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Actin Assembly and Cell-to-Cell Transmission by Enterohemorrhagic and Enteropathogenic E. coli

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Actin ‘comet’ tails generated by intracellular microbial pathogens have been useful tools for deciphering the cellular mechanisms of actin nucleation and their roles in pathogenesis. Two extracellular bacteria, Enteropathogenic and Enterohemorrhagic *E. coli* (EPEC and EHEC), offer unique models for determining how cells control actin dynamics, because each pathogen signals across the host cell plasma membrane to reorganize actin into a protrusive ‘pedestal.’ EPEC and EHEC generate pedestals using distinct signaling pathways, but each results in actin nucleation by N-WASP and the Arp2/3 complex. EPEC pedestal assembly relies on the tyrosine phosphorylated bacterial effector protein Tir to recruit the Nck1/Nck2 adaptor proteins and N-WASP, while EHEC uses the multivalent effector protein EspF_U to directly activate N-WASP. However, these pedestal assembly mechanisms are not fully understood, and the function of pedestals in pathogenesis is unclear. To address these questions, I characterized bacterial interactions with several host cell types. I found that EPEC and EHEC exhibited similar actin-based ‘surfing’ motility on fibroblasts, but on polarized epithelial cells, EHEC EspF_U promoted faster motility and greater levels of colonization than EPEC Tir. Furthermore, I defined a pathway of cell-to-cell spread in which EspF_U-expressing bacteria use Arp2/3-dependent actin assembly to move to cell junctions, where they replicate and infect neighboring cells. Lastly, I found that EPEC, but not EHEC, assembles pedestals using the actin nucleator mDia1 from the formin family. Collectively, these results highlight differences in the mechanisms of actin-based motility used by EPEC and EHEC and reveal a method for extracellular spread mediated by intracellular actin polymerization.
Actin Assembly and Cell-to-Cell Transmission by
Enterohemorrhagic and Enteropathogenic \textit{E. coli}

Katrina B. Velle

B.S., University of Massachusetts-Dartmouth, 2012

A Dissertation
Submitted in Partial Fulfillment of the Requirements for Degree of Doctor of Philosophy at the University of Connecticut 2018
APPROVAL PAGE

Doctor of Philosophy Dissertation

Actin Assembly and Cell-to-Cell Transmission by Enterohemorrhagic and Enteropathogenic \textit{E. coli}

Presented by
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University of Connecticut
2018
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Chapter 1. Introduction

1.1 Overview and Significance

Precisely timed and placed actin networks are responsible for orchestrating many cellular processes. Microbial pathogens that hijack actin to form cytosolic ‘comet tails’ have been paramount to uncovering the regulators and mechanisms driving actin polymerization. While these studies of intracellular bacteria have been extremely valuable, they do not represent the actin dynamics that occur at the plasma membrane. In contrast, Enterohemorrhagic *Escherichia coli* (EHEC) and Enteropathogenic *E. coli* (EPEC) manipulate actin while remaining extracellular, and therefore represent ideal models for studying the actin assembly underlying membrane protrusions. Interestingly, EHEC and EPEC each use distinct signaling pathways to polymerize actin, so dissecting these mechanisms could reveal different strategies used by healthy cells to assemble actin at membranes.

EHEC and EPEC are closely related pathogens that cause severe diarrheal diseases in humans. EHEC is the leading cause of bloody diarrhea, hemolytic uremic syndrome, and pediatric kidney failure in the US, and is acquired from the consumption of contaminated beef and leafy vegetables. EPEC presents a greater concern in underdeveloped countries, where it is a leading cause of diarrhea and death in children under the age of two. Infection with EHEC or EPEC manifests as attaching and effacing (A/E) lesions in the large or small intestines, respectively. A/E lesions are characterized by the loss of intestinal microvilli, a tight attachment of bacteria to the host cell membrane, and the accumulation of an F-actin ‘pedestal’ beneath the bacteria. These pedestals allow both EHEC and EPEC to ‘surf’ on the surface of host cells, however the pathogenic function of pedestals has remained unclear. Therefore, defining the role of actin assembly in pathogenesis is important for uncovering new potential drug targets to treat these infections.
1.2 Actin Assembly

The actin cytoskeleton is responsible for controlling many cellular processes including endocytosis, vesicular trafficking, and cell motility. To drive these processes, globular (G-) actin monomers must polymerize into filamentous (F-) actin polymers. Actin filaments are polar, with a barbed (+) end that readily incorporates monomers, and a pointed (-) end that does not support rapid growth. Actin polymerization is kinetically unfavorable \textit{in vitro}, as G-actin dimer and trimer ‘nuclei’ are unstable. In cells, \textit{de novo} actin polymerization requires proteins called nucleators to overcome this kinetic barrier (Reviewed in (Campellone and Welch, 2010; Dominguez, 2016)). This section details the mechanisms and regulation of three types of nucleators (i) Arp2/3 complex, (ii) formins, and (iii) tandem WH2 domain-containing nucleators.

1.2.1 Arp2/3 complex-mediated actin nucleation and branching

The Arp2/3 complex was the first major actin nucleator to be described. Its nucleation activity was characterized using \textit{Listeria monocytogenes}, which was found to rely on Arp2/3 complex activation to polymerize actin comet tails (Welch et al., 1997; Welch et al., 1998). The seven-subunit complex is comprised of actin related proteins 2 and 3 (Arp2 and Arp3), as well as the additional subunits ArpC1-5. To assemble an actin filament \textit{de novo}, Arp2/3 complex typically binds to the side of an existing mother filament, where Arp2 and Arp3 mimic a barbed end to support the addition of monomers (Fig 1A). Arp2/3 complex remains at the pointed end of the filament, capping it and maintaining the connection to the mother filament, thereby generating F-actin networks with 70° Y- branches (Mullins et al., 1998; Rouiller et al., 2008) (Fig 1A). The Arp2/3 complex is an inefficient nucleator by itself, but it can be activated by proteins known as nucleation promoting factors (NPFs). NPFs include Wiscott-Aldrich Syndrome Protein (WASP) family members, as well as atypical NPFs, like cortactin and WISH/SPIN90/DIP1. Cortactin contains an acidic N-terminal motif that activates Arp2/3 complex, as well as a proline-rich domain important for protein-protein interactions, although its main function is to regulate
branching (Schnoor et al., 2018). WISH is unique in that it activates Arp2/3 complex without the requirement of a preexisting filament and promotes linear filament nucleation (Wagner et al., 2013).

In human cells, eight WASP-family members from four sub-classes function to promote Arp2/3 complex-mediated nucleation: Wiskott-Aldrich syndrome protein (WASP) and neural WASP (N-WASP); WASP-family verprolin homolog (WAVE1-3); WASP and SCAR homolog (WASH); and WASP homolog associated with actin, membranes, and microtubules (WHAMM) and junction-mediating regulatory protein (JMY) (Reviewed in (Alekhina et al., 2017; Campellone and Welch, 2010)). These proteins share conserved C-terminal WCA domains, in which the WH2 domain...
binds G-actin, and the Connector and Acidic regions bind the Arp2/3 complex. While the C-termini are conserved, differences in the N-termini allow for locational and functional specificity (Table 1-1) (Alekhina et al., 2017). For instance, N-WASP localizes near the plasma membrane, where it activates the Arp2/3 complex to promote functions including endocytosis and phagocytosis (Benesch et al., 2005; Dart et al., 2012). To achieve N-WASP-mediated Arp2/3 activation, however, N-WASP itself must first be relieved from an autoinhibited state, which is maintained through an interaction between its Autoinhibitory (AI) region and the C and A domains (Kim et al., 2000). Activation is typically achieved through synergistic binding of Cdc42 to the CRIB domain and PI(4,5)P₂ binding to a basic motif (Rohatgi et al., 2000). Other molecules also activate N-WASP, including SH3-domain containing proteins like Nck1 and Nck2 which bind to the Proline Rich Domain (PRD) (Rohatgi et al., 2001) (Fig 1A).

When active N-WASP binds Arp2/3 complex, it promotes a conformational change that brings Arp2 and Arp3 subunits into a short pitch formation resembling a barbed end (Robinson

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<td>Phagocytosis</td>
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<td></td>
<td>Cell motility</td>
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<td></td>
<td>(present exclusively in hematopoietic cells)</td>
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<td>N-WASP</td>
<td>Filopodia formation</td>
<td>(Benesch et al., 2005; Dart et al., 2012; Snapper et al., 2001)</td>
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<td>Autophagy</td>
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<td>JMY</td>
<td>Anterograde Golgi trafficking</td>
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<td>CTTN</td>
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Table 1-1. NPFs activate Arp2/3 complex to orchestrate a variety of cellular functions.
et al., 2001; Rouiller et al., 2008). The WH2 domains of N-WASP are proposed to deliver actin monomers to Arp2 and Arp3, where they bind and generate an actin nucleus. It was also demonstrated that activation of Arp2/3 complex is best achieved when engaged by two WCA domains, with one bound to Arp3, and another on ArpC1 and Arp2 (Padrick et al., 2011). This multimerization promotes optimal activation of Arp2/3 complex.

In addition to its importance for regulating host processes (Table 1-1), many pathogens manipulate the Arp2/3 complex activation pathway to polymerize actin comet tails (Truong et al., 2014; Welch and Way, 2013). For instance, *Listeria monocytogenes* uses its bacterial NPF ActA to mimic WCA domains and directly activate Arp2/3 complex at the bacterial surface (Domann et al., 1992; Kocks et al., 1992; Welch et al., 1997; Welch et al., 1998), while *Shigella flexneri* and EHEC work one step upstream, activating N-WASP with the bacterial effectors IcsA (Bernardini et al., 1989; Suzuki et al., 1998) and EspF_U (Campellone et al., 2004; Campellone et al., 2008a; Garmendia et al., 2004), respectively. Acting even further upstream, vaccinia virus uses the viral membrane protein A36 (Frischknecht et al., 1999; Scaplehorn et al., 2002) and EPEC uses its translocated receptor, Tir, to recruit host adaptor proteins containing SH3 domains, including Nck1 and Nck2, to activate N-WASP and promote Arp2/3 complex mediated actin polymerization (Campellone et al., 2002; Gruenheid et al., 2001). These pathogen-mediated assembly pathways have been useful tools for understanding actin dynamics and remodeling in healthy cells.

1.2.2. *Formin driven nucleation*

Formins (reviewed in Breitsprecher and Goode, 2013; Schönichen and Geyer, 2010)) nucleate actin using a distinct mechanism from Arp2/3 complex, and instead of nucleating branched actin networks and capping the pointed end of the filament, formins polymerize linear filaments and move processively with the barbed end (Higashida et al., 2004; Pruyn et al., 2002; Sagot et al., 2002). Thus, formins have the capacity for at least three major actin related activities:
nucleation, barbed end filament binding, and elongation of filaments. Formins are defined by a conserved FH2 domain, and the 15 human formins have been classified into seven groups: formin (FMN1-2), diaphanous (containing mDia1-3), formin-related gene in leukocytes (FRL1-3), delphilin, disheveled-associated activator of morphogenesis (DAAM1-2), formin homology domain-containing protein (FHOD1 and FHOD3), and inverted formin (INF1-2) (Higgs and Peterson, 2005). While all these subclasses contain conserved FH2 domains, as well as proline-rich profilin-binding FH1 domains, they vary greatly in their nucleation efficiencies, regulation, localization, and other cytoskeleton-associated activities (Table 1-2).

The FH2 domain is essential for the nucleation activity of formins (Pring et al., 2003; Pruyne et al., 2002) and dimerizes such that these domains form donut-shaped head-to-tail rings, with a large enough diameter to accommodate an actin filament (Fig 1B) (Otomo et al., 2005; Shimada et al., 2004; Xu et al., 2004). Early studies of the formin Bni1 from budding yeast suggested a nucleation mechanism by which the FH2 domain stabilizes actin dimers to form a nucleus, either through consecutive binding of monomers, or simultaneous binding of an unstable actin dimer (Pring et al., 2003). However, profilin-bound actin has been shown to inhibit nucleation of both yeast Bni1 (Paul and Pollard, 2008) and mouse mDia1 (Li and Higgs, 2003), and the prevalence of profilin-actin in cells has added controversy to this model of nucleation (Chesarone et al., 2010). While profilin inhibits in vitro nucleation activity of purified FH2 domains, it promotes nucleation by FH1-FH2 constructs, indicating a potential for FH1 to participate in nucleation at high profilin concentrations (Pring et al., 2003). Less controversial is profilin’s role in elongation, in which the FH1 domain binds profilin-actin and rapidly delivers it to the barbed end for incorporation into the filament (Paul and Pollard, 2008; Vavylonis et al., 2006). When a new monomer is added, one of the FH2 domains is thought to “step” onto the new monomer, and alternate with the next addition, allowing processive capping to occur (Higashida et al., 2004; Otomo et al., 2005).
Diaphanous related formins, including Dia, Daam, FMNL, and FHOD families, are autoinhibited by an interaction between the DAD and DID regions (Higgs and Peterson, 2005). This autoinhibition can be relieved by Rho GTPase binding, for example between RhoA and the GTPase binding domain at the N-terminus of mDia1 (Li and Higgs, 2003; Watanabe et al., 1997) (Fig 1B). Many other interactions that were not previously appreciated by in vitro biochemical assays using FH1-FH2 fragments are currently being uncovered, and it is clear that some formins rely on additional cofactors, which are now being referred to as NPFs (Breitsprecher and Goode, 2013). For instance, full length mDia1 is a poor nucleator, and in

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<td>Focal adhesions, lamellipodial formation, cell motility</td>
<td>(Dettenhofer et al., 2008)</td>
</tr>
<tr>
<td>FMN2</td>
<td>Endocytosis</td>
<td>(Lian et al., 2016)</td>
</tr>
<tr>
<td>mDia1 (hDia1, DIAPH1)</td>
<td>Lamellipodial assembly, Membrane ruffling, phagocytosis, cell motility</td>
<td>(Brandt et al., 2007; Isogai et al., 2015)</td>
</tr>
<tr>
<td>mDia2 (hDia3, DIAPH3)</td>
<td>Filopodial assembly, vesicle trafficking, lamellipodial protrusions</td>
<td>(Young et al., 2015)</td>
</tr>
<tr>
<td>mDia3 (hDia2, DIAPH2)</td>
<td>Invadapodia</td>
<td>(Lizárraga et al., 2009)</td>
</tr>
<tr>
<td>FRL1/FMNL1</td>
<td>Lipid droplet structure</td>
<td>(Pfisterer et al., 2017)</td>
</tr>
<tr>
<td>FRL2/FMNL3</td>
<td>Filopodial formation, cell-cell adhesions, lamellipodial protrusions</td>
<td>(Gauvin et al., 2015; Kage et al., 2017; Young et al., 2015)</td>
</tr>
<tr>
<td>FRL3 (FMNL2, FHOD3)</td>
<td>Filopodia, lamellipodia</td>
<td>(Kage et al., 2017)</td>
</tr>
<tr>
<td>Delphilin</td>
<td>Glutamate receptor signaling</td>
<td>(Miyagi et al., 2002)</td>
</tr>
<tr>
<td>DAAM1</td>
<td>Focal adhesions, filopodial assembly, stress fibers</td>
<td>(Jaiswal et al., 2013)</td>
</tr>
<tr>
<td>DAAM2</td>
<td>Sarcomere assembly</td>
<td>(Ajima et al., 2015)</td>
</tr>
<tr>
<td>FHOD1/FHOS</td>
<td>Focal adhesions, cell motility</td>
<td>(Iškratsch et al., 2013)</td>
</tr>
<tr>
<td>FHOD3</td>
<td>Sarcomere organization</td>
<td>(Kan-O et al., 2012)</td>
</tr>
<tr>
<td>INF1 (WHIF1, FHDC1)</td>
<td>Stress fibers</td>
<td>(Thurston et al., 2012)</td>
</tr>
<tr>
<td>INF2</td>
<td>Mitochondrial division, Focal adhesions, stress fibers</td>
<td>(Chakrabarti et al., 2018)</td>
</tr>
</tbody>
</table>

Table 1-2. Formin family proteins are responsible for actin dynamics driving many cellular processes.
cells relies on the tandem WH2-domain containing protein APC to nucleate actin. APC remains associated with the pointed end, while mDia1 functions in elongation, separating from APC and moving processively with the barbed end (Breitsprecher et al., 2012). A similar cooperation also takes place with the *Drosophila* formin cappuccino and the WH2 domain-containing protein spire (Quinlan et al., 2007), which is detailed in the next section. Furthermore, some formins have been shown to bundle actin filaments, sever filaments, or directly bind microtubules, potentially allowing for cross talk between these two cytoskeletal systems. These findings suggest there are many more functions for formins than originally appreciated (Breitsprecher and Goode, 2013).

While the hijacking of Arp2/3 complex-mediated nucleation by pathogens has been studied extensively over the past few decades, the manipulation of formins by pathogens is only beginning to be understood. Since 2010, *Shigella flexneri* (Heindl et al., 2010) and *Listeria monocytogenes* (Fattouh et al., 2015) have been shown to manipulate diaphanous formins for protrusions and cell-to-cell transmission, and vaccinia virus was found to use FHOD1 for comet tail formation, actin-based motility, and spread (Alvarez and Agaisse, 2013). Additionally, *Rickettsia* from the spotted fever group express Sca2, a formin-like bacterial effector that polymerizes long comet tails in the cytosol (Haglund et al., 2010; Reed et al., 2014). Collectively, these findings demonstrate that the manipulation of multiple actin assembly pathways including formins is an emerging trend in pathogen actin-based motility (Truong et al., 2014).

1.2.3. Tandem WH2-domain containing nucleators: Spire and Cobl

A third class of nucleators, those containing G-actin-binding WH2 repeats, is not as well understood as Arp2/3 complex or formins. These proteins are thought to function by clustering actin monomers together to generate an actin nucleus, although a wide variety of additional roles including filament capping and severing have been described. This family includes APC,
Leiomodin, Spire1-2, and cordon-bleu (Cobl) (Table 1-3). Additionally, the WASP-family protein JMY possesses three WH2 domains and can nucleate linear filaments as a tandem WH2-domain containing nucleator. This section will focus on two of the best characterized members, Spire and Cobl.

Spire was initially described as a nucleator in *Drosophila melanogaster* and was the first of this class of nucleator to be identified (Quinlan et al., 2005). *Drosophila* spire, as well as the mammalian orthologs Spire1 and Spire2, possess four G-actin-binding WH2 domains, that are separated by short linker regions. These WH2 domains bind G-actin monomers to form one side of a long pitch helix (Quinlan et al., 2005; Rebowski et al., 2008). *In vitro*, spire is slightly more potent than formin nucleators, but less active than Arp2/3 complex activated by ActA (Quinlan et al., 2005). In *D. melanogaster*, spire mutants exhibit the same oocyte and embryonic defects as *cappuccino* mutants, and it was uncovered that the two proteins interact (Rosales-Nieves et al., 2006). Cappuccino (Capu) is a *Drosophila* formin (Fmn1 in humans), and its C-terminus interacts with the N-terminus of Spire, allowing dimerization (Quinlan et al., 2007). This interaction stimulates the nucleation activity of Spire, potentially by bringing the two long pitch helices together to form a nucleus (Fig 1 C). However, the nucleation activity and barbed end binding ability of Capu is inhibited by Spire binding. In mammals, Spire1 and Spire2 have been found to cooperate with Fmn1 and Fmn2 in a similar fashion (Pechlivanis et al., 2009; Quinlan et al., 2007).

Cordon-bleu (Cobl) contains three WH2 domains, with a 65-residue linker sequence between the second and third WH2 domains. Initial data supported a model of nucleation in which the WH2 domains promote the formation of an actin trimer nucleus, in which the long linker enables the assembly of the correct conformation to form a barbed end (Ahuja et al., 2007). However, *in vitro* experiments revealed that a single WH2 domain with a short N-terminal lysine rich sequence were sufficient to induce nucleation, indicating that an actin trimer does not necessarily drive nucleation (Husson et al., 2011). The crystal structure of Cobl’s first two WH2
repeats with actin monomers revealed a different potential mechanism of nucleation, in which the barbed end is initially blocked, and monomers slowly add to the pointed end until a conformational change induced by ATP hydrolysis exposes the barbed end. Subsequent pointed end disassembly is then proposed to release Cobl from the filament (Chen et al., 2013).

*In vitro* experiments also revealed that Cobl has the capacity to perform a dynamic range of functions in addition to nucleation, including promoting ATP-actin addition to the barbed end, severing filaments, and sequestering ADP-actin monomers (Husson et al., 2011). These functions were recently visualized in cells by live imaging microscopy, in which fluorescently labeled Cobl associated with pointed ends of dynamic, linear filament bundles, and localized to filament sides prior to disassembly (Grega-Larson et al., 2016). These activities are likely important for Cobl’s cellular function of polymerizing actin in microvilli (Grega-Larson et al., 2015; Wayt and Bretscher, 2014).

