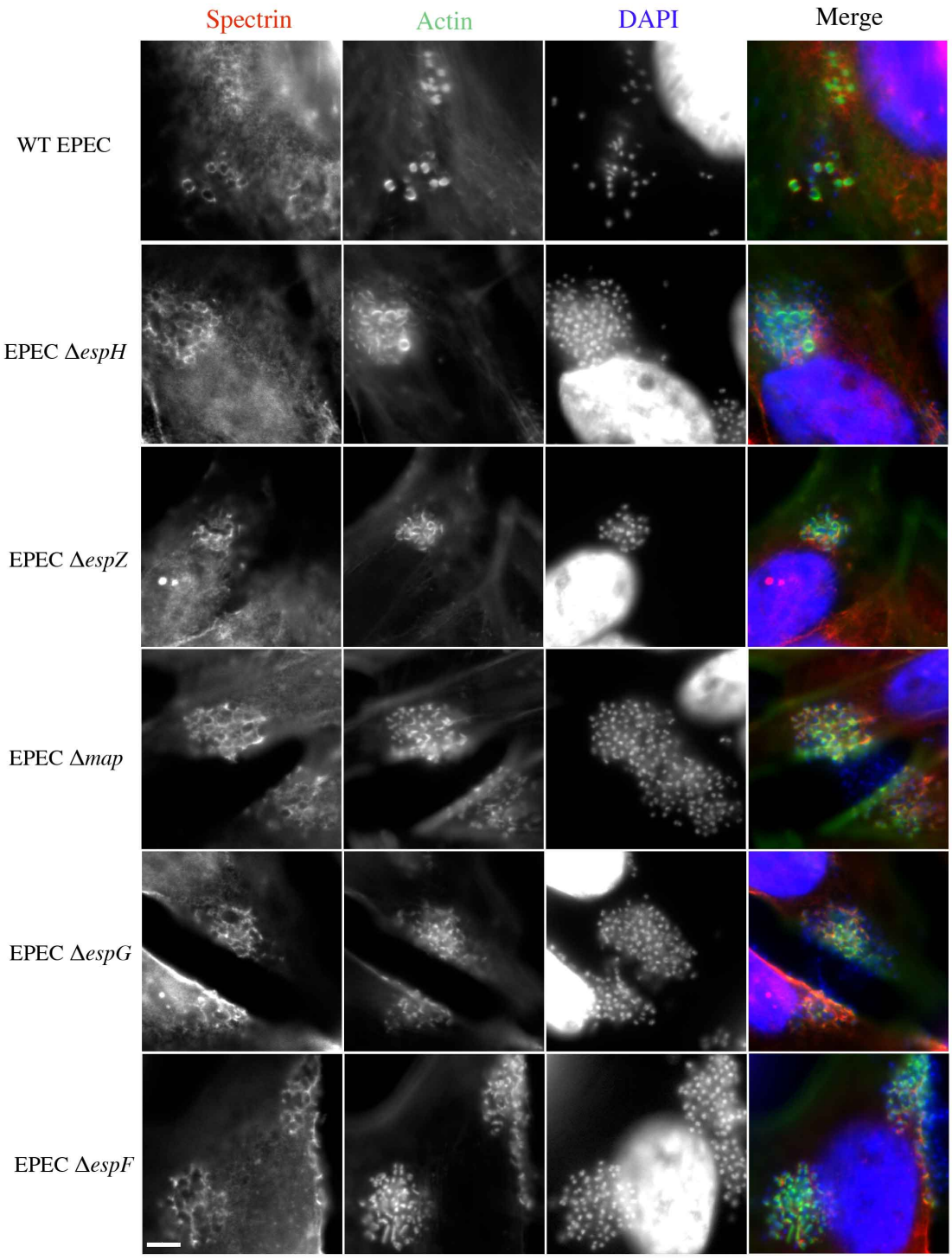

Spectrin
Actin
DAPI
Merge
WT EPEC
EPEC Δ*espH*
EPEC Δ*espZ*
EPEC Δ*map*
EPEC Δ*espG*
EPEC Δ*espF*

Figure 2-11S Spectrin recruitment to pedestals generated during other EPEC effector mutants that are also involved in efficient pedestal formation.

HeLa cells were infected with EPEC or EPEC effector mutants and immunolocalized with an anti-spectrin antibody and co-localized with actin and DAPI. Figure shows immunolocalization of spectrin during infections with WT EPEC, EPEC Δ*espH*, EPEC Δ*espZ*, EPEC Δ*map*, EPEC Δ*espG*, EPEC Δ*espF*. Spectrin is recruited to all mutant pedestals. Scale bars are 5 μm.

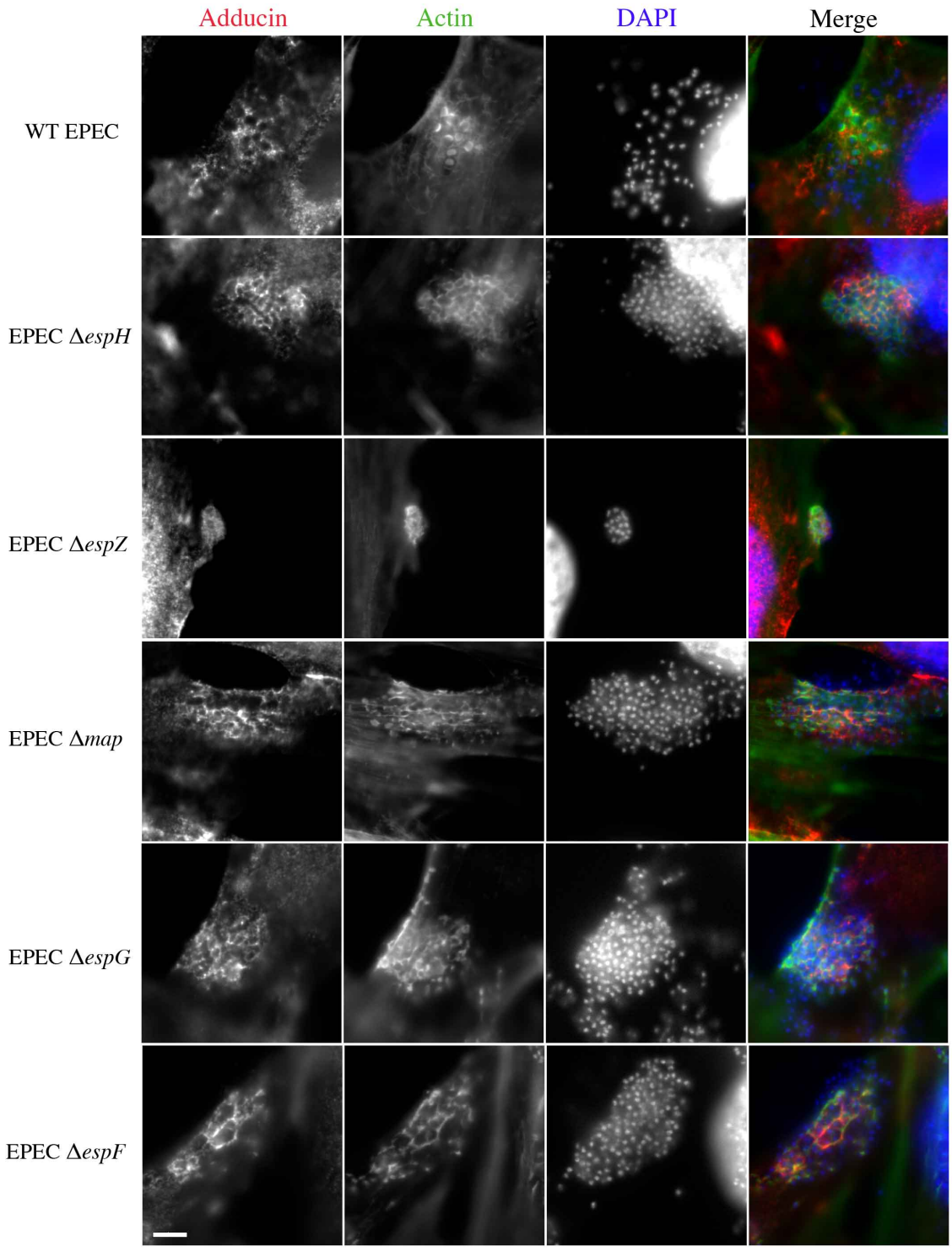

Adducin
Actin
DAPI
Merge
WT EPEC
EPEC Δ*espH*
EPEC Δ*espZ*
EPEC Δ*map*
EPEC Δ*espG*
EPEC Δ*espF*

Figure 2-12S Immunolocalization of adducin, actin and DAPI during infections with EPEC effector mutants on HeLa cells.

The figure shows immunolocalization of adducin to pedestals of WT EPEC, EPEC Δ*espH*, EPEC Δ*espZ*, EPEC Δ*map*, EPEC Δ*espG*, EPEC Δ*espF*. Adducin is recruited to all mutant pedestals. Scale bars are 5 μm.

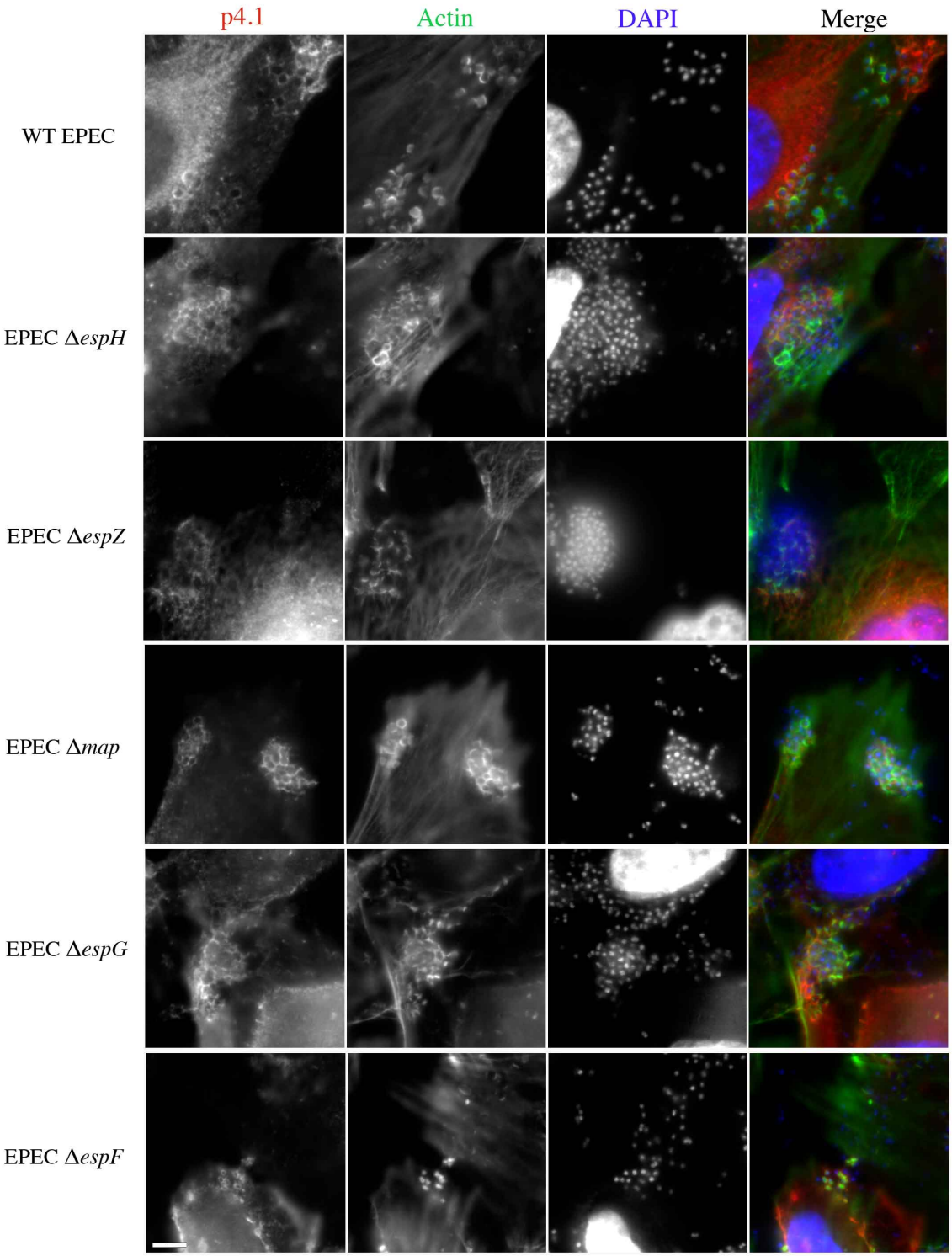

p4.1
Actin
DAPI
Merge
WT EPEC
EPEC Δ*espH*
EPEC Δ*espZ*
EPEC Δ*map*
EPEC Δ*espG*
EPEC Δ*espF*

Figure 2-13S P4.1 actin and DAPI co-localization during infections with EPEC effector mutants.

Figure showing recruitment of p4.1 to pedestals of WT EPEC, EPEC Δ*espH*, EPEC Δ*espZ*, EPEC Δ*map*, EPEC Δ*espG*, EPEC Δ*espF*. P4.1 is recruited to all mutant pedestals. Scale bars are 5 μm.

