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Functional Elements of $EspF_U$, an Enterohemorrhagic *E. coli* Effector that Stimulates Actin Assembly: A Dissertation

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FUNCTIONAL ELEMENTS OF ESPF$_U$, AN ENTEROHEMORRHAGIC *E. COLI* EFFECTOR THAT STIMULATES ACTIN ASSEMBLY

A Dissertation Presented

By

BRIAN MICHAEL SKEHAN

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MD/PhD Program in Biomedical Sciences

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ABSTRACT

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is an attaching and effacing pathogen that upon attachment to host cells, induce characteristic attaching and effacing lesions and formation of F-actin rich pedestals beneath sites of bacterial attachment. EHEC harbors a Type III secretion system through which it delivers dozens of effectors into the host cell. The two secreted effectors critical for EHEC-mediated actin pedestal formation are the translocated intimin receptor (Tir) and $EspF_U$. $EspF_U$ consists of an N-terminal secretion signal and a C-terminus containing six tandem 47-residue proline-rich repeats, each of which can bind and activate the actin nucleation promoting factor N-WASP. Structural and functional analyses described here have identified the mechanism of N-WASP activation by $EspF_U$ and the minimal domains and specific residues required for this activity. While $EspF_U$ and Tir are the only bacterial effectors required for F-actin pedestal formation, recruitment of $EspF_U$ to Tir is mediated by an unidentified putative host factor. To identify the host factor responsible for linking these two effectors, a combination of *in vitro* and functional assays were used to identify the host factor, IRTKS and the residues required for these interactions were defined. Further, the presence of at least two 47-residue repeats in all characterized clinical isolates of canonical EHEC strains led us to address the minimal requirements for $EspF_U$ functional domains to promote recruitment to Tir and N-WASP activation. Here we show that two proline-rich elements of $EspF_U$ are required for recruitment of $EspF_U$ by IRTKS to sites of bacterial attachment. Furthermore, once artificially clustered at the membrane, a single N-WASP binding element of $EspF_U$ can induce actin pedestal formation.

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CHAPTER I

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) is an important food-borne pathogen in North America, Europe, and Japan (Nataro and Kaper 1998) that is responsible for at least 20,000 cases of intestinal disease per year in the U.S. alone. In addition, EHEC infection is associated with the life-threatening hemolytic uremic syndrome (HUS) (Karmali 2004; Karch, Tarr et al. 2005), the triad of hemolytic anemia, thrombocytopenia, and renal failure due to the production of shiga-like toxin (Stx), a potent inhibitor of protein synthesis (O'Brien, Tesh et al. 1992), by bacteria localized in the intestine. Ten percent of EHEC-infected children under the age of 10 develop HUS, making it a leading cause of pediatric renal failure in the U.S. (Karmali 2004). Serotype O157:H7 is the most common cause of EHEC infection and is thought to encompass the most virulent EHEC strains.

Study of host-pathogen interactions of enterohemorrhagic *E. coli*, enteropathogenic *E. coli* (EPEC), the rabbit enteropathogenic *E. coli* (RPEC), the mouse enteropathogen *Citrobacter rodentium* (Schauer and Falkow 1993; Marches, Nougayrede et al. 2000; Deng, Vallance et al. 2003; Mundy, MacDonald et al. 2005), and others reveal that, upon colonization of the intestinal epithelium, these pathogens induce the formation of characteristic attaching and effacing (AE) lesions (Knutton, Baldwin et al. 1989; Donnenberg and Whittam 2001; Kaper, Nataro et al. 2004). These lesions consist of effacement of the microvilli, tight attachment to the underlying epithelial cell, and generation of filamentous F-actin rich “pedestals” that lift the bacteria above the plane of

the cell membrane (Staley, Jones et al. 1969; Ulshen and Rollo 1980; Moon, Whipp et al. 1983). The ability to induce these characteristic lesions is essential for efficient colonization as disruption of the genes required for AE lesion formation has been shown to diminish colonization and disease in experimental animals (Donnenberg, Tzipori et al. 1993; Donnenberg, Yu et al. 1993; Schauer and Falkow 1993; Tzipori, Gunzer et al. 1995; Marches, Nougayrede et al. 2000; Ritchie, Thorpe et al. 2003).

Generation of actin pedestals is dependent upon translocation of bacterial effector proteins into the mammalian host via a type III secretion apparatus, a needle-like structure that spans the inner and outer membranes and protrudes from the bacterial surface (Dean, Maresca et al. 2005; Garmendia, Frankel et al. 2005; Hayward, Leong et al. 2006; Dean and Kenny 2009). This secretion system is encoded within a ~35 kb pathogenicity island known as the locus of enterocyte effacement (LEE) which is absolutely required for intestinal colonization and AE lesion formation (Jarvis, Giron et al. 1995; Elliott, Wainwright et al. 1998; Perna, Mayhew et al. 1998). Attached to the needle-like structure is a long filamentous-like protein, EspA, that is thought to serve as a conduit for the translocation of effector proteins (Ebel, Podzadel et al. 1998; Daniell, Takahashi et al. 2001; Cleary, Lai et al. 2004) through a pore in the mammalian cell membrane formed by two other translocated effectors, EspB and EspD (Sekiya, Ohishi et al. 2001; Ogino, Ohno et al. 2006). The EHEC LEE is homologous to several other Type III secretion systems found in a variety of pathogens that alter the actin cytoskeleton of mammalian cells including *Shigella*, *Salmonella,* and *Yersinia*, which induce actin assembly to promote cell invasion (Coburn, Sekirov et al. 2007) and the closely related EPEC LEE required for intimate host cell attachment (Sperandio, Kaper et al. 1998).

In addition to the Type III secretion system, the LEE encodes several effectors that are dependent upon this secretion system, including the translocated intimin receptor (Tir). Tir integrates into the host cell membrane in a hairpin loop conformation and serves as the receptor for the bacterium through binding to the bacterially expressed outer membrane adhesin intimin, encoded in the same bacterial operon in the LEE (Jerse, Yu et al. 1990; de Grado, Abe et al. 1999; Batchelor, Prasannan et al. 2000). Intimin binds the extracellular domain of Tir, an interaction absolutely required for attachment and colonization (Kenny, DeVinney et al. 1997; Kenny and Finlay 1997; Deibel, Kramer et al. 1998). Clustering of the receptor Tir beneath sites of bacterial attachment, through Tir-intimin interactions, permits the cytoplasmic Tir-N and Tir-C domains to coordinate the downstream signaling cascade inducing actin-rich pedestal formation (Campellone, Rankin et al. 2004; Caron, Crepin et al. 2006; Hayward, Leong et al. 2006). The N-terminus of Tir has been shown to interact with actin binding proteins such as α-actinin and cortactin that may play a role in actin assembly (Goosney, DeVinney et al. 2000; Cantarelli, Takahashi et al. 2002; Allen-Vercoe, Waddell et al. 2006). Important to note however, is that Tir derivatives lacking the N-terminus, when ectopically expressed, can still induce pedestal formation by EHEC or EPEC due to the recruitment of the actin assembly machinery by key residues in the Tir C-terminus (Campellone, Rankin et al. 2004; Campellone, Brady et al. 2006; Brady, Campellone et al. 2007).

A central component of actin assembly by EHEC is the Arp2/3 pathway, a heptameric complex that creates branched actin filament networks in eukaryotic cells (Goley and Welch 2006; Stradal and Scita 2006). This complex is tightly regulated by nucleation promoting factors (NPFs) such as WASP (Wiskott Aldrich Syndrome Protein)

and its homolog, the ubiquitously expressed N-WASP (Neuronal-Wiskott Aldrich Syndrome Protein) (Cory, Cramer et al. 2003; Millard, Sharp et al. 2004) . N-WASP exerts its effect on Arp2/3 through its C-terminal VCA (verprolin cofilin acidic) domain which binds and activates the complex. N-WASP is normally in an autoinhibited conformation where the alpha-helical C region of the VCA domain is bound to the GTPase binding domain (GBD) (Kim, Kakalis et al. 2000; Panchal, Kaiser et al. 2003). This interaction is stabilized by other host proteins such as WIP (WASP interacting protein) which binds to the N-terminal WASP-homology-1 (WH-1) domain in N-WASP (Ho, Rohatgi et al. 2001). A small basic region in the GBD domain of N-WASP can be bound by small phosphoinositides such as $PI(4,5)P_2$, and the CRIB (Cdc42-Rac interactive binding) region in the GBD is bound by small Rho-GTPases, including Cdc42 that can globally destabilize the autoinhibited conformation of N-WASP, disrupting VCA-GBD binding, and releasing the VCA domain to activate Arp2/3 (Kim, Kakalis et al. 2000; Prehoda, Scott et al. 2000; Rohatgi, Nollau et al. 2001; Takenawa and Suetsugu 2007). SH3-containing proteins such as Nck, Grb2, and Toca-1 can bind to the proline rich region of N-WASP immediately upstream of the VCA domain to similarly disrupt the VCA-GBD interaction providing an additional mechanism for activation of the Arp2/3 complex (Buday, Wunderlich et al. 2002; Takano, Toyooka et al. 2008). Importantly, both N-WASP and Arp2/3 are found localized to EHEC and EPEC induced actin pedestals (Kalman, Weiner et al. 1999; Goosney, DeVinney et al. 2001; Campellone, Robbins et al. 2004) and cells deficient for N-WASP or overexpressing dominant-negative forms of N-WASP (Campellone, unpublished observation) are unable

to support actin assembly induced by EPEC or EHEC infection (Garmendia, Carlier et al. 2006).