<table>
<thead>
<tr>
<th>Name</th>
<th>Actin Related Functions</th>
<th>Selected References</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Focal adhesions, cell migration</td>
<td>(Juanes et al., 2017)</td>
</tr>
<tr>
<td>Leiomodin</td>
<td>Sarcomere assembly (exclusively present in muscle cells)</td>
<td>(Chereau et al., 2008)</td>
</tr>
<tr>
<td>JMY</td>
<td>Anterograde Golgi trafficking, Autophagy</td>
<td>(Coutts and La Thangue, 2015; Schlüter et al., 2014)</td>
</tr>
<tr>
<td>Spire1-2</td>
<td>Vesicle trafficking</td>
<td>(Schuh, 2011)</td>
</tr>
<tr>
<td>Cobl</td>
<td>Microvilli assembly</td>
<td>(Grega-Larson et al., 2015; Wayt and Bretscher, 2014)</td>
</tr>
</tbody>
</table>

**Table 1-3.** Tandem WH2 domain containing nucleators assemble actin for specific cellular processes.

While there are no apparent examples of pathogen-driven manipulation of host WH2-domain containing nucleators, *Vibrio parahaemolyticus* and *Vibrio cholerae* secrete WH2-domain containing effector proteins VopL (Liverman et al., 2007) and VopF (Tam et al., 2007), respectively. These bacterial effectors are important for disrupting host actin and allowing invasion, however, there remains controversy about the biochemical functions of VopL/VopF. X-ray scattering data argues that VopF does not bind the pointed end (Avvaru et al., 2015), contrary to a previously published crystal structure (Yu et al., 2011) and suggests that VopF sequesters actin monomers (Avvaru et al., 2015), while other studies from the same lab suggest
VopF also uncaps filaments to promote elongation (Pernier et al., 2013). Recent work using multicolor total internal reflection microscopy has shown that VopL and VopF nucleate by briefly associating with the pointed end of filaments. When profilin was added, most VopL/F still associated with the pointed end, but a small fraction was observed capping the barbed end and preventing elongation (Burke et al., 2017). Given these actin-manipulating activities of VopL/F, it appears there is no pathway of actin nucleation that pathogens cannot hijack or mimic.
1.3 EPEC- and EHEC-driven actin assembly

Many pathogens have evolved strategies to hijack the actin cytoskeleton of their host. While most of these pathogens are intracellular bacteria that form comet tails, EPEC and EHEC are unique as they remain extracellular throughout infection, signaling across the plasma membrane to polymerize actin into pedestals. While this positions EPEC and EHEC as ideal models for studying the actin dynamics that underlie membrane protrusions, understanding pedestal formation is additionally important due to its role in disease. Because the ability to form A/E lesions is critical for colonization, and there are no effective treatments for EHEC-associated diseases, defining the molecular and cellular basis of pathogenesis is crucial to uncovering new therapies. Interestingly, while EPEC and EHEC are closely related, and the outcome of both actin assembly pathways is N-WASP activation and thus Arp2/3 complex-mediated nucleation, the molecular mechanisms driving N-WASP activation are quite different. This section will detail the major EPEC and EHEC pathways of pedestal assembly, as well as the current understanding of alternative methods for polymerizing actin into pedestals.

1.3.1. EPEC pathways of pedestal formation

EPEC and EHEC each use a type 3 secretion system (T3SS) to translocate dozens of bacterial effector proteins into host cells (Wong et al., 2011). Importantly, EPEC and EHEC each inject their own version of Tir (translocated intimin receptor), which forms a hairpin loop in the host cell plasma membrane, and binds to intimin on the bacterial surface, mediating a tight interaction between the bacterium and host cell (de Grado et al., 1999; DeVinney et al., 1999; Gruenheid et al., 2001). Tir and intimin are each essential for actin assembly (DeVinney et al., 1999; Jerse et al., 1990; Kenny, 1999), as well as for infection in animal models (Deng et al., 2003; Donnenberg et al., 1993; Marches et al., 2000; Ritchie et al., 2003; Tzipori et al., 1995).

Although the EPEC and EHEC pathways of pedestal assembly each begin with translocating Tir and end with N-WASP-mediated Arp2/3 complex activation, the signaling
cascades that result in recruitment of N-WASP are distinct (Fig 1-2). These differences stem from the cytoplasmic C-termini of EPEC and EHEC Tir, which are only 44% identical, compared to >60% identical in the rest of the proteins (Perna et al., 1998). EPEC Tir becomes tyrosine phosphorylated at residue 474 by Src and Abl family kinases (Kenny, 1999; Phillips et al., 2004; Swimm et al., 2004), and a 12 amino acid sequence in Tir containing Y474 is necessary and sufficient for recruiting the SH2/SH3 adaptor proteins Nck1 and Nck2 (collectively Nck) (Campellone et al., 2002; Gruenheid et al., 2001). The SH2 domain of Nck binds phosphorylated Y474 on Tir, and the SH3 domains can bind the PRD of N-WASP for activation (Gruenheid et al., 2001; Rohatgi et al., 2001). The second SH3 domain also has the capacity to recruit N-WASP by binding WIP, which forms a stable complex with N-WASP in cells (Antón et al., 1998). N-WASP recruitment to Tir then drives Arp2/3 complex activation for pedestal assembly (Kalman et al., 1999; Lommel et al., 2001).

1.3.2. EHEC pathways of pedestal formation

EHEC pedestal assembly differs significantly from EPEC in that EHEC Tir does not become tyrosine phosphorylated (DeVinney et al., 1999). Instead, the C-terminus of EHEC Tir contains a distinct 12-residue peptide that is necessary for pedestal formation (Campellone, 2006). Within this region is an asparagine-proline-tyrosine (NPY458) tripeptide, which binds to the IRSp53/MIM homology domain (IMD) of host I-BAR-family proteins IRTKS and IRSp53 (Brady et al., 2007; Vingadassalom et al., 2009; Weiss et al., 2009). The BAR proteins contain SH3 domains which are capable of activating N-WASP (Campellone et al., 2012; Kim et al., 2008), but in pedestals mainly function to recruit a second critical EHEC effector, EspFu (also called TccP) (Vingadassalom, 2009; Weiss, 2009; Campellone, 2004; Garmendia, 2004). EspFu contains an N-terminal type three secretion signal, and 2-8 (depending on strain) 47-residue repeats, each composed of an SH3-interacting proline-rich sequence, and a short α-helix capable of binding N-WASP (Campellone et al., 2004; Campellone et al., 2008a; Cheng et al.,
EspF<sub>U</sub> binding activates N-WASP outcompeting the CA domains for binding to the autoinhibitory (AI) region, resulting in potent stimulation of Arp2/3 complex to nucleate actin into a pedestal (Campellone et al., 2008a; Cheng et al., 2008; Sallee et al., 2008) (Fig.1-2). Clustering of Tir-EspF<sub>U</sub> fusion proteins containing at least two 47-residue repeats in the host plasma membrane is sufficient to induce actin polymerization (Campellone et al., 2008a). Furthermore, additional repeats act in an additive fashion to enhance polymerization (Campellone et al., 2008a; Sallee et al., 2008). In animal models, mutants lacking EspF<sub>U</sub> colonize less efficiently (Ritchie et al., 2008), suggesting that actin assembly has an important function in pathogenesis.

Fig 1-2. EHEC and EPEC manipulate host Arp2/3 complex to polymerize actin pedestals. EHEC (left, purple) triggers actin assembly using an NPY458 sequence in Tir, which binds IRSp53/MIM homology domains of host I-BAR proteins like IRTKS/IRSp53. The SH3 domain of IRTKS/IRSp53 recruits the EHEC effector EspF<sub>U</sub>, which contains six 47 residue repeats (R1-R6) that each contain an SH3-interacting proline-rich sequence and a short α-helix that can bind and relieve N-WASP’s autoinhibition to activate the Arp2/3 complex. EPEC Tir (right, green) is phosphorylated by host cell kinases at Y474 to allow the recruitment of adaptor proteins Nck1 and Nck2 (drawn C-terminus to N-terminus). The SH3 domains of the Nck proteins activate N-WASP alone, or in a complex with WIP (not shown).
1.3.3. Alternative pathways of pedestal formation

The mechanisms described above are the best studied and likely represent the most prominent pathways of pedestal formation for EHEC and EPEC. Nevertheless, both pathogens are able to exploit alternative pathways of actin assembly. For instance, in the absence of Nck, approximately 25% of adherent EPEC retain the ability to form actin pedestals (Campellone and Leong, 2005). This Nck-independent pathway of pedestal assembly is largely dependent on Tir Y474 being phosphorylated, because less than 5% of adherent bacteria can form pedestals using a Y474F Tir point mutant. This residual ability involves a second tyrosine residue of EPEC Tir, Y454, which also becomes phosphorylated, although at a lower efficiency (Campellone and Leong, 2005). When phosphorylated, Y454 is able to recruit PI3K, which phosphorylates the phosphoinositide PI(4,5)P2 (Sason et al., 2009; Selbach et al., 2009). This alters the membrane composition, enriching it in PI(3,4,5)P3. The subsequent recruitment of the inositol-5-phosphatase SHIP2 by Tir residues Y483 and Y511 converts PI(3,4,5)P3 to PI(3,4)P2 in the membrane and influences pedestal morphology (Smith et al., 2010).

Y454 is also part of an NPY sequence, which is homologous to the NPY of EHEC Tir (Brady et al., 2007; Campellone and Leong, 2005), and can recruit IRSp53 similarly (Weiss et al., 2009). Given that IRSp53 can directly activate N-WASP, and that this tripeptide confers low efficiency pedestal formation (Brady et al., 2007), it remains possible that pedestal assembly could be driven by a Tir NPY454-IRSp53-N-WASP signaling mechanism, although this has not been directly tested.

EHEC also seems to have usurped multiple pathways of actin assembly independent of N-WASP. It was originally thought that activation of N-WASP by EspF_U was required to form pedestals, as pedestal formation is abolished on one mouse N-WASP knock-out cell line (Lommel et al., 2004). However, a more thorough investigation found that murine N-WASP is required for efficient type three secretion, but not for EspF_U-mediated pedestal formation (Vingadassalom et al., 2010). This study used KC12, a strain of EPEC which was engineered to
express the EHEC versions of Tir and intimin, as well as EspF_U. Infection of an independent N-WASP knockout cell line with this strain revealed that 95% of bacteria that translocated Tir and EspF_U could still form pedestals. Additional experiments demonstrated that this N-WASP-independent pathway required the Arp2/3 complex, but did not involve recruitment of other WASP family members (Vingadassalom et al., 2010). It is possible that EHEC achieves Arp2/3 complex activation by relying on the atypical nucleation factor, cortactin, which has been shown to positively influence pedestals (Cantarelli et al., 2006), although the precise mechanism of N-WASP-independent actin assembly has yet to be elucidated.
1.4. Research Goals

EPEC and EHEC are important human pathogens, and while actin assembly is known to be important for surfing motility in vitro (Sanger et al., 1996; Shaner et al., 2005) and colonization in some animals (Ritchie et al., 2008), the precise role of actin pedestals in infection has never been defined in the 30 years since their discovery. Given the clinical relevance of A/E lesions, the first aim of this thesis was to determine the pathogenic functions of actin pedestals. This is addressed in Chapter 2.

While understanding the mechanisms underlying actin pedestal biogenesis has the inherent benefit of uncovering potential therapeutic targets, it can also reveal how actin polymerization is normally regulated at membrane protrusions. An emerging trend in the field of actin dynamics is that cooperation of multiple nucleators is often necessary to assemble complex actin networks (Dominguez, 2016). Additionally, it is becoming clear that other actin-manipulating pathogens hijack the activities of multiple nucleators (Truong et al., 2014). Therefore, the second aim of this work was to investigate the potential for cooperation between nucleators in EPEC and EHEC pedestals. This is addressed in Chapter 3.
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host cell membrane but is not tyrosine phosphorylated. *Infect Immun* 67, 2389–2398.


Schlüter, K., Waschbüsch, D., Anf, M., Hügging, D., Kind, S., Hänisch, J., Lakisic, G.


Chapter 2. Extracellular motility and cell-to-cell transmission of enterohemorrhagic *E. coli* is driven by EspF_U-mediated actin assembly

(Velle and Campellone, 2017)

2.1 Abstract:

Enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC) are closely-related pathogens that attach tightly to intestinal epithelial cells, efface microvilli, and promote cytoskeletal rearrangements into protrusions called actin pedestals. To trigger pedestal formation, EPEC employs the tyrosine phosphorylated transmembrane receptor Tir, while EHEC relies on the multivalent scaffolding protein EspF_U. The ability to generate these structures correlates with bacterial colonization in several animal models, but the precise function of pedestals in infection remains unclear. To address this uncertainty, we characterized the colonization properties of EPEC and EHEC during infection of polarized epithelial cells. We found that EPEC and EHEC both formed distinct bacterial communities, or “macrocolonies,” that encompassed multiple host cells. Tir and EspF_U, as well as the host Arp2/3 complex, were all critical for the expansion of macrocolonies over time. Unexpectedly, EspF_U accelerated the formation of larger macrocolonies compared to EPEC Tir, as EspF_U-mediated actin assembly drove faster bacterial motility to cell junctions, where bacteria formed a secondary pedestal on a neighboring cell and divided, allowing one of the daughters to disengage and infect the second cell. Collectively, these data reveal that EspF_U enhances epithelial colonization by increasing actin-based motility and promoting an efficient method of cell-to-cell transmission.
2.2 Introduction:

Many pathogens reorganize the cytoskeleton of their host cells during the course of infection. These include the intracellular bacteria *Listeria monocytogenes* and *Shigella flexneri*, which generate filamentous actin “comet tails” that propel the bacteria through the cytosol, drive the formation of membrane protrusions, and ultimately spread the infection to neighboring cells (Ireton, 2013; Kuehl et al., 2015; Welch and Way, 2013). Enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC) are also capable of reorganizing actin, but these pathogens remain extracellular and signal across the plasma membrane to create structures called attaching and effacing (A/E) lesions (Hayward et al., 2006). A/E lesions are characterized by intimate attachment of the bacteria to the host cell membrane, a loss of microvilli, and assembly of filamentous actin “pedestals” beneath the bacteria (Knutton et al., 1989; Moon et al., 1983). The ability to form these lesions correlates with pathogenesis, because EHEC and EPEC mutants that are unable to adhere intimately to host cells fail to colonize or cause disease in animal models (Deng et al., 2003; Donnenberg et al., 1993a; Marchès et al., 2000; Ritchie et al., 2003; Tzipori et al., 1995), intestinal explants (Schuller et al., 2007), and human volunteers (Donnenberg et al., 1993b; Tacket et al., 2000). Since the discovery of pedestals nearly three decades ago (Knutton et al., 1989), the mechanisms of actin assembly within these structures have been fairly well characterized. However, the function of pedestals in the cellular basis of disease remains relatively unclear.

To generate actin pedestals, EHEC and EPEC each use a type three secretion system (T3SS) for injecting effector proteins into mammalian host cells (Wong et al., 2011). Among the numerous effectors is the translocated intimin receptor, Tir, which is inserted into the plasma membrane and binds to the adhesin intimin expressed on the bacterial surface, thereby forming a tight attachment to the host cell (DeVinney et al., 1999; Kenny et al., 1997). The EPEC version of Tir becomes tyrosine phosphorylated at residue 474 by host cell kinases (Kenny, 1999; Phillips et al., 2004; Swimm et al., 2004) and binds to the adaptor proteins Nck1 and Nck2.
which recruit N-WASP (Kalman et al., 1999), an activator of the Arp2/3 complex that promotes actin nucleation to form a pedestal (Lommel et al., 2001). The EHEC version of Tir does not become tyrosine phosphorylated (DeVinney et al., 1999), but recruits the host proteins IRTKS (Vingadassalom et al., 2009) and IRSp53 (Weiss et al., 2009) which interact with the EHEC effector protein EspF	extsubscript{U}, a multivalent and potent activator of N-WASP (Campellone et al., 2004a; Campellone et al., 2008a; Cheng et al., 2008; Garmendia et al., 2004; Sallee et al., 2008). Although EPEC and EHEC pedestals are triggered by different signaling mechanisms, they are morphologically indistinguishable and contain many of the same host factors (Goosney et al., 2001; Lai et al., 2013), leading to the widespread belief that their pathogenic functions are similar or equivalent (Lai et al., 2013).

Early studies suggested that intimate attachment to the plasma membrane by EPEC may play a role in evading phagocytosis (Goosney et al., 1999) or that the intimin-Tir-actin interactions function to anchor the bacteria to the host cell by linking them to the cytoskeleton (Goosney et al., 2000). However, additional work demonstrated that actin pedestals are dynamic and drive a form of actin-based motility that allows the bacteria to "surf" on top of cultured cells (Sanger et al., 1996; Shaner et al., 2005). More recent studies implied that actin assembly enhances effector entry either directly or indirectly (Battle et al., 2014; Vingadassalom et al., 2010), while another has indicated that Tir tyrosine signaling is important for colonization in vivo (Crepin et al., 2010). Furthermore, actin pedestals appear to promote more stable attachments to cultured cells or to the intestinal mucosa in animal models (Battle et al., 2014; Mallick et al., 2014; Ritchie et al., 2008). For example, an EHEC strain capable of intimate adherence but deficient in EspF	extsubscript{U}-mediated actin pedestal assembly was less abundant in the intestines of experimentally infected infant rabbits and gnotobiotic piglets (Ritchie et al., 2008). Most strikingly, the use of Citrobacter rodentium strains to model EPEC/EHEC infections revealed that intestinal N-WASP knock-out mice were resistant to infection, and that bacteria harboring a tyrosine-to-phenylalanine Tir mutant colonized the colon in wild type mice less
efficiently (Mallick et al., 2014). Despite this progress, the precise cellular basis for how pedestals promote or enhance colonization has yet to be clearly defined.

The current study focused on characterizing the roles of pedestals in anti-phagocytosis, bacterial motility, and epithelial cell colonization. Our findings indicate that EHEC and EPEC pedestals serve similar functions with respect to resisting phagocytosis by macrophages and enabling actin-based motility on non-polarized cells. However, on polarized epithelial cells, EHEC pedestals confer a colonization advantage, allowing for the formation of large “macrocolonies” that encompass several host cells. This EHEC-specific advantage stems from faster EspF\textsubscript{U}-driven motility and a previously unrecognized mechanism of cell-to-cell bacterial transfer.
2.3 Materials and Methods:

**Bacterial and mammalian cell culture**

All bacterial strains are described in Table 2-1. In each pedestal-proficient bacterium, the main pedestal-driving effector is encoded behind the effector’s own promoter on a low copy number plasmid (Campellone et al., 2002; Campellone et al., 2004a). 24 h prior to all infections, bacterial cultures were inoculated from single colonies into LB broth with appropriate antibiotics and grown shaking at 37°C for 8-9 h. Cultures were then diluted 1:500 in DMEM + 100 mM HEPES, pH 7.4, and grown standing overnight at 37°C + 5% CO₂ to enhance the production of the T3SS and its effectors.

HeLa (University of Massachusetts Medical School) (Campellone et al., 2004a) and JEG-3 cells (American Type Culture Collection: ATCC) were each maintained as subconfluent monolayers in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotic/antimycotic. HeLa cells were seeded at approximately 5x10⁵ cells/ml onto glass coverslips 24 h before infections, while JEG-3 cells were seeded at approximately 5x10⁵ cells/ml either onto glass coverslips or into 35 mm glass-bottom dishes (MatTek) and allowed to grow for 24-48 h post confluency. THP-1 monocytes (ATCC) were grown in suspension in RPMI 1640 supplemented with 10% FBS, antibiotic/antimycotic, and 0.05 mM 2-mercaptopetoethanol. Cells seeded at a density 2x10⁵ cells/ml were activated to a macrophage-like state by adding 40 ng/ml phorbol 12-myristate 13-acetate (PMA) to the media, and allowing the cells to adhere to glass coverslips in 24-well plates for approximately 72 h (Park et al., 2007) before infections. NIH3T3 cells (University of California Berkeley, cell culture facility) stably expressing mCherry-βactin (Campellone et al., 2008b) were maintained in DMEM supplemented with 10% FBS, antibiotic/antimycotic, and 500 μg/ml G418. 48 h before infection, cells were seeded at approximately 5x10⁵ cells/ml onto 35 mm glass-bottom dishes in media lacking G418, and 16 h prior to infection were induced to express mCherry-actin with 7.6 mM sodium butyrate. C2BBe1 (referred to as Caco-2) cells (ATCC) were maintained in subconfluent monolayers in DMEM.
supplemented with 10% FBS, antibiotic/antimycotic, and 0.01 mg/ml human transferrin. To create polarized monolayers (Peterson and Mooseker, 1992), cells were grown to confluency on glass coverslips or aclar (for EM) in 24-well plates, and given half media changes every 1-2 days for at least 2 weeks prior to infections. All cells were grown at 37°C + 5% CO₂.

**Infections and chemical inhibitors**

For phagocytosis assays, activated THP-1 cells were washed twice with warm phosphate buffered saline (PBS) and infected with bacteria diluted 1:1000 in RPMI + 3.5% FBS + 20mM HEPES to achieve a multiplicity of infection (MOI) of ~3. Plates were centrifuged at 172 x g for 5 min to synchronize infections. Infected cells were incubated at 37°C + 5% CO₂ for 15-150 min. At various time points cells were washed 3 times with warm PBS and fixed for 20 min using 2.5% paraformaldehyde (PFA) prior to performing outside-inside staining, or fixed for 5 min in methanol prior to performing LAMP-1 staining.