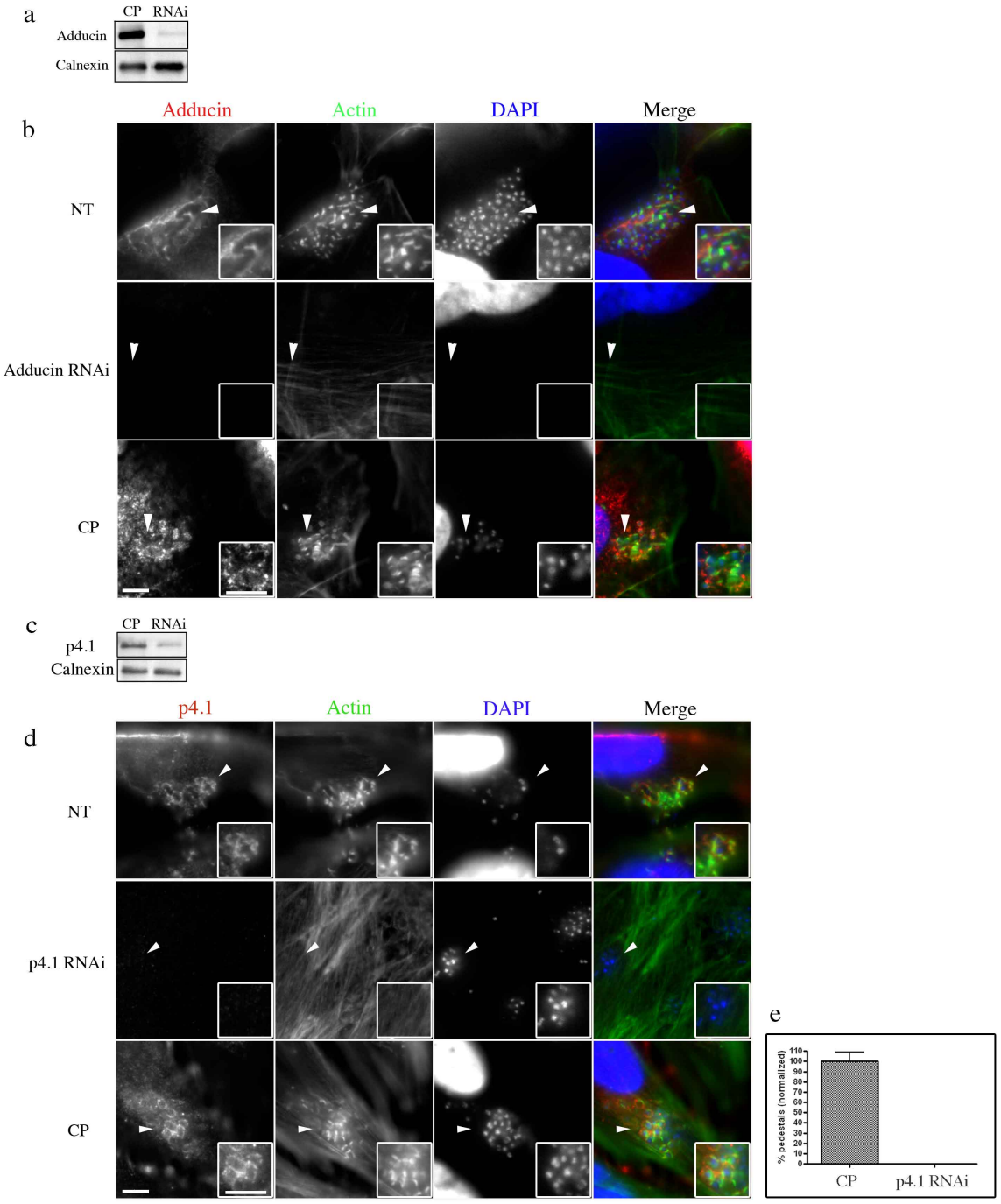

a
CP
RNAi
Adducin
Calnexin
b
Adducin
Actin
DAPI
Merge
NT
Adducin RNAi
CP
c
CP
RNAi
p4.1
Calnexin
d
p4.1
Actin
DAPI
Merge
NT
p4.1 RNAi
CP
e
% pedestals (normalized)
110
100
90
80
70
60
50
40
30
20
10
0
CP
p4.1 RNAi

Figure 2-14S Adducin and p4.1 are crucial for EPEC attachment and pedestal formation respectively.

(**a**) Adducin was knocked-down in host cells. (**b**) EPEC infected cells were labeled with adducin, actin and DAPI. Bacteria did not attach to adducin RNAi cells, but attached and generated pedestals in cells with no treatment (NT) and control pool (CP) siRNA treated cells. (**c**) Western blot confirming p4.1 was knocked down using siRNA (RNAi). Cells were infected with wild-type EPEC and pedestals counted. (**d**) Immunofluorescent images and (**e**) quantification of the number of bacteria forming pedestals. For each treatment, 3 independent experiments were run; for microscopy counts n = 3, error bars show s.e.m. No stats run due to a complete absence of pedestals generated in infected RNAi samples. Scale bars are 5 μm.

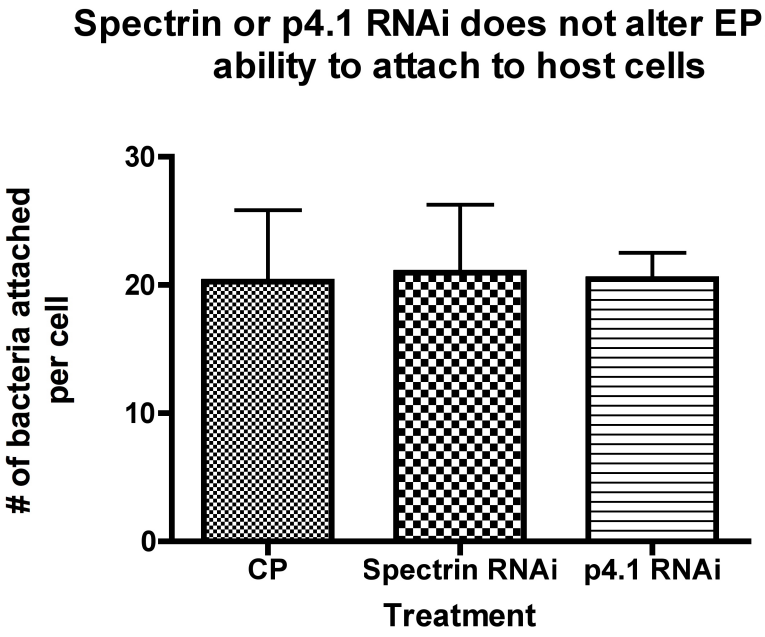
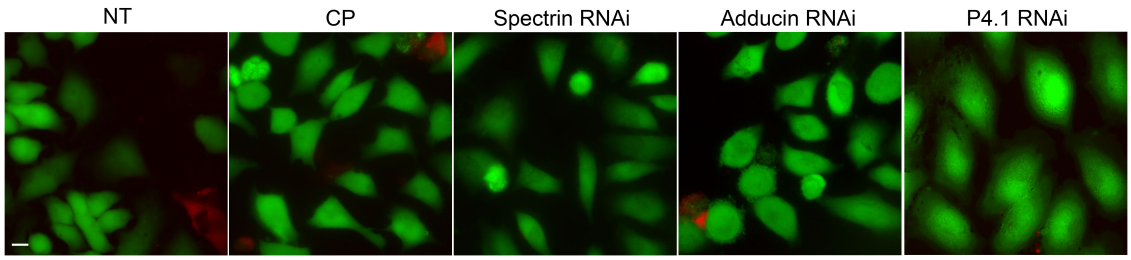

Spectrin or p4.1 RNAi does not alter EPEC's ability to attach to host cells
# of bacteria attached per cell
30
20
10
0
CP
Spectrin RNAi
p4.1 RNAi
Treatment

Figure 2-15S Spectrin or p4.1 knockdowns do not influence the ability of EPEC to attach to the host cell.

HeLa cells were transfected with control pool (CP), spectrin or p4.1 siRNA, then infected with EPEC for 6 hours. The average number of bacteria attached to each cell was then counted. Each experiment was run in triplicate and 30 host cells were counted per treatment (n=3). The means of each treatment were not statistically significant ($P<0.05$). Error bars show s.e.m.


NT
CP
Spectrin RNAi
Adducin RNAi
P4.1 RNAi

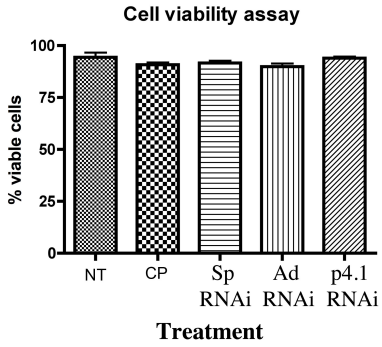

Cell viability assay
% viable cells
100
75
50
25
0
NT
CP
Sp RNAi
Ad RNAi
p4.1 RNAi
Treatment

Figure 2-16S Viability of cells is unaltered by various siRNA treatments.

Hela cells were left untreated (NT=no treatment) or treated with control pool (CP), spectrin (Sp), p4.1, or adducin (Ad) siRNA identically to our infection siRNA protocols. The cells were stained with a cell viability probe (Invitrogen). Green cells represent viable cells, red cells represent dead cells. For each treatment, 3 independent experiments were run (n=3). Total cell viability of each treatment was quantified by counting 200 cells in each sample. The means of each treatment are not statistically significant ($P<0.05$). Error bars show s.e.m. Scale bar is 5 μm.

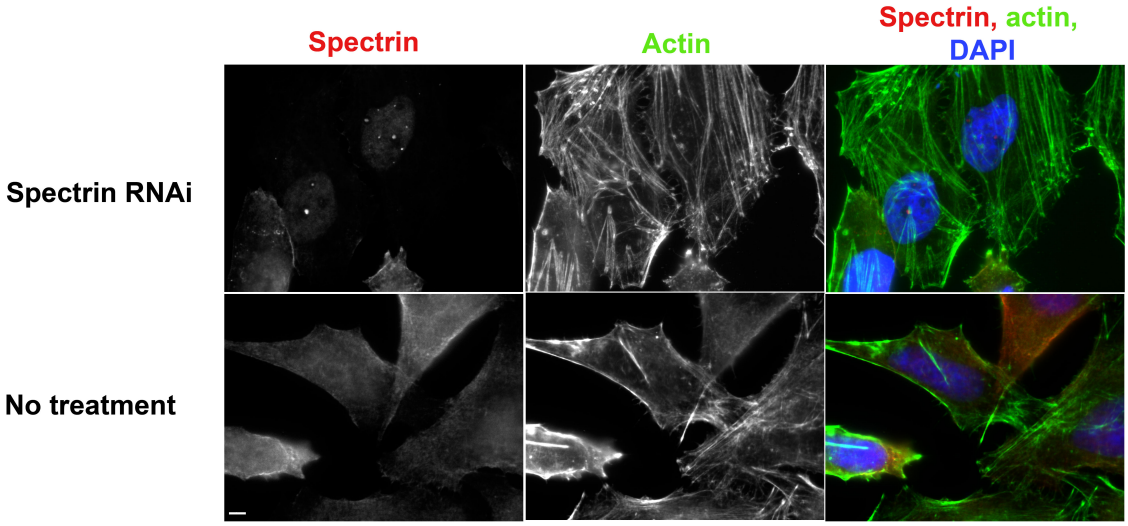

Spectrin
Actin
Spectrin, actin,
DAPI
Spectrin RNAi
No treatment

Figure 2-17S Actin cytoskelton morphology is unaltered during spectrin knockdown.

HeLa cells were treated with spectrin siRNA for 48 hours. Cells were stained for actin, spectrin and DAPI. In the absence of β_2-spectrin, the actin cytoskeleton morphology appears normal, with characteristic cortical actin and stress fibers present in the cells. Scale bar is 5μm.

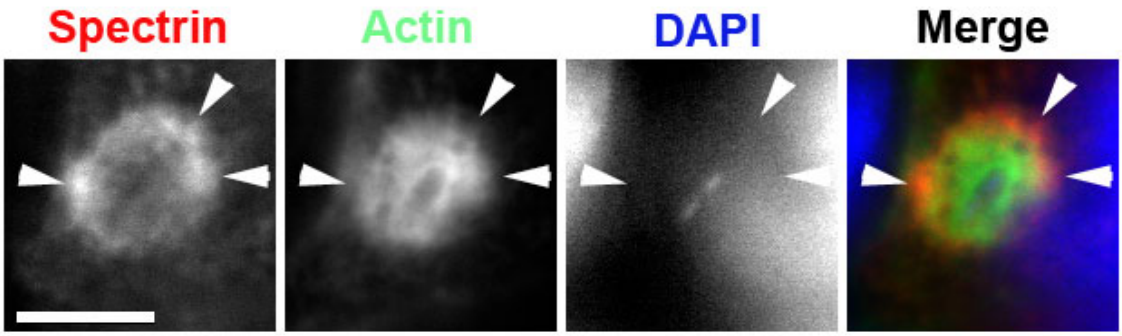

Spectrin
Actin
DAPI
Merge

Figure 2-18S Spectrin is recruited to membrane ruffles during *S.* Typhimurium invasion of Caco-2 cell monolayers.

Polarized Caco-2 cells were infected with *S.* Typhimurium for 15 minutes and immunolocalized with spectrin, actin and DAPI. Spectrin is localized to *Salmonella* invasion sites on polar Caco cells. Arrows indicated regions where spectrin is present peripheral to actin at the membrane ruffles. Scale bar is 5μm.