Several other microbial pathogens have developed means to impact the Arp2/3 actin assembly pathway. *Listeria* binds and activates Arp2/3 directly through its ActA protein to initiate actin comet-tail formation and cell to cell propulsion (Boujemaa-Paterski, Gouin et al. 2001). *Shigella* independently recruits Toca-1 (transducer of Cdc42-dependent actin assembly) and N-WASP to activate Arp2/3 and disrupt the actin cytoskeleton (Leung, Ally et al. 2008). *Vaccinia* virus protein A36R shares homology with EPEC Tir and is similarly phosphorylated at two tyrosine residues to recruit Nck and Grb2, which can both activate N-WASP and induce actin assembly (Frischknecht, Cudmore et al. 1999; Scaplehorn, Holmstrom et al. 2002). EPEC has been shown to utilize Tir-mediated actin assembly to intimately attach to host cells and induce rapid polymerization of F-actin to move across the surface of host cells (Sanger, Chang et al. 1996; Shaner, Sanger et al. 2005). Together, these data show a wide variety of pathogens have developed similar strategies to colonize and proliferate in the host utilizing the Arp2/3 actin assembly pathway.

Two closely related pathogens, EPEC and EHEC share 70% similarity in Tir and harbor nearly identical LEE pathogenicity islands, although replacement of EPEC Tir with EHEC Tir is insufficient to promote actin assembly in EPEC (DeVinney, Puente et al. 2001; Kenny 2001; Campellone, Giese et al. 2002). A key difference in the two Tir proteins is a tyrosine residue at position 474 in EPEC Tir that is phosphorylated early during infection. This phosphorylation induces recruitment of the SH2-domain containing protein Nck, an adaptor molecule that binds to phosphorylated tyrosine

residues and can bind and activate N-WASP through its SH3 domains, initializing a downstream signaling cascade that activates Arp2/3 complex and results in actin pedestal formation (Kenny 1999; Gruenheid, DeVinney et al. 2001; Campellone, Rankin et al. 2004; Rivera, Briceno et al. 2004). Lacking this important tyrosine residue, EHEC Tir is not phosphorylated and has been shown to induce actin assembly in the absence of Nck (Gruenheid, DeVinney et al. 2001; Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004). While ectopic expression and clustering of EPEC Tir is sufficient to induce actin assembly (Campellone, Giese et al. 2002), EHEC Tir does not share this phenotype. Screening and deletion of additional pathogenicity islands in the EHEC genome revealed a second, non-LEE encoded effector that plays a critical role in actin assembly. The protein identified, $EspF_U$ (also known as TccP), was shown to be secreted by the Type III secretion system and localize to tips of EHEC-mediated actin pedestal (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004). Deletion of this protein in EHEC resulted in a ~95% decrease in the ability to induce EHEC-mediated actin assembly. As the name indicates, $EspF_U$ shares significant homology with a LEE encoded effector found in many AE pathogens, EspF (Cheng, Skehan et al. 2008), that has been shown to bind and activate N-WASP (Alto, Weflen et al. 2007), but EspF cannot substitute for $EspF_U$ during actin pedestal formation.

$EspF_U$ from the sequenced O157:H7 strain EDL933 consists of an N-terminal Type III secretion signal followed by 6 tandem 47-residue proline-rich repeats in the C-terminus. Yeast two-hybrid assays indicated that a single proline-rich repeat of $EspF_U$ can efficiently bind the $N\text{-}WASP_{GBD}$, a known activator of Arp2/3 and required for actin assembly during EHEC infection (Campellone, Robbins et al. 2004; Garmendia, Phillips

et al. 2004; Campellone, Cheng et al. 2008). Tir, $EspF_U$, and N-WASP form a complex that can be immunoprecipitated from mammalian cells (Campellone, Robbins et al. 2004) but yeast two-hybrid and gel overlay assays did not reveal direct interaction between Tir and $EspF_U$ or Tir and N-WASP, suggesting that recruitment of $EspF_U$ and N-WASP to Tir requires another factor. Ectopic expression and clustering of EHEC Tir and $EspF_U$ is sufficient to induce actin assembly in the absence of bacterial infection (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004), suggesting that the putative factor responsible for recruitment of $EspF_U$ and N-WASP to Tir is likely of host origin.

Recently, a group of SH3-domain containing proteins have been shown to modulate membrane deformation and induce actin assembly due to the presence of a BAR-related domain (Bin, amphiphysin, Rvs167-) that binds $PI(4,5)P_2$ to bind and deform membranes (Takenawa and Suetsugu 2007). These proteins can serve as adaptor molecules that induce a change in the curvature of the membrane and recruit other NPFs through their C-terminal SH3 domains. The shape of these BAR-related domains is the determining factor in the effect on membrane deformation. BAR and F-BAR domains are concave and induce membrane invagination similar to that which occurs during endocytosis. Conversely, I-BAR domains (inverse-BAR domains, also known as IMDs for IRSp53-MIM homology domains) induce membrane protrusions, and two such proteins, IRSp53 and IRTKS (insulin receptor tyrosine kinase substrate) have been shown to localize to the tips of EHEC-mediated actin pedestals (Saarikangas, Zhao et al. 2009; Vingadassalom, Kazlauskas et al. 2009; Weiss, Ladwein et al. 2009). Furthermore, $PI(4,5)P_2$, known to bind both N-WASP and BAR-domain containing proteins has been shown to accumulate beneath sites of bacterial attachment early after infection (Sason,

Milgrom et al. 2009), indicating that these proteins may form a complex with $PI(4,5)P_2$ that plays an integral role in actin pedestal formation. Overexpression of either IRSp53 or IRTKS in mammalian cells results in the formation of membrane protrusive actin-rich structures resembling filopodia at the plasma membrane, but the mechanisms by which these actin structures develop is still largely unknown (Millard, Bompard et al. 2005; Scita, Confalonieri et al. 2008).

The Tir-intimin interaction is critical for cell attachment and colonization as loss-of-function mutants are deficient for cell attachment in animal models. It has been proposed that actin pedestal formation may inhibit phagocytosis, providing AE pathogens an opportunity to temporarily evade the immune system. The tight attachment and "surfing" (dynamic polymerization and breakdown of F-actin beneath the cell surface induced by EPEC, permitting movement across the surface of the host cell and perhaps cell to cell spread) (Sanger, Chang et al. 1996), might be indicative of increased dissemination dependent upon actin assembly, an observation underscored by the relative size and morphology of EHECΔ*espF*$_U$ microcolonies seen on the intestinal walls in an infant rabbit model compared to wild type EHEC (Ritchie, Brady et al. 2008). A better understanding of the mechanisms of actin assembly induced by these pathogens may provide a means to better understand the significance of the process.

EHEC mediated actin assembly is a complex multifactorial process requiring coordination and integration of both bacterial and host signaling factors (Figure 1). Despite intense study of the actin assembly pathway in EHEC as well as other AE pathogens, many questions remain and some are addressed here.

1. Numerous host cell proteins from a variety of different gene families have been shown to modulate the activity of N-WASP and therefore impact Arp2/3 dependent actin assembly. The EHEC effector protein $EspF_U$ has been shown to bind and activate N-WASP in-vitro and co-localizes with N-WASP and Arp2/3 in actin pedestals. How, specifically, does $EspF_U$ modulate N-WASP activity during the course of an infection?
2. Tir and $EspF_U$ have been shown to be the only bacterially expressed co-factors required for EHEC mediated actin assembly and co-localize when Tir is clustered despite the inability to show any direct interaction in-vitro. By what mechanism is $EspF_U$ recruited to Tir and what are the key residues responsible for this interaction?
3. The 47-residue proline-rich repeats in the C-terminus of $EspF_U$ appear to have two different functions, recruitment to Tir and activation of N-WASP. How many repeats are required for efficient function of each of these activities?