For bacterial motility assays, NIH3T3 cells were washed twice with PBS and infected with bacteria diluted 1:200 into DMEM + 3.5% FBS + 20 mM HEPES to achieve an MOI of ~6. Infected cells were rotated for 5 min to disperse the bacteria before incubation for 3-5 h. Cells were then washed twice with PBS, given fresh media, and imaged for 2-3 h at 26°C in an environmental chamber (Okolab). Live imaging with Arp2/3 complex inhibitors (Hetrick et al., 2013) was performed by adding 50 µM CK666 + 50 µM CK869 (Calbiochem) or equivalent doses of DMSO. The addition of DMSO or Arp2/3 complex inhibitors did not restrict bacterial growth compared to media alone. JEG-3 cells were imaged at 37°C between 3 and 8 h post infection.

For colonization assays, polarized Caco-2 monolayers were washed twice and infected with overnight cultures diluted 1:33 in DMEM + 3.5% FBS + 20 mM HEPES for an MOI of ~10. Plating assays were performed to verify equal input for all bacterial strains by CFU counts. Plates were centrifuged and subjected to PBS washes and media changes every 1 h to remove
unbound bacteria. After 6 h, cells were washed 3 times with PBS, and fixed with 3.7% PFA for 30 min. Colonization assays with EHEC strains were infected as described above, but PBS washes and media changes were completed every 2 h, and the total infection was carried out for 8 h. Experiments involving Arp2/3 inhibitors were completed by pretreating monolayers for 15 min with 50 µM CK666 + 50 µM CK869 and using fresh media with inhibitors throughout the infection process.

**Fluorescence microscopy**

For standard immunofluorescence, fixed cells were washed 3 times with PBS, permeabilized for 2 min with 0.1% TritonX-100, washed 3 times with PBS, and blocked using 1% FBS + 1% bovine serum albumin (BSA) in PBS + 0.02% NaN₃ for a minimum of 30 min. Cells were probed with primary antibodies diluted in blocking buffer at concentrations listed in Table 2-2 for 35-40 min. Cells were then washed 3 times with PBS and treated with secondary antibodies and/or DAPI and phalloidin for 35 min, at concentrations listed in Table 2-2. Cells were then washed 3 times with PBS, and mounted in Prolong Gold anti-fade. For outside-inside staining, fixed THP-1 cells were blocked in 1% BSA + 1% FBS + 1% normal goat serum (NGS) + 0.02% NaN₃ in PBS for 30 min. Cells were incubated in mouse anti-LPS for 40 min, followed by 3 PBS washes, and Alexa 555 anti-mouse antibodies for 40 min, followed by 3 PBS washes. Cells were then permeabilized with 0.1% Triton X-100 for 2 min, washed 3 times, and re-blocked for 30 min. The same primary antibody was used again for 40 min, but Alexa488 anti-mouse was used as a secondary antibody for 40 min, and cells were also stained with DAPI, similar to previous experiments (Celli et al., 2001). After washing, coverslips were mounted in Prolong Gold anti-fade. All fixed and live cells were imaged using a Nikon Eclipse Ti microscope equipped with a Plan Apoλ 100x 1.45 NA objective, a Plan Fluor 20x 0.5 NA objective, an Andor Clara-E camera, and a computer running NIS Elements software. Image analyses were performed using ImageJ. Live phase-contrast imaging as well as mCherry and GFP fluorescence of infected
NIH3T3 cells was performed using the 100x objective, and images were captured at 30 s intervals. Live imaging of JEG-3 cells was performed using a 20x phase-contrast objective, and images were acquired at 30 or 45 s intervals.

**Electron Microscopy:**

Caco-2 cells were fixed in 2% glutaraldehyde + 2.5% paraformaldehyde in 0.1 M Na cacodylate buffer (Shifrin Jr. et al., 2014) + 1.5 mM CaCl₂ + 1.5 mM MgCl₂, pH 7.4 with 0.2% tannic acid (Burgess, 1982). The primary fixation was carried out for 15 min at room temperature, followed by replacement with fresh fixative and incubation for 1 h at room temperature. Monolayers were washed in 0.1 M Na cacodylate buffer + 1.5 mM CaCl₂ + 1.5 mM MgCl₂, pH 7.4 twice for 20 min, and a third time overnight at 4°C. Osmium postfixation was performed on all samples using 1% Osmium tetroxide (Shifrin Jr. et al., 2014) and 0.8 % K₃Fe(CN)₆ in 0.1 M Na cacodylate buffer + 1.5 mM CaCl₂ + 1.5 mM MgCl₂, pH 7.4. Samples were rinsed twice with distilled water for 15 min, and transmission electron microscopy (TEM) samples were subjected to en bloc fixation in 1% aqueous uranyl acetate (Shifrin Jr. et al., 2014) for 80 min, followed by two 10 min washes. All samples were dehydrated through a graded ethanol series (30%, 50%, 70%, 95%, 100%, 100%) using 10 min wash times, and scanning electron microscopy (SEM) samples were then pulled for critical point drying. TEM samples continued on to 2 10 min acetone washes and were embedded in Embed 812 + Araldite 506 + DDSA + 1.5% DMP-30 by using 1:1 resin:acetone for 90 min, 3:1 resin:acetone for ~15 h and 100% resin twice in 2 h incubation steps. Resin was polymerized in a 60°C oven for 49 h. Aclar was removed from the resin, and the tissue was reembedded in resin for 49 h. 100 nm ultrathin sections were cut using a diamond knife on a Leica microtome. Sections were lifted onto 200-mesh copper grids, and stained for 8 min with 2% aqueous uranyl acetate, followed by 2 min in 2.5% lead citrate. TEM was carried out using a Technai Spirit electron microscope, operated at 80 kV. SEM was performed using a FEI Nova NanoSEM 450 microscope.
Data analysis and statistics

Motility assays were quantified using ImageJ software. Movement was tracked with the MTrackJ plugin, and tracking plots were generated using the chemotaxis tool plugin. Colony sizes were measured using the threshold feature followed by the analyze particles feature. In most cases, the lower limit of colony sizes was set to 100 µm². Exceptions included time courses with short time points, the use of inhibitors, or experiments which included strains that were severely deficient at colony formation (KC12Δtir strains), which required lowering the limit to 50 or 25 µm². Statistics on data sets with 3 or more conditions were performed using one-way ANOVAs followed by Tukey’s post-hoc test unless otherwise indicated. P-values for data sets including 2 conditions were determined using unpaired t tests unless otherwise noted. For phagocytosis assays (Fig 2-2 D), SE was calculated based on the number of coverslips examined. For motility quantifications in which all pedestals on a single cell were averaged (Fig 2-3 C, 2-3 F), SE was calculated based on the number of cells examined. For motility assays in which the mean of all pedestals was calculated (Fig 2-11 C, 2-12 D), SE was calculated based on the number of pedestals examined. SE values for the fraction of cells moving (Fig 2-3 D) and for the directional persistence (Fig 2-3 E) were calculated from the total number of pedestals, and the total number of moving pedestals, respectively. The SEs for colony size in Fig 2-5 F and 2-8 B were calculated based on the number of coverslips, while SDs and SEs in Fig 2-6 B-D were based on the number of experiments. For smaller sample sizes (Fig 2-6 F and Fig 2-11 B), and for Fig 2-8 E and 2-8 G, the number of colonies was used to generate SD and SE. Finally, quantifications of the % area covered (Fig 2-6 E and Fig 2-8 C) were averaged by field of view (FOV), and the total number of FOVs was used for SD and SE determination.
### Table 2-1: Strains used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Strain + Plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT EPEC</td>
<td>JPN15/pMAR7</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt; derivative of EPEC E2348/69 (O127:H6)</td>
<td>(Jerse et al., 1990)</td>
</tr>
<tr>
<td>EPEC Y474&lt;sup&gt;*&lt;/sup&gt;</td>
<td>KC14+pKC17</td>
<td>EPECΔtir+pHA-tir(WT)</td>
<td>(Campellone et al., 2002)</td>
</tr>
<tr>
<td>EPEC Y474F</td>
<td>KC14+pKC142</td>
<td>EPECΔtir+pHA-tir(Y474F)</td>
<td></td>
</tr>
<tr>
<td>EPECΔtir</td>
<td>KC14</td>
<td>EPECΔtir</td>
<td></td>
</tr>
<tr>
<td>EPECΔT3SS</td>
<td>KC30</td>
<td>EPECΔT3SS</td>
<td>(Murphy and Campellone, 2003)</td>
</tr>
<tr>
<td>EPEC+GFP</td>
<td>WT EPEC+pAT113</td>
<td>EPEC+pEGFP</td>
<td>This study, (Fortinea et al., 2000)</td>
</tr>
<tr>
<td>EPEC+EspF&lt;sub&gt;U&lt;/sub&gt;</td>
<td>WT EPEC+pKC471</td>
<td>EPEC+pEspF&lt;sub&gt;U&lt;/sub&gt;-Myc</td>
<td>This study</td>
</tr>
<tr>
<td>EPEC+vector</td>
<td>WT EPEC+pKC469</td>
<td>EPEC+pMyc</td>
<td></td>
</tr>
<tr>
<td>WT EHEC</td>
<td>TUV93-0</td>
<td>Stx&lt;sup&gt;−&lt;/sup&gt; derivative of EDL933 (O157:H7)</td>
<td>(Campellone et al., 2002)</td>
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<td>KC44+pKC471</td>
<td>EHECΔespF&lt;sub&gt;U&lt;/sub&gt;+pEspF&lt;sub&gt;U&lt;/sub&gt;-Myc</td>
<td>(Campellone et al., 2004a)</td>
</tr>
<tr>
<td>EHECΔespF&lt;sub&gt;U&lt;/sub&gt;+vector</td>
<td>KC44+pKC469</td>
<td>EHECΔespF&lt;sub&gt;U&lt;/sub&gt;+pMyc</td>
<td></td>
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<tr>
<td>KC12</td>
<td>KC12</td>
<td>EPECΔtir-cesT-eeae::EHEC-HA-tir-cesT-eeae</td>
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</tr>
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<td>KC12+pKC471</td>
<td>KC12+pEspF&lt;sub&gt;U&lt;/sub&gt;-Myc</td>
<td>(Campellone et al., 2004a)</td>
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<tr>
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<td>KC12+pMyc</td>
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<td>KC26</td>
<td>KC12Δtir</td>
<td>(Campellone et al., 2002)</td>
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<td>KC12Δtir+EspF&lt;sub&gt;U&lt;/sub&gt;</td>
<td>KC26+pKC471</td>
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<td>This study</td>
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<td>KC12Δtir+pMyc</td>
<td>This study</td>
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Table 2-2: Antibodies and molecular probes used in this study.

<table>
<thead>
<tr>
<th>Target</th>
<th>Antibody/Probe</th>
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<th>Concentration</th>
<th>Fixation</th>
<th>Company</th>
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<tr>
<td>LPS</td>
<td>anti-Lipopolysaccharide</td>
<td>Mouse</td>
<td>2 µg/ml</td>
<td>PFA</td>
<td>Abcam</td>
</tr>
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<td>LPS/bacteria</td>
<td>Rabbit serum</td>
<td>Rabbit</td>
<td>1%</td>
<td>PFA</td>
<td>Covance</td>
</tr>
<tr>
<td>O157</td>
<td>O157 Antiserum</td>
<td>Rabbit</td>
<td>0.01%</td>
<td>PFA</td>
<td>Difco</td>
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<td>Mouse</td>
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<td>PFA</td>
<td>Life Technologies</td>
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<td>Microvilli</td>
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<td>Rabbit</td>
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<td>PFA</td>
<td>Cell Signaling Technologies</td>
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<td>HA-Tir</td>
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<td>Covance</td>
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<tr>
<td>HA-Tir</td>
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<td>PFA</td>
<td>Covance</td>
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<td>Sigma</td>
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<td>Early lysosomes</td>
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<td>Santa Cruz Biotechnologies</td>
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<td><strong>Secondary Antibodies:</strong></td>
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<td></td>
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<tr>
<td>Mouse IgG</td>
<td>Alexa555,568,488 anti-</td>
<td>Goat</td>
<td>4 µg/ml</td>
<td>PFA or</td>
<td>Life Technologies</td>
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<tr>
<td></td>
<td>mouse</td>
<td></td>
<td></td>
<td>Methanol</td>
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<tr>
<td>Rabbit IgG</td>
<td>Alexa555,568,488 anti-</td>
<td>Goat</td>
<td>4 µg/ml</td>
<td>PFA or</td>
<td>Life Technologies</td>
</tr>
<tr>
<td></td>
<td>rabbit</td>
<td></td>
<td></td>
<td>Methanol</td>
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<td><strong>Other:</strong></td>
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<tr>
<td>F-actin</td>
<td>Alexa488-Phalloidin</td>
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<td>2 U/ml</td>
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<td>Life Technologies</td>
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<td>DNA</td>
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<td>PFA or</td>
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</tr>
<tr>
<td></td>
<td>phenylindole (DAPI)</td>
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<td></td>
<td>Methanol</td>
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2.4 Results:

2.4.1 EPEC and EHEC pedestals can be compared directly using engineered EPEC strains

EPEC is able to infect cultured cells better than EHEC in vitro (Brady et al., 2011; Cantey and Moseley, 1991), and EHEC has a more extensive repertoire of effector proteins than EPEC (Wong et al., 2011). Therefore, to study phenotypic differences stemming specifically from the divergent pedestal assembly pathways, we used EPEC strains engineered to express epitope-tagged versions of Tir or EspF_U that generate pedestals by either the EPEC or EHEC mechanism. Actin pedestal assembly driven by EPEC Tir relies heavily upon the phosphorylation of tyrosine 474, so to enable immunostaining of EPEC Tir variants capable or incapable of Y474 phosphorylation, we employed two strains of EPEC engineered with chromosomal deletions of tir that harbor low copy number plasmids encoding HA-tagged wild type Tir or an HA-tagged Y474F point mutant (Campellone et al., 2002). In agreement with previous results showing that Y474 is required for >95% of actin pedestal formation (Campellone and Leong, 2005; Campellone et al., 2004b; Gruenheid et al., 2001), these two strains, referred to as EPEC Y474* and EPEC Y474F (Fig 2-1A), were each capable of translocating HA-Tir into HeLa cells, but only EPEC Y474* triggered actin polymerization into intensely-staining pedestals (Fig 2-1B).

In contrast to EPEC, the EHEC pathway of actin polymerization is largely dependent on clustering of the multivalent effector protein, EspF_U (Campellone et al., 2008a). To directly compare bacteria proficient for the EspF_U-driven mechanism of pedestal assembly to bacteria utilizing EPEC Tir, we employed an EPEC strain encoding HA-tagged EHEC Tir and EHEC intimin in place of the endogenous chromosomal copies of the EPEC genes for Tir and intimin (Campellone et al., 2002). Referred to as KC12, this strain was transformed with a low copy number plasmid encoding myc-tagged EspF_U (KC12+EspF_U) to enable pedestal formation (Fig 2-1A). KC12 harboring an empty vector (KC12+vector) was used as a pedestal-deficient control strain (Campellone et al., 2004a). As expected, both KC12 derivatives translocated HA-Tir into
HeLa cells, but intense actin pedestals were only assembled by the EspFu-expressing bacteria (Fig 2-1B). These strains, isogenic with the exception of the pedestal effectors, were well suited to characterize the functions of the different actin assembly pathways on multiple cell types.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tir</th>
<th>Intimin</th>
<th>EspFu</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT EPEC</td>
<td>🔹</td>
<td>🔹</td>
<td></td>
</tr>
<tr>
<td>EPEC Y474*</td>
<td>HA</td>
<td>🔹</td>
<td></td>
</tr>
<tr>
<td>EPEC Y474F</td>
<td>HA</td>
<td>🔹</td>
<td></td>
</tr>
<tr>
<td>WT EHEC</td>
<td>🔹</td>
<td>🔹</td>
<td></td>
</tr>
<tr>
<td>KC12+EspFu</td>
<td>HA</td>
<td>🔹</td>
<td>EspFu</td>
</tr>
<tr>
<td>KC12+vector</td>
<td>HA</td>
<td>🔹</td>
<td></td>
</tr>
</tbody>
</table>

Fig 2-1. EHEC and EPEC pedestals can be compared directly using engineered EPEC strains.
(A) EPEC Y474*, EPEC Y474F, KC12+EspFu, and KC12+vector are all EPEC strains engineered to express HA-tagged versions of Tir and/or myc-tagged EspFu to reflect the WT EPEC or WT EHEC pathways of pedestal assembly. Green and purple boxes represent EPEC and EHEC proteins, respectively. The asterisk indicates phosphotyrosine residue 474. (B) HeLa cells were infected for 3 h with the indicated strains, fixed, and stained to visualize LPS, HA-Tir, and F-actin. Scale bar, 10 µm.

2.4.2 The EPEC and EHEC mechanisms of pedestal assembly both provide anti-phagocytic functions

Intestinal macrophages serve as an initial line of innate defense in the gut, as they phagocytose bacteria and help maintain tissue homeostasis (Gross et al., 2015). Macrophages are also known to rely on N-WASP and the Arp2/3 complex to form protrusions used to engulf bacteria (Rougerie et al., 2013). To test whether the direct manipulation of the actin nucleation machinery by EHEC or EPEC might prevent phagocytosis, human THP-1 monocytes were activated to a macrophage-like state and infected with the strains of KC12 or EPEC that could or could not form pedestals. KC12+EspFu and EPEC Y474* both retained the capacity to translocate Tir and form pedestals on this cell type, while KC12+vector and EPEC Y474F were only capable of Tir translocation (Fig 2-2 A). To differentiate external bacteria from total
bacteria, outside-inside staining was performed. These experiments revealed that some bacteria were internalized by THP-1 cells (Fig 2-2 B). LAMP-1 staining of cells infected in parallel confirmed that internalized bacteria were associated with lysosomes (Fig 2-2 C),

![Image](https://example.com/image.png)

**Fig 2-2.** The EPEC and EHEC mechanisms of pedestal assembly both provide anti-phagocytic functions.

(A) Activated THP-1 macrophages were infected for 3.5 h, fixed, and stained to visualize DNA, HA-Tir, and F-actin. Scale bar, 10 µm. (B) Activated THP-1 cells infected for 90 min were differentially stained to determine the total number of cell-associated bacteria (red), and the number of external bacteria (green). Scale bar, 10 µm. (C) Activated THP-1 cells infected for 90 min with EPEC were fixed and stained for DNA, bacteria, and LAMP-1. Scale bar, 10 µm; inset scale bar, 1 µm. (D) The % of internalized bacteria was quantified at the depicted times. Each data point represents the mean (+/- SE) calculated from 4-6 coverslips with 200-300 total cells spanning at least 3 experiments. **p<0.01, *** p<0.001 (ANOVA, Tukey post-hoc tests).
suggesting that the intracellular bacteria were indeed phagocytosed and targeted for degradation.

To determine if there was a discernable difference in phagocytosis of the KC12 and EPEC strains, differential outside-inside staining of infected cells was used to quantify the proportions of extracellular versus intracellular bacteria at various time points (Fig 2-2 D). Extracellular KC12+EspF\textsubscript{U} and EPEC Y474* were both found in greater quantities than their pedestal deficient counterparts between 30 and 150 min post infection (Fig 2-2 D). Despite recruiting the actin assembly machinery by different mechanisms, resistance to internalization by KC12+EspF\textsubscript{U} and EPEC Y474* was nearly identical, as approximately 75% of each strain remained extracellular at 90 min. In contrast, the pedestal-deficient strains KC12+vector and EPEC Y474F were internalized in amounts equivalent to a strain that completely lacked the type 3 secretion system (EPEC\textDelta T3SS) (Fig 2-2 D). Thus, the ability to form pedestals by either the EHEC or EPEC pathway of actin assembly allows for increased resistance to phagocytosis.

### 2.4.3 Both mechanisms of actin pedestal assembly promote motility and exploration of the cell surface

EPEC and EHEC actin pedestals were shown to enable bacterial movement, or “surfing” on host cells many years ago, and EPEC was initially found to move at a maximum speed of 4.2 \( \mu \text{m/min} \) (Sanger et al., 1996). A follow-up study determined that actin polymerization rates within pedestals ranged between 0.2 and 1.0 \( \mu \text{m/min} \) (Shaner et al., 2005). However, aside from those preliminary measurements, the properties of EPEC and EHEC movement have not been directly compared. To explore the similarities and differences in motility among bacteria forming EPEC Tir-dependent pedestals versus EspF\textsubscript{U}-dependent pedestals, NIH3T3 fibroblasts stably expressing mCherry-actin were infected with EPEC or KC12 derivatives, and examined live (Fig 2-3 A, Video S1). The general movements of actin pedestals that we observed were consistent
Fig 2-3. The actin pedestals assembled by KC12+EspF<sub>U</sub> and EPEC Y474* promote motility and exploration of the host cell surface.