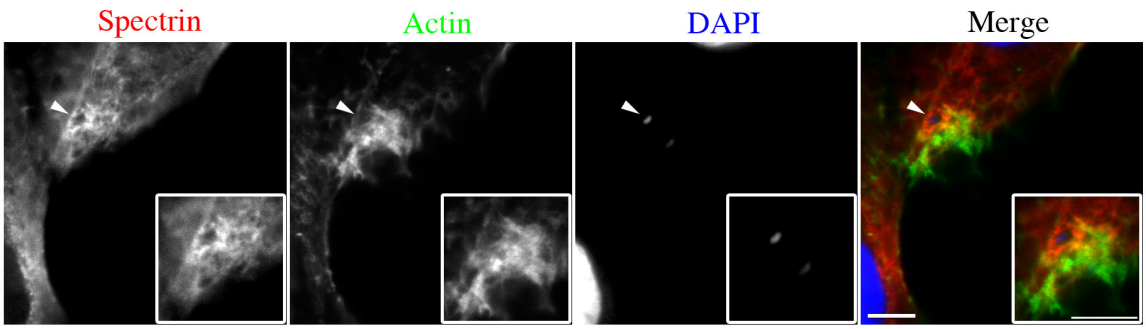

Spectrin
Actin
DAPI
Merge

Figure 2-19S Spectrin is present at regions of *S.* Typhimurium membrane ruffles independent of actin.

Immunolocalization of spectrin, actin and DAPI during infection of HeLa cells with *S.* Typhimurium. Arrowhead and inset identify a site of invasion, demonstrating spectrin recruitment at site of bacterial invasion that are independent of actin in certain regions. Scale bars are 5μm.

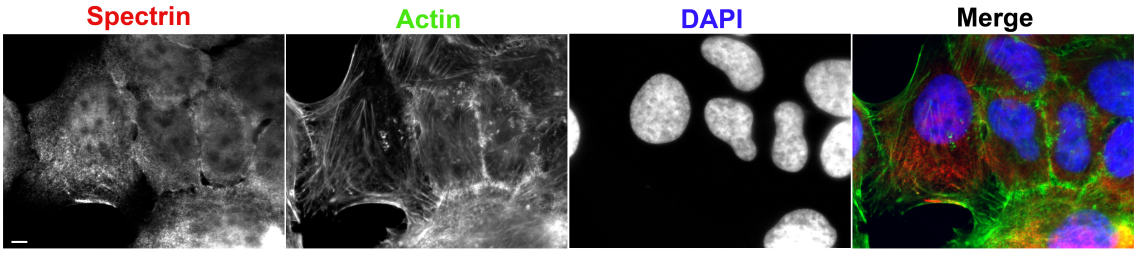

Spectrin
Actin
DAPI
Merge

Figure 2-20S Examples of actin cytoskeletal network in regions where spectrin is absent in uninfected cells.

Uninfected HeLa cells were stained for actin, spectrin, and DAPI. Stress fibers and the cell cortex are present with actin in the absence of spectrin. Scale bar is 5μm.

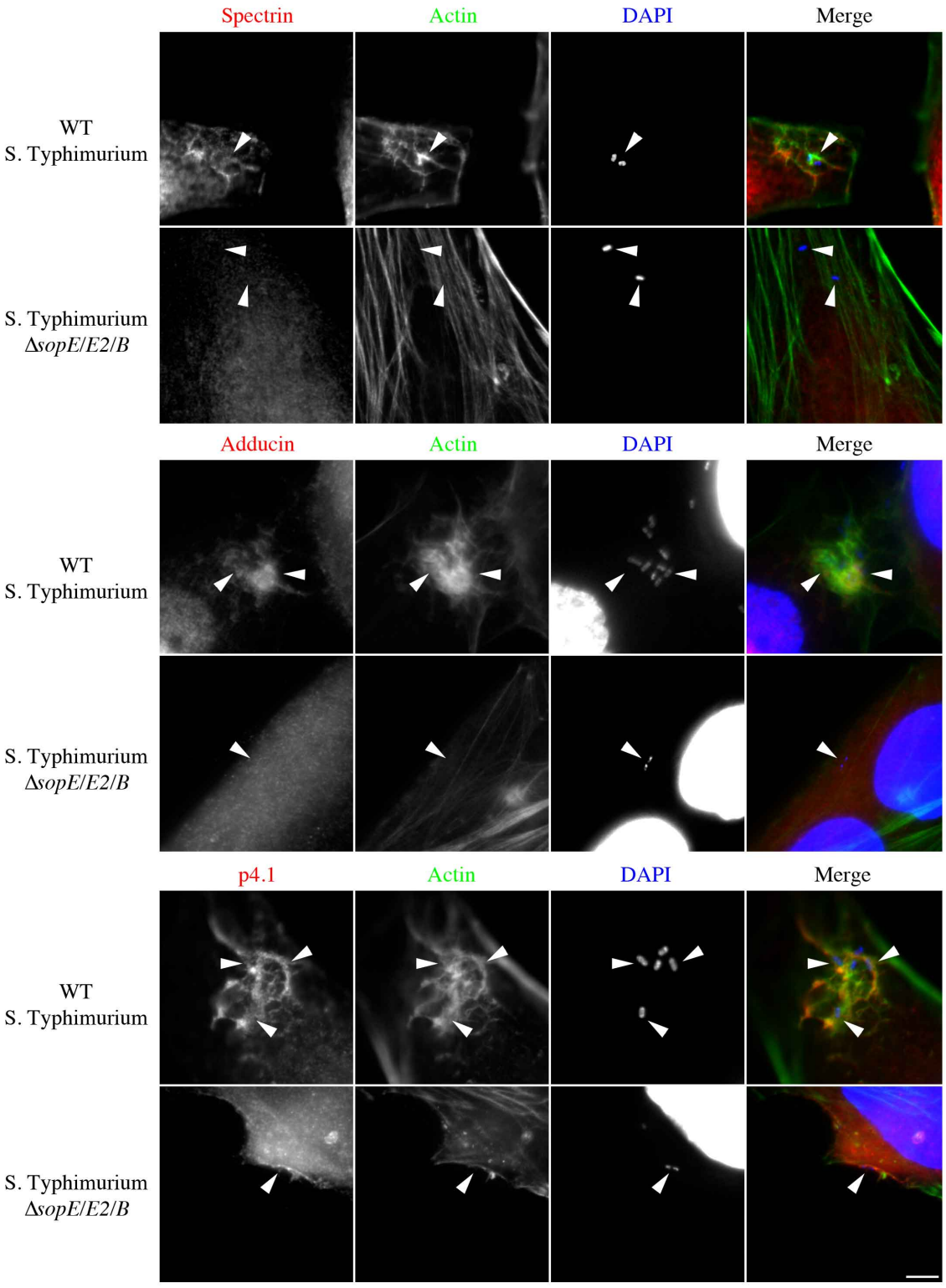

Spectrin
Actin
DAPI
Merge
WT
S. Typhimurium
S. Typhimurium
ΔsopE/E2/B
Adducin
Actin
DAPI
Merge
WT
S. Typhimurium
S. Typhimurium
ΔsopE/E2/B
p4.1
Actin
DAPI
Merge
WT
S. Typhimurium
S. Typhimurium
ΔsopE/E2/B

Figure 2-21S Membrane ruffles are needed for spectrin cytoskeletal protein recruitment.

Immunofluorescence images of spectrin, adducin and p4.1 with DAPI and actin during infection with *Salmonella* Δ*sopE/E2/B* mutant compared to WT *Salmonella*. Arrows indicate areas of interest. *S.* Typhimurium Δ*sopE/E2/B* did not generate membrane ruffles and did not recruit spectrin cytoskeletal proteins. Scale bar is 5μm.

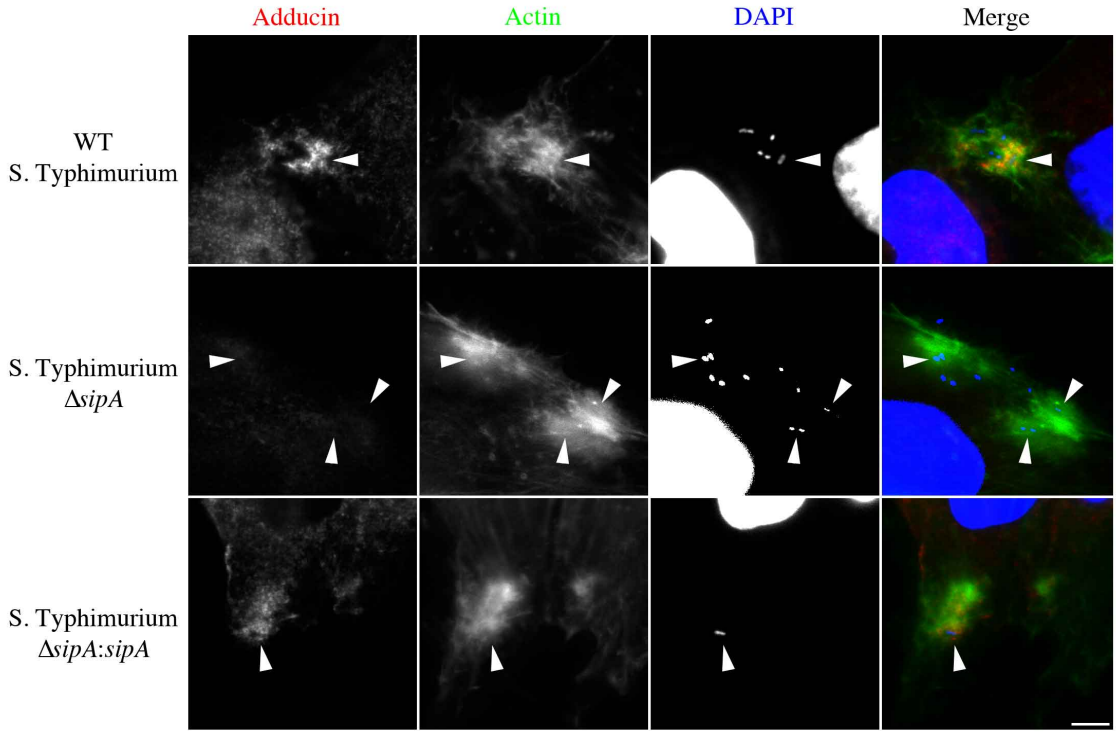

Adducin
Actin
DAPI
Merge
WT
S. Typhimurium
S. Typhimurium
ΔsipA
S. Typhimurium
ΔsipA:sipA

Figure 2-22S SipA is required for adducin recruitment to membrane ruffles.

Adducin was immunolocalized during *S*. Typhimurium Δ*sipA* infections. Images show a lack of adducin recruitment to invasion sites with actin-rich membrane ruffling on HeLa cells infected with *S*. Typhimurium Δ*sipA*. Complemented *S*. Typhimurium Δ*sipA:sipA* rescued the wild-type phenotype. Scale bar is 5μm.

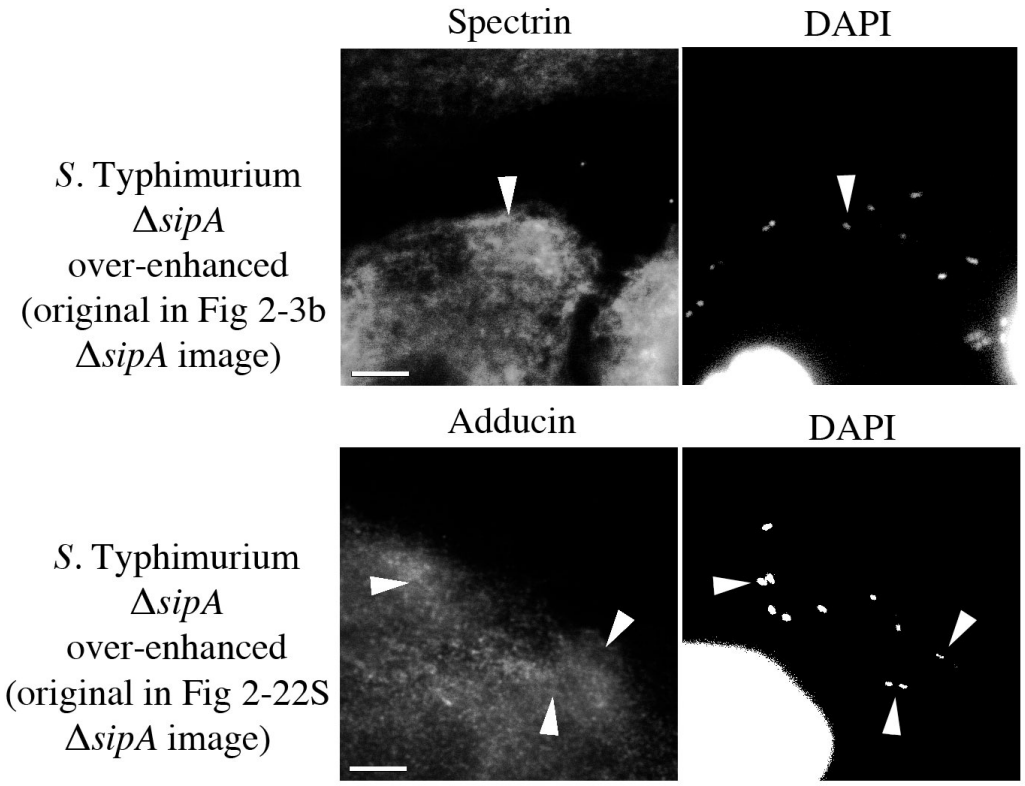
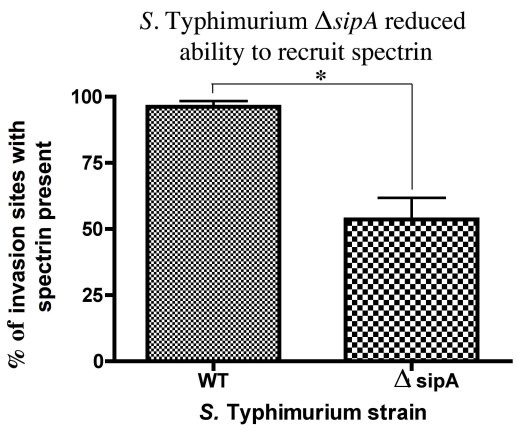

Spectrin
DAPI
S. Typhimurium ΔsipA over-enhanced (original in Fig 2-3b ΔsipA image)
Adducin
DAPI
S. Typhimurium ΔsipA over-enhanced (original in Fig 2-22S ΔsipA image)

Figure 2-23S *S.* Typhimurium Δ*sipA* infections with over-enhanced images to indicate that a cell was present in the S. Typhimurium Δ*sipA* panels in figures 2-3b and S22.