A better understanding of the actin assembly mechanisms induced by EHEC and its effectors could provide further insight into the significance of these actin-rich structures seen during the course of an infection by AE forming pathogens as well as provide a model system for the regulation of actin dynamics in host cells.

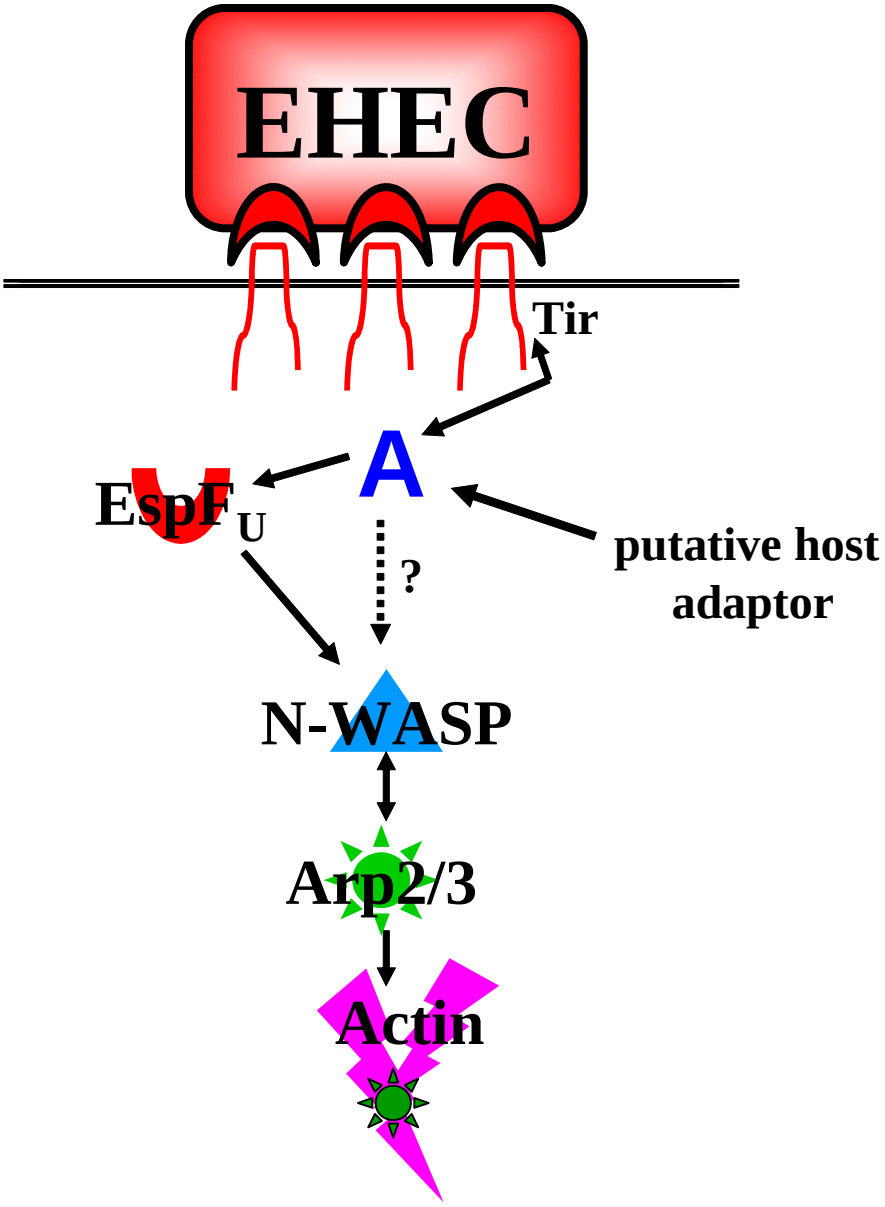

Figure 1. Current model of Tir-mediated actin assembly by EHEC.

CHAPTER II

STRUCTURAL MECHANISM OF WASP ACTIVATION BY THE ENTEROHEMORRHAGIC *E. COLI* EFFECTOR ESPF$_U$

Summary

During infection enterohemorrhagic Escherichia coli (EHEC) usurp the actin cytoskeleton of eukaryotic cells by injecting the EspF$_U$ protein into the host cytoplasm. EspF$_U$ controls actin by activating members of the Wiskott-Aldrich Syndrome Protein (WASP) family. Here we show that EspF$_U$ binds the autoinhibitory GTPase binding domain (GBD) in WASP proteins and displaces it from the activity-bearing VCA domain. This interaction potently activates WASP and N-WASP in vitro and induces localized actin assembly in cells. In the solution structure of the GBD-EspF$_U$ complex, EspF$_U$ forms an amphipathic helix that binds the GBD, mimicking interactions of the VCA in autoinhibited WASP. Thus, EspF$_U$ activates by competing directly for the VCA binding site on the GBD. This mechanism is distinct from that used by the eukaryotic activators Cdc42 and SH2 domains, which globally destabilize the GBD fold to release the VCA (Kim, Kakalis et al. 2000; Torres and Rosen 2003; Buck, Xu et al. 2004). Such diversity of mechanism in WASP proteins is distinct from other multi-modular systems, and may result from the intrinsically unstructured nature of the isolated GBD and VCA elements. The structural incompatibility of the GBD complexes with EspF$_U$ and Cdc42/SH2 plus high affinity EspF$_U$ binding enable EHEC to potently hijack the eukaryotic cytoskeletal machinery.

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Figure 1. A single repeat of $EspF_U$ activates WASP/N-WASP with high potency.
Hui-Chun Cheng and Dr. Michael Rosen - Department of Biochemistry and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center

Figure 2. Structures of the WASP GBD in complex with different ligands.
Hui-Chun Cheng and Dr. Michael Rosen - Department of Biochemistry and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center

Figure 3. $EspF_U$ residues that are predicted to bind the GBD or truncation of the extended arm of 1R33 contribute to interactions between $EspF_U$ and the GBD.
Brian Skehan and Dr. John Leong – University of Massachusetts Medical School

Figure 4. $EspF_U$ induces actin pedestal formation.
Brian Skehan, Douglas Robbins and Dr. John Leong – University of Massachusetts Medical School

Figure 5. N-WASP binding mutations in $EspF_U$ significantly impair actin assembly in a highly efficient clustering model.
Brian Skehan, Douglas Robbins and Drs. Kenneth Campellone and John Leong – University of Massachusetts Medical School

Introduction

Pathogenic bacteria often target the host actin cytoskeleton during infection to assist entry, cell-to-cell spreading, and intimate attachment to host membranes (Gruenheid and Finlay 2003; Galan and Cossart 2005; Caron, Crepin et al. 2006; Hayward, Leong et al. 2006; Munter, Way et al. 2006). EHEC (serotype O157:H7) is a food-born human pathogen that causes severe diarrhea and hemolytic uremic syndrome (Rangel, Sparling et al. 2005). During infection, EHEC binds eukaryotic host intestinal epithelial cells and secretes Tir (Translocated Intimin Receptor) and $EspF_U$ (also known as TccP), into the host cell (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004). The secreted Tir localizes in the host plasma membrane, and upon clustering by binding to the bacterial outer surface protein intimin, recruits $EspF_U$ by its carboxy-terminal cytoplasmic domain (Campellone, Brady et al. 2006). $EspF_U$ in turn then hijacks members of the Wiskott-Aldrich Syndrome Protein (WASP) family, which locally stimulate the actin nucleation factor, the Arp2/3 complex (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004; Lommel, Benesch et al. 2004; Campellone, Brady et al. 2006). Together, these interactions induce the formation of an actin-rich membrane protrusion beneath the site of EHEC attachment, termed an actin pedestal (DeVinney, Stein et al. 1999; Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004).

WASP proteins activate Arp2/3 complex through a C-terminal VCA domain (Higgs and Pollard 2001). In free WASP, this activity is blocked through intramolecular interactions with a central GTPase binding domain (GBD). Structural and biochemical studies have shown that the GBD sequesters an amphipathic helix in the C region of the

VCA that is necessary for activation of Arp2/3 complex, resulting in autoinhibition (Kim, Kakalis et al. 2000; Panchal, Kaiser et al. 2003; Kelly, Kranitz et al. 2006). WASP can be allosterically activated by several intracellular ligands, including Cdc42 and (for phosphorylated WASP) SH2 domains (Rohatgi, Ma et al. 1999; Torres and Rosen 2003). These ligands bind to WASP and N-WASP in a manner that is structurally incompatible with the autoinhibited fold of the GBD. Thus, they activate by globally destabilizing the GBD, consequently releasing the VCA (Buck, Xu et al. 2001; Torres and Rosen 2003; Buck, Xu et al. 2004).