(A) NIH3T3 cells stably expressing mCherry-actin were infected with EPEC expressing GFP for 3 h and imaged live for 45 min. Scale bar, 10 μm. (B) mCherry-actin expressing cells were infected with the indicated strains for 3 h and imaged live for 18-20 min. 15-20 bacteria per host cell for each strain were tracked, and data from representative cells were plotted such that points starting at t = 0 were centered at the origin. (C) Bacterial motility rates were quantified from cells infected as in (B). Each bar represents the mean speed (+/- SE) of bacteria on 6-20 host cells (95-293 total bacteria). ** p<0.01, *p<0.05 (ANOVA, Tukey post-hoc tests). (D) The fraction of pedestals that were considered moving was quantified, using the speed of the pedestal deficient counterpart strain as a minimum cutoff to define movement. Each bar represents the mean (+/- SE) from 227-320 pedestals. p=0.3 (Fisher’s exact test). [Legend continued on next page]
with previous descriptions (Sanger et al., 1996), as some pedestals remained fairly stationary, while others translocated along the cell surface (Fig 2-3 A).

We next tracked the movements of individual bacteria and plotted them so that the starting point at time = 0 was placed at the graph origin (Fig 2-3 B). These experiments illustrated that pedestal proficient bacteria explored the cell surface to a greater extent than pedestal deficient strains, with KC12+EspF_U and EPEC Y474* typically traveling through areas with radii of 6-7 µm over an 18 min period, compared to radii of <4 µm for the pedestal deficient KC12+vector and EPEC Y474F bacteria (Fig 2-3 B). Measurements of the speeds of each strain further demonstrated that KC12+EspF_U and EPEC Y474* moved at very similar average rates (0.36 and 0.39 µm/min, respectively) and maximum rates (4.14 and 4.20 µm/min, respectively), and were each twice as fast as their pedestal deficient counterparts KC12+vector and EPEC Y474F (average rates of 0.17 and 0.19 µm/min, respectively), whose limited movements could be largely attributed to the fluidity of the plasma membrane or cell stretching (Fig 2-3 C). Additionally, KC12 and EPEC derivatives completely lacking Tir showed restricted movements similar to those of KC12+vector and EPEC Y474F, (Fig 2-3 B-C), indicating that intimate adherence by the latter strains in the absence of a pedestal does not provide a measurable motility advantage. However, strains lacking Tir were still faster than the T3SS mutant, suggesting that the T3SS mutant did not interact with the plasma membrane extensively enough to be influenced by the movement of the underlying cell. (Fig 2-3 B-C).

To assess potential motility differences between the pedestals of KC12+EspF_U and EPEC Y474*, live imaging data was subjected to further quantitative analyses. Based on pedestal tracking, and using the average speeds of pedestal deficient strains as background
values for defining movement, the fraction of pedestals classified as moving was calculated. By these criteria, equivalent fractions of KC12+EspF_U and EPEC Y474* pedestals were scored as moving (Fig 2-3 D), indicating that both the EPEC Tir-dependent mechanism and the EspF_U-dependent mechanism of actin polymerization promote similar frequencies of motility. Additionally, directional persistence was measured by dividing the displacement of each pedestal by the total path length, making a value of 1 a directly linear route. Both sets of pedestals had values just over 0.4, indicating that KC12+EspF_U and EPEC Y474* display comparable degrees of directional persistence (Fig 2-3 E). Finally, to ensure that the same trend of pedestal-associated motility that we observed for KC12+EspF_U was exhibited by bona fide EHEC, movements of WT EHEC, as well as EHECΔespF_U mutant strains harboring either the EspF_U-myc plasmid or an empty vector control were examined. As expected, the presence of EspF_U allowed for significantly faster motility than the pedestal deficient strain (Fig 2-3 F).

Collectively, these data highlight numerous similarities between the EHEC and EPEC pedestal assembly pathways in motility and exploration of the surface of non-polarized cells.

2.4.4 KC12 and EPEC strains form “macrocolonies” that grow over time on polarized epithelial cells

While our macrophage and fibroblast infections revealed many parallels in function between EspF_U- and phospho-Tir-mediated pedestals on these cell types, the ultimate target host cells for EPEC and EHEC are polarized intestinal epithelial cells. To explore potential pedestal-related similarities and differences on epithelial monolayers, we infected polarized JEG-3 (human placental epithelial) and Caco-2 (human colonic epithelial) cells. Staining with antibodies to Ezrin and ZO-1 confirmed that in contrast to nonpolarized HeLa epithelial cells, JEG-3 and Caco-2 cells contained microvilli and formed tight junctions (Fig 2-4 A). As expected, 6 h of infection resulted in disassembly of microvilli, especially in cells with high bacterial burdens (Fig 2-4 B). However, ZO-1 staining was not extensively disrupted at this time point.
Fig 2-4. KC12+EspF<sub>U</sub> disrupts microvilli of polarized cells, but does not significantly alter tight junctions.

(A) HeLa, JEG-3, or Caco-2 cells were left uninfected or infected with KC12+EspF<sub>U</sub> for 6 h. Cells were fixed and stained with phalloidin to detect F-actin, Ezrin antibodies to stain microvilli, ZO-1 antibodies to visualize tight junctions, and DAPI to label DNA. Scale bar, 25 µm. (B) Polarized Caco-2 monolayers were infected with KC12+EspF<sub>U</sub> for 6 h, fixed, and stained to detect ZO-1 (bottom) or Ezrin (top), in addition to bacterial LPS, DNA, and F-actin. Areas of low (first and third rows) and high (second and fourth rows) bacterial burdens were imaged from the same coverslip for each staining condition. Scale bar, 50 µm.

(Fig 2-4 A), and disruption did not correlate with bacterial load (Fig 2-4 B). Immunostaining of adherent bacteria and imaging at high magnification confirmed the presence of actin pedestals (Fig 2-5 A).

Interestingly, imaging at low magnification revealed an unexpected colonization phenotype. KC12+EspF<sub>U</sub> and EPEC Y474* formed discrete infection foci, or “macrocolonies,” on the polarized cell monolayers (Fig 2-5 B). These were reminiscent of colonies formed on an agar plate or plaques generated by intracellular pathogens. We chose the term macrocolonies to distinguish these structures from the definition of microcolonies as multiple loci of infection on
Fig 2-5. **KC12+EspF<sub>U</sub> and EPEC Y474* form macrocolonies that grow over time on polarized epithelial cells.**

(A) Polarized Caco-2 monolayers were infected for 6 h, fixed, and stained for LPS, DNA, and F-actin. Scale bar, 25 µm; inset 2.5 µm. (B) Cells infected in (A) with KC12+EspF<sub>U</sub> or EPEC were imaged at a lower magnification. Scale circles have areas of 100, 500, and 1000 µm<sup>2</sup>. (C) Polarized Caco-2 monolayers were mock infected (top panels) or infected for 6 h with KC12+EspF<sub>U</sub> (bottom panels), and visualized by scanning electron microscopy. The inset highlights a portion of a macrocolony. Scale bars, 10 µm; inset, 1 µm. (D) Cells infected in parallel with those in (C) were visualized by transmission electron microscopy. The inset shows a cross-section of a pedestal. Scale bars, 2 µm. (E) Polarized Caco-2 monolayers were infected for 3, 5, or 7 h, fixed, and stained to visualize bacteria, DNA, and F-actin. Scale circles, 100, 500, 1000 µm<sup>2</sup>. (F) Macrolony sizes were quantified from cells infected as in (E). Each bar represents the mean (+/- SE) of macrocolony sizes calculated from 3-6 coverslips (85-2309 colonies). Macrolonies over 25 µm<sup>2</sup> were included in quantification. *p<0.05, ***p<0.001 (unpaired t tests).
a single cell, or localized adherence patterns driven by bundle-forming pili (Mills et al., 2013; Scaletsky et al., 1984), because they were larger and spanned several host cells.

To obtain a higher resolution view of KC12+EspFU macrocolonies, electron microscopy was conducted on uninfected and infected polarized Caco-2 cells. Scanning electron micrographs confirmed the immunostaining results, in that the uninfected cells were extensively microvilliated. But, after infection, patches lacking microvilli became apparent while some microvilli had coalesced towards bacteria (Fig 2-5 C), as demonstrated previously for EPEC infections (Shifrin Jr. et al., 2014). Similar to our fluorescence microscopy results, bacteria were found in distinct macrocolonies on the monolayer (Fig 2-5 C). Transmission electron microscopy of samples prepared in parallel allowed for sectioning through a macrocolony, and revealed that bacteria generated pedestals (Fig 2-5 D). Collectively, these findings indicate that EPEC and KC12 strains colonize polarized monolayers by forming discrete infection foci, or macrocolonies, which encompass multiple host cells.

To further characterize macrocolony biogenesis on Caco-2 cells, we fixed cells at various time points after infection (Fig 2-5 E). At 3 h, colonies were small and were often restricted to one host cell. By 5 h, colonies had increased in size, and a striking difference in colony growth between strains began to emerge, with KC12+EspFU macrocolonies growing significantly larger than EPEC Y474* macrocolonies (Fig 2-5 F). By 7 h, this difference was exacerbated, as the average size of KC12+EspFU colonies was nearly three-fold larger than those formed by EPEC Y474* (Fig 2-5 E-F). Although macrocolonies of both strains grew over time, the faster expansion kinetics exhibited by KC12+EspFU highlights a significant and unexpected colonization difference arising from the two different pedestal assembly pathways.
Fig 2-6. EHEC Tir and EspFU promote more efficient colonization of polarized epithelial cells than EPEC Tir.

(A) Polarized Caco-2 monolayers were infected with KC12 and EPEC strains for 6 h, fixed, and stained to visualize bacteria, F-actin, and DNA. Scale circles, 100, 500, 1000 µm². (B) Macrocolony sizes >100 µm² from experiments described in (A) were measured and binned into size groups. Each bar represents the mean number of macrocolonies (+/- SE) from 3 experiments, spanning 225-275 fields of view (FOV) and 4658-8434 colonies. All p-value significance is in reference to the 100-250 µm² bins. *p<0.05, **p<0.01 (ANOVA, Tukey post-hoc tests). (C) Data collected in (B) were reorganized to compare the

[Legend continued on next page]
strains within each category. Bars represent the mean (+/- SD) for 3 experiments, of the % of colonies falling into each bin. Asterisks are in reference to KC12+EspFr. *p<0.05, **p<0.01 (ANOVA, Tukey post-hoc tests). (D) Macrocolony sizes measured from part (B) were averaged. Each bar represents the mean (+/- SD) from 3 experiments. All p-values are in reference to KC12+EspFr. (E) Experiments were performed as in (A), but for 7 h. Each bar represents the mean (+/- SE) of the % of monolayer area infected for 58-60 FOVs. (F) The number of infected cells per macrocolony was calculated. Each bar represents the mean (+/- SE) calculated from 238-497 macrocolonies, taken from 17-19 representative fields of view from the images quantified in (E). *p<0.05, **p<0.01 (ANOVA, Tukey post-hoc tests).

2.4.5 EHEC Tir and EspFr promote more efficient colonization of polarized epithelial cells than EPEC Tir

To further investigate the difference in macrocolony size between KC12+EspFr and EPEC Y474*, polarized Caco-2 monolayers were infected with these strains, and subjected to frequent washes and media changes to remove unbound bacteria and promote the development of macrocolonies exclusively from bacteria that adhered within the first hour of infection. After 6 h, monolayers were fixed and analyzed by fluorescence microscopy, and the area of each macrocolony over 100 µm² was measured. In agreement with the time course data, quantification and histogram analysis revealed that KC12+EspFr colonies were notably larger than EPEC Y474* colonies (Fig 2-6 A-D). This difference was especially apparent when considering structures over 1000 µm², because these made up 16% of all colonies for KC12+EspFr, but <4% of those for EPEC Y474* (Fig 2-6 A-C). After 7 h of infection, this difference in colony size translated to a larger infected area of the monolayer (Fig 2-6 E), and a greater number of infected cells per macrocolony (Fig 2-6 F), despite indistinguishable bacterial multiplication rates under these culture conditions (Fig 2-7).

To examine if pedestals play a role in determining macrocolony size, cells were also infected with KC12+vector and EPEC Y474F. KC12+vector macrocolonies were significantly smaller than those of their pedestal proficient counterpart (Fig 2-6 A-D), and while KC12+EspFr
had approximately equal numbers of macrocolonies of all size ranges, KC12+vector had many more colonies in the small 100-250 μm² range than in categories above 500 μm² (Fig 2-6 A-B). Furthermore, KC12+vector infected a smaller percentage of the monolayer, and fewer host cells per macrocolony (Fig 2-6 E-F), indicating that EspFₚ enhances colony size. In contrast, EPEC Y474* did not have any colony size advantage over EPEC Y474F. The size distributions for these two strains were very similar (Fig 2-6 B), and there was no significant difference in average size, area infected, or the number of cells infected per macrocolony (Fig 2-6 D-F).

![Graph showing CFU/mL over time and Maximum Division Rate](image)

**Fig 2-7. KC12 and EPEC strains divide at similar rates in suspension and on cells.**

(A) Bacteria grown in infection media were diluted and plated every 90 min to determine the number of Colony Forming Units (CFUs). Each data point represents the mean number of CFUs (+/- SD) from 4 experiments. (B) JEG-3 cells were infected for 6 h with the indicated strains and imaged live. Individual bacteria were tracked over time to determine the amount of time between consecutive divisions and calculate the maximum division rate. Each point represents a single bacterium, with the mean (+/- SD) indicated in black.

Surprisingly, KC12+vector infected a larger area of the monolayer and more cells per colony than any of the EPEC Tir-expressing strains, suggesting an important role for the EHEC version of Tir in enhancing macrocolony size. As expected, the T3SS mutant yielded very petite colonies, with 70% falling into the small 100-250 μm² range (Fig 2-6 A-C). Collectively, these
data suggest that the EHEC mechanism of pedestal assembly provides a colonization advantage over the EPEC pathway of actin polymerization in infections of polarized intestinal epithelia.

2.4.6 EHEC Tir, EspF\textsubscript{U}, and the host Arp2/3 complex drive KC12 macrocolony expansion

To further characterize the role of EHEC Tir in colonization, KC12 and KC12\textDelta tir strains harboring EspF\textsubscript{U}-myc or control plasmids were used to infect Caco-2 monolayers. These variants allowed for an assessment of the individual and combined contributions of Tir and EspF\textsubscript{U} to colonization. Again, KC12+EspF\textsubscript{U} formed larger macrocolonies and infected a greater fraction of the epithelial tissue than KC12+vector (Fig 2-8 A-C). In addition, both strains with tir deletions formed smaller colonies than the Tir-expressing strains (Fig 2-8 A-B). EspF\textsubscript{U} was unable to rescue this defect of KC12\textDelta tir (Fig 2-8 A-B), suggesting that EspF\textsubscript{U} requires Tir to contribute to colonization. Collectively, these results show that EHEC Tir can promote the development of macrocolonies and that its effects are enhanced by EspF\textsubscript{U}.

To ensure that EspF\textsubscript{U} plays an equally important role in colonization by EHEC, Caco-2 monolayers were infected with WT EHEC, as well as an EspF\textsubscript{U} deletion strain and a complemented strain expressing EspF\textsubscript{U}-myc. Macrocolonies of EHEC\textDelta espF\textsubscript{U}+vector were significantly smaller than WT EHEC, and this deficiency was rescued by the EspF\textsubscript{U}-myc plasmid (Fig 2-8 D-E). Since EspF\textsubscript{U} is capable of interacting with the EPEC version of Tir to promote Nck-independent actin polymerization (Brady et al., 2007), we further hypothesized that adding EspF\textsubscript{U} to EPEC might enhance EPEC macrocolony size. In fact, infection of polarized Caco-2 monolayers with EPEC+EspF\textsubscript{U} confirmed that EspF\textsubscript{U} localized to EPEC pedestals (Fig 2-9 A), and revealed that EPEC+EspF\textsubscript{U} macrocolonies were 25% larger than those formed by WT EPEC (Fig 2-8 F-G). However, KC12+EspF\textsubscript{U} still produced the largest macrocolonies, underscoring the importance of EHEC Tir (Fig 2-8 G). These data indicate that EspF\textsubscript{U} is able to enhance macrocolony size using either the EHEC or EPEC version of Tir.
EspF<sub>U</sub> can enhance macrocolony size using either the EHEC or EPEC versions of Tir.

(A) Polarized Caco-2 monolayers were infected for 6 h with the indicated KC12 and KC12Δtir strains, fixed, and stained to visualize bacteria, DNA, and F-actin. (B) Experiments described in (A) were quantified. Each bar represents the mean macrocolony size (+/- SE) calculated from 6 coverslips (2025-3179 colonies). (C) The experiments in (B) were also used to quantify the % of monolayer area infected. Each bar represents the mean (+/- SE) from 59-60 FOVs. (D-E) Polarized Caco-2 monolayers were infected with EHEC strains for 8 h, fixed, and stained as in (A). Bars represent the mean macrocolony size (+/- SE) calculated from 315-617 macrocolonies. (F-G) Polarized Caco-2 monolayers were infected with EPEC strains with or without EspF<sub>U</sub>. Each bar represents the mean macrocolony size (+/- SE) calculated from 1163-2722 macrocolonies. KC12+EspF<sub>U</sub> is shown in purple. For all panels, scale circles, 100, 500, 1000 µm<sup>2</sup>. ** p <0.01, *** p <0.001 (ANOVA, Tukey post-hoc tests). To allow for a sufficient number of Δtir colonies that could be analyzed, colonies larger than 50 µm<sup>2</sup> were included in quantification, unlike Fig 2-6 where 100 µm<sup>2</sup> was the lower limit.
Fig 2-9. EspFₜ and Tir can colocalize even if delivered by independent bacteria.
(A) Polarized Caco-2 monolayers were infected with EPEC+EspFₜ or KC12+EspFₜ, fixed and stained for EspFₜ-myc, F-actin, and DNA. Scale bar, 10 µm. (B) JEG-3 monolayers were co-infected for 6 h with equal amounts of EPEC Y474* and EHECΔespFₜ+EspFₜ, fixed, and stained for HA-Tir (which is only tagged in EPEC), EspFₜ-myc (which is only expressed by EHEC), F-actin, and DNA. Areas of isolated EHEC bacteria (i), isolated EPEC bacteria (ii), or mixed infection (iii) are shown in insets. Colocalization between HA-Tir and EspFₜ-myc is indicated with arrowheads, indicating that bacteria can share pedestal effectors during co-infection. Scale bars, 50 µm, inset 5 µm. (C) JEG-3 monolayers were co-infected and fixed as in (B), but stained for EHEC O157 in addition to HA-Tir. Colocalization between HA-Tir and O157 was not observed, suggesting that Tir is not effectively transferred from EPEC to EHEC. Scale bars, 50 µm, inset 12.5 µm. (D) JEG-3 monolayers were co-infected with EPEC Y474* and EPEC+EspFₜ, fixed, and stained for HA-Tir (which is only tagged in EPEC Y474*) and EspFₜ-myc (which is only expressed in EPEC+EspFₜ). Colocalization indicates that EPEC strains can efficiently share HA-Tir and EspFₜ. Scale bar, 5 µm.
Because EHEC Tir and EspF trigger pedestal assembly and increase macrocolony size, we speculated that disrupting pedestal formation on the host side by inhibiting the Arp2/3 complex could diminish colonization in a way that resembled what was seen with the EHEC effector mutants. To test this, we examined the effects of treating Caco-2 cells with the Arp2/3 complex inhibitors CK666 and CK869 during infection with KC12+EspF. The inhibitors did not alter the morphology of tight junctions or microvilli on Caco-2 cells, but did interfere with pedestal assembly as expected (Fig 2-10 A-F). In addition, the Arp2/3 complex inhibitors restricted the size of KC12+EspF macrocolonies compared to DMSO-treated controls (Fig 2-11 A-B). To determine if this inhibition could be caused by limiting actin based motility, mCherry-

Fig 2-10. Arp2/3 inhibitors do not disrupt Caco-2 microvilli or tight junctions, but do inhibit actin pedestal formation.

(A-B) Polarized Caco-2 monolayers were treated with either DMSO or CK666+CK869 for 6 h, fixed, and stained for DNA, F-actin, and either Ezrin or ZO-1. Scale bar, 50 µm. (C) Polarized Caco-2 monolayers were pretreated for 15 min with DMSO or CK666+CK869, then infected for 6 h with KC12+EspF, in the presence of either DMSO or inhibitors. Cells were fixed and stained for F-actin and bacteria. Scale bar, 10 µm. (D) NIH3T3 cells were infected with KC12+EspF and treated with DMSO or CK666+CK869 for 4 h, fixed, and stained for HA-Tir, F-actin, and bacteria. Scale bar, 10 µm. (E) The % of HA-Tir positive bacteria that were associated with actin pedestals from experiments in (D) was calculated. Each bar represents the mean (+/- SE) calculated from 15 cells, each harboring up to 50 bacteria. (F) The relative intensity of pedestals associated with HA-Tir positive bacteria was calculated and normalized to an adjacent pedestal-free area of the cell equal to 1. Each bar represents the mean (+/- SE) calculated from 9 cells harboring up to 40 pedestals per cell. *p<0.05, ***p<0.001 (unpaired t tests).
actin expressing NIH3T3 cells were treated with DMSO or CK666/CK869, and motility was measured. As expected, KC12+EspF_U moved at less than half the speed on CK666/CK869 treated cells as on DMSO treated cells (Fig 2-11 C). Taken together with the smaller macrocolony size and slower motility exhibited by EspF_U-deficient bacteria, these data are consistent with the idea that actin-based motility driven by EHEC Tir and EspF_U plays a key role in the extent of tissue colonization.