Background spectrin and adducin host cell levels are presented. Scale bars are 5μm.


*. Typhimurium ΔsipA reduced ability to recruit spectrin
*
100
75
50
25
0
% of invasion sites with spectrin present
WT
ΔsipA
S. Typhimurium strain

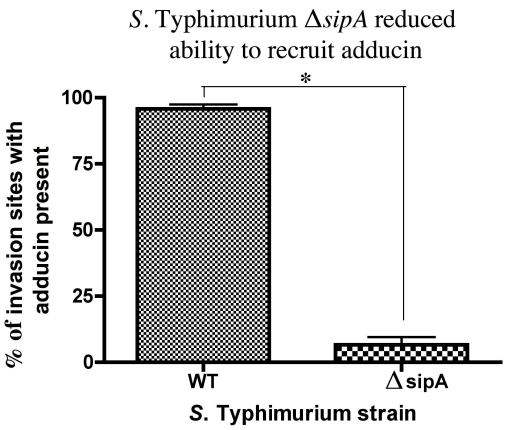

*. Typhimurium ΔsipA reduced ability to recruit adducin
*
100
75
50
25
0
% of invasion sites with adducin present
WT
ΔsipA
S. Typhimurium strain

Figure 2-24S *S*. Typhimurium Δ*sipA* infections showed reduced ability to recruit spectrin and adducin.

Quantification of spectrin and adducin recruitment to sites of *S*. Typhimurium Δ*sipA* invasion as compared to WT *S*. Typhimurium invasion. Invasion sites were identified by actin-rich membrane ruffles around attached bacteria, then observed for spectrin or adducin recruitment to those sites. Each experiment was performed in triplicate (n=3), counting 100 actin-based invasion sites. The means of the WT versus Δ*sipA* infection are significant ($P<0.0001$). Error bars show s.e.m.

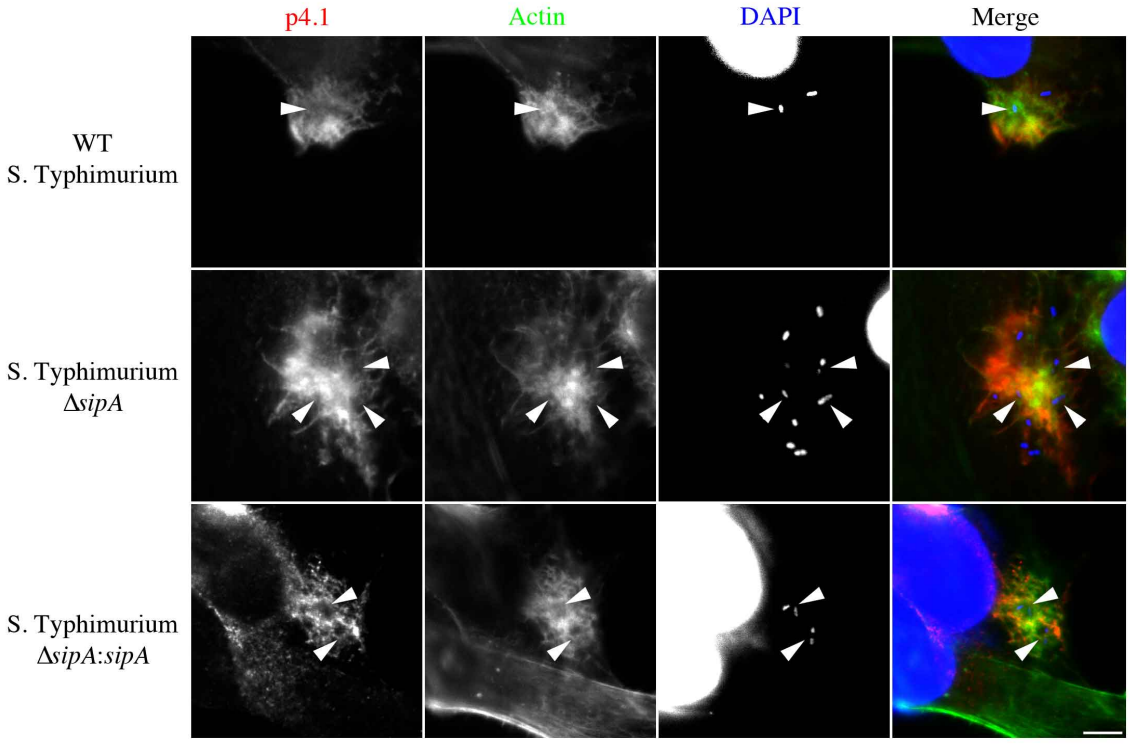

p4.1
Actin
DAPI
Merge
WT
S. Typhimurium
S. Typhimurium
ΔsipA
S. Typhimurium
ΔsipA:sipA

Figure 2-25S P4.1 accumulation is unaltered by a mutation in *sipA*.

S. Typhimurium Δ*sipA* infected HeLa cells recruited protein 4.1 to membrane ruffles during invasion. P4.1 localization was maintained during *S.* Typhimurium wild-type, Δ*sipA,* or Δ*sipA:sipA* infected cells. Scale bar is 5μm.

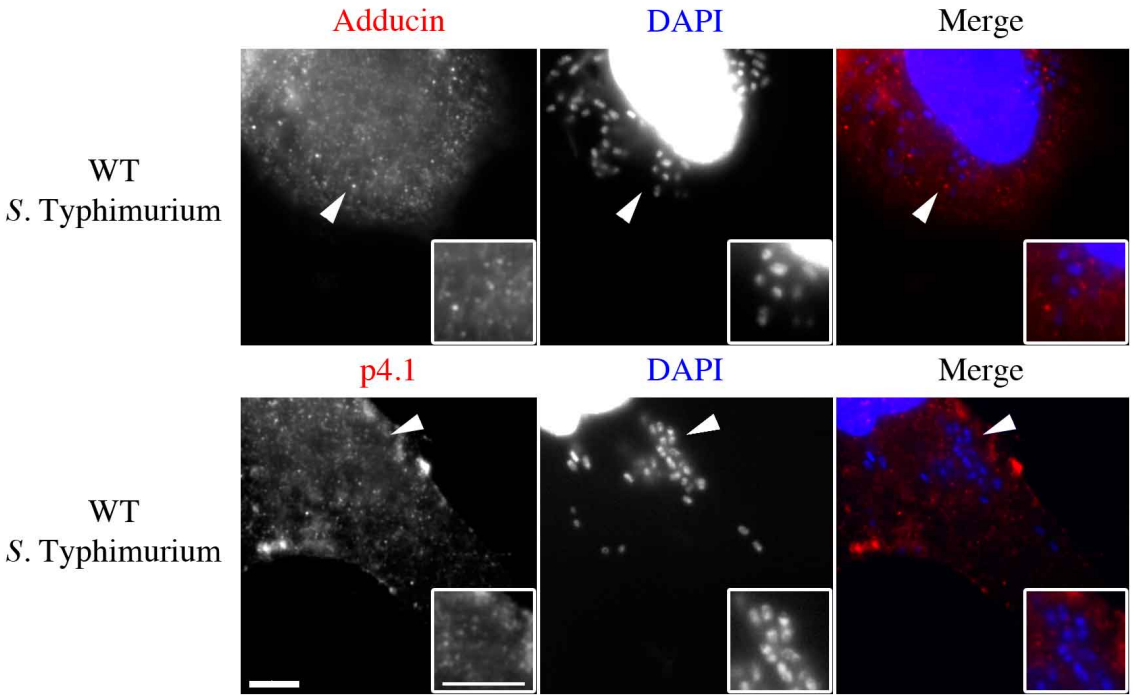

Adducin
DAPI
Merge
WT
S. Typhimurium
p4.1
DAPI
Merge
WT
S. Typhimurium

Figure 2-26S Adducin and p4.1 are not recruited to SCV's.

S. Typhimurium infected HeLa cells were immunolocalized with anti-adducin or anti-p4.1 antibodies together with DAPI at 90 minutes post infection. No accumulation of adducin or protein 4.1 was detected around internalized *Salmonella*. Areas of interest are indicated by arrowheads and highlighted in insets. Scale bars are 5μm.

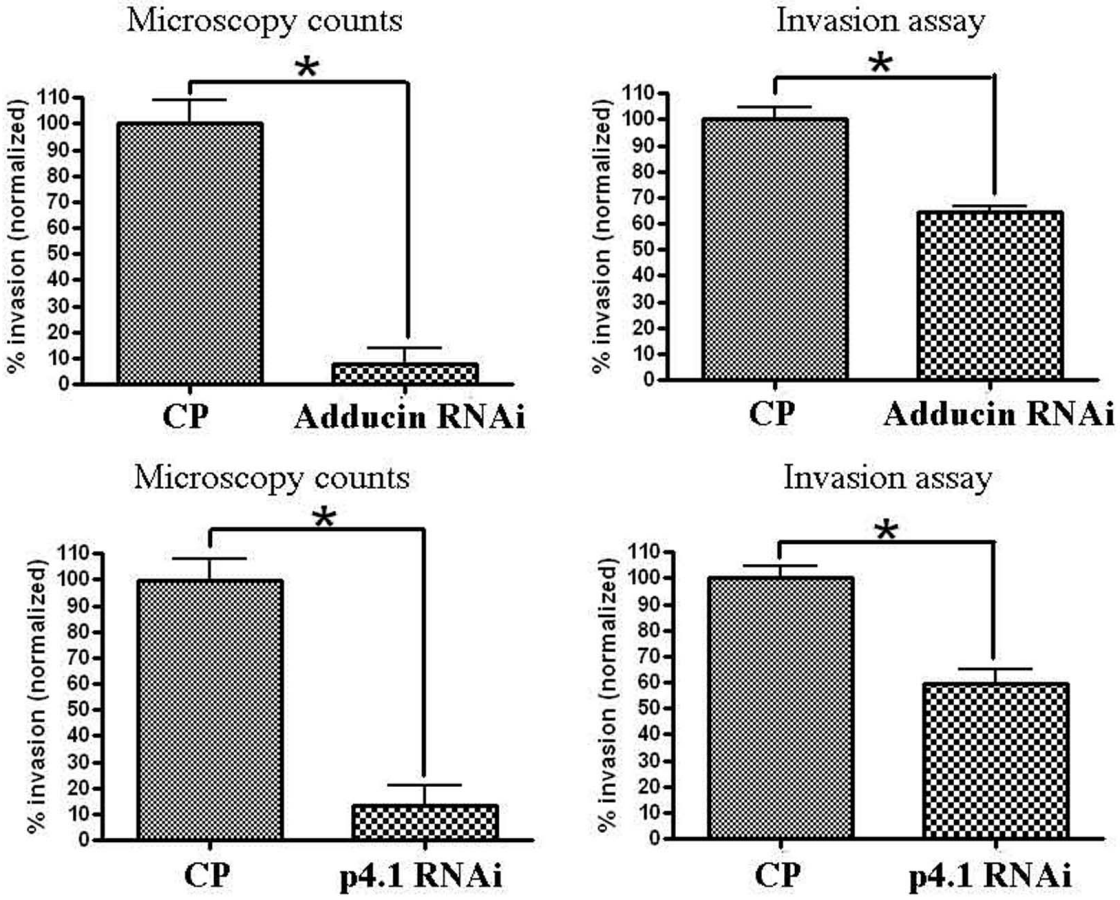

Microscopy counts
*
% invasion (normalized)
110
100
90
80
70
60
50
40
30
20
10
0
CP
Adducin RNAi
Invasion assay
*
% invasion (normalized)
110
100
90
80
70
60
50
40
30
20
10
0
CP
Adducin RNAi
Microscopy counts
*
% invasion (normalized)
110
100
90
80
70
60
50
40
30
20
10
0
CP
p4.1 RNAi
Invasion assay
*
% invasion (normalized)
110
100
90
80
70
60
50
40
30
20
10
0
CP
p4.1 RNAi

Figure 2-27S Adducin and p4.1 are crucial for efficient *S*. Typhimurium invasion.

Adducin and p4.1 were individually knocked down in HeLa cells and infected with wild-type *S*. Typhimurium. Samples were assayed my microscopic counts and invasion assays and compared to non-targeting control pools (CP) of siRNAs. For each treatment, 3 independent experiments were run; for microscopy counts n = 3, error bars show s.e.m, *P<0.0001 for all statistics. Microscopy counts focused on cells with complete knockdown, counting total number of internalized bacteria. Invasion assays involved typical gentamicin survival assay.