$EspF_U$ is composed of an N-terminal signal sequence necessary for translocation into the eukaryotic host, followed by 2-7 nearly identical 47 residue repeats (Fig. 1A) (Garmendia, Ren et al. 2005). Each full repeat consists of an N-terminal 27 residue hydrophobic segment and a C-terminal 20 residue proline-rich segment. Previous studies showed that $EspF_U$ can bind the N-WASP GBD and activate N-WASP toward Arp2/3 complex in actin assembly assays *in vitro* (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004; Garmendia, Carlier et al. 2006). A similar sequence is found in the related bacterial effector EspF, which also can activate N-WASP (Alto, Weflen et al. 2007). Here we show the structure of $EspF_U$ in a complex with the WASP GBD, and using this data. The alpha helical region of $EspF_U$ that binds the GBD, as well as specific contact residues, were shown to be critical for binding and activation of WASP/N-WASP *in vitro* and *in vivo*.

Experimental procedures

$EspF_U$ expression and purification

$EspF_U$ and WASP proteins were expressed in bacteria as either His6- or GST-fusions, and purified by affinity and conventional chromatographies. Bovine Arp2/3 complex and rabbit skeletal muscle actin were purified as described (Cooper and Pollard 1982; Higgs, Blanchoin et al. 1999).

Biochemical Assays

All biochemical assays were performed in KMEI buffer (10 mM imidazole pH 7.0, 50 mM KCl, 1 mM MgCl2, and 1 mM EGTA), or KMEI plus 5 mM β-mercaptoethanol at 25 °C.

Actin Assembly

Actin assembly assays were performed as described (Higgs, Blanchoin et al. 1999), with 4 μM actin (5% pyrene labeled) and 10 nM Arp2/3 complex. Filament barbed end concentrations were calculated as described (Leung, Morgan et al. 2006).

Chemical Denaturation

GBD- $EspF_U$ fusion proteins (10 μM) were denatured with guanidine hydrochloride. Tryptophan fluorescence emission (λex = 295 nm, λem = 320 nm, 355 nm) was fit to a six parameter equation (Pace 1990) to yield ΔGunfolding.

Isothermal Titration Calorimetry

$EspF_U$ proteins were titrated into N-WASPC in a Microcal VP-ITC microcalorimeter. Baseline was corrected for $EspF_U$ addition to buffer. Data were fit to a single site binding model.

Yeast Two-hybrid Analyses

The two hybrid analyses were performed as described (Liu, Radhakrishnan et al. 2002) . DNA oligonucleotides encoding $EspF_U$ residues 268-281 (1R14), 268-285 (1R18), 268-295 (1R28), 268-297 (1R30), 268-314 (1R) were cloned into the EcoRI and BamHI sites of pGAD424. DNA fragments encoding the GTPase binding domain of N-WASP (GBD) were cloned into the EcoRI and BamHI sites of pGAD424 and pBTM116 respectively. ONPG assays were performed as described (Campellone, Robbins et al. 2004).

Mammalian Cell Culture and Immunofluorescence Microscopy

To create transfection plasmids expressing Tir- $EspF_U$ fusions (all N-terminally HA-tagged), DNA fragments encoding $EspF_U$ R14, R30, R33, R47, R_2 (repeats 4-5) and point mutants were cloned into the KpnI and BamHI sites of pHN-TirΔC1. Mouse fibroblast-like cells were cultured in six-well plates in DMEM plus 10% FBS and transfected with 1 μg of plasmid per well for 12-16 h using Lipofectamine Plus reagent (Invitrogen) (Campellone, Robbins et al. 2004). Cells were reseeded onto 12-mm glass coverslips to achieve 50–75% confluency after an additional 24 h of growth. Transfected cells expressing TirΔC-R_2 proteins were treated with an E. coli expressing intimin for 3.5 hours. Cells expressing TirΔC fused to a single $EspF_U$ repeat were treated with a 1:200 dilution of antibodies raised against the extracellular domain of EHEC Tir (anti-TirM, gift from A. Donohue-Rolfe, Tufts University School of Veterinary Medicine) for 30 min, washed to remove excess antibody, and then treated with S. aureus particles (Pansorbin; Calbiochem) for a further 2.5 h. In all cases, monolayers were fixed in PBS+4% paraformaldehyde for 30 minutes and permeabilized with 0.1% Triton-X-100 as described (Campellone, Giese et al. 2002). Cells were then treated with HA.11 (diluted 1:500 in PBS+1%BSA) or HA.11 and α-N-WASP (1:500) (gift from M. Kirschner) for

30 minutes prior to washing and addition of Alexa488 goat anti-mouse antibody (1:200; Molecular Probes) or Alexa568 goat anti-rabbit antibody (1:150). F-actin was identified by staining with Alexa568-phalloidin (1:100; Molecular Probes).

NMR Spectroscopy and Structure Calculation

The structure of the complex of GBD (residues 242-310) and R33 (residues 1-33 in Fig. 1) was determined in iterative fashion based on NOE, dihedral and hydrogen bond restraints using ARIA 2.130.

Results

To understand $EspF_U$ -mediated activation, we examined fragments containing the full 47-residue fifth repeat (R47, Fig. 1) and an N-terminal 33-residue fragment lacking most of the proline-rich sequence (R33). In Arp2/3-mediated pyrene-actin assembly assays, R47 and R33 appreciably and similarly enhanced the activities of autoinhibited N-$WASP_C$ (Fig. 1B). Titration of R47 into N- $WASP_C$ produced a monotonic increase in actin assembly rate (Fig. 1C), which reached a plateau value similar to that of the N-WASP VCA. Thus, the endpoint of the titration produces a state which biochemically resembles free N-WASP VCA. Fitting the data to a single-site binding isotherm yields a K_{Act} (the concentration of $EspF_U$ R47 required for half-maximal activation in actin assembly assays) of 24 nM, approximating the dissociation constant (K_D) of R47 for N-$WASP_C$ as 35 nM (data not shown). $EspF_U$ binds N-WASP more than 100-fold more strongly than Cdc42 does (Leung and Rosen 2005), resulting in much greater potency in actin assembly assays (Fig. 1C). This much higher affinity should enable $EspF_U$ to hijack N-WASP away from its endogenous regulators *in vivo* (Leung and Rosen 2005).

Cdc42 and SH2 domains activate WASP by physically displacing the VCA from the GBD (Kim, Kakalis et al. 2000; Torres and Rosen 2003). $EspF_U$ R33 disrupted GBD-VCA interactions similarly (data not shown), indicating that it also binds the GBD in a manner that is incompatible with the autoinhibitory interactions. The combined data show that $EspF_U$ R33 is functionally equivalent to Cdc42 and SH2 domains: all three ligands activate WASP/N-WASP by releasing the VCA from the GBD.

The WASP GBD is largely unstructured on its own, but folds to a well-defined conformation upon binding VCA (Kim, Kakalis et al. 2000). ^{1}H-^{15}N and ^{1}H-^{13}C amide and methyl transverse relaxtion-optimized spectroscopy (TROSY) NMR spectra showed that $EspF_U$ R33 or a five-repeat fragment, $R47_5$, also induced folding of the GBD (data not shown). Reciprocally, the GBD induces folding of the unstructured R33 (data not shown). Titrations of R33 and $R47_5$ into GBD saturated at 1:1 and 0.2:1 $EspF_U$:GBD stoichiometry, respectively, showing that each repeat can bind N-WASP. The endpoint spectra are strikingly similar, indicating that the GBD folds to the same structure on each repeat, and that the molecular nature of allosteric activation can be understood through analysis of the R33 complex.