Fig 2-11. The Arp2/3 complex is important for macrocolony size and actin-based motility. (A) Polarized Caco-2 monolayers were treated with DMSO or the Arp2/3 inhibitors CK666+CK869 (CK+CK), infected with KC12+EspF_U, fixed, and stained for bacteria. Scale circles, 100, 500, 1000 µm². (B) The average colony size was quantified from experiments performed in (A). Each bar represents the mean (+/− SE) calculated from 640-706 colonies spanning 4-6 coverslips from 3 experiments. Colonies larger than 25 µm² were included in analysis. *** p<0.001 (unpaired t test). (C) NIH3T3 cells expressing mCherry-actin were infected with KC12+EspF_U and treated with DMSO or CK666+CK869. Mean speeds (+/− SE) were calculated based on pedestals from 13-15 cells per condition from at least 3 experiments (185-220 bacteria). **p<0.01, (unpaired t test).

2.4.7 KC12+EspF_U moves faster than EPEC Y474* on polarized cells and uses motility to spread from cell-to-cell

Although our data implicate Tir and EspF_U in spreading infection across a cell monolayer, this process has never been directly visualized. To better understand the mechanism of colony growth, we performed live cell imaging to monitor macrocolony development over time. JEG-3 cells were utilized as host cells because they polarize more rapidly than Caco-2 cells and are easier to image by phase-contrast microscopy. Tracking macrocolony growth over time revealed that colony expansion is partially driven by bacterial replication and spreading outwards towards
cell junctions, thereby promoting infection of neighboring cells (Fig 2-12 A, Video S2). Further time lapse analyses (Video S2) revealed that KC12+EspF<sub>U</sub> bacteria often migrated out of

![Image of cell junctions and bacteria migration](image)

**Fig 2-12.** KC12+EspF<sub>U</sub> moves faster than EPEC Y474* on polarized cells and uses motility to spread efficiently from cell-to-cell. (A) JEG-3 cells were infected with KC12+EspF<sub>U</sub> for 6 h and imaged for 2.5 h using phase-contrast microscopy. Selected frames (taken from Video S2) show the expansion of a macrocolony. Scale bar, 10 µm. (B) Additional images (from Video S2) show a cell-cell junction (white dashed line), and a bacterium (white arrowhead) traveling on a pedestal (white arrow in inset). The highlighted bacterium divided, and one daughter adhered to the adjacent cell (black arrowhead). The bacterium then divided again on the same cell (gray arrowhead). Scale bar = 5 µm. (C) NIH3T3 cells expressing mCherry-actin were infected with WT EHEC for 4 h and imaged live. Selected frames from live imaging show a bacterium on an mCherry-actin pedestal (red arrowhead) reaching a nearby cell and dividing. Phase-dense pedestals can be seen on the second cell for both bacteria (white arrows), while only one possesses an mCherry-actin pedestal still connected to the original cell. Scale bar, 50 µm, inset 5 µm. (D) Bacteria were categorized as free, in macrocolonies, or at junctions based on their localization (NQ= not quantifiable). Each bar represents the mean speed (+/- SE) for 30 bacteria for each condition, with the exception of junction bacteria, where n=15 bacteria. ** p<0.01 (ANOVA, Tukey post-hoc tests).
colonies and eventually reached cell junctions. Bacteria moving on pedestals appeared to pause at these junctions, where they replicated to cause infection of the neighboring cell (Fig 2-12 B). To visualize this process in more detail, and verify that EHEC employs the same mechanism of transmission, NIH3T3 fibroblasts expressing mCherry-actin were infected with WT EHEC for and imaged live (Fig 2-12 C). A bacterium moving on a pedestal containing mCherry-actin (Fig 2-12 C, arrowhead) contacted a nearby cell that was not expressing mCherry-actin. This difference in mCherry-actin expression allowed us to distinguish the pedestals between the two host cells, as phase-contrast microscopy revealed that the bacterium with an mCherry-labeled pedestal formed a phase-dense pedestal on the second cell (Fig 2-12 C, top arrow) and divided, while maintaining the original pedestal. After division, one bacterium had two pedestals on the two different cells, while the other daughter was only associated with the second cell via a phase-dense pedestal (Fig 2-12 C, bottom arrow). These observations provide the first evidence for how extracellular EPEC/EHEC spread beyond initially infected cells while remaining intimately attached to the plasma membrane.

In the course of these studies, we noticed that adherent bacteria exhibited different movement behaviors based on their associations with colonies or junctions, so we quantified motility of KC12+EspF \(_U\) within macrocolonies, at cell-cell junctions, or “free,” (not in colonies or at junctions). Free bacteria moved significantly faster than bacteria in tightly-packed colonies or at junctions (Fig 2-12 D). In an attempt to uncover why EPEC colonies do not grow as large on polarized cells as KC12 macrocolonies, EPEC movement was also assessed. While KC12+EspF \(_U\) and EPEC Y474\(^*\) bacteria in macrocolonies displayed similar speeds (Fig 2-12 D), the frequency of EPEC Y474\(^*\) reaching junctions and replicating between 6 and 8 h of infection was too low to be quantified (Video S3). Surprisingly, and in contrast to fibroblast data (Fig 2-3), the population of free EPEC Y474\(^*\) moved significantly slower than free KC12+EspF \(_U\) (Fig 2-12 D). The faster speed of KC12+EspF \(_U\) provides one explanation for why these bacteria are better able to migrate out of bacterial clusters, move to junctions, and infect more cells than EPEC.
Y474*. Thus, EHEC Tir and EspF_U confer an advantage over EPEC Tir in motility and cell-to-cell spreading that is specific to polarized epithelia.

2.4.8 EspF_U-dependent actin pedestals allow for an efficient pathway of cell-to-cell transmission

Fig 2-13. EspF_U-dependent actin pedestals allow for an efficient pathway of cell-to-cell transmission.

(A) JEG-3 and HeLa cells infected for 6 h and 5 h, respectively, were stained to show bacteria (blue), F-actin (red), and HA-Tir (green). Individual events were ordered into the following sequence based on live imaging in Fig 2-12: bacteria (i) use pedestals to protrude and contact an uninfected neighboring cell, (ii) translocate effectors including Tir (arrowheads) into the second cell, (iii) polymerize a second pedestal (arrows), and (iv) use the secondary pedestal to dock bacteria at junctions as the bacteria divide. Scale bars, 3 µm. (B) The proposed model is based on data from experiments in Fig 2-12 and 2-13, and is shown to incorporate every step of the infectious life cycle. Green circles represent translocated effectors, red lines indicate F-actin in pedestals, and green ovals represent Tir.
To better elucidate how cell-to-cell spreading occurs when KC12+EspF\textsubscript{U} pauses at a cell junction, we fixed infected JEG-3 cells and stained for bacteria, F-actin, and HA-Tir. Additionally, we infected, fixed, and stained a highly infectable HeLa cell line that was seeded at an optimal density for easily distinguishing the boundaries between cells. These experiments allowed us to outline a straightforward pathway for cell-to-cell transmission. We detected (1) bacteria on pedestals protruding towards neighboring cells, (2) bacteria with a pedestal on one cell and Tir in a neighboring cell, (3) bacteria with two pedestals on two different cells, and (4) bacteria that had divided and maintained pedestals on two different cells (Fig 2-13 A). Ordering these events, and pairing them with live imaging data, we infer that the bacteria use actin-based surfing motility to escape densely-packed bacterial communities, move towards junctions with uninfected cells, contact and inject effectors into the adjacent cell, polymerize a second pedestal, divide, and eventually disengage from the first cell to complete the infection cycle by moving onto a new cell (Fig 2-13 B).
2.5 Discussion:

EPEC and EHEC pedestals were discovered decades ago, but our understanding of their cellular functions has remained elusive. Early on, it was hypothesized that pedestals act as an anchor to maintain attachment to the host cell via direct cytoskeletal linkage (Goosney et al., 2000), or that intimate adherence via Tir and intimin prevents phagocytosis (Goosney et al., 1999). More recent studies have focused on the importance of Tir signaling in intestinal colonization (Crepin et al., 2010), or of actin polymerization in maintaining cell attachment and thereby enhancing type 3 translocation (Battle et al., 2014; Mallick et al., 2014; Vingadassalom et al., 2010). It has also been speculated that surfing on pedestals could aid in the spreading of infection and the development of bacterial niches (Lai et al., 2013). However, live examinations of EPEC and EHEC infections are limited (Sanger et al., 1996; Shaner et al., 2005; Shifrin Jr. et al., 2014), so how actin-based surfing motility could contribute to pathogenesis was not clear. The studies and speculations thus far have also not directly considered functional differences between the EHEC and EPEC mechanisms of pedestal assembly, so it was unknown if either of the two actin polymerization pathways have distinct advantages. In the current work, we defined similar roles for EHEC and EPEC pedestals in resisting phagocytosis and promoting motility on fibroblasts. Most importantly, we uncovered differences in colonization of polarized epithelial cells and delineated a pedestal-based mechanism of cell-to-cell transmission by EHEC.

It is well established that EPEC and EHEC are able to resist phagocytosis, and several effector proteins such as EspF, EspB, EspH, and EspJ have been implicated directly or indirectly in this capacity (Celli et al., 2001; Dong et al., 2010; Goosney et al., 1999; Iizumi et al., 2007; Marches et al., 2008; Quitard et al., 2006; Tahoun et al., 2011). EPEC strains lacking only Tir were previously shown to be phagocytosed less than a T3SS mutant, supporting a pedestal-independent mechanism of phagocytotic evasion (Celli et al., 2001). In contrast to these results, we found that the capacity to polymerize actin into pedestals positively correlates with
resistance to phagocytosis, and that pedestal deficient strains were phagocytosed at the same level as a T3SS mutant (Fig 2-3 D), suggesting that translocated effectors previously proposed to play an anti-phagocytic role may not be able to exert their effects in the absence of a pedestal. Our results also indicate that both the EspF_U-mediated and phospho-Tir-mediated pathways of actin polymerization confer the same level of resistance to phagocytosis, agreeing with the common belief that similar form yields similar function for pedestals.

Pathogen motility assays on fibroblasts also revealed conserved pedestal functions, as both KC12+EspF_U and EPEC Y474* were capable of undergoing surfing motility at average speeds of approximately 0.35 µm/min, and maximum speeds of 4.2 µm/min. Although these are much slower than the actin-based motility rates of intracellular pathogens such as Listeria and Shigella, which average 12-36 µm/min depending on the experimental system (Dabiri et al., 1990; Goldberg and Theriot, 1995; Welch and Way, 2013), the surfing motility of KC12+EspF_U and EPEC Y474* allows for an exploration of the cell surface that surpasses that of pedestal deficient strains. Surprisingly, motility on polarized epithelial cells differed between EspF_U-driven and phospho-Tir-driven actin polymerization, with KC12+EspF_U moving significantly faster than EPEC Y474*. Therefore, it is tempting to speculate that the EspF_U mechanism of pedestal assembly may somehow produce more force to enable better movement, specifically on polarized cells.

Studying colonization kinetics on polarized epithelial cells exposed another significantly different outcome of the EHEC and EPEC pedestal assembly pathways, as we were able to characterize the phenomenon of macrocolony formation. These extracellular infection foci are reminiscent of the plaques formed by intracellular Listeria and Shigella (Fattouh et al., 2015; Kuehl et al., 2014). For the E. coli strains, discrete macrocolonies grew over time and encompassed more cells. However, KC12+EspF_U colonies grew significantly faster and larger, covering more host cells than EPEC Y474*. Additionally, our data show that the introduction of EspF_U into WT EPEC can enable those bacteria to also form larger colonies. Interestingly, the
size of macrocolonies is positively correlated with the speed of surfing motility on polarized cells, with a faster strain able to produce larger colonies. This is the first evidence that speed may be related to cell-to-cell spreading for pathogenic *E. coli*.

Intracellular pathogens that manipulate the actin cytoskeleton derive a clear advantage from actin based motility, as it promotes cell-to-cell transmission without exposure to the extracellular immune system (Kuehl et al., 2015; Welch and Way, 2013). However, whether surface-associated EPEC and EHEC could benefit from surfing had never been explored, and no mechanisms for cell-to-cell spread have been proposed. How EHEC colonize the intestine so efficiently has been a long-standing question, and our data provide new insight into colonization by describing how EHEC solves the complex problem of cell-to-cell transmission. Live imaging and fluorescence microscopy revealed that KC12+EspFU use faster surfing motility to leave macrocolonies and reach cell junctions, where they contact a neighboring cell, translocate effectors, polymerize a second pedestal, and divide. This stepwise cellular mechanism of spread across the host intestinal tissue allows the bacteria to remain attached to the epithelial surface at all times, which would effectively reduce the risk of washing away during intestinal contractions and diarrhea.

Consistent with these results, disease models using infant rabbits and gnotobiotic piglets have demonstrated that EHEC which express EspFU expand to greater numbers in the intestine than EHEC lacking EspFU (Ritchie et al., 2008). Intriguingly, the discrete colonization patterns of EHEC that were observed in piglet intestines *in vivo* (Ritchie et al., 2008) closely resemble the macrocolonies that we visualized on polarized monolayers *in vitro*. These observations, combined with the finding that KC12+EspFU forms larger colonies than EPEC Y474*, raises the question of whether the advantage conferred by EspFU would manifest in an *in vivo* competition assay. However, EspFU from WT EHEC is able to trans-complement EspFU-deficient bacteria during co-infection in rabbits and on HeLa cells (Ritchie et al., 2008). Similarly, we found that EspFU injected by EHEC can incorporate into EPEC pedestals during co-infections of polarized
monolayers *in vitro* (Fig 2-9). Thus, deciphering the distinct benefits of EHEC EspF_U *in vivo* will require more innovative approaches in the future.

Finally, our findings prompt the question of how EHEC Tir and EspF_U cause faster motility and macrocolony expansion than EPEC Tir. It is plausible that EspF_U is a more potent activator of N-WASP than Nck1 and Nck2 because it is better at multimerizing N-WASP. Canonical EspF_U has six 47-residue repeats, each with the potential to recruit the N-WASP-WIP complex via a short α helix that activates N-WASP through binding its autoinhibitory region (Campellone et al., 2008a; Cheng et al., 2008; Sallee et al., 2008). Each repeat also possesses proline-rich motifs that bind the SH3 domains of IRTKS, IRSp53, and TOCA-1 to allow recruitment to Tir or enhanced N-WASP activation (Campellone et al., 2012; Vingadassalom et al., 2009; Weiss et al., 2009). *In vitro*, each individual repeat can contribute to faster polymerization, although only two are required for efficient pedestal formation in cells (Campellone et al., 2008a; Cheng et al., 2008; Garmendia et al., 2006). In contrast, the Nck adaptor proteins recruited by EPEC Tir each possess three SH3 domains, all of which are able to bind the proline rich domain of N-WASP (Gruenheid et al., 2001; Rohatgi et al., 2001), and include one that can bind to WIP (Antón et al., 1998). These differences in ability to multimerize N-WASP may impact the structure or function of the pedestal, as multivalency has been shown to promote phase separation both in cells and using purified proteins (Banjade and Rosen, 2014; Hyman et al., 2014; Li et al., 2012). Since increasing valency within a system results in phase separation at lower concentrations (Li et al., 2012), EspF_U may promote phase transitions at lower concentrations than would be necessary for Nck1/2, leading to more efficient signaling to the Arp2/3 complex. It also remains possible that the differential recruitment of several host proteins by EHEC Tir and EspF_U versus EPEC Tir significantly affects motility and cell-to-cell spreading. Future work will likely shed light on how EHEC’s mechanism of pedestal formation provides motility, colonization, and transmission advantages over EPEC’s signaling pathway, and whether EPEC Tir provides its own set of benefits.
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Motil Cytoskelet. 60, 104–120.


3.1 Abstract

Enteropathogenic and Enterohemorrhagic *E. coli* (EPEC and EHEC) polymerize host actin into pedestal protrusions as a critical step in their pathogenesis. This activity makes EPEC and EHEC ideal models to study the actin dynamics that underlie membrane protrusions. EPEC relies on tyrosine phosphorylation of the bacterial effector Tir to recruit the Nck1/Nck2 adaptor proteins, which bind N-WASP, a potent Arp2/3 complex activator. In contrast, EHEC depends on the effector EspFu to activate N-WASP. Although these core pathways of actin pedestal assembly are well-characterized, the potential contributions of additional nucleation factors are unknown. We investigated the cooperation between Arp2/3 complex and other classes of nucleators using chemical inhibitors, siRNAs, and knock-out cell lines. We found that chemical inhibition of formins impairs actin pedestal assembly, motility, and colonization for bacteria using the EPEC, but not the EHEC, mechanism of actin polymerization. We also identified mDia1 as the formin contributing to EPEC pedestal biogenesis, as it localizes to pedestals and its depletion decreases pedestal formation. Collectively, our data suggest that mDia1 cooperates with the Arp2/3 complex to polymerize actin in EPEC pedestals.
3.2 Introduction

Manipulation of the host actin cytoskeleton is a common tactic used by pathogens and is often observed in the form of comet tails which propel intracellular bacteria through the cytosol (Welch and Way, 2013) and promote their cell-to-cell transmission (Lamason and Welch, 2017). Pathogen motility is frequently driven by activation of the Arp2/3 complex, through either bacterial (Welch, 1998; Welch et al., 1997) or host actin nucleation-promoting factors (NPFs) (Suzuki et al., 1998). As a result, several bacteria including Listeria and Shigella have become useful tools for studying the regulation of actin dynamics (Rolhion and Cossart, 2017).

Enteropathogenic Escherichia coli (EPEC) and Enterohemorrhagic E. coli (EHEC) are also capable of reorganizing host actin into protrusions, but these pathogens remain extracellular to form actin pedestals (Knutton et al., 1989; Moon et al., 1983). Pedestals promote actin-based “surfing” motility (Sanger et al., 1996; Shaner et al., 2005), which is important for cell-to-cell spread (Velle and Campellone, 2017). Because EPEC and EHEC signal across the plasma membrane to activate the host actin nucleation machinery, they represent an ideal model for studying the cytoskeletal dynamics that underlie cellular protrusions (Hayward et al., 2006).

To trigger actin pedestal assembly, EPEC and EHEC translocate effector proteins into the host cell using a type 3 secretion system (T3SS) (Wong et al., 2011). One effector, Tir (translocated intimin receptor), adopts a hairpin conformation in the plasma membrane and binds intimin on the surface of the bacterium, enabling tight binding of EPEC and EHEC to the plasma membrane (Devinney et al., 1999; Kenny et al., 1997). For EPEC, tyrosine residue 474 within the cytoplasmic region of Tir is phosphorylated by host cell kinases (Kenny, 1999; Phillips et al., 2004; Swimm et al., 2004), to trigger binding of the adaptor proteins Nck1 and Nck2 (Campellone et al., 2002; Gruenheid et al., 2001). The Nck adaptors in turn recruit N-WASP, an NPF, resulting in actin assembly via the Arp2/3 complex (Kalman et al., 1999; Lommel et al., 2001). EHEC-mediated pedestal biogenesis differs from that of EPEC because it does not rely on tyrosine phosphorylation or Nck1/Nck2 (Campellone et al., 2002; Devinney et al., 1999).
Instead, EHEC Tir binds host BAR proteins including IRTKS (Vingadassalom et al., 2009) and IRSp53 (Weiss et al., 2009) to recruit an additional bacterial effector protein called EspFU (Campellone et al., 2004; Garmendia et al., 2004), which activates N-WASP to achieve Arp2/3 complex-mediated actin assembly (Campellone et al., 2008a; Cheng et al., 2008; Sallee et al., 2008).

EPEC and EHEC pedestals serve several potential pathogenic purposes, ranging from phagocytosis resistance to epithelial colonization (Battle et al., 2014; Goosney et al., 1999; Mallick et al., 2014). Recently, actin pedestals were shown to allow the formation of large, two-dimensional bacterial aggregates called macrocolonies (Velle and Campellone, 2017). A macrocolony encompasses multiple epithelial cells and likely originates from a single adherent bacterium which multiplied and used Arp2/3-mediated actin-based motility to reach and infect neighboring cells. This series of events allows the bacteria to effectively spread infection without dissociating from the epithelia (Velle and Campellone, 2017). These findings indicate that Arp2/3 complex-driven actin assembly is important for the expansion of macrocolonies and the extent of colonization during infection.