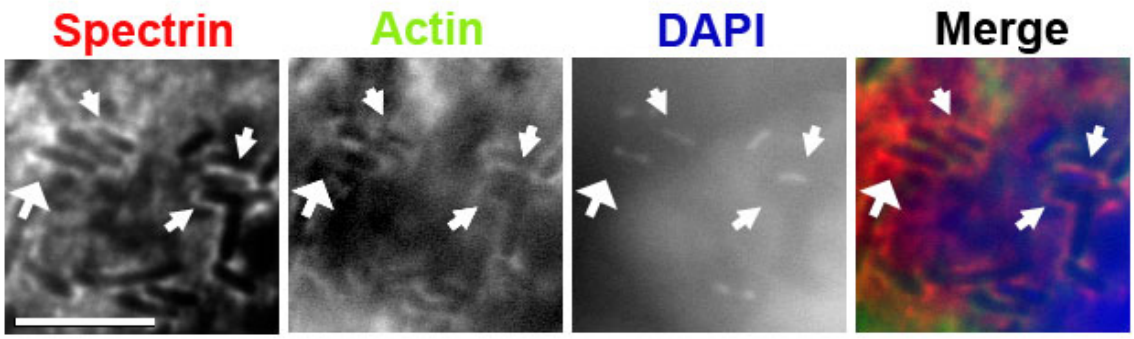

Spectrin
Actin
DAPI
Merge

Figure 2-28S Spectrin is recruited to *L. monocytogenes* at the initial stages of comet tail formation in polar Caco-2 cells.

Caco-2 monolayers were infected with *L. monocytogenes* for 30 minutes and stained for spectrin, actin and DAPI. Large arrows show areas where spectrin was recruited to bacterial membranes without actin, while small arrows show co-localization of spectrin and actin at the bacterial membrane. Scale bar is 5mm.

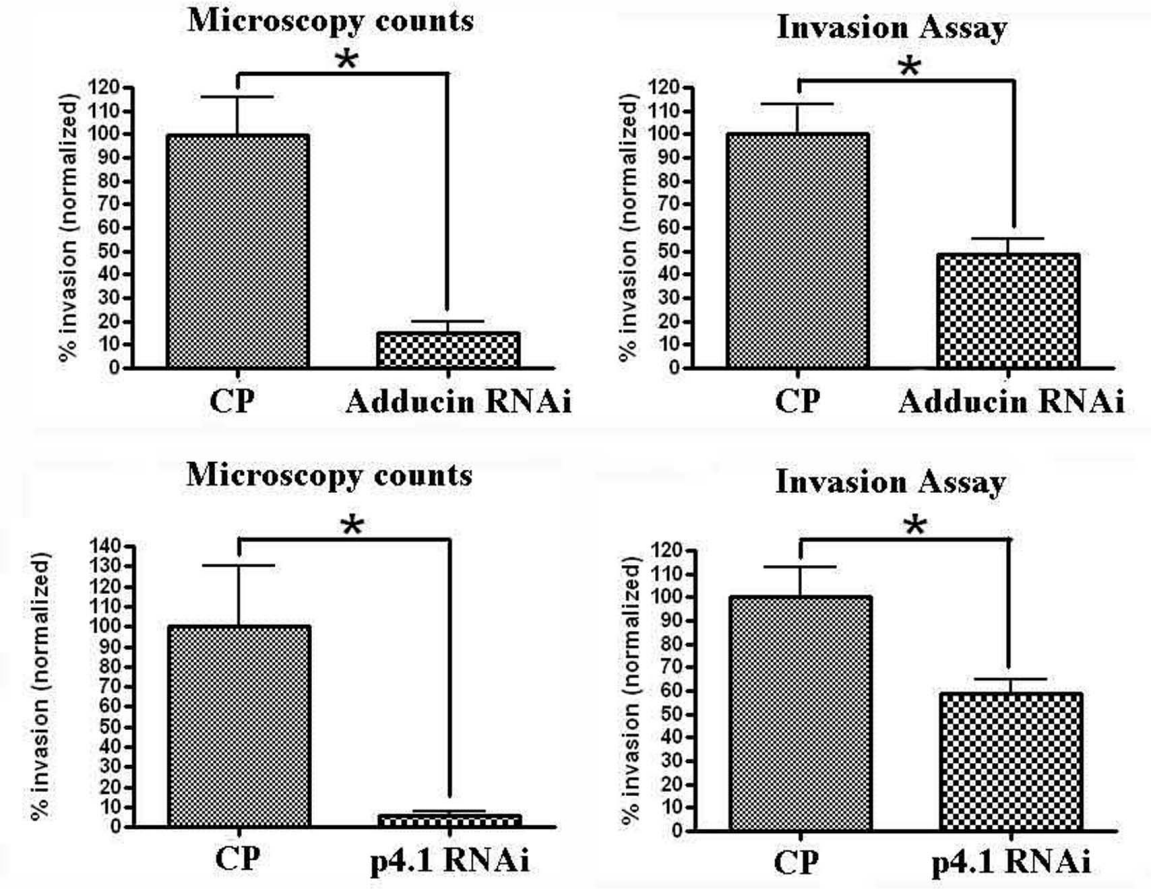

Microscopy counts
*
% invasion (normalized)
120
110
100
90
80
70
60
50
40
30
20
10
0
CP
Adducin RNAi
Invasion Assay
*
% invasion (normalized)
120
110
100
90
80
70
60
50
40
30
20
10
0
CP
Adducin RNAi
Microscopy counts
*
% invasion (normalized)
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
CP
p4.1 RNAi
Invasion Assay
*
% invasion (normalized)
120
110
100
90
80
70
60
50
40
30
20
10
0
CP
p4.1 RNAi

Figure 2-29S Adducin and p4.1 are crucial for efficient Listeria invasion.

Adducin, p4.1 or control pool (CP) non-targetting RNAi treated HeLa cells were infected with *L. monocytogenes* and quantified by microscopy and invasion assays. For each treatment, 3 independent experiments were run; for microscopy counts n = 3, error bars show s.e.m, *P<0.0001 for all statistics. Microscopy counts focused on cells with complete knockdown, counting total number of internalized bacteria. Invasion assays involved typical gentamicin survival assay.

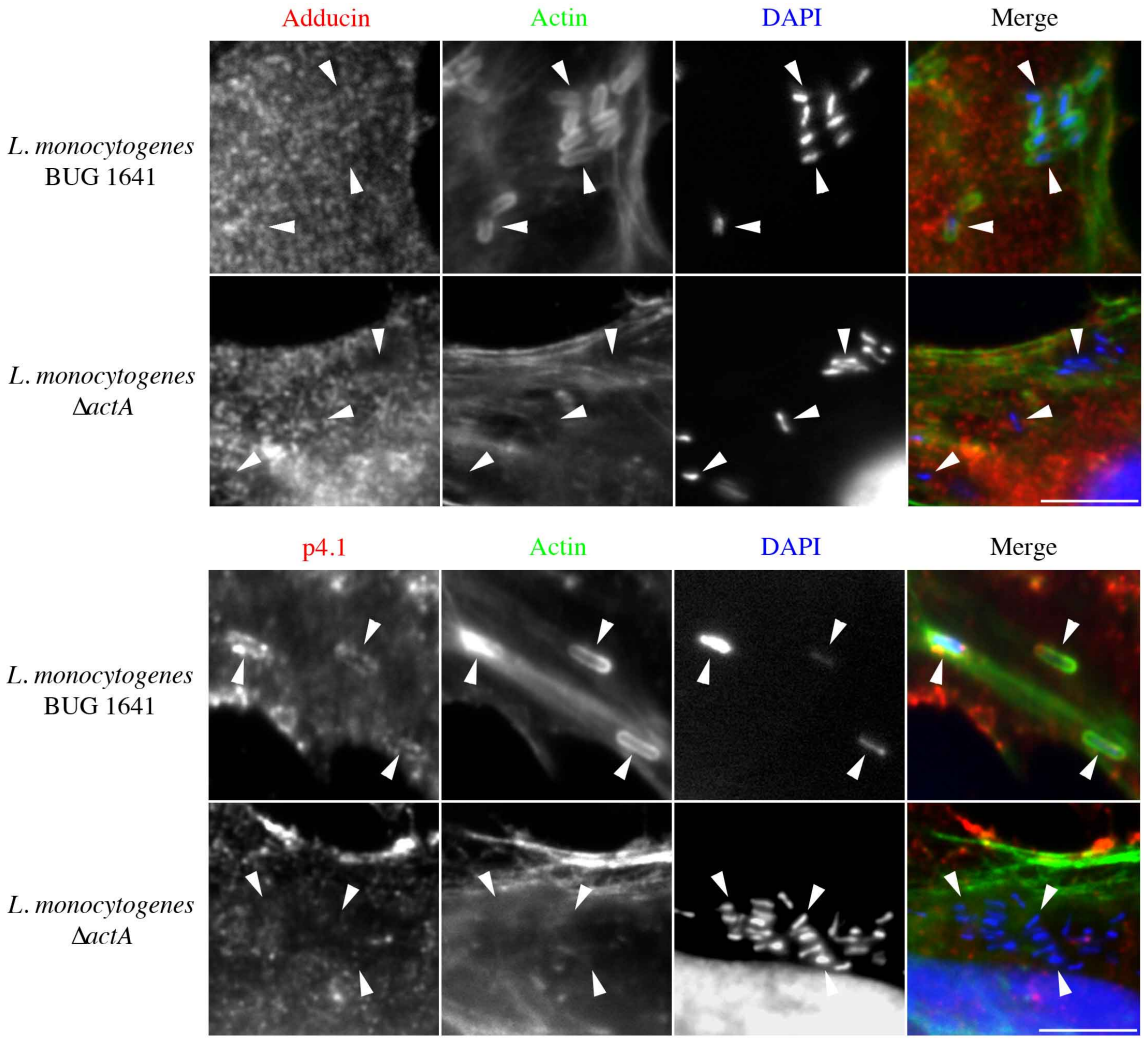

Adducin
Actin
DAPI
Merge
L. monocytogenes BUG 1641
L. monocytogenes ΔactA
p4.1
Actin
DAPI
Merge
L. monocytogenes BUG 1641
L. monocytogenes ΔactA

Figure 2-30S ActA is needed for p4.1 recruitment to sites of *L. monocytogenes* comet tail formation but does not influence the lack of adducin recruitment.

Immunoflourescence images depicting p4.1, adducin, actin and DAPI at 90 minutes post infection. *L. monocytogenes* Δ*actA* infections show no actin, adducin or p4.1 recruitment, whereas wild-type *L. monocytogenes* (BUG 1641) containing *actA* recruits actin and p4.1, but not adducin. Scale bars are 5μm.

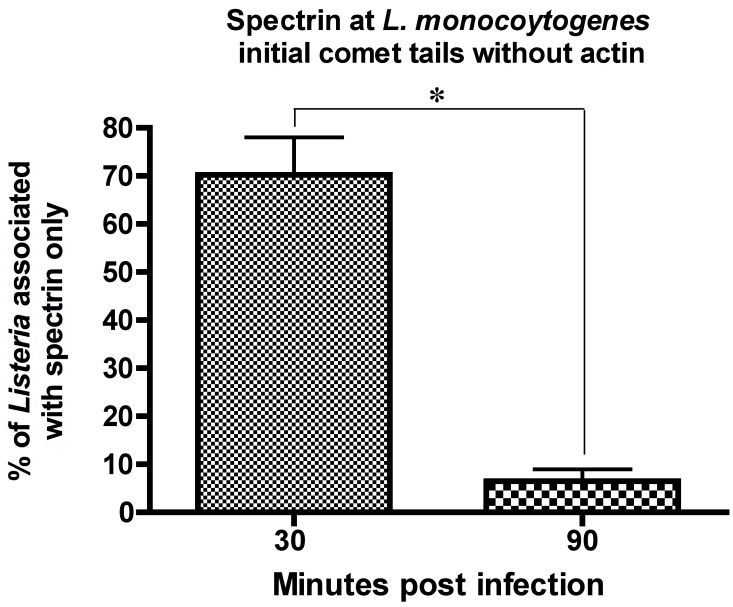

Spectrin at L. monocoytogenes
initial comet tails without actin
*
% of Listeria associated
with spectrin only
80
70
60
50
40
30
20
10
0
30
90
Minutes post infection

Figure 2-31S Quantification of spectrin localized at internalized *L. monocytogenes* in the absence of actin.

HeLa cells infected for 30 or 90 minutes with *L. monocytogenes* were immunolocalized with spectrin, actin and DAPI. Internalized bacteria associated with spectrin were quantified and compared to bacteria associated with both spectrin and actin. The graph depicts the percentage of bacteria associated with spectrin alone at various time points. 100 internalized bacteria were counted per experiment. Each experiment was run in triplicate (n=3). The means of the two data sets are significantly different ($P<0.05$). Error bars show s.e.m.