We determined the solution structure of the WASP GBD in complex with $EspF_U$ R33 using NMR spectroscopy (Fig. 2). The complex can be considered in three stacked structural layers (Kim, Kakalis et al. 2000). The first layer is composed of a short N-terminal i+4 β hairpin (β1, 252-253; β2, 257-258, two backbone hydrogen bonds between D253 and G257) and α1 helix (265-274) from the GBD. The second layer lies behind the first and consists of GBD helices α2-α4 (α2, 278-281; α3, 284-296; α4, 300-306), which have a roughly planar C-shaped arrangement. The third layer is an amphipathic α5 helix

(3-14) and an extended arm (16-20) from $EspF_U$. These elements contact the back of the GBD and are roughly antiparallel to α3 and α2, respectively (Fig. 2A). On the basis of the absence of long-range ^1H-^1H nuclear Overhauser effects (NOEs) and low heteronuclear ^1H-^{15}N NOEs WASP residues 242-249 and $EspF_U$ residues 21-33 seem to be largely disordered in solution (data not shown). The $EspF_U$ relative, EspF, is expected to bind the GBD through an analogous amphipathic α5 helix, but may not use an extended arm, as these C-terminal residues are not conserved.

The complexes of the GBD with R33 and the C region of the VCA (GBD-C) are highly similar (Fig. 2). In both cases, ligand binding causes the GBD to fold into a β-hairpin and four helices. The backbone root mean squared deviation (r.m.s.d.) between the average GBD coordinates in the two structures is only 0.95 Å (residues 247-310). This structural similarity is reflected in high chemical shift similarity: the average GBD chemical-shift differences are 0.015 parts per million (p.p.m.) for HN, 0.062 p.p.m. for Hα, 0.043 p.p.m. for Hβ, 0.21 p.p.m. for Cα, and 0.30 p.p.m. for Cβ. The largest chemical shift differences between the two complexes occur in α2, which contacts the extended arm of $EspF_U$ in the GBD-R33 complex, but is solvent exposed in the GBD-C complex. The WASP C helix and $EspF_U$ helix bind the GBD at the same site and orientation through analogous hydrophobic contacts involving similar sequences (Fig. 1A). $EspF_U$ makes further contacts to the GBD through its C-terminal arm, which are not seen in the bound C region helix in autoinhibited WASP. These contacts contribute to the much higher WASP affinity of $EspF_U$ compared to its eukaryotic counterparts (Leung and Rosen 2005). The combined structural and biochemical data lead to a clear and simple mechanism for WASP and N-WASP activation by a single repeat element of $EspF_U$:

displacement of the VCA through competitive binding to a common site on the folded GBD.

To qualitatively explore the significance of the C-terminal tail of EspFu 1R33 in binding to the GBD, we used a yeast two hybrid assay to analyze the interactions between the N-WASP GBD (residues 151-273) and a series of EspFu 1R fragments (Figure 3A). The EspFu 1R constructs and the N-WASP GBD were fused to the Gal4 transcription activation domain and the LexA DNA binding domain, respectively, and co-expressed in yeast strain L40, containing a lacZ reporter. Interactions were scored by quantifying β-galactosidase activity. The fragments containing the full arm (1R, 1R30 and 1R27) all show substantially greater activity in this assay than those where the arm is truncated (1R18 and 1R14) (Figure 3A). Although this assay should not be interpreted quantitatively, the data qualitatively support the notion that the C-terminal arm contributes to interactions with the GBD. Next, we C-terminally fused these proteins to the WASP GBD through a short linker. Because the GBD and R33 are only folded when bound to each other, the thermodynamic stability of the fusions reports on the relative strengths of interaction between the two elements.

We mutated three conserved hydrophobic residues, V4, L8 and L12, which lie at the center of the interface between α5 and the GBD. To initially test the interaction of the point mutants with the GBD, the L12A ($R30^{VL}$*) was examined in the yeast two-hybrid assay for binding to the GBD. Indeed, mutation of this residue completely abrogated the interaction in this system (Figure 3B). The L12A ($R33^{VL}$*) and V4A/L8A ($R33^{**L}$) mutants have substantially reduced stability relative to wild type in the GBD-R33 fusions (Fig. 1D), demonstrating the importance of α5 in the interaction of N-WASP with $EspF_U$.

Truncations from R33 to R30 or R27 decrease stability of these fusions by ~2 kcal/mol^{-1}. Thus, residues beyond Ala30 contribute thermodynamically to the GBD-R33 interaction, despite the absence of discrete structure in these regions. Similar contributions to affinity through disordered regions have also been observed in other systems (Volkman, Prehoda et al. 2002). Truncation of all (R14) or part (R18) of the $EspF_U$ C-terminal arm decreases the stability further, indicating that the arm contributes appreciably to binding of the GBD to $EspF_U$. These unfolding studies mirror direct binding measurements. R33 and R18 bind N-$WASP_C$ with K_D values of 35 and 4,000 nM, respectively (Fig. 1D), corresponding to a difference in free energy of 2.9 kcal/mol^{-1}. This value agrees closely with the stability difference between the two GBD-fusions (2.6 kcal/mol^{-1}; Fig. 1D). Furthermore, these differences in binding correspond well to the activities of the $EspF_U$ mutants in N-WASP-mediated actin assembly assays, in which activity decreases together with the strength of interaction (Figs. 1B, D).

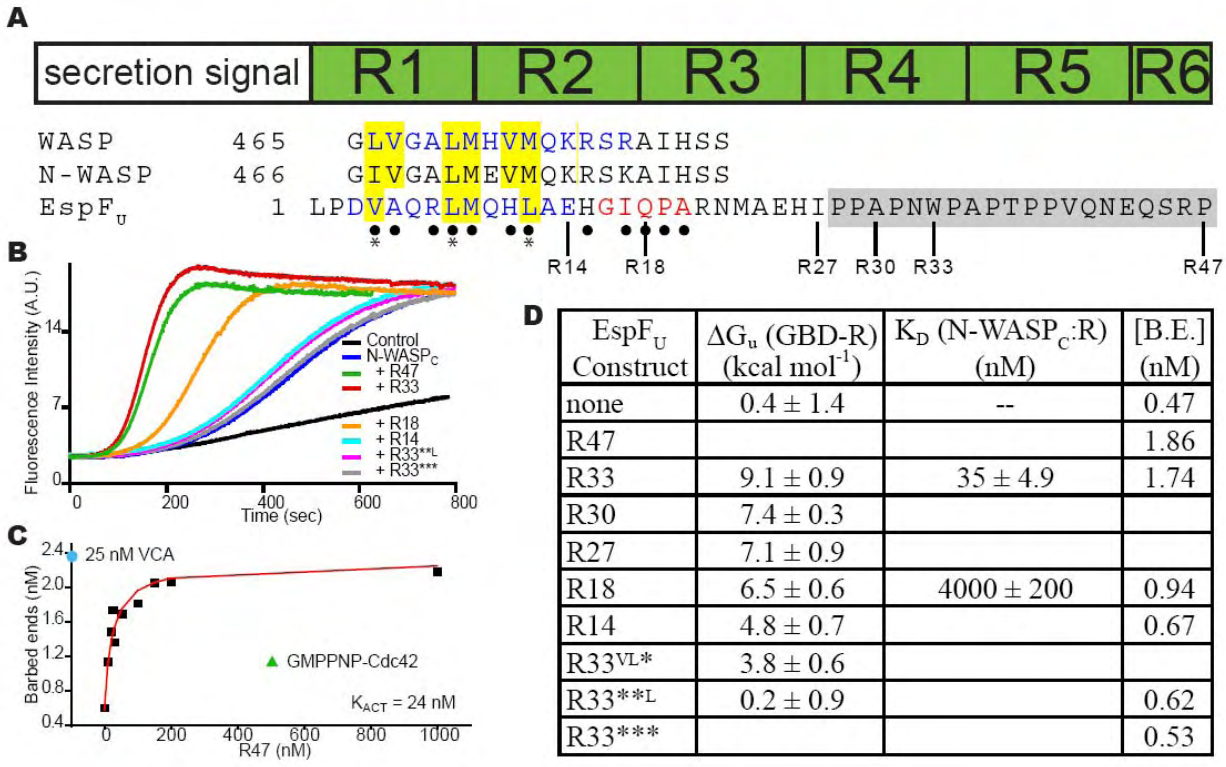
To test how the biophysical properties of $EspF_U$ -N-WASP interaction relate to biological function, we examined the ability of $EspF_U$ derivatives to induce localized actin assembly in mammalian cells. To uncouple Tir-mediated recruitment of $EspF_U$ from $EspF_U$ function in pedestal generation after recruitment, we replaced the C-terminal cytoplasmic domain of Tir with two repeats of $EspF_U$ (giving rise to TirΔC-$R47_2$). We expressed TirΔC-$R47_2$ in mouse fibroblast-like cells, where it inserted into the plasma membrane, and then clustered it by adding *E. coli* expressing intimin. Immunostaining showed that foci of clustered Tir and N-WASP coincided with bound bacteria (Fig 4A). Similarly, F-actin strongly colocalized with Tir. In contrast, a control Tir derivative (TirΔC) lacking cytoplasmic elements did not recruit N-WASP or produce actin pedestals

(Fig. 4A). Mutation of Leu12 to Ala in both $EspF_U$ repeats (giving rise to TirΔC-$R47_2^{VL}$*) also abolished both actin assembly and N-WASP recruitment (Fig. 4A), consistent with the decreased binding of this mutant to N-WASP *in vitro* (Fig. 1).