It is becoming increasingly apparent that the Arp2/3 complex does not function alone as a nucleator and instead cooperates with other classes of actin nucleators to both assemble complex cellular structures (Dominguez, 2016; Isogai et al., 2015), and to promote pathogen-driven actin polymerization (Truong et al., 2014). For instance, it is well established that *Listeria monocytogenes* activates the Arp2/3 complex using the bacterial NPF ActA (Domann et al., 1992; Kocks et al., 1992; Welch, 1998), *Shigella flexneri* uses the bacterial N-WASP activator IcsA (Bernardini et al., 1989; Suzuki et al., 1998), and vaccinia virus relies on the viral membrane protein A36 to bind the Nck1/2 and Grb2 adaptors (Frischknecht et al., 1999; Scaplehorn et al., 2002). However, recent studies have uncovered roles for formin nucleators in actin tails and pathogen-driven membrane protrusions. Specifically, protrusion formation and cell-to-cell transmission of *Listeria* and *Shigella* were shown to be negatively impacted by the
knockdown or inhibition of Diaphanous-related formins (Fattouh et al., 2015; Heindl et al., 2010), suggesting that the formin family of nucleators contribute to the motile force required for protruding into neighboring cells. Furthermore, actin comet tails generated by vaccinia virus were found to rely on the formin FHOD1 in addition to N-WASP and Arp2/3 for actin assembly, motility, and cell-to-cell spread (Alvarez and Agaisse, 2013). Additionally, *Rickettsia parkeri* was observed to undergo a switch in motility from Arp2/3 complex dependence early in infection to formin-mediated motility late in infection (Reed et al., 2014), relying on the bacterial NPF RickA (Gouin et al., 2004; Jeng et al., 2004) followed by the formin-like nucleator Sca2 (Haglund et al., 2010). A Sca2 mutant strain displayed a more severe cell-cell spreading defect than a RickA mutant, but either pathway could function to promote motility to the cell periphery (Reed et al., 2014). Taken together, these studies reveal that exploitation of multiple actin nucleators or actin assembly pathways may be necessary for efficient pathogen-driven actin assembly and cell-to-cell transmission.

In the current study, we examined the role of formins and other actin nucleators in both the EPEC and EHEC actin polymerization pathways. Collectively, our results support a model in which the formin mDia1 contributes to Arp2/3-complex-driven actin assembly in pedestals generated specifically by EPEC.
3.3 Materials and Methods

3.3.1 Bacterial and Mammalian Cell Culture

EPECΔTir + pHA-Tir (Campellone et al., 2002) and KC12+EspF (Campellone et al., 2004) strains were streaked from glycerol stocks onto LB plates containing 35 µg/ml kanamycin and 100 µg/ml ampicillin and used within 2 weeks for host cell infections. 24 h prior to infection, single colonies were grown in LB + antibiotics with shaking at 37°C for 8–9 h. Cultures were then diluted 1:500 in DMEM + 100 mM HEPES, pH 7.4, with antibiotics and grown standing overnight 37°C in 5% CO₂.

HeLa cells, NIH3T3 cells stably expressing mCherry-β-actin, and C2BBe1 (referred to as Caco-2) cells were maintained and seeded as previously described (Velle, 2017). Caco2 cells were maintained with half media changes every 48 h for two weeks post confluency to generate polarized monolayers. Haploid cell lines (Horizon Genomics) were maintained as subconfluent monolayers in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Gibco). Tamoxifen-inducible ArpC2 knockout mouse fibroblasts (Rotty, 2015) were maintained in DMEM (with 4.5 g/L glucose + L-Glutamine + 110 mg/L sodium pyruvate) supplemented with 1x GlutaMAX (Gibco), 10% FBS, and 1x antibiotic/antimycotic (Gibco). To obtain knockout (KO) and control populations, cells were treated with 2 µM 4-hydroxy-tamoxifen (4-OHT) (Sigma) or an equivalent amount of DMSO for 6 days, including a media change to add fresh 4-OHT or DMSO on day 3. After 6 days, ArpC2 KO cells and control cells were returned to normal media and used within 14 days. All cells were grown at 37°C + 5% CO₂.

3.3.2 Infections

Infections were performed as previously described (Velle, 2017). Briefly, cells were washed twice with PBS and infected with bacteria diluted in DMEM + 3.5% FBS + 20mM HEPES, pH 7.4 to achieve MOIs of 3-10, depending on the host cell line.
**3.3.3 Chemical inhibitors, RNAi, and transfections**

HeLa cells and Caco2 monolayers were treated with 50 µM CK666 + 50 µM CK869 (Calbiochem), 25 µM SMIFH2 (Tocris), 4 µM Wiskostatin (Sigma), or equivalent volumes of DMSO for 15 min prior to infection. During infections, media containing bacteria and inhibitors was added to HeLa cells and Caco2 monolayers, and the latter cells were washed with PBS and given fresh inhibitor-containing media every hour during the course of infection. NIH 3T3 cells expressing mCherry-βactin (Campellone et al., 2008b) were infected prior to treatment with the same concentrations described above, and live imaging was completed 15-120 min after the addition of the inhibitors.

RNA and DNA transfections were performed using RNAiMAX or Lippofectamine-LTX reagents (Invitrogen). To clone GFP-mDia1, mDia1 plasmid DNA (variant BC143413, Dharmaco) was PCR amplified as a Kpn1-Not1 fragment using primers ATCATCGGTACCATGGAGCCGCCGGAG, and ATCATCGCGGCGCTTATTAGCTTGCACGGCCAACCAACTC and ligated into the vector pKC-EGFP-C1 (Campellone et al., 2008b). The plasmid was maintained in E. coli XL-1 Blue. For transient expression of GFP-mDia1, 100 ng of GFP-mDia1 plasmid was transfected in 6 well plates. Sigma MISSION siRNAs (see Table 3-1) or Sigma MISSION universal negative control #1 were used at 40 nM for RNAi experiments. Targets were selected based on HeLa cell expression data cataloged on the Human Protein Atlas (https://www.proteinatlas.org/cell).

**3.3.4 Fluorescence Microscopy**

Immunofluorescence microscopy was performed as previously described (Velle, 2017), and all antibodies and molecular probes are listed in Table 3-2. Briefly, cells seeded onto glass coverslips were fixed in 3.7% PFA for 30 min, washed with PBS, permeabilized with 0.1% TritonX-100, washed, and incubated in blocking buffer (1% FBS + 1% BSA in PBS + 0.02%
NaN$_3$) for 30 min. Primary antibodies against HA, LPS, or mDia1 were diluted in blocking buffer and cells were probed for 40 min. Cells were washed and treated with Alexa Fluor 488, 555, 568, or 647 conjugated goat anti-rabbit or goat anti-mouse secondary antibodies and/or DAPI and Alexa Fluor 488 labeled phalloidin for 40 min, followed by washes and mounting in Prolong Gold anti-fade reagent. All fixed and live cells were imaged using a Nikon Eclipse Ti microscope equipped with Plan Apoλ 100x 1.45 NA, 60x 1.40 NA, and Plan Fluor 20x 0.5 NA objectives, an Andor Clara-E camera, and a computer running NIS Elements software. Live phase-contrast imaging as well as mCherry visualization of infected NIH3T3 cells was performed using the 60x objective, and images were captured at 30 s intervals. All image processing was completed in ImageJ, and the mTrackJ and Cell Counter plugins were used for analysis. Statistical analysis of data sets was performed using Graphpad Prism software, and all statistical tests are noted in the figure legends.

3.3.5 Immunoblotting

To determine the levels of mDia1, Arp3, ArpC2, and GAPDH, cells were grown in 6-well plates and collected using 2 mM EDTA in PBS. Cell pellets were resuspended in lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 1% TritonX-100, with 1mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin, leupeptin, pepstatin, and chymostatin), diluted in Laemmli buffer, and loaded onto 8% or 10% polyacrylamide gels for SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (GE Healthcare), blocked for 30 min in PBS + 5% milk (or TBS + 5% milk + 1% Tween-20 for mDia1 staining), and exposed to primary antibodies diluted in blocking buffer overnight at 4 °C, plus a further 2 h at room temperature. Membranes were washed thrice with PBS + 5% Tween-20 (PBS-T, or TBS-T for mDia1 staining), and HRP- or IR-conjugated secondary antibodies were diluted in blocking buffer and incubated with the membrane for 1-2 h. Membranes were then washed thrice in PBS-T or TBS-T. Bands were detected using a LI-
COR Odyssey Fc imaging system. Western blots were analyzed using LI-COR image studio and ImageJ.

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3.4 Results

3.4.1 Chemical inhibition of formins impairs pedestal formation by EPEC but not KC12+EspFU

EPEC and EHEC have different repertoires of effectors and different capacities for infecting cultured cell lines (Brady et al., 2011; Cantey and Moseley, 1991). To directly compare the actin polymerization pathways exploited by EPEC and EHEC, we employed two well-characterized strains, EPEC Y474* (referred to hereafter as EPEC) and KC12+EspFU (Velle and Campellone, 2017). EPEC differs from the wild type in that it has a chromosomal deletion of tir and a low copy number plasmid encoding an HA-tagged version of Tir (Campellone et al., 2002). KC12+EspFU is an EPEC strain that acts as a surrogate for EHEC because it was engineered to have the EHEC versions of intimin and HA-tagged EHEC Tir, as well as a low copy plasmid encoding the effector EspFU (Campellone et al., 2004). Thus, the EPEC and KC12+EspFU strains are isogenic except for their pedestal effectors and can be used to examine the differences in pedestal assembly pathways.

Arp2/3 complex is thought to be critical for all pathways of pedestal assembly. RNAi-mediated knockdown of Arp2/3 complex or overexpression of the N-WASP WCA domain, which has a dominant negative effect by sequestering and/or ectopically activating Arp2/3, impairs pedestal formation by both EPEC and EHEC (Vingadassalom et al., 2010). N-WASP is essential for EPEC pedestal assembly (Lommel et al., 2001; Vingadassalom et al., 2010), and although some N-WASP deficient mouse cells do not support EHEC pedestal assembly (Lommel et al., 2004), others can form pedestals when EHEC Tir and EspFU are either delivered by KC12 or directly expressed in the knockout cells (Vingadassalom et al., 2010). Therefore, we expect inhibition of either the Arp2/3 complex or N-WASP to completely block or inhibit pedestal assembly by EPEC as well as KC12+EspFU. The roles of other nucleation factors, like formins, are unknown in the context of EPEC and EHEC infection.

To determine the contributions of Arp2/3 complex, N-WASP, and formins to actin assembly in pedestals, HeLa cells were pretreated with either DMSO as a control, or the Arp2/3
inhibitors CK666 and CK869, the N-WASP inhibitor Wiskostatin, and/or the broad formin inhibitor SMIFH2. The cells were then infected with EPEC or KC12+EspF\textsubscript{U} and stained to detect HA-Tir, F-Actin, and DNA (Fig 3-1A). The fraction of bacteria that translocated Tir and formed a pedestal was assessed, and the F-actin intensity was quantified at the x-y locations of HA-Tir
staining and normalized to an adjacent Tir-free area of the cell to determine the relative F-actin levels. Treatment with CK666+CK869 caused a 33% reduction in the average percentage of EPEC associated with pedestals, and a 60% reduction in KC12+EspF associated with pedestals (Fig 3-1 B-C). Furthermore, Arp2/3 complex inhibition resulted in significantly dimmer pedestals than DMSO-treated controls for both strains (Fig 3-1 D-E). Wiskostatin had similar effects on EPEC pedestals, but did not cause as severe of a reduction in the fraction of KC12+EspF associated with pedestals (Fig 3-1 B-C). Cells infected with KC12+EspF displayed a bimodal distribution, with some cells showing near control levels of pedestal assembly and another population showing a severe reduction in pedestals (Fig 3-1C).

Interestingly, inhibition of formins using SMIFH2 caused a 20% reduction in pedestal formation and significantly dimmer pedestals for EPEC but not KC12+EspF, which was generally unaffected by SMIFH2 treatment (Fig 3-1 B-E). Furthermore, the reduction in EPEC pedestal intensity with Arp2/3 complex inhibition was exacerbated by simultaneous formin inhibition (Fig 3-1D). Other treatment combinations did not strengthen any of the deficiencies in pedestal formation or intensity. These results provide the first evidence that formins may be involved in EPEC pedestal assembly.

3.4.2 Actin-based motility by EPEC is restricted by chemical inhibition of formins

Actin pedestal-based motility is important for cell-to-cell transmission, and EPEC surfing has been shown to rely heavily on the ability of Tir to become tyrosine phosphorylated at residue 474 (Velle and Campellone, 2017), presumably to trigger a Nck1/2-N-WASP-Arp2/3 complex actin polymerization pathway. To determine if the pedestal defects observed with SMIFH2 treatment impacts motility, mCherry-Actin expressing NIH3T3 cells were infected, treated with inhibitors, and subjected to live imaging. Bacteria associated with pedestals were tracked over time and pedestal speeds were calculated using movies spanning 20-30 min. EPEC pedestals moved on DMSO-treated cells at an average speed of 1.02 µm/min, with individual pedestal
speeds ranging from 0.40 - 2.09 µm/min (Fig 3-2 A, left). Treatment with CK666+CK869 reduced the average speed by more than half, to 0.47 µm/min (range: 0.21 – 0.64 µm/min), and Wiskostatin resulted in a similar reduction in average speed to 0.53 µm/min (range: 0.24 – 1.10 µm/min). SMIFH2 treatment also significantly inhibited motility, although not to the same degree, as the average speed was reduced to 0.73 µm/min (range: 0.34 – 1.13 µm/min) (Fig 3-1A, left).

Similar to the results in Fig 3-1, KC12+EspF_U motility was only impacted by inhibition of Arp2/3 complex or N-WASP, and not by SMIFH2 treatment (Fig 3-1A, right). These results suggest that

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**Fig 3-2. Chemical inhibition of formins impairs EPEC motility and colonization.** (A) NIH3T3 cells stably expressing mCherry-actin were infected with EPEC (left) or KC12+EspF_U (right) for 3.5-4.0 h, treated with the indicated inhibitors, and imaged live for 20-30 min. Bacteria with pedestals were tracked over time (top panels, scale bar, 2 µm) to determine actin-based motility rates (lower panels). Each point represents a single bacterium associated with a pedestal, and lines show the mean speed +/- SEM (n = 25-40 pedestals, 3-4 cells per condition). *** p < 0.001 (ANOVA, Tukey post-hoc tests). (B) Polarized Caco-2 monolayers were pretreated with the indicated inhibitors for 15 min prior to and during infection with EPEC or KC12+EspF_U for 6 h. Monolayers were then fixed and treated with antibodies to detect LPS (red), phalloidin to visualize F-actin (green), and DAPI to label DNA (blue). Scale bar, 25 µm. (C-D) Experiments shown in (B) were quantified. Each bar represents the mean macrocolony area +/- SEM (n = 70-124 EPEC colonies, 750-1177 KC12+EspF_U colonies). Only macrocolonies larger than 100 µm² were included in analysis. * p < 0.05, ** p < 0.01, *** p<0.001 (ANOVA, Dunnett’s multiple comparison test).
Formin-mediated actin polymerization contributes to actin-based motility driven by the pedestals of EPEC, but not EHEC.

3.4.3 **EPEC colonization is significantly impaired only when both the Arp2/3 complex and formin nucleators are inhibited**

Because motility was positively correlated with macrocolony size and epithelial colonization (Velle and Campellone, 2017), we next sought to determine if macrocolony biogenesis was impacted by treatment with the inhibitors of Arp2/3 complex, N-WASP, or formins. Polarized Caco2 cell monolayers were pretreated with DMSO or inhibitors and infected for 6 h, with hourly washes and media changes to promote colonization only from adherent bacteria. Monolayers were then fixed, stained, and imaged at a low magnification to visualize macrocolonies. In line with previous results (Velle and Campellone, 2017), EPEC consistently formed smaller macrocolonies than KC12+EspF_U (Fig 3-2 B). Although treatment with CK666+CK869, Wiskostatin, or SMIFH2 individually reduced EPEC macrocolony size to some extent, only pairwise combinations of CK666+CK869 and SMIFH2 or Wiskostatin and SMIFH2 resulted in statistically significantly smaller macrocolonies (Fig 3-2 C). In contrast, KC12+EspF_U colonies were unaffected by SMIFH2 treatment and combining SMIFH2 with either CK666+CK869 or Wiskostatin did not further the deficiencies in colony size beyond what was observed with Arp2/3 or N-WASP inhibition alone (Fig 3-2 D). These data suggest that KC12+EspF_U macrocolony size is largely dictated by the N-WASP-Arp2/3 complex pathway of actin assembly, whereas the cooperation between Arp2/3 complex and formins promote colonization by EPEC.

3.4.4 **Knockdown of mDia1 results in a pedestal defect unique to EPEC**

SMIFH2 is a broad inhibitor of actin nucleation by formin FH2 domains (Rizvi et al., 2009), so to determine which specific formins could be functioning in EPEC pedestals, we performed a small
scale screen using HeLa cells treated with pairs of siRNAs to DAAM1, FHOD1, INF2, FMNL2, mDia1, mDia2, and mDia3. In addition to targeting these formins, we also used siRNAs to the tandem-WH2 domain-containing nucleators cordon-bleu (Cobl) and Spire 1 and 2 (Spire1, Spire2). Lastly, we examined additional Arp2/3 complex interacting proteins in our screen, including Cortactin (CTTN), which was previously reported to contribute to EPEC and EHEC pedestal formation (Cantarelli et al., 2002; Cantarelli et al., 2006), WISH/SPIN90/DIP1, which activates Arp2/3 complex to promote the nucleation of unbranched filaments (Wagner, 2013), and JMY, a WASP family nucleation promoting factor that can also nucleate as a tandem-WH2 domain-containing protein (Zuchero et al., 2009).

On control-siRNA-treated HeLa cells, 90% of EPEC and 86% of KC12+EspF_U generated pedestals, and, as expected, siRNAs targeting the Arp2/3 complex or N-WASP significantly diminished pedestal formation by both strains by 32-48% (Fig 3-3 A-C). In agreement with previous studies, siRNAs to Cortactin negatively impacted the EspF_U-dependent pathway of actin polymerization (Cantarelli et al., 2006), however EPEC pedestals were unaffected. Targeting of JMY, WISH, Cobl, Spire1 or Spire2 did not cause any significant defects in pedestal biogenesis by either strain (Fig 3-3 B and C). Among the formins, DAAM1 targeting resulted in a modest (10%) reduction in EPEC pedestal formation, while knocking down mDia1 (also called DIAPH1 or hDia1) caused a more obvious inhibition of pedestal assembly, reflected in an approximately 25% reduction in pedestal formation efficiency (Fig 3-3 B). Because this latter defect was more significant and specific to EPEC pedestals, we investigated the role of this formin further.

Independent siRNAs targeting mDia1 were each efficient at depleting cellular mDia1 levels when assessed by immunofluorescence microscopy (Fig 3-4 A-B) or western blotting (Fig 3-4 C). Each mDia1 siRNA reduced pedestal formation by EPEC by over 30%, but neither one affected pedestal assembly by KC12+EspF_U (Fig 3-4A, D). To more clearly relate cellular mDia1 levels to pedestal formation efficiency, the percentage of EPEC or KC12+EspF_U that had
successfully formed pedestals on control or mDia1-depleted cells was plotted against the mDia1 staining intensity on those cells (Fig 3-4E-F). For EPEC, the amount of mDia1 present in the cell positively correlated with the percentage of bacteria forming pedestals, but KC12+EspF_U formed pedestals more than 60% of the time regardless of mDia1 levels. Finally, to more closely examine pedestals that were formed on mDia1-depleted cells, the F-actin pixel intensity was

**Fig 3-3.** siRNAs targeting Arp2/3 complex, N-WASP, or mDia1 inhibit actin pedestal formation by EPEC. (A) HeLa cells were treated with siRNA pairs to the indicated targets, infected with EPEC for 4 h, fixed, and stained with antibodies to detect HA-Tir (magenta) and phalloidin to visualize F-actin (green). Scale bar, 10 µm. (B-C) The % of adherent, Tir-positive EPEC (B) or KC12+EspF_U (C) that were associated with pedestals was quantified from experiments performed in (A). Each bar shows the mean % (+/- SEM) of pedestal-forming bacteria, while black data points represent mean %s from each infected cell that harbored 10-50 bacteria (n = 16-46 cells). * p < 0.05, *** p<0.001 (ANOVA, Dunnett’s multiple comparison test).
Fig 3-4. mDia1 depletion inhibits actin pedestal assembly by EPEC but not by KC12+EspF\textsubscript{U}. (A) HeLa cells were treated with control siRNAs or independent siRNAs targeting mDia1 and infected with EPEC or KC12+EspF\textsubscript{U} for 4 h. Cells were fixed and treated with antibodies to detect HA-Tir (red) and mDia1 (green), and with phalloidin to visualize F-actin (magenta). Scale bar, 10 µm. (B) The whole cell fluorescence intensity for mDia1 from experiments shown in (A) was measured. Each point represents the average pixel intensity of a single cell, and black lines show the mean intensity (+/- SD) for 28-32 cells. *** p<0.001, ns = not significant (ANOVA, Tukey post-hoc tests). (C) Lysates from cells treated...
in parallel to those in (A) were analyzed by immunoblotting with antibodies to detect mDia1 and GAPDH. Densitometry was calculated from 3 experiments and was normalized to GAPDH. mDia1 levels in the control cells were set to 100. (D) The % of adherent bacteria (determined by HA-Tir staining) associated with pedestals was quantified from experiments performed in (A). Each point represents a single infected cell (n = 15-20 cells) harboring 10-50 bacteria, and lines display the mean (+/- SD). *** p<0.001, ns = not significant (ANOVA, Tukey post-hoc tests). (E-F) The whole-cell intensity of mDia1 staining (for control and mDia1 depleted cells) was plotted against the % of EPEC (E) or KC12+EspF_U (F) forming pedestals on that cell. Each point represents a single cell (n = 45-46 cells). Data were analyzed by linear regression analysis, and linear trend lines are displayed on the plot with p values describing if the slopes are significantly non-zero. (G) Cells were treated with control siRNAs or siRNAs targeting mDia1 or ArpC4. After fixation and staining as in (A), lines were drawn through the pedestal forming region (indicated by Tir staining) and F-actin intensity along the 3 µm line was plotted. All lines were normalized so that a distance of 0 represents the brightest fluorescence of HA-Tir, with the bacteria positioned to the left of 0. Points represent the mean fluorescence of F-actin (+/- 95% CI) (n=15 pedestals per condition from 3-4 cells).

plotted along the length of the pedestal (Fig 3-4G). In control siRNA treated cells, actin pedestals were strong and peaked immediately adjacent to HA-Tir. However, targeting the Arp2/3 complex or mDia1 diminished this peak in actin intensity, with intensity values less than half that of control cells. These data indicate that EPEC can only efficiently assemble pedestals when mDia1 is present in the host cell.