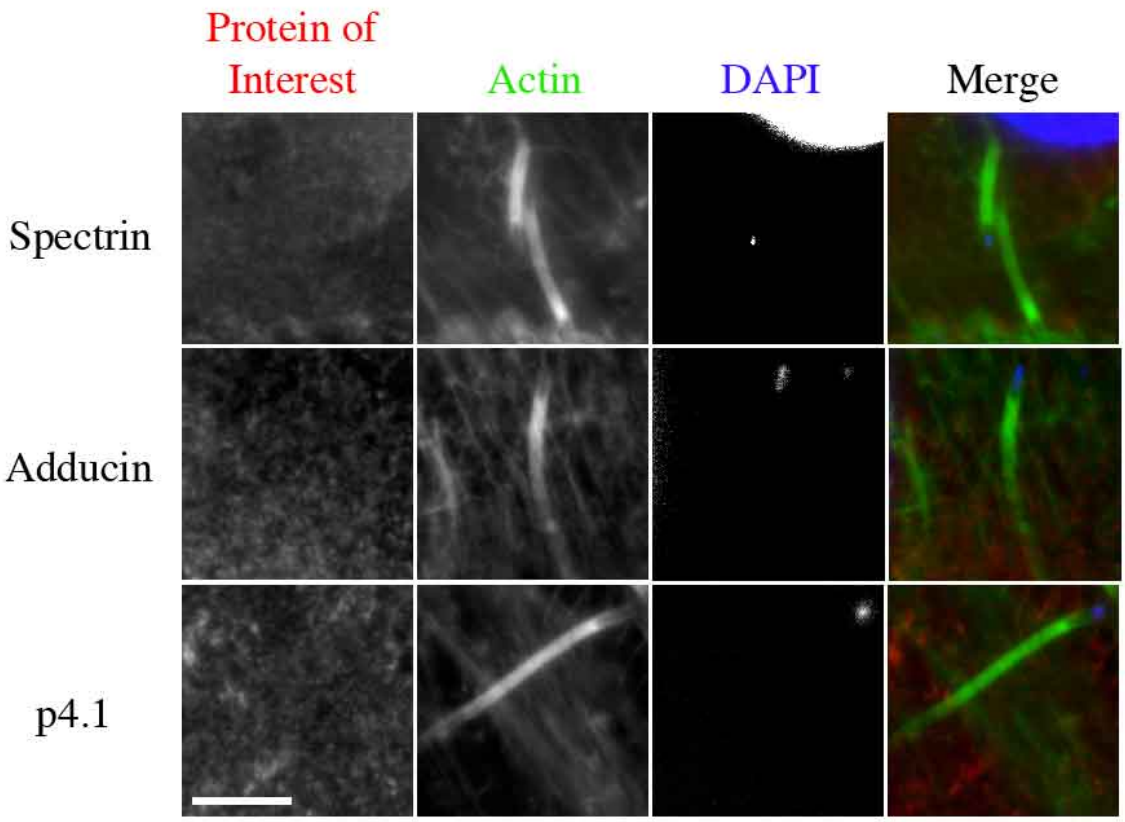

Protein of Interest
Actin
DAPI
Merge
Spectrin
Adducin
p4.1

Figure 2-32S Spectin cytoskeletal components are absent from established *L. monocytogenes* comet tails.

Spectin, adducin, and p4.1 together with actin and DAPI were labeled on *L. monocytogenes* infected HeLa cells 3 hours post infection. None of the spectrin cytoskeletal proteins were recruited to comet tails. Scale bars are 5 μm.

3: General Discussion

The exploitation of the actin cytoskeletal network has been extensively studied and shown to be necessary for EPEC, *S.* Typhimurium and *L. monocytogenes* pathogenesis. Yet the spectrin based cytoskeleton had not been examined during these infections. To investigate whether these microbes altered the spectrin cytoskeletal network, I investigated the core structural filamentous protein β_2-spectrin, as well as the spectrin accessory proteins α-adducin and p4.1. My work demonstrated that each organism usurps these spectrin cytoskeletal components during key stages of their pathogenesis.

I investigated the generation of actin-rich pedestals during EPEC infections and found that siRNA mediated knockdown of spectrin cytoskeletal components nearly completely inhibited pedestal formation. There are three possible explanations for this: 1) Tir recruitment to the plasma membrane was affected, 2) the recruitment/localization of host cell proteins involved in pedestal formation had been altered, or 3) Tir phosphorylation was inhibited. The detailed mechanisms by which Tir is targeted and recruited to the host cell plasma membrane remain elusive. However, it has been established that the trans-membrane domains of Tir are sufficient for membrane insertion [273]. Furthermore, upon membrane insertion, Tir proteins are clustered by intimin in the host cell membrane and this event is necessary for pedestal formation [48,274]. The spectrin cytoskeleton is well established as a membrane protein-sorting machine, overseeing the correct

localization of a diverse group of membrane-associated proteins. It is possible that the spectrin scaffold may be necessary for Tir localization to the membrane. Upon Tir translocation into the host cytosol, spectrin cytoskeletal proteins may direct Tir to the plasma membrane for insertion. Spectrin and associated proteins are involved in microtubule-based transport of membrane-associated proteins through either dynactin complexes or kinesin motor proteins [275,276,277,278]. For example, E-cadherin localization to lateral membranes of polar epithelial cells is reliant on both spectrin and intact microtubules for transport to the membrane [221]. Spectrin is thought to provide a link between various membrane proteins and the microtubule network during intracellular transport [221]. Thus, it is possible that spectrin may direct Tir to the plasma membrane via a microtubule-based mechanism at the initial stages of infection prior to pedestal formation. To test this, I could immunolocalize Tir during infection of cells with spectrin knockdown to examine if Tir is appropriately localized beneath attached bacteria. It may be that the bacteria are only forming initial attachments via the BFP, and thus are not exhibiting Tir-intimin binding at the plasma membrane. We could also treat cells with microtubule disrupting reagents such as colchicine or nocodazole [279] to examine if Tir localization is microtubule dependant.

The fact that spectrin cytoskeletal proteins help localize proteins to the plasma membrane is not exclusive to affecting Tir recruitment. Spectrin, adducin or p4.1 knockdown may affect any number of proteins that are crucial for pedestal formation. It is possible that during EPEC infection of spectrin/adducin/p4.1 knocked down cells, that the host cell proteins normally recruited to (and involved

in) pedestal formation are unable to localize to the site of EPEC attachment. Because actin polymerization at sites of EPEC attachment is inhibited during spectrin knockdown, proteins involved in actin polymerization such as nck, WASP, and Arp2/3 may be inhibited from recruitment. During membrane remodelling for cell migration, spectrin localizes basal to actin at the leading edge of filopodia and lamellipodia together with actin-remodelling proteins such as EVL [221]. Our findings of spectrin and adducin localization at the base of pedestals, colocalizing with (and basal to) actin (Figure 2-5, Appendix 1 and 2), suggests that the spectrin cytosketon may be providing a similar function for EPEC pedestals as it does during cell migration processes. The spectrin network is crucial to membrane-remodelling events. When spectrin cytoskeletal components are disrupted, cell migration is inhibited [280]. Similarily, during pedestal formation, spectrin is found basal to actin and may be supporting the actin reorganizing machinery. Without spectrin, proteins may be unable to remodel actin, and the corresponding EPEC pedestals would not be formed. In addition to investigating Tir, future research should examine if any of these pedestal-associated proteins are not localized to the site of EPEC attachment when spectrin cytoskeletal proteins are knocked down.

The third mechanism by which pedestal generation may be affected by spectrin cytoskeletal knockdown is through the phosphorylation of Tir Y474. Upon insertion in the host cell plasma membrane, Tir is clustered by intimin at the host cell membrane and phosphorylated at Y474 culminating in the polymerization of actin [48]. Tir phosphorylation is enabled by the N-terminal polyproline region (PPR). Tir PPR is necessary for the recruitment of host cell kinases, such as the Tec,

Src and Abl family kinases, which phosphorylate Tir Y474 [50]. During infections with Tir Y474F mutants, Tir molecules are unable to be phosphorylated, are unable to recruit Nck and pedestals are not formed [47,50,51]. PPRs interact with SH3 domains of proteins, such as those found within Abl/Tec family kinases, which researchers have suggested is the mechanism by which Tir N-terminal PPR recruits host cell kinases to phosphorylate the C-terminal Tir Y474 [50]. However, the SH3 domains of Tec and Abl family kinases are dispensable for their recruitment to sites of EPEC attachment, suggesting other mechanisms are involved [50]. The spectrin scaffold has previously been shown to interact with various kinases and play important roles in localizing them to various membrane domains [281,282,283,284]. One such example is the formation of a complex between spectrin, RACK1, and protein kinase C (PKC), a complex mediated by the interaction of the spectrin and PKC PH domains with RACK1 [283]. RACK1 serves as an anchoring protein for PKC and is thought to bring PKC within close proximity to target substrates at the plasma membrane [283]. In our EPEC model, spectrin (and possibly RACK1) may similarly utilize PH domain interactions to target host cell kinases to the plasma membrane to phosphorylate Tir Y474 at the initial stages of pedestal formation. In support, both Tec and Abl kinases contain PH domains that could provide a link to the spectrin network [285,286]. To test these ideas, we could first investigate the phosphorylation status of Tir (using methods described in [50]) during EPEC infections of cells with spectrin knockdowns compared to control cells. We could also probe EPEC infected cell lysates with Tir antibodies to look for co-immunoprecipitation of spectrin and host cell kinases with Tir.

My studies investigating *S.* Typhimurium infections showed that the spectrin cytoskeleton is necessary for *S.* Typhimurium invasion. The inhibition of *S.* Typhimurium invasion during spectrin or adducin knockdown, suggests that these proteins are key components of *S.* Typhimurium induced membrane ruffling and invasion. These results may be explained by previously established roles for spectrin/adducin during membrane ruffling and cell migration events. Adducin function is tightly regulated by its phosphorylation status at 3 key residues; Thr 445, Thr 480, and Ser 726. When adducin is phosphorylated at Thr 445 and 480, adducin binds actin with much higher affinity and facilitates spectrin recruitment [280]. Conversely, phosphorylation of adducin at Ser 726 inhibits adducin from recruiting spectrin to actin [287]. Rho-kinase, which is activated by Rho-GTPase, phosphorylates adducin at Thr 445 and Thr 480 to induce membrane ruffling and cell migration. Substitution of adducin Thr 445/480 to Ala 445/480, results in inhibition of adducin phosphorylation, and subsequent membrane ruffling and motility are abolished [280]. Those findings may provide insight into the mechanism of membrane ruffling and *S.* Typhimurium based invasion. Through *S.* Typhimurium SopE/E2/B activation of Rho-GTPases, and downstream Rho-kinase activation, adducin may be phosphorylated and initiate spectrin recruitment to actin at the site of invasion. As with cell migration processes, adducin-spectrin-actin relations may be necessary for membrane ruffling during *S.* Typhimurium invasion. I was unable to identify any membrane ruffles at sites of *S.* Typhimurium attachment on knocked down cells with undetectable levels of spectrin or adducin.

Spectrin and adducin recruitment was influenced by the SipA effector, as identified during *S.* Typhimurium Δ*sipA* infections showing partial loss of spectrin/adducin presence, while maintaining actin recruitment. SipA is required for efficient entry into cultured epithelial cells [253], while infection of calf ileal loops demonstrated that *Salmonella* SipA mutants are significantly attenuated for symptoms of diarrhea [288]. The SipA effector is known to directly bind and stabilize actin filaments. In binding F-actin, SipA protects actin from gelsolin mediated severing [160]. Gelsolin is a multifunctional protein capable of both severing F-actin as well as capping barbed ends [289,290]. Gelsolin and other depolymerization proteins are crucial for actin polymerization events at the plasma membrane. While monomers of actin are encouraged to join the assembling actin filaments at the leading edge of lamellipodia and filopodia, gelsolin and other proteins depolymerize actin at distal sites of the membrane protrusion to maintain a reservoir of actin monomers at the leading edge [291]. While it has not been observed directly, it is expected that gelsolin may attempt to sever actin filaments at *S.* Typhimurium membrane ruffles [160]. Gelsolin also competes with the spectrin-associated protein adducin to bind actin, whereby the presence of gelsolin displaces adducin from actin. As a consequence, occupation of actin by gelsolin disassembles the spectrin-actin network [233]. Adducin not only directly binds and stabilizes fast growing ends of actin, but also strongly promotes spectrin-actin interactions [292]. Therefore, it is plausible that if SipA inhibits gelsolin at sites of invasion, adducin would be free to bind actin and promote spectrin-actin interactions. Whereas when SipA is absent, gelsolin is not excluded, but rather may be recruited to invasion sites,

inhibiting adducin activity. Our results have shown that infections with *S.* Typhimurium Δ*sipA* were reduced in their ability to recruit adducin and spectrin to sites of invasion, potentially because SipA was not there to impede gelsolin from excluding adducin. To test that hypothesis, I could immunolocalize gelsolin and adducin during *S.* Typhimurium WT and Δ*sipA* infections to see if there is any difference in the two proteins presence/absence at sites of invasion. I could also perform *S.* Typhimurium Δ*sipA* infections of cells with gelsolin knockdowns, to potentially restore adducin and spectrin recruitment to sites of invasion. Furthermore, gelsolin (and its downstream affects on adducin and spectrin) has no known effect on p4.1, and therefore we would expect no changes in p4.1 recruitment to sites of *S.* Typhimurium invasion, whether or not SipA is present. Our results showed that p4.1 recruitment was maintained throughout infections with *S.* Typhimurium Δ*sipA*. The presence of p4.1 at ruffles in the absence of spectrin was unanticipated, as there has yet to be any identified role for p4.1 independent from the spectrin network. Thus our results are interesting and suggest a potential role for p4.1 independent of the spectrin cytoskeleton.