To examine the activity of $EspF_U$ truncation mutants, we modified this assay to analyze derivatives of one $EspF_U$ repeat unit by clustering Tir-EspFU fusions using fixed *Staphylococcus aureus* and anti-Tir antibody. Perhaps due to the highly efficient antibody clustering used in this assay, the $R47^{VL}$* maintained the ability to induce actin assembly and was not significantly different from wild type (Figure 5B). This is not completely surprising as our data showed that the free energy of unfolding of GBD- $EspF_U$ fusion with the $R47^{VL}$* mutation indicated there was still low level interaction between the mutant and the GBD *in vitro* (3.8 kcal/mol^{-1}; Figure 1D). In contrast, the data in Figure 1A showed that the triple V4A/L8A/L12A mutant, R33*** showed no ability to bind and activate N-WASP *in vitro* so the corresponding mutation was made in 1R TirΔC-R47*** and when tested for function, was unable to assemble actin in this assay (Figure 5A,B). Truncation of R47 to R33 decreased activity, perhaps owing to the loss of most of the proline-rich region, which in EspF is known to bind to an SH3-containing protein (Alto, Weflen et al. 2007) (Fig. 4B). R30 and R27 (Fig. 4) showed even lower actin assembly levels, consistent with the diminished N-$WASP_C$ binding by these proteins compared to R33 (Fig. 1). Furthermore, R14, which bound N-$WASP_C$ only very weakly (Fig. 1), did not promote actin assembly above background levels. Together, these data show that the ability of $EspF_U$ derivatives to promote actin assembly *in vivo* reflects the physical interactions between $EspF_U$ and WASP *in vitro*.

Discussion

We have described the autoinhibited GBD-VCA structure as composed of three layers, analogous to the three layers of the GBD-R33 structure, with the C region of the VCA substituting for R33 (Kim, Kakalis et al. 2000). Three structurally distinct mechanisms for relieving autoinhibition of WASP/N-WASP are now known that involve attacks on distinct regions of this fold. Cdc42 globally destabilizes the GBD by binding to the first layer of structure. Similarly, following phosphorylation of the conserved Tyr291 and Tyr256 in WASP and N-WASP respectively, SH2 domains can also destabilize the GBD by binding to this site in the second layer. EHEC has evolved the simplest and structurally most direct means of releasing the VCA: $EspF_U$ binds to the GBD-VCA interface, displacing the VCA from the folded GBD by structural competition. Of the three known allosteric mechanisms, only this latter one is akin to those found in most other autoinhibited multi-domain systems, in which activation is driven by rearrangements of intact functional modules. The diversity of WASP regulation probably originates from the intrinsically unstructured nature of the GBD, which enables its broad binding specificity. Because the folded GBD is structurally incompatible with Cdc42/SH2 binding, $EspF_U$ can displace the VCA while simultaneously preventing interactions with eukaryotic activators, thus sequestering WASP proteins away from their normal signaling pathways. This, coupled with very high affinity binding, allows EHEC to subvert the host actin cytoskeleton to its own ends.



EspF$_U$ Construct	ΔG_u (GBD-R) (kcal mol^{-1})	K_D (N-WASP$_C$:R) (nM)	[B.E.] (nM)
none	0.4 ± 1.4	--	0.47
R47			1.86
R33	9.1 ± 0.9	35 ± 4.9	1.74
R30	7.4 ± 0.3		
R27	7.1 ± 0.9		
R18	6.5 ± 0.6	4000 ± 200	0.94
R14	4.8 ± 0.7		0.67
R33^{VL}*	3.8 ± 0.6		
R33**^{L}	0.2 ± 0.9		0.62
R33***			0.53

Figure 1. A single repeat of EspF$_U$ activates WASP/N-WASP with high potency. **A.** Sequence alignment of WASP and N-WASP VCA C regions and the fifth repeat element of EspF$_U$. Helix residues in the autoinhibited GBD-C structure and GBD-R33 complex are blue. Residues in the extended EspF$_U$ arm are red. Aligned hydrophobic residues are boxed in yellow. EspF$_U$ residues that contact the GBD are indicated by •. Sites of EspF$_U$ mutations used in panels B and D are indicated by *. Proline-rich motif is boxed in gray. The C-termini of EspF$_U$ single repeat constructs used throughout this work are indicated below the sequence. **B.** Pyrene-actin fluorescence measured during assembly of 4 μM actin (5% pyrene-labeled) plus 10 nM Arp2/3 complex (black) plus 25 nM N-WASP$_C$ (blue) plus 500 nM of: R47 (green), R33 (red), R18 (orange), R14 (cyan), R33**L (pink) or R33*** (V4A/L8A/L12A, grey). **C.** Concentration of filament barbed ends produced during assembly of 4 μM actin by 10 nM Arp2/3 complex, 25 nM N-WASP$_C$ and increasing concentrations of R47 (black squares; red curve shows fit to single site binding isotherm) or 500 nM Cdc42-GMPPNP (green triangle). Blue circle shows barbed ends produced by actin plus Arp2/3 plus 25 nM N-WASP VCA. **D.** For EspF$_U$ proteins listed, table shows free energy of unfolding of GBD- EspF$_U$ fusion, dissociation constant for binding to N-WASP$_C$, and actin filament barbed ends produced by assays in panel B. R33^{VL}* (L12A) indicates a mutated derivative of R33. In "none" row, ΔGu represents melting of the isolated GBD, B.E. represents assays performed with only N-WASP$_C$ and Arp2/3 complex.

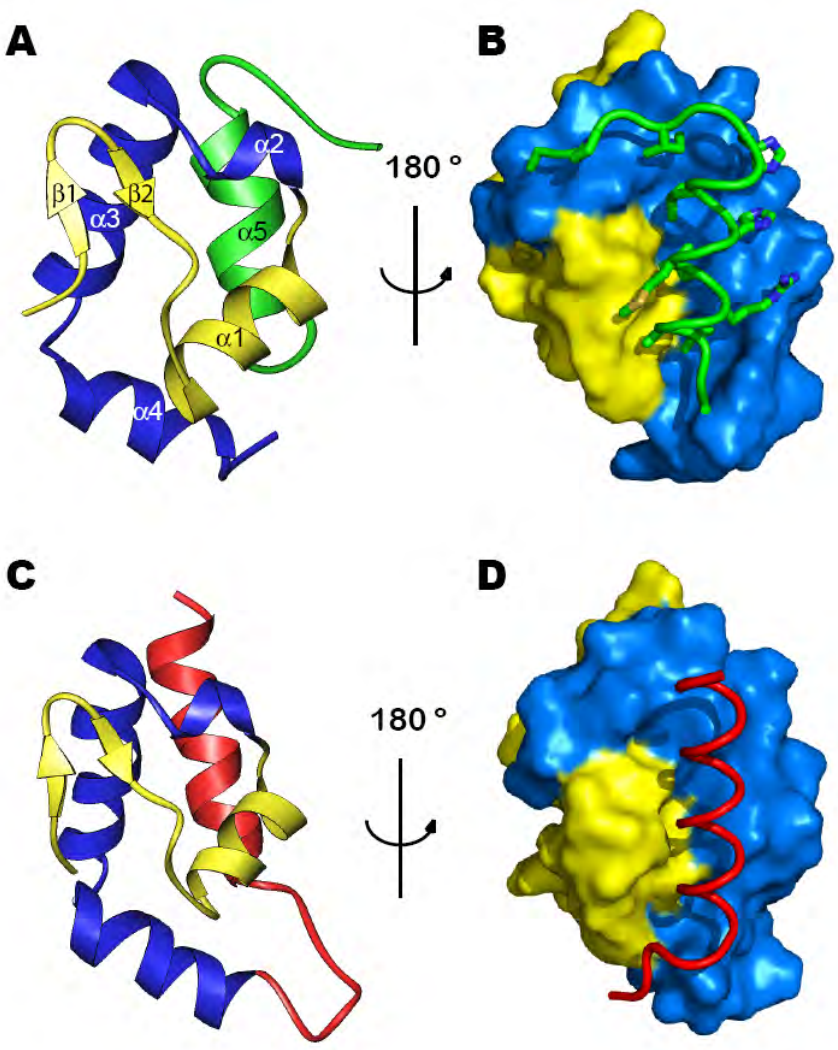


Figure 2. Structures of the WASP GBD in complex with different ligands.
A, B. Complex of WASP GBD and $EspF_U$ R33. Structure is colored: layer 1 (WASP 250-276), yellow; layer 2 (WASP 277-310), blue; layer 3 ($EspF_U$ 2-20), green. In B, GBD is shown as surface representation, R33 is shown as a ribbon with sticks for sidechains that contact the GBD. Views are related by 180° rotation about a vertical axis. **C, D.** Autoinhibited WASP (GBD-C7). GBD colored as in A; C region of WASP VCA is red. Representation and views are as in panels A and B.