3.4.5 Arp2/3 complex is essential for EPEC and KC12+EspF_U pedestal assembly.

We next sought to determine if the contribution of mDia1 to pedestals is dependent or independent of the Arp2/3 complex. Because cells treated with chemical inhibitors of Arp2/3 complex or siRNAs to target Arp2/3 complex were still capable of forming pedestals beneath about 50% of bacteria, it was unclear if mDia1 was responsible for this degree of pedestal formation, or if the ability to make pedestals under these conditions is due to residual Arp2/3 complex activity. To distinguish between these possibilities, we infected cells completely lacking the Arp2/3 complex. Tamoxifen-inducible ArpC2 knockout (KO) mouse fibroblasts (Rotty, 2015) were treated with 4-hydroxy-tamoxifen (4OHT) for 6 days to fully deplete Arp2/3 complex prior to comparison with DMSO-treated Arp2/3-proficient control cells (Fig 3-5). EPEC and
KC12+EspF\textsubscript{U} readily formed pedestals on the DMSO-treated cells but did not form any pedestals on the ArpC2 KO cells. Interestingly, adherent EPEC was sometimes associated with intense F-Actin staining in ring-like structures around clusters of Tir translocation (Fig 3-5 B, inset). Nevertheless, these results imply that the pedestals which formed during CK666/CK869 or siRNA treatment relied on residual Arp2/3 complex activity, and therefore any effects of mDia1 on EPEC pedestals still absolutely requires N-WASP (Lommel et al., 2001; Vingadassalom et al., 2010) and Arp2/3 complex.

**Fig 3-5. The Arp2/3 complex is essential for actin pedestal assembly.** (A) Tamoxifen-inducible ArpC2 Floxed mouse fibroblasts were treated with DMSO (-) or 4OHT (+) for the indicated number of days. >6 indicates that tamoxifen or DMSO was removed after 6 days, and cells were cultured for an additional 6 days before collection. Cell lysates were analyzed by immunoblotting with antibodies to detect ArpC2, Arp3, and GAPDH. (B) DMSO-treated (ArpC2 Flox) and 4OHT-treated (ArpC2 KO) cells from the >6 d condition were infected with EPEC or KC12+EspF\textsubscript{U} for 4 h, fixed, and stained with antibodies to detect HA-Tir (magenta), phalloidin to visualize F-actin (green), and DAPI to detect DNA (blue). Scale bar, 25 µm; inset, 2.5 µm.

**3.4.6 mDia1 localizes to EPEC pedestals**

Because mDia1 has an apparent positive role in EPEC pedestals, we next sought to determine its localization. In the experiments performed in Figure 3-4A, mDia1 localized to a subset of EPEC pedestals, although not every pedestal showed mDia1 staining. To more closely assess mDia1 localization, infected HeLa cells were again treated with antibodies to detect mDia1 and
Fig 3-6. mDia1 localizes to EPEC pedestals.
(A) HeLa cells were infected for 4 h, fixed, and stained with antibodies to detect mDia1 and HA-Tir, and with phalloidin to visualize F-actin. Scale bar, 10 µm. (B) HeLa cells transiently expressing GFP-mDia1 were infected with EPEC for 4 h, fixed, and stained with antibodies to detect HA-Tir and phalloidin to visualize F-actin. Scale bar, 10 µm. (C) The % of Tir-positive bacteria associated with an enrichment in mDia1 staining was calculated from control siRNA experiments in Fig 4A. Each point represents a single infected cell, and lines show the mean +/- SD (n = 16-20 cells). * p<0.05 (unpaired t test). (D) HeLa cells were infected, fixed, and stained as in (A). Lines were drawn through pedestals to measure pixel intensity profiles for HA-Tir (not shown), F-actin, and mDia1. The brightest pixel from Tir staining was set to a distance of 0 for each pedestal (n=15 pedestals, 4 cells). Each point represents the mean pixel intensity (+/- 95% CI).

HA-Tir, as well as phalloidin to visualize F-actin, and examined by confocal microscopy. In parallel, HeLa cells transiently expressing GFP-mDia1 were infected and stained for HA-Tir and
F-actin. Similar recruitment to pedestals was observed with both antibody staining and with the GFP-tagged protein (Fig 3-6 A-B).

Because mDia1 was not enriched at all EPEC pedestals, we quantified the fraction of EPEC pedestals that showed distinguishable mDia1 antibody staining. Additionally, we compared this value to KC12+EspF₀ pedestals, which do not appear to rely on mDia1 for actin pedestal polymerization. Using control siRNA treated cells that were infected with 10-50 bacteria, pedestals were scored as mDia1 positive or negative, and the average percentage of bacteria in each category was calculated on a per-cell basis. mDia1 was enriched in a subset of pedestals generated by both EPEC (43.2%) and KC12+EspF₀ (30.9%), and there was a slight yet statistically significant preference for EPEC pedestals (Fig 3-6 C).

Next, by plotting the pixel intensity profiles of HA-Tir, F-actin, and mDia1 staining along the length of the pedestal, we examined the position of mDia1 within the pedestal. The brightest pixel in the HA-Tir channel was set to a distance of 0 to compare the intensities across several EPEC pedestals. On average, F-actin intensity peaked 0.13 µm after Tir, while mDia1 staining peaked 0.19 µm later than F-actin (Fig 3-6 D). This could indicate that the actin polymerized by mDia1 in pedestals is further from the bacterium than the actin nucleated by Arp2/3 complex, which typically localizes throughout the pedestal, including the tip (Vingadassalom et al., 2010). Collectively, these data show that mDia1 has a slight preference for actin pedestals generated by EPEC over EHEC, and that its localization is strongest at the base of pedestals.
3.5 Discussion
Pathogens such as *Listeria* and *Shigella* are often employed as tools to better understand actin dynamics and uncover new pathways and regulators of actin assembly, yet their utility for modeling actin polymerization at the plasma membrane is limited by the fact that they are cytosolic. By remaining extracellular throughout infection, EPEC and EHEC represent ideal models to study actin rearrangements triggered by transmembrane signaling cascades (Hayward et al., 2006). While the pathways of EHEC and EPEC pedestal assembly have been thoroughly characterized (see (Campellone, 2010; Lai et al., 2013) for reviews), the potential contributions of actin nucleation factors outside of the Arp2/3 complex and WASP family have never been directly assessed. Furthermore, the coordination of multiple nucleators has been observed to orchestrate a variety of cellular functions, forming specifically timed and placed actin networks such as those found in lamellipodia (Isogai, 2015). In light of this, and findings of cooperation between Arp2/3 complex and formins in *Shigella*, *Listeria*, and vaccinia protrusions (Heindl, 2010; Fattouh, 2014; Alvarez, 2013), we examined whether some level of cooperation would exist in EPEC and EHEC pedestals. Our results indicate that the formin mDia1 contributes to Arp2/3 complex-mediated actin assembly in the pedestals of EPEC but not EHEC.

Inhibiting formin activity with SMIFH2 resulted in EPEC-specific pedestal defects. Fewer bacteria formed pedestals, the pedestals that did form contained less F-actin, and actin-based motility was significantly slower. Moreover, formins also had an apparent role in colonization, as inhibitors of the Arp2/3 complex, N-WASP, or formins did not impact EPEC colony size by themselves, but simultaneously inhibiting both the N-WASP-Arp2/3 and formin pathways reduced macrocolony size. Taken together, these findings suggest that the cooperation of Arp2/3 complex with formins is important for EPEC cell-to-cell spreading.

Using a small siRNA screen, we identified mDia1 as the formin mostly responsible for the EPEC pedestal defects that were observed with SMIFH2. Although targeting DAAM1 also
resulted in a significant decrease in pedestal formation, this phenotype was not as strong or as significant as the one caused by depletion of mDia1. Further, by plotting the fraction of pedestals formed per cell against the whole cell mDia1 staining intensity for knockdown and control cells, we were able to show a positive correlation between the cellular level of mDia1 and the percent of bacteria generating pedestals. These results parallel the findings that *Listeria monocytogenes* and *Shigella flexneri* rely on formins from the diaphanous subfamily (which includes mDia1, mDia2, mDia3) in addition to Arp2/3 complex for protrusion formation and cell-to-cell transmission (Fattouh et al., 2015; Heindl et al., 2010).

Somewhat surprisingly, EPEC did not show any phenotype when FHOD1 was targeted in the siRNA screen. This was unexpected because Vaccinia virus, which triggers a similar Nck-dependent signaling cascade to EPEC, was found to manipulate Rac1 and FHOD1 for actin tail assembly, motility, and cell-to-cell spreading (Alvarez and Agaisse, 2013). Canonical Vaccinia actin tail assembly relies on tyrosine phosphorylation of the viral membrane protein A36 by host cell Src and Abl family kinases (Frischknecht et al., 1999). Phosphorylated Y112 binds the adaptor proteins Nck1 and Nck2, which recruit N-WASP and WIP as a complex (Donnelly et al., 2013; Scaplehorn et al., 2002). Phosphorylation of a second residue, Y132, promotes the recruitment of another adaptor, Grb2, which may contribute to N-WASP activation or work to stabilize these complexes (Scaplehorn et al., 2002; Weisswange et al., 2009). These mechanisms of actin assembly are strikingly similar to the pathway to actin polymerization promoted by EPEC Tir, which is phosphorylated on two similarly spaced residues, Y454 and Y474. Although EPEC pedestals do not recruit Grb2 (Campellone and Leong, 2005), phosphorylated Y474 recruits Nck1 and Nck2, which bind and activate N-WASP with or without WIP (Campellone et al., 2002; Garber et al., 2012; Gruenheid et al., 2001). It is possible that Grb2 somehow promotes FHOD1 recruitment in the case of Vaccinia virus, potentially explaining why EPEC does not employ this nucleator. It is also possible that other Vaccinia proteins or EPEC effectors influence FHOD1 localization and function.
Formin-related changes in pedestal assembly were exclusive to EPEC, as neither SMIFH2 treatment nor siRNA targeting of formins decreased pedestal formation by KC12+EspF\textsubscript{U}. Although mDia1 has no obvious role in the EHEC pathway of pedestal assembly, it does localize to roughly 30% of pedestals, which is only marginally lower than the 43% of EPEC pedestals with mDia1 enrichment. Therefore, it is possible that mDia1 is recruited to both types of pedestals but is only activated by signaling mechanisms stemming from EPEC Tir. Alternatively, it is possible that mDia1 contributes to EHEC pedestal assembly, but that its effects are dwarfed by the activity of EspF\textsubscript{U}, a multivalent effector protein capable of activating multiple N-WASP molecules to achieve extraordinarily high levels of Arp2/3 complex activation (Campellone et al., 2008a; Ritchie et al., 2008; Sallee et al., 2008).

In the case of EPEC Tir signaling, a few potential candidates could mediate recruitment of mDia1. For example, WISH is capable of interacting with Nck1 and Nck2, as well as mDia1 and the Arp2/3 complex (Lim et al., 2001; Satoh and Tominaga, 2001; Wagner et al., 2013), which made it an attractive possible intermediate. However, inconsistent with this hypothesis, targeting WISH in our siRNA screen did not cause any pedestal phenotypes. Another possibility is that small G-proteins recruit and activate mDia1 in pedestals, which is also how they are suggested to function in \textit{Listeria} protrusions (Fattouh et al., 2015). Yet another interesting candidate is IQGAP, a large scaffolding protein that can bind actin, activate N-WASP, and interact with Cdc42 and Rac1 (Le Clainche et al., 2007). IQGAP can stimulate filopodial protrusions (Swart-Mataraza et al., 2002), and is involved in \textit{Salmonella} invasion (Brown et al., 2007). Furthermore, IQGAP is recruited to nephrin in kidney podocyte foot processes (Rigothier et al., 2012). Given the similarities between phosphorylated Tir and nephrin in recruiting Nck1 and Nck2 to activate N-WASP (Blasutig et al., 2008; Jones et al., 2006), this could draw another parallel between pathogen-induced pedestal protrusions and the actin dynamics in healthy cells. IQGAP1 localizes to EPEC pedestals, and actin pedestal assembly in IQGAP-deficient MEFs is reduced by about 40% (Brown et al., 2008). Finally, \textit{in vitro} experiments revealed that the N-
terminus of IQGAP is capable of binding EPEC Tir directly (Brown et al., 2008), while the C terminus of IQGAP1 has been shown to bind the DID domain of mDia1 to drive cell migration and phagocytosis (Brandt et al., 2007). Whether IQGAP1 actually links EPEC Tir to mDia1 in pedestals awaits further investigation.

Although the mechanism of mDia1 recruitment remains to be determined, our data support a model in which mDia1 contributes to Arp2/3 complex-mediated actin assembly in EPEC pedestals. Based on its localization within the pedestal at the distal portion, mDia1 may nucleate mother filaments that the Arp2/3 complex can use as seeds for branching. Alternatively, but less likely, is the possibility that mDia1 serves to elongate Arp2/3-nucleated filaments. In either case, these results join EPEC with a growing number of pathogens that have the capacity to manipulate multiple host cell actin nucleation pathways. In the future, EPEC is likely to shed further light on how cells control actin dynamics at membrane protrusions.
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Chapter 4. Discussion

4.1 Overview

Many pathogens hijack the host-cell actin nucleation machinery for their own benefit. This is often observed in the form of “comet tails,” which propel intracellular bacteria and viruses through the cytosol (See Table 4-1) and allow for direct cell-to-cell spread in which the pathogen is protected from the host humoral immune system by remaining intracellular. To achieve this, actin-based motility drives the pathogen to the plasma membrane, where it can protrude into a healthy, neighboring cell, resolve into a vacuole, and then escape to begin a new infectious cycle. This basic series of events was first characterized for *Listeria monocytogenes* nearly 30 years ago in a landmark publication that was among the first to describe bacterial-driven actin manipulation (Tilney and Portnoy, 1989). Since then, numerous bacteria and viruses have been found to use similar strategies, and a wide variety of molecular mechanisms have been defined for manipulation of host actin nucleators (Fig 4-1) (Lamason and Welch, 2017; Truong et al., 2014; Welch and Way, 2013).

Notably, the study of actin hijacking by pathogens has advanced the field of actin dynamics, as the actin polymerization activities N-WASP and Arp2/3 complex were both initially described using *Shigella* (Suzuki et al., 1998) and *Listeria* (Welch et al., 1997), respectively. Therefore, the study of actin manipulation by pathogens not only has the inherent benefits of discovering new targets for treatments, but these pathogens are also useful tools for understanding actin dynamics in healthy cells. EPEC and EHEC fit into this framework as they represent important human pathogens, as well as ideal models for the study of actin protrusions at membranes (Hayward, 2006). My work has contributed to the field by defining the functions of actin pedestals, detailing a pathway of cell-to-cell transmission, and characterizing the cooperation between Arp2/3 complex and the formin mDia1 in EPEC pedestals.

This section will review the actin assembly and cell-to-cell spreading mechanisms employed by *Listeria, Shigella, Vaccinia virus, Rickettsia, and Burkholderia*, and will conclude
with a more thorough discussion of the EPEC and EHEC polymerization and dissemination pathways.

<table>
<thead>
<tr>
<th>System</th>
<th>Effectors</th>
<th>Average Speed</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>ActA</td>
<td>~ 20 µm/min</td>
<td>Reed, 2014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.2-12 µm/min</td>
<td>Theriot, 1992</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>IcsA</td>
<td>~7.1 µm/min</td>
<td>Heindl, 2010</td>
</tr>
<tr>
<td><em>Vaccinia virus</em></td>
<td>A36</td>
<td>~ 60 µm/min</td>
<td>Alvarez, 2013</td>
</tr>
<tr>
<td><em>Rickettsia parkeri</em></td>
<td>RickA</td>
<td>~15 µm/min</td>
<td>Reed, 2014</td>
</tr>
<tr>
<td></td>
<td>Sca2</td>
<td>~ 23 µm/min</td>
<td></td>
</tr>
<tr>
<td><em>Burkholderia mallei</em></td>
<td>BmBimA</td>
<td>~ 30 µm/min</td>
<td>Benanti, 2015</td>
</tr>
<tr>
<td><em>B. pseudomallei</em></td>
<td>BpBimA</td>
<td>~ 60 µm/min</td>
<td></td>
</tr>
<tr>
<td><em>B. thailandensis</em></td>
<td>BtBimA</td>
<td>~30 µm/min</td>
<td></td>
</tr>
<tr>
<td>EPEC</td>
<td>Tir</td>
<td>~1 µm/min</td>
<td>Velle, 2017</td>
</tr>
<tr>
<td>EHEC</td>
<td>Tir+ EspF$_U$</td>
<td>~1 µm/min</td>
<td></td>
</tr>
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Table 4.1. AMB rates and effectors for select pathogens.

4.2. *Listeria:*

*Listeria monocytogenes* is a gram positive facultative intracellular pathogen. Although infection is typically not severe and results in food poisoning symptoms, Listeriosis can be life-threatening for immunocompromised individuals and can cause serious complications for pregnant women (Hernandez-Milian and Payeras-Cifre, 2014). Therefore, *Listeria* research is not only valuable to the study of actin dynamics, but it is also a medically relevant pathogen.

*Listeria* is one of the best characterized pathogens for the study of actin-based motility. Transmission electron microscopy first revealed that *Listeria* induces actin polymerization in the form of a comet tail, and the physiological importance of this was demonstrated by observing a cell-to-cell spreading defect in Cytochalasin-D treated cells (Tilney and Portnoy, 1989). ActA was then identified as the bacterial surface protein responsible for actin polymerization (Domann et al., 1992; Kocks et al., 1992) and was later found to induce nucleation via activation of the host Arp2/3 complex (Welch, 1998; Welch et al., 1997) by mimicking a WASP-family protein using N-Terminal A, W, and C domains (Fig 4-1) (Skoble et al., 2000). The continued actin polymerization at one pole of the bacterium generates the force required to push it through the cytosol, leaving the actin tail behind to disassemble (Sanger et al., 1992; Theriot et al.,
To successfully infect a healthy, neighboring cell, *Listeria* first protrudes into an adjacent cell using actin to elongate the protrusion before resolving into a double membrane vacuole in the next cell. Once in the neighboring cell, *Listeria* escapes the vacuole by degrading the membrane with listeriolysin O (Gaillard et al., 1987) to begin a new infectious cycle (Robbins et al., 1999; Tilney and Portnoy, 1989).

The minimum requirements for reconstituting *Listeria* actin-based motility *in vitro* have been defined as actin, Arp2/3 complex, ADF/cofilin, and capping protein, but movement is enhanced by including profilin, α-actinin, and VASP (Loisel et al., 1999). To assemble an actin network with these basic components, ActA activates Arp2/3 complex using the Acidic and Connector (also called cofilin homology) regions and binds an actin monomer with a WH2 domain, similar to a WASP-family protein (Lauer et al., 2001; Skoble et al., 2000; Welch, 1998). ActA also has a central proline rich region that can bind VASP (Chakraborty et al., 1995; Smith et al., 1996), which contributes to polymerization by interacting with barbed ends, where it recruits and delivers profilin-actin (Pasic et al., 2008). While capping protein is important to prevent elongation at barbed ends no longer associated with *Listeria*, VASP prevents capping protein from blocking elongation within the comet tail (Pasic et al., 2008; Samarin et al., 2003). ADF/cofilin is responsible for filament disassembly to retain a pool of G-actin monomers, which can then bind profilin (Loisel et al., 1999). Finally, α-actinin can contribute to filament bundling to stabilize F-actin (Dold et al., 1994). While these requirements have been well-defined *in vitro*, cell-to-cell transmission of *Listeria* relies on additional host factors not required in these reconstitution assays, or even comet tail formation in the cytosol. For instance, the serine/threonine kinase CSNK1A1 and the actin disassembly promoting protein AIP1 are each dispensable for comet tails, but both are important for cell-to-cell spreading (Chong et al., 2011; Talman et al., 2014).