The involvement of the spectrin cytoskeleton during *L. monocytogenes* clathrin-mediated internalization contradicts the traditional views of clathrin-mediated endocytosis (CME). During traditional CME events, the spectrin cytoskeleton is disassembled at sites of membrane invagination and scission [265,266]. Localized dismantling of the spectrin meshwork at clathrin pits is achieved by Annexin VI and is proposed to aid in vesicle scission at the plasma membrane [266]. By blocking Annexin VI activity on spectrin, or by stabilizing the

spectrin cytoskeleton through treatment with anti-spectrin serum, clathrin mediated endocytosis is significantly reduced [266]. Because of the similarities between traditional CME and *L. monocytogenes* clathrin-mediated internalization, I would have expected spectrin to be excluded from *Listeria* invasion sites. However, as I have shown, spectrin is distinctly recruited to the site of invasion. Furthermore, spectrin was necessary for efficient invasion, directly contrasting the traditional CME model. It has been previously established that differences exist between traditional CME and *Listeria* uptake. One specific example is the absence of the key clathrin adaptor protein AP-2 from sites of *Listeria* uptake [188]. AP-2 is a well-established clathrin-associated protein thought to be required for traditional clathrin mediated uptake [293]. Yet, AP-2 is dispensable for *L. monovytogenes* invasion [188]. These and other differences have lead researchers to propose that clathrin may provide a non-traditional role during *L. monocytogenes* uptake [202]. My research provides further evidence that major differences exist between *Listeria* clathrin-mediated invasion and traditional CME.

I found that the spectrin cytoskeleton is targeted independently from actin during EPEC, *S.* Typhimurium, and *L. monocytogenes* during various pathogenic events. Spectrin was observed in the absence of actin at regions such as the basal region of EPEC pedestals, during *S.* Typhimurium Δ*sipA* invasion, and by ActA during *L. monocytogenes* initial comet tail formation. The independent localization, together with the observed inhibition of pathogenesis upon siRNA knockdown of spectrin components, make the spectrin cytoskeleton a potential therapeutic target for treatments of these diseases. There are a list of hormones, small molecule

inhibitors, and host signalling pathways, which all have the ability to target, down-regulate, or modify activities of the spectrin cytoskeletal network. An example is the heparin binding cytokine pleiotrophin, which initiates protein kinase C (PKC) phosphorylation of adducin at Ser 726 (and Ser 713), leading to adducin deactivation and dismantling of the spectrin cytoskeleton [287]. There are many other established PKC activators, such as arachidonic acid, oleic acid, PEP005, Brytostatin and a number of other commercially available activators that may induce similar effects on adducin phophorylation [294,295,296,297,298]. By activating PKC, initiating adducin phosphorylation and spectrin cytoskeletal dissolution, it is possible that EPEC, *S.* Typhimurium and *L. monocytogenes* pathogenesis may be inhibited. To test this, I performed some preliminary research and observed an 82% reduction in EPEC pedestal formation when EPEC was incubated with Hela cells in the presence of pleiotrophin (Appendices 4-6). Furthermore, I observed a marked reduction in *S*. Typhimurium's ability to produce membrane ruffles during invasion of Hela cells in the presence of nmol concentrations of pleiotrophin (Appendix 7). I have yet to quantify if there are any reductions in *S*. Typhimurium or *L. monocytogenes* invasion when cells are treated with pleiotrophin. Potential pitfalls of such treatments would be the undesired off target affects of PKC activation. PKC is involved in many cellular processes, ranging from controlling assembly and disassembly of cell signalling complexes, mediating cell polarity, and regulating cell migration processes [299]. By increasing PKC activity, we would be drastically altering many host cell processes in addition to the spectin cytokeleton. Thus, we would be unable to exclude other host cell responses in contributing to any changes

we observe in pathogenesis. Furthermore, the targeted disruption of the spectrin cytoskeleton may have dramatic effects on epithelial barrier function, and may exacerbate barrier dysfunction during infection *in vivo*. Yet, intraperitoneal injection of mice with pleiotrophin is not associated with any severe side effects, suggesting the treatment may not be as obtrusive as predicted [300]. Irrespective of potential off targeted affects, PKC activating molecules may further characterize the spectrin cytoskeleton's role during pathogenesis, and represent a possible strategy to target the spectrin cytoskeleton in attempt to treat these bacterial diseases going forward.

The requirement of spectrin cytoskeletal proteins for EPEC, *S.* Typhimurium and *L. monocytogenes* infections not only provides insight into each organism's pathogenesis, but may also suggest another mechanism for symptoms of diarrhea. Spectrin is necessary for the plasma membrane localization of many epithelial ion transporters and pumps. Ion channels and pumps maintain the electro-chemical gradient that is necessary for the transport of water across the intestinal barrier [301]. When these channels are disrupted, the electro-chemical gradient is lost, leading to water loss at the epithelial lining [15,21,301]. The massive spectrin cytoskeletal manipulation during EPEC, *S.* Typhimurium, and *L. monocyotgenes* infection may contribute to the downstream effects of water loss and associated symptoms of diarrhea. When spectrin is disrupted and recruited to sites of pathogenesis, it is possible that spectrin cytoskeletal function is compromised. Upon disruption, ion channel localization may be compromised, exacerbating symptoms of diarrhea. Interestingly, infection of calf ileal loops with *Salmonella sipA* mutants severely attenuates symptoms of diarrhea [288]. Taken together with my findings that SipA

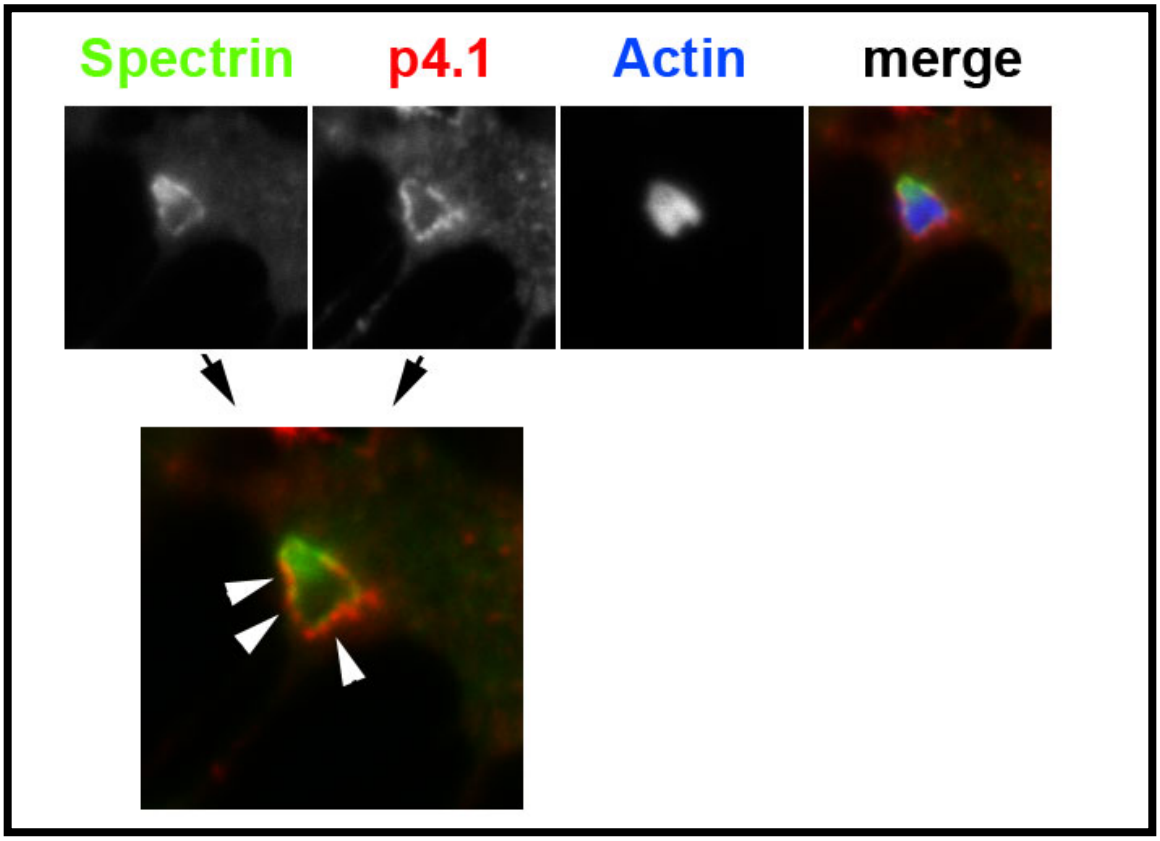
appears to have a major role in spectrin cytoskeletal remodelling, these findings may support the notion that spectrin remodelling may contribute to symptoms of diarrhea.

My studies have identified the spectrin cytoskeleton as a novel target of both an A/E bacterial disease as well as invasive bacterial infections. These findings not only identify the spectrin machinery as crucial for each organism's pathogenesis, but also shed light on spectrin cytoskeletal functions within eukaryotic cells. The next step will be to identify some of the specific functions of the spectrin scaffold for these diseases including; its involvement in EPEC Tir membrane insertion and phosphorylation, potential targeting of adducin by Rho kinases during *S.* Typhimurium invasion, and finally the function provided during clathrin-mediated *L. monocytogenes* invasion as well comet tail formation. Ultimately, research would benefit from identifying ways to specifically inhibit each pathogen's ability to commandeer the spectrin cytoskeleton, a process I have identified as essential to their pathogenesis.

Appendices

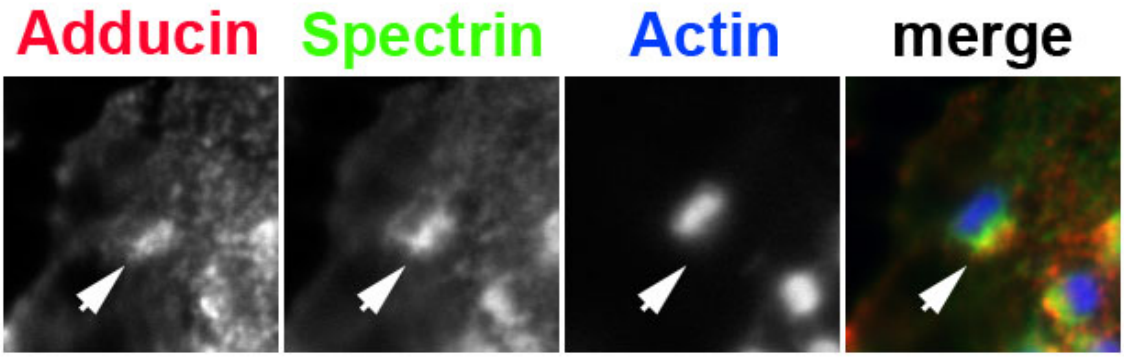
Appendix 1: In-depth look at spectrin and p4.1 at EPEC pedestal

Spectrin, p4.1 and Actin were immunlocalized during 6-hour EPEC JPN15 infections. The EPEC JPN15 strain produces large pedestals and they do not form microcolonies, allowing in-depth visualization of EPEC pedestals. Spectrin is shown localizing to the base of the actin pedestal, while p4.1 localizes to the periphery of the pedestal. White arrows show p4.1 localization peripheral to spectrin and actin within the pedestal.


Spectrin
p4.1
Actin
merge

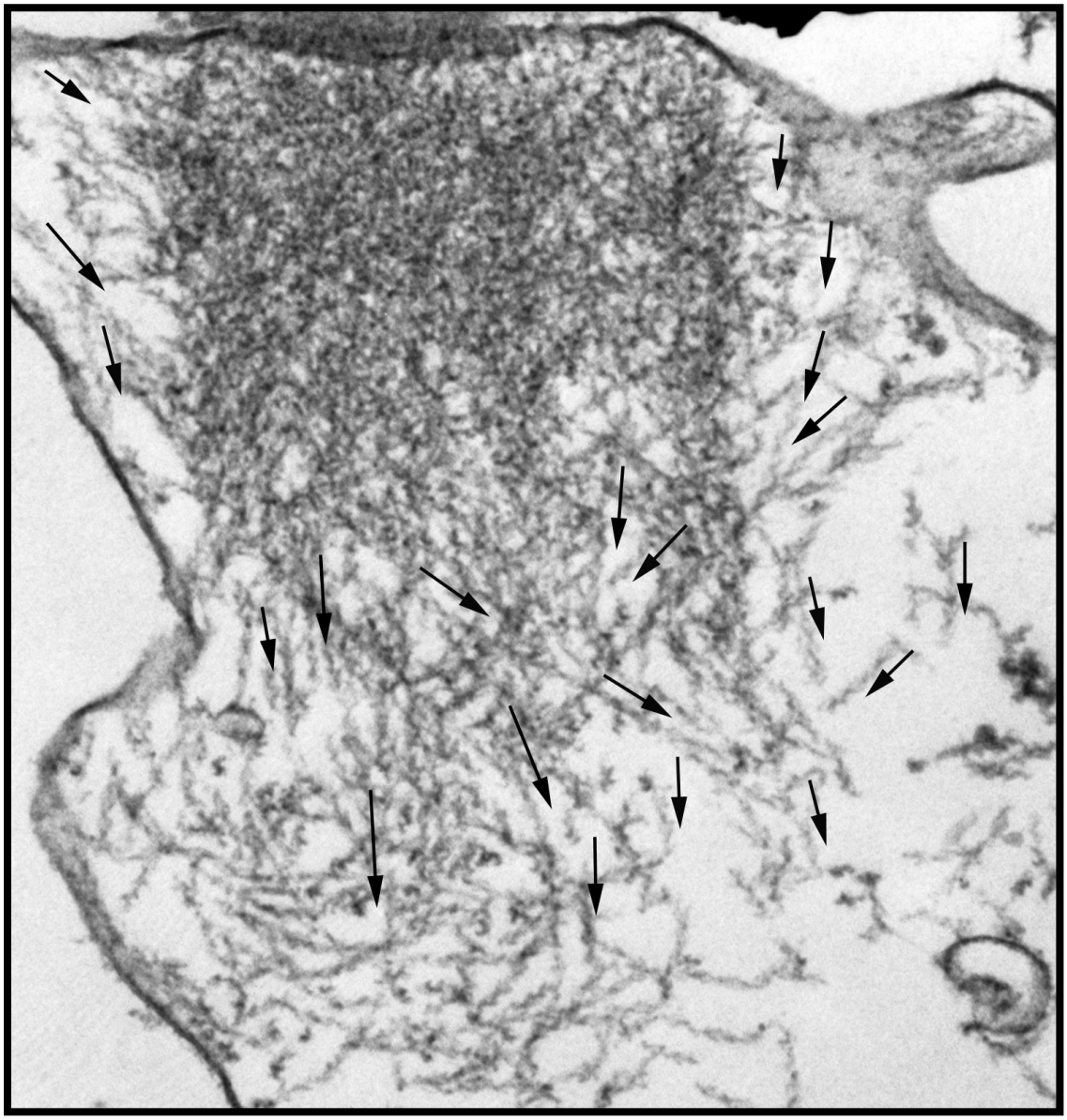
Appendix 2: In-depth look at adducin at EPEC pedestal

HeLa cells were infected with EPEC JPN15 for 6 hours. Adducin was visualized with spectrin, localizing to the base of the pedestal. Actin staining identified pedestals.