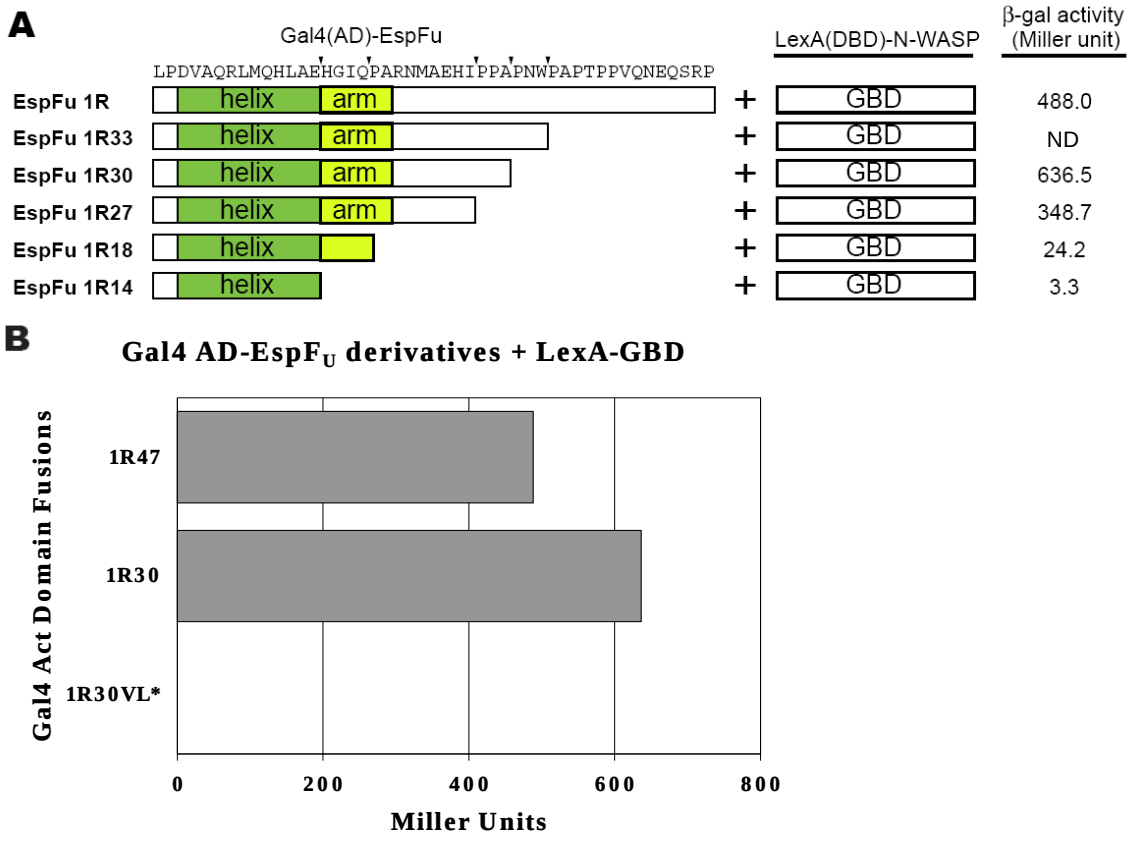


Figure 3. EspF$_U$ residues that are predicted to bind the GBD or truncation of the extended arm of 1R33 contribute to interactions between EspF$_U$ and the GBD.
A, B. Binding interactions between N-WASP GBD and EspF$_U$ 1R fragments observed in a yeast-two hybrid system. EspF$_U$ 1R fragments are shown schematically. Plasmids encoding the N-WASP GBD fused to the LexA DNA-binding domain and EspF$_U$ fragments fused to the Gal4 transcriptional activation domain were co-transformed into a yeast two-hybrid reporter strain. β-galactosidase activity was assessed as an average of three co-transformants. Similar results were seen in other transformations. **B.** A point mutation L12A in an EspF$_U$ derivative (1R30^{VL}*) blocks binding to GBD.

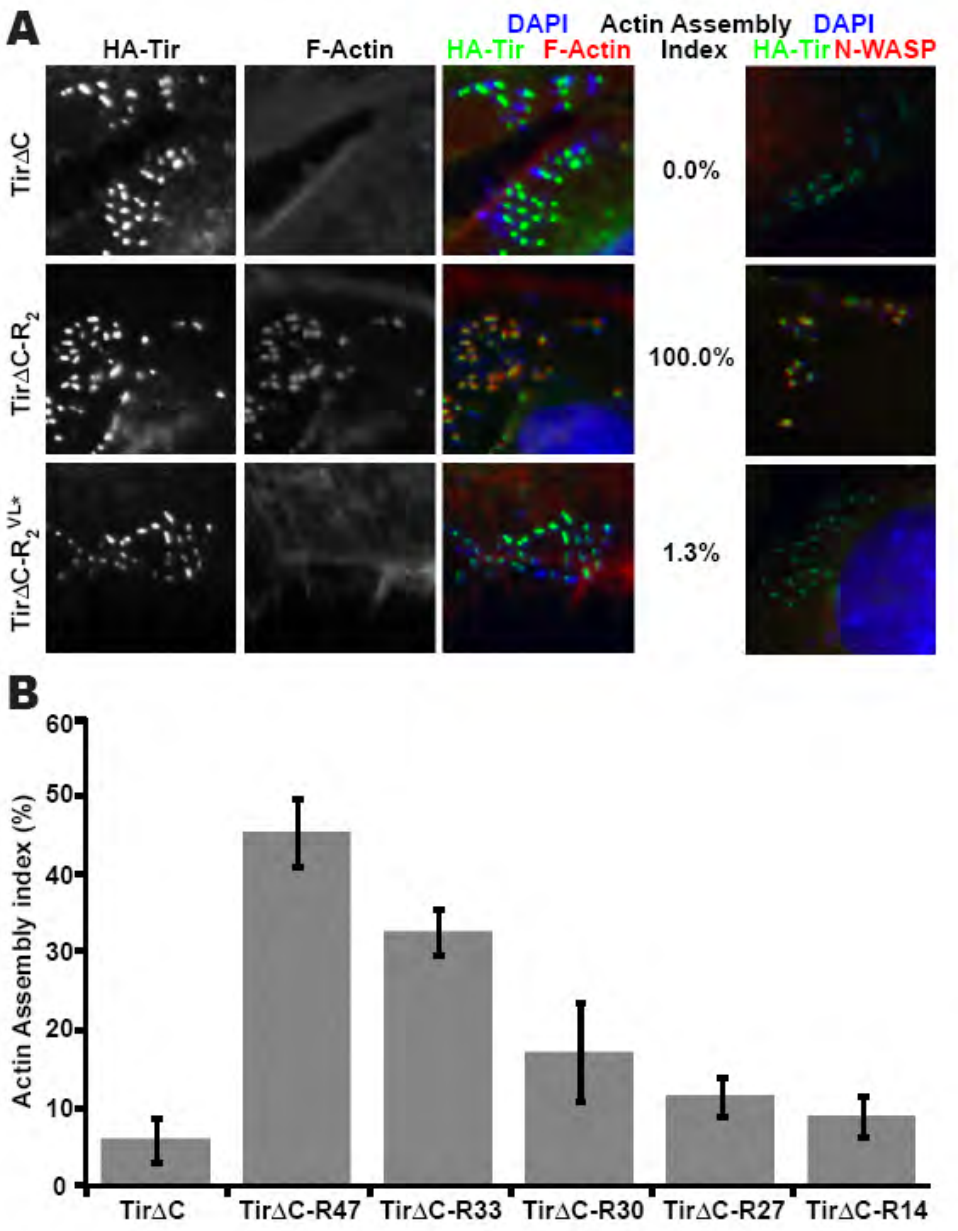


Figure 4. $EspF_U$ induces actin pedestal formation.
A. Cells expressing the TirΔC, TirΔC-R_2 or TirΔC-R_2^{VL*} (with N-terminal HA-tags) were challenged with E. coli expressing intimin, and stained with anti-HA antibody (green) and Alexa568-phalloidin (red, left panels) or anti-N-WASP antibody (red, right panels). Co-localization is yellow in merged images. The % of transfected cells harboring at least five F-actin foci was quantified (Actin Assembly Index). Bacteria were visualized by DAPI staining. **B.** Cells expressing Tir-repeat proteins were challenged with anti-Tir antibody and S. aureus particles and Actin Assembly Index was determined. In A and B data represent mean +/- SD from at least two samples with 20-50 cells examined per sample.

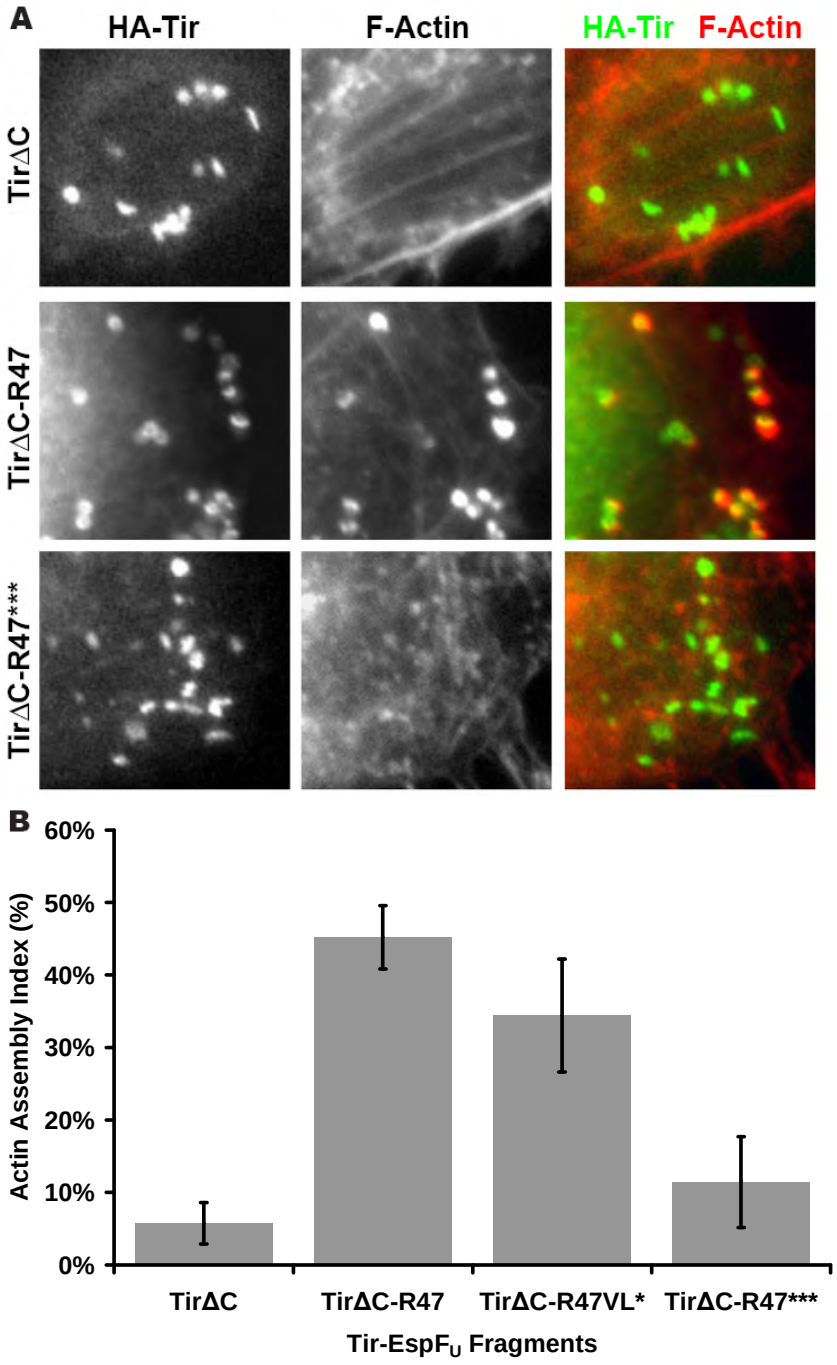


Figure 5. N-WASP binding mutations in $EspF_U$ significantly impair actin assembly in a highly efficient clustering model.

A,B. Cells expressing the TirΔC, TirΔC-R47, TirΔC-R47^{VL}*, or TirΔC-R47*** (with N-terminal HA-tags) were challenged with anti-Tir antibody and S. aureus particles, and stained with anti-HA antibody (green) and Alexa568-phalloidin (red). Colocalization is yellow in merged images. The % of transfected cells harboring at least five F-actin foci was quantified (Actin Assembly Index). Bacteria were visualized by DAPI staining. Data represent mean +/- SD from at least two samples with 20-50 cells examined per sample.

CHAPTER III

AN ESSENTIAL ACTIVITY OF THE ESPF$_U$ PROLINE-RICH SEQUENCE IS IRTKS BINDING AND RECRUITMENT OF THE ESPF$_U$ NWASP-BINDING HELIX TO TIR

Summary

Enterohemorrhagic *Escherichia coli* O157:H7 translocates two effectors to trigger localized actin assembly in mammalian cells, resulting in filamentous actin pedestals. One effector, the translocated intimin receptor (Tir), is localized in the plasma membrane and clustered upon binding the bacterial outer membrane protein intimin. The second, the proline-rich effector EspF$_U$ (aka TccP) activates the actin nucleation-promoting factor N-WASP, and is recruited to sites of bacterial attachment by a mechanism dependent on an Asn-Pro-Tyr (NPY458) sequence in the Tir C-terminal cytoplasmic domain. Tir, EspF$_U$, and N-WASP form a complex in infected cells, but neither EspF$_U$ nor N-WASP bind Tir directly, suggesting involvement of another protein(s) in complex formation. Screening of the human SH3 proteome for the ability to bind EspF$_U$ identified the SH3 domain of insulin receptor tyrosine kinase substrate (IRTKS), a factor known to regulate the cytoskeleton. Replacement of the C-terminus of Tir with the IRTKS SH3 domain resulted in a fusion protein competent for EspF$_U$-mediated actin assembly *in vivo*. A second domain of IRTKS, the IRSp53/MIM homology domain (IMD), bound to Tir in a manner dependent on the C-terminal NPY458 sequence, thereby recruiting IRTKS to sites of bacterial attachment. The IRTKS-mediated recruitment of EspF$_U$ is dependent upon a minimum of two proline-rich regions and can be inhibited by alanine substitution

of a residue conserved in all six C-terminal repeats, W33. Artificial clustering of $EspF_U$ reveals that the proline-rich region of $EspF_U$ is not required to induce actin assembly and only a single alpha-helical domain of $EspF_U$ is required to induce actin assembly in this system. Thus, enterohemorrhagic *E. coli* translocates two effectors that bind to distinct domains of a common host factor to promote the formation of a complex that triggers robust actin assembly at the plasma membrane.

Acknowledgements

This chapter is the result of collaboration among members of our lab and contains material previously published in (Vingadassalom, Kazlauskas et al. 2009). As such, I would like to acknowledge the appropriate people for their contribution to the work presented here.

Figure 1. IRTKS-SH3 domain binds to a single $EspF_U$ repeat and IRTKS-IMD binds to TirC.
Brian Skehan, Loranne Magoun and Drs. Didier Vingadassalom and John Leong

Figure 2. IRTKS is recruited to sites of bacterial attachment in an $EspF_U$-independent manner, and clustering of its SH3 domain is sufficient to trigger pedestal formation.
Lindsay Soll, Douglas Robbins and Drs. Didier Vingadassalom and John Leong

Figure 3. Genetic depletion of IRTKS inhibits $EspF_U$-dependent pedestal formation.
Drs. Didier Vingadassalom and John Leong

Figure 4. A Tir-$EspF_U$ fusion protein harboring a single N-WASP-binding helix flanked by IRTKS-binding sequences can promote actin assembly.
Brian Skehan, Douglas Robbins and Dr. John Leong

Figure 5. A single $EspF_U$ repeat can stimulate actin pedestal formation when fused to Tir residue 505 near the Tir C-terminus.
Brian Skehan