Although the ActA-Arp2/3 pathway is the most well-characterized for actin comet tail assembly, a role for formins in *Listeria* protrusion formation was recently uncovered (Fattouh et
al., 2015). This is not entirely surprising, as many nucleators have been shown to cooperate to assemble actin networks in cells (Dominguez, 2016). RNAi-mediated knockdown of mDia1-3, or the Rho-GTPases that activate these formins results in a cell-to-cell spreading defect, although there is no impact on comet tail formation (Fattouh et al., 2015). This finding is consistent with electron microscopy studies, which have shown branched actin proximal to the bacteria, but parallel actin bundles in the distal portion of the protrusion (Sechi et al., 1997). Taken with the studies mentioned above, it is clear that the cytoskeletal requirements for *Listeria monocytogenes* motility in the cytosol and the requirements for cell-to-cell spreading are quite different, although Arp2/3 complex is critical for both activities. The precise molecular function formins perform in protrusions and cell-to-cell spreading will require more work.

### 4.3. Shigella:

*Shigella flexneri* is responsible for shigellosis in developing countries. Like *L. monocytogenes*, *S. flexneri* forms actin comet tails that allow for rapid motility in the cytosol, which was first observed in 1968, although the role of actin was not appreciated until decades later (Ogawa et al., 1968). To form these comet tails, *S. flexneri* relies on the bacterial membrane protein IcsA (also called VirG), which was first defined as a protein required for cell-to-cell spreading, and was later shown to promote actin assembly (Bernardini et al., 1989; Makino et al., 1986). IcsA activates host N-WASP to promote Arp2/3 complex-mediated actin polymerization, and was initially thought to mimic Cdc42 binding to activate N-WASP (Egile et al., 1999; Suzuki et al., 1998). However, recent work demonstrated that IcsA binds the AI region of N-WASP and not the CRIB domain, mimicking and outcompeting the CA domains for binding in a similar manner to EspFu in EHEC (Fig 4-1) (Mauricio et al., 2017).

The same study that defined the minimal requirements to reconstitute *Listeria* motility in vitro also examined *Shigella*, using an *E. coli* strain expressing IcsA. Similar to ActA-mediated actin assembly, IcsA-driven polymerization requires actin, Arp2/3 complex, ADF/cofilin, and
capping protein, in addition to N-WASP (Loisel et al., 1999). Comet tail formation in cells, however, involves the host F-BAR protein TOCA-1 to efficiently activate N-WASP (Leung et al., 2008), as well as tyrosine kinases Abl and Btk that phosphorylate N-WASP (Burton et al., 2005; Dragoi et al., 2013). Furthermore, Myosin X is important for protrusion formation and cell-to-cell spread (Bishai et al., 2013), and the mechanism of engulfment, which normally occurs near tricellular junctions, involves clathrin, epsin-1, and dynamin (Fukumatsu et al., 2012). These additional requirements highlight the distinctions between the mechanisms of actin-based motility used by _Listeria_ and _Shigella_.

**Fig 4-1.** There are multiple pathways to activate N-WASP and/or Arp2/3 complex. N-WASP autoinhibition can be relieved by the binding of host proteins (blue) or pathogen derived proteins (purple) to basic, CRIB, AI, and/or PRD regions (Green arrows). Vaccinia virus A36 and EPEC Tir recruit adaptor proteins to activate N-WASP. _Listeria_ ActA and _Rickettsia_ RickA mimic WASP-family proteins to activate Arp2/3 complex. Protein domains are not to scale. WH1, WASP homology 1 domain; B, basic; CRIB, Cdc42-Rac interactive binding; AI, autoinhibitory; PRD, proline rich domain; W, WASP homology 2 domain; C, connector; A, acidic; WIP, WASP interacting protein

While there are stark differences in requirements beyond the core set of proteins for Arp2/3 activation, _Listeria_ and _Shigella_ share another commonality— the requirement of formins
for protrusion and dissemination. While formins have no apparent role in Shigella comet tails, depletion of diaphanous formins (specifically, mDia1 and mDia2) or the expression of the DID domain of mDia1, which has a dominant negative effect, restricts protrusion formation and cell-to-cell spreading (Heindl et al., 2010). The authors speculated that the force generated by linear filament assembly perpendicular to the plasma membrane, which can reach >1.3 pN per filament, could generate the >10 pN of force required to induce a membrane protrusion (Heindl et al., 2010; Kovar and Pollard, 2004), however further work is required to determine the location and orientation of filaments nucleated by mDia1 and mDia2. Another question that remains is how Shigella transitions from Arp2/3 complex-mediated actin tail assembly to mDia-dependent protrusion formation, and if these two pathways directly cooperate. A clearer understanding of these mechanisms will contribute to the growing network of interactions observed between nucleators.

4.4. Vaccinia virus

Vaccinia virus is an Orthopoxvirus closely related to Variola virus, the causative agent of smallpox. Vaccinia virus was first observed to associate with microvilli-like cellular projections in 1976 (Stokes, 1976), but the importance of actin was not uncovered until decades later (Cudmore et al., 1995). As an obligate intracellular pathogen, the life cycle of Vaccinia virus differs from the facultative intracellular pathogens discussed above. The infectious form of Vaccinia virus is extracellular enveloped virus (EEV), which bind host cells and enter via membrane fusion (Law et al., 2006) or macropinocytosis followed by fusion (Mercer and Helenius, 2008; Mercer et al., 2010). Once in the cytoplasm, virions uncoat, and DNA is replicated and packaged into new virions, or intracellular mature viruses (IMVs). IMVs can become wrapped in membrane derived from the Golgi (Sivan et al., 2016), generating intracellular enveloped viruses (IEVs), which travel on microtubules to reach the cell periphery. IEVs then fuse with the plasma membrane, where they either dissociate from the cell as EEVs,
or fuse but remain attached to the cell as cell-associated enveloped virus (CEVs). This section will focus on CEVs, which are the form that hijack actin nucleation machinery for motility and cell-to-cell spreading.

A36 is a viral protein expressed on the outer IEV membrane, and after fusion to become a CEV, is positioned in the host plasma membrane beneath the virion. A36 is essential for actin-based motility, and the signaling mechanism is strikingly similar to that of EPEC Tir (Fig 4-2). A36 is tyrosine phosphorylated at residues 112 and 132 by Src and Abl family kinases. Phosphorylated Y112 recruits the adaptor proteins Nck1 and Nck2 (Frischknecht et al., 1999; Scaplehorn et al., 2002), while phosphorylated Y132, unlike EPEC, recruits the adaptor Grb2 (Scaplehorn et al., 2002). Phosphorylated Y112 and Nck1/2 represent the primary signaling pathway, as they are sufficient for actin comet tail formation, although tail formation is not completely abolished unless both Y112 and Y132 are mutated (Frischknecht et al., 1999; Weisswange et al., 2009). Nck1 and Nck2 recruit N-WASP via its constitutive binding partner WIP, which ultimately results in Arp2/3 complex activation (Donnelly et al., 2013). While phosphorylation of Y132 is not required for actin tail assembly, it can enhance polymerization by recruiting Grb2. Interestingly, Grb2 recruitment also relies on the PRD of N-WASP, and the primary function of Grb2 is thought to be the stabilization of the Nck-WIP-N-WASP complex (Weisswange et al., 2009), although in the absence of Y112, Y132 is responsible for a low level of actin tail assembly (Frischknecht et al., 1999). Collectively, these findings demonstrate the A36 signaling is not a straightforward and stepwise process, but rather a signaling platform able to interact simultaneously with different N-WASP activators.

Recently, a role for the formin FHOD1 was uncovered in Vaccinia virus actin tails (Alvarez and Agaisse, 2013). In line with the findings of formin-mediated nucleation in Listeria and Shigella, formin activity is also important for Vaccinia cell-to-cell spread. However, unlike these intracellular bacteria, FHOD1 is additionally important for comet tail formation and motility. RNAi-mediated depletion of FHOD1 resulted in approximately 50% fewer CEVs with actin tails,
and a 30% decrease in speed. Furthermore, knockdown of FHOD1, its activator Rac1, or profilin all resulted in fewer actin tails, slower motility, and decreased cell-to-cell spreading.

**Fig 4-2. Vaccinia virus and EPEC employ similar signaling cascades for actin polymerization.** Vaccinia virus A36 (top panel) is phosphorylated on Y112 and Y132, recruiting Nck1/2 and Grb2, respectively. The second SH3 domain of Nck (drawn C terminus to N terminus) recruits WIP-bound N-WASP, which is activated by SH3-PRD interactions. Grb2 binds phosphorylated Y132 and stabilizes the Nck-WIP-N-WASP complex. In addition to Arp2/3 activation, Vaccinia also recruits the formin FHOD1, which could either nucleate seed filaments for Arp2/3 complex to branch from (shown above), or elongate Arp2/3 nucleated filaments (not shown). EPEC Tir (bottom panel) is phosphorylated on Y454 and Y474. Y474 mediates Nck1/2 recruitment, which can activate N-WASP via its PRD. Similar to Vaccinia and FHOD1, mDia1 may seed filaments for, or elongate filaments from the Arp2/3 complex.
(Alvarez and Agaisse, 2013). This was the first evidence that in addition to importance for dissemination, a host formin-mediated pathway could contribute to Arp2/3 complex-mediated motility. While it is clear that FHOD1 is contributing to actin polymerization in Vaccinia tails, more research is required to determine if its main function is to nucleate filaments for Arp2/3 complex to branch from, or if it is elongating filaments nucleated by Arp2/3 complex.

4.5. Rickettsia:

Rickettsia are gram-negative obligate intracellular bacteria, and the genus contains approximately 30 species that are further classified based on disease. The spotted fever group (SFG) includes R. rickettsii, R. conorii, and R. parkeri, and are responsible for spotted fever and eschar-associated rickettsioses in mammals infected through arthropod vectors. SFG Rickettsia spp. are distinct from Shigella and Listeria in that they possess two effectors for actin manipulation. Temporal regulation of these effectors allows Rickettsia to undergo a switch in the mechanism of actin-based motility between early and late infection (Reed et al., 2014). Early motility, 15-60 minutes after cellular invasion, is mediated by the effector RickA, which acts as an NPF to activate the Arp2/3 complex (Gouin et al., 2004; Jeng et al., 2004) and produces short, curved tails. However, late motility at times greater than 24 hours post infection relies on Sca2, which acts as a formin to nucleate linear actin filaments (Haglund et al., 2010), generating long, relatively straight actin tails. Surprisingly, although RickA and Sca2 mutants each cause a spreading defect in plaque forming assays, neither of these actin-manipulating mechanisms is directly responsible for cell-to-cell spreading. The RickA/Sca2 spreading defects likely reflect the inability of the bacteria to reach the cell periphery, as this is a crucial step. However, once the bacteria reach the plasma membrane, they no longer associate with actin tails. Instead, Rickettsia employs the secreted effector Sca4, which can bind host vinculin to reduce intercellular tension and promote uptake (Lamason et al., 2016).
Although it is possible that different actin assembly pathways function simultaneously in *Listeria*, *Shigella*, and Vaccinia virus, it is extremely unlikely that there is direct cooperation between RickA- and Sca2-mediated actin assembly in *Rickettsia* actin tails, because the expression of these effectors is so temporally distinct. However, SFG *Rickettsia* spp. represent yet another example of pathogen reliance on multiple actin polymerization mechanisms.

4.6. *Burkholderia*:

The genus *Burkholderia* includes the opportunistic human pathogens *B. mallei* and *B. pseudomallei*, and the nonpathogenic species *B. thailandensis*. All three bacteria possess orthologs of the effector protein BimA (named BmBimA, BpBimA, and BtBimA, respectively), which are responsible for actin comet tail formation (Kespichayawattana et al., 2000; Sitthidet et al., 2010; Stevens et al., 2005a; Stevens et al., 2005b). However, the molecular mechanisms of BimA induced actin assembly differ between these species. BmBimA and BpBimA mimic Ena/VASP proteins to independently polymerize actin, while BtBimA acts as an NPF to activate Arp2/3 complex (Benanti et al., 2015; Sitthidet et al., 2010). These different pathways lead to distinct comet tail morphology, as BmBimA and BpBimA nucleate linear actin filaments, resulting in long tails due to continued elongation and bundling of filaments, while BtBimA results in shorter, curved tails from branched actin assembly (Benanti et al., 2015). This represents the first and only known instance of pathogens mimicking Ena/VASP proteins.

The cell-to-cell spreading mechanism of *Burkholderia* differs substantially from the pathogens previously described. Although actin-based membrane protrusions similar to *Listeria* have been observed (Kespichayawattana et al., 2000), and *Burkholderia* can escape phagocytic vacuoles like the other pathogens discussed (Harley et al., 1998a), cell-to-cell spread is not mediated by elongation and resolution of protrusions. Instead, *Burkholderia* uses a type 6 secretion system to promote membrane fusion (Schwarz et al., 2014; Toesca et al., 2014), resulting in the formation of “multinucleated giant cells” (Harley et al., 1998b;
Kespichayawattana et al., 2000). Although actin polymerization does not directly cause cell-to-cell transmission, actin-based motility is still critical for allowing the bacteria to reach cell junctions to promote fusion.

4.7. EPEC and EHEC
The work presented in my thesis provides insight into both the pathogenic function of EPEC and EHEC actin pedestal assembly, as well as the mechanisms that underlie actin polymerization. Prior to my work, it was unclear why EPEC and EHEC formed actin pedestals, and the activities of other nucleators in pedestals had never been investigated. Therefore, I sought to characterize the functions of actin pedestals on a variety of host cell types, and to determine if other host nucleators were also manipulated during infection using chemical inhibitors and siRNAs. Together, my findings have addressed the questions of why and how EHEC and EPEC induce actin rearrangements into pedestals.

Although actin pedestals were first described in 1989 (Knutton et al., 1989), and actin-based motility was observed in the mid 1990’s (Sanger et al., 1996; Shaner et al., 2005), the function of actin pedestals had remained elusive. It was known that actin assembly was important for colonization (Ritchie et al., 2008), maintaining mucosal attachment, and enhancing type 3 secretion (Battle et al., 2014; Mallick et al., 2014), but the potential for actin-based motility to contribute to cell-to-cell spreading was unexplored. By live imaging infections of polarized epithelial monolayers, I was able to directly visualize the transmission process (Fig 4-3). To promote colonization and spread, actin-based motility drives EHEC to a cell-cell junction, where it injects effectors into a neighboring, healthy cell. Then, a second pedestal is assembled, stalling motility until the bacteria divides, at which point one of the daughters dissociates from the junction, promoting infection of the adjacent cell. EspF_U drives faster actin-based motility on polarized cells than EPEC Tir-induced motility, leading to more efficient cell-to-cell spread and the formation of large two-dimensional bacterial aggregates called macrocolonies.
encompass multiple host cells. Since the bacteria never detach from the host plasma membrane, this mechanism could promote very efficient infection in vivo, as a single bound bacterium can form the seed of a macrocolony by transferring stepwise to adjacent cells. While this mechanism is distinct from the pathway of cell-to-cell spread for intracellular pathogens, the protrusions that occur at junctions and the pause in motility is similar to that observed for Listeria and Shigella.

One of the questions that remains about EHEC and EPEC colonization is why EHEC EspF_U-dependent motility is faster. The answer to this likely lies in the different pedestal assembly pathways, because although both rely on Tir to signal to the cytoskeleton, the mechanisms used by EPEC Tir and EHEC Tir are distinct. EPEC Tir is phosphorylated on residue Y474 by host cell kinases (Kenny, 1999; Phillips et al., 2004; Swimm et al., 2004), promoting the binding of Nck1 and Nck2 (collectively referred to as Nck) via Nck’s SH2 domain (Campellone et al., 2002). Nck also has three SH3 domains, which all have the capacity to bind the PRD of N-WASP, while the second SH3 domain has the added ability to recruit WIP, a constitutive N-WASP binding partner (Antón et al., 1998; Gruenheid et al., 2001; Rohatgi et al., 2001). Further, recent work has uncovered the existence of a short amphipathic helix in the linker between the first and second SH3 domains of Nck, which interacts with the autoinhibitory region in the GBD, outcompeting the interaction between N-WASP’s WCA domain and the AI (Okrut et al., 2015), however it is unknown if this occurs in the context of EPEC Tir-mediated polymerization.

Meanwhile, EHEC Tir lacks the equivalent Y474 residue, and instead of employing Nck, uses the additional effector EspF_U to directly activate N-WASP (Campellone et al., 2004; Garmendia et al., 2004). Importantly, EspF_U typically harbors six 47-residue repeats, each with a proline rich region that allows recruitment to Tir via host BAR proteins, as well as a short, amphipathic helix that mimics and outcompetes N-WASP’s CA domains for binding to the AI region, allowing for extraordinary levels of N-WASP activation (Campellone et al., 2008; Cheng
et al., 2008; Sallee et al., 2008). EspFu’s added level of multivalency over Nck could therefore be responsible for the differences in motility and colonization.

Chapter 3 of my thesis focused on defining the contributions of additional nucleators to pedestal biogenesis. This investigation allowed another parallel to be drawn between the bacteria and viruses discussed in previous sections and EPEC, in that the use of formins is widespread. Specifically, I have shown that the EPEC actin assembly pathway is enhanced by the formin mDia1. Although Arp2/3 complex is absolutely essential for pedestal assembly, mDia1 contributes to pedestal biogenesis, as it localizes to pedestals and knockdown results in fewer, less intense actin pedestals. This formin was also found to contribute to actin protrusions and cell-to-cell spread for Listeria and Shigella, but, like Vaccinia virus and FHOD1, mDia1 likely contributes to motility and actin pedestal polymerization. It was somewhat surprising that RNAi-mediated knockdown of FHOD1 had no effect on EPEC pedestals, as the Vaccinia virus protein A36 and EPEC Tir use strikingly similar signaling mechanisms (Fig 4-2). While phosphotyrosine signaling and Nck are central to both pathways, the recruitment of Grb2 to Vaccinia and the requirement of WIP represent differences that could be responsible for reliance on different formins. Although these pathways seemingly depend on different formin subfamilies, the potential for Vaccinia virus to promote mDia1 nucleation cannot be excluded until it is directly assessed. Another surprising result was that EHEC pedestals do not seem to rely on formins, as EHEC pedestals were unaffected by chemical inhibitors as well as siRNAs. Therefore, the polymerization mediated by mDia1 represents a new and exciting difference between these closely-related pathogens.

Together, these findings address why and how EHEC and EPEC polymerize actin pedestals. Interestingly, my work has uncovered additional differences between these pathogens stemming solely from the pedestal assembly pathways, reflecting the wide variety of actin signaling networks that exist in mammalian cells.
1. Initial Adherence
2. Effector Translocation
3. Pedestal Assembly
4. Bacterial Multiplication
5. Motility to Neighboring Cell & Effector Translocation
6. Formation of 2nd Pedestal
7. Bacterial Division & Macrocolony Biogenesis

Fig 4-3. Actin pedestals allow for a stepwise pathway of cell-to-cell transmission. After initial adherence, effector translocation, and pedestal assembly (1-3), EHEC multiplies to seed a macrocolony (4). Once contact with a neighboring cell is established and effectors are injected (5), a second pedestal is polymerized, causing a pause in motility (6). Bacterial division releases one of the daughters from the cell junction, while maintaining the pedestal on newly infected cell (7), extending the borders of the macrocolony (4-7).
4.8 Future Directions:
There are additional questions prompted by these results that would be exciting opportunities to explore in the future. For instance, why EspF₀-driven motility and colonization is more effective than the EPEC Tir mediated pathway, how mDia1 is recruited to EPEC and activated, and why EHEC seems to be alone in not requiring formins are interesting questions that could drive future work.

To investigate the discrepancy in colonization levels between EPEC and EHEC, assessing the in vitro activation of Arp2/3 complex and N-WASP by EPEC Tir and EHEC EspF₀ would be an important first step. Pyrene-actin assembly assays could be employed to directly compare actin polymerization rates driven by these distinct mechanisms. Additionally, to determine if the macrocolony phenotypes observed on polarized monolayers are reflective of in vivo infections, the colonization experiments could be performed as competition assays using infant rabbits as a model system. Together, these in vitro and in vivo experiments could further clarify the molecular mechanism and physiological relevance of these phenotypes, respectively.

While my work revealed that the formin mDia1 is recruited to EPEC pedestals, the mechanisms of recruitment and activation remain to be elucidated. Additionally, mDia1 appears to be recruited to some EHEC pedestals, although siRNA targeting of mDia1 does not show a phenotype. This could indicate that mDia1 contributes to nucleation in EHEC pedestals, but in significantly lower levels than EspF₀. An important follow up study would include determining what other host proteins could recruit mDia1, and if mDia1 actually contributes to actin pedestal assembly for both strains. Furthermore, this study could be extended to Vaccinia virus, which has a strikingly similar mechanism of N-WASP activation to EPEC. These studies would provide mechanistic insight into the cooperation between formins and Arp2/3 complex in pedestals, and the findings could be applied to future investigations of actin nucleator cooperation in membrane protrusions.
This work has characterized how and why EPEC and EHEC hijack the host actin cytoskeleton to polymerize pedestals. The future investigations that may stem from these findings will provide further insight into the mechanisms and physiological relevance of both actin pedestals and host nucleator cooperation.
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