Adducin
Spectrin
Actin
merge

Appendix 3: S1 myosin fragment staining of actin in EPEC pedestals

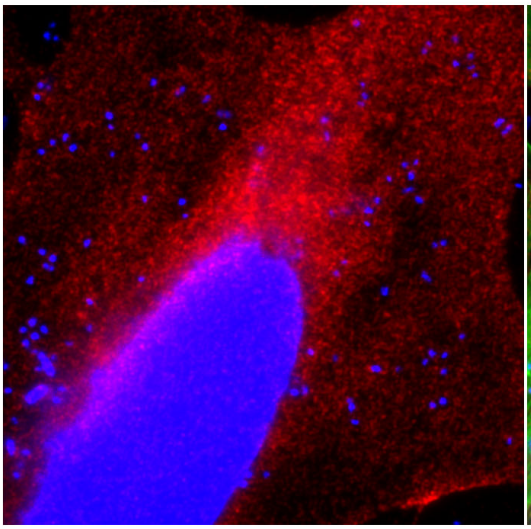
The S1 fragment of myosin motor proteins was extracted from rat muscle and utilized to identify actin filament polarity within EPEC pedestals using transmission electron microscopy. Image identifies the barbed ends of actin arranged toward the site of EPEC attachment (top of picture) as well as toward the periphery of the pedestal, orientated toward the membrane.



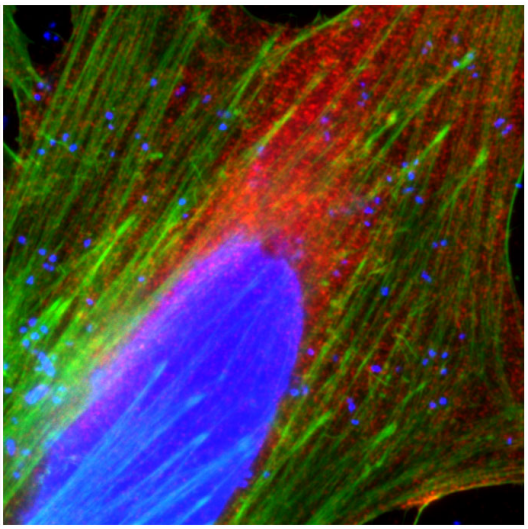
Appendix 4: Pleiotrophin treated Hela cells for 6 hour EPEC infection

HeLa cells were incubated with medium supplemented with 100nM of pleiotrophin (the spectrin cytoskeletal disrupting cytokine) and infected with EPEC for a 6-hour infection. Left image depicts the disrupted, fragmented morphology of spectrin during treatment. The right image shows attached EPEC were unable to initiate pedestals to form in the presence of pleiotophin.

Spectrin and DAPI



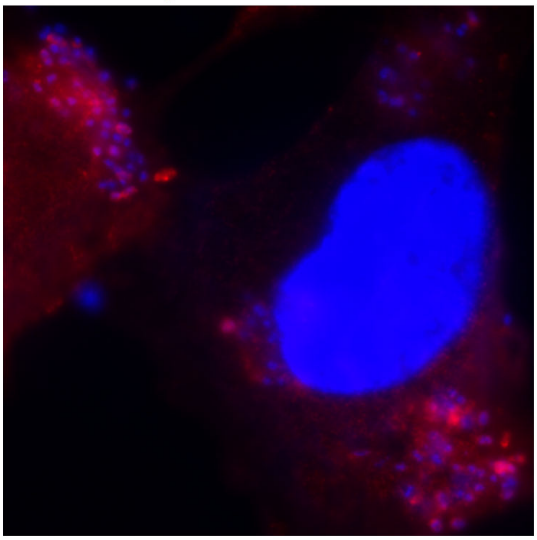
Spectrin, Actin and Dapi



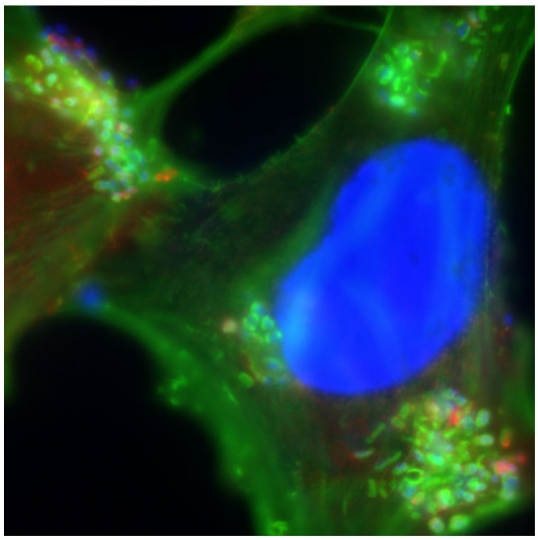
Appendix 5: 6 Hour EPEC infection without pleiotrophin

Control to show EPEC produced pedestals under identical conditions as in Appendix 4 but in the absence of the pleiotrophin cytokine.

Spectrin and DAPI

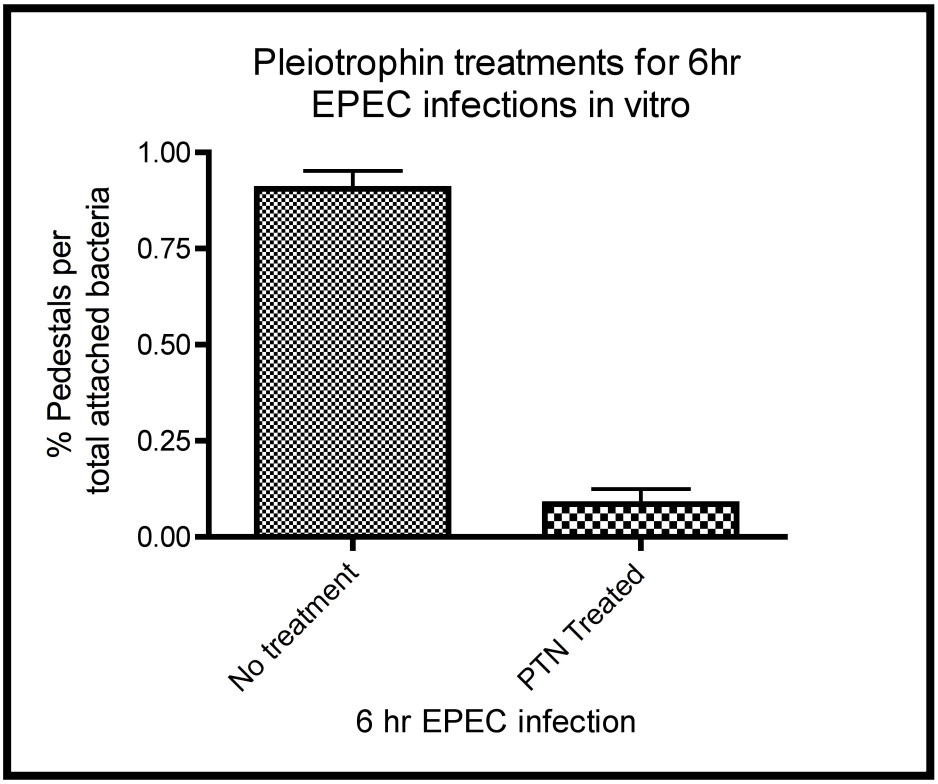


Spectrin, actin and DAPI



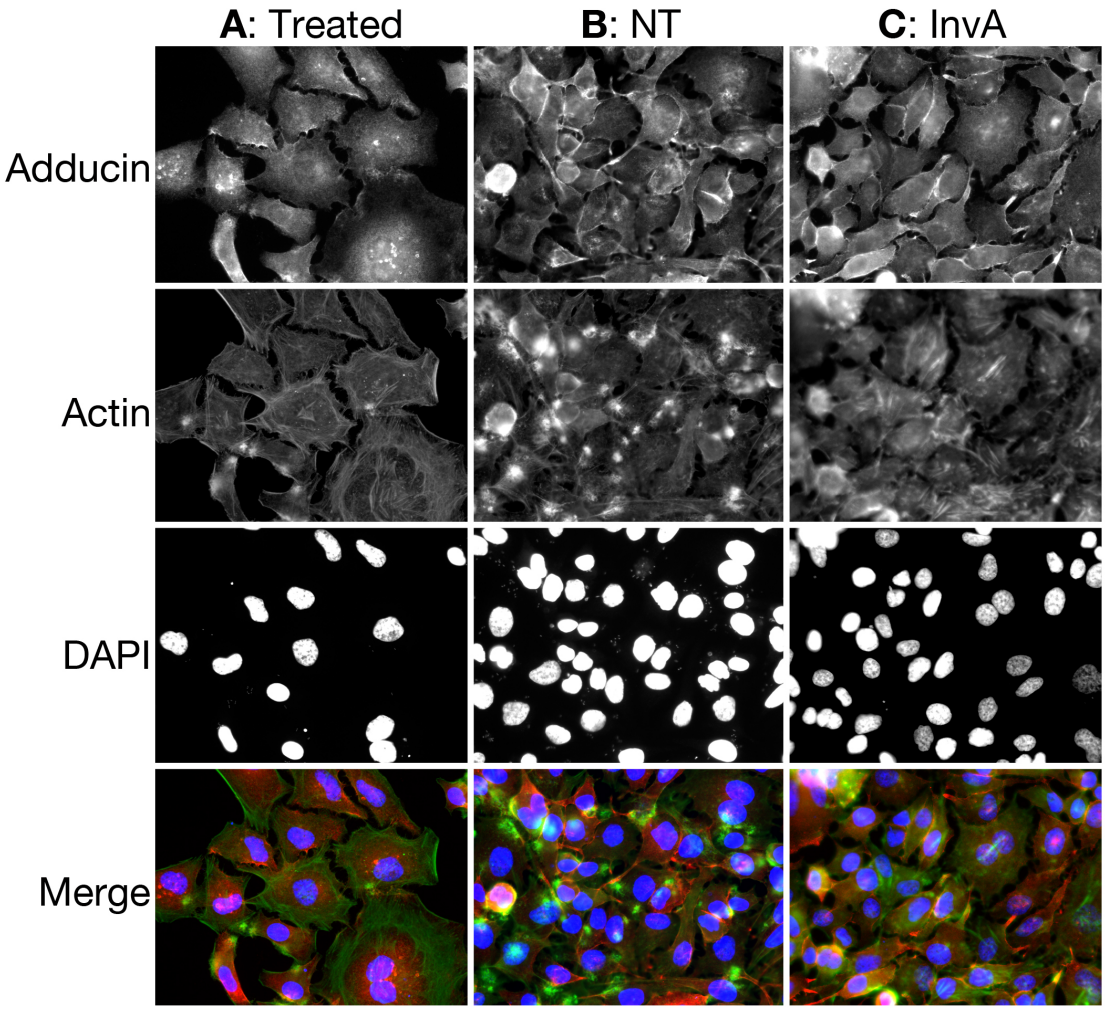
Appendix 6: Quantifying EPEC pedestals in presence of pleiotrophin

HeLa cells were infected with EPEC for 6 hours. Ability of attached EPEC to produce pedestals was quantified. Untreated cells (no treatment) infection levels were compared to infection levels in the presence of pleiotrophin (PTN treated). A drastic reduction in EPEC infection was observed in the presence of pleiotrophin.


Pleiotrophin treatments for 6hr
EPEC infections in vitro
% Pedestals per
total attached bacteria
1.00
0.75
0.50
0.25
0.00
No treatment
PTN Treated
6 hr EPEC infection

Appendix 7: Pleiotrophin treated HeLa cells for 30 minute Salmonella infection

HeLa cells were infected with Salmonella strains for 20 minutes, and immunolocalized for adducin, actin and DAPI. Cells were either treated with 100nM pleiotrophin, or left untreated. (A) Ability of *S.* Typhimurium to induce actin-rich membrane ruffling was severely attenuated in the presence of pleiotrophin compared to (B) no treatment (NT). The non-invasive *Salmonella* (InvA) strain was used as a control to show adducin is not recruited to site of *Salmonella* attachment.


A: Treated
B: NT
C: InvA
Adducin
Actin
DAPI
Merge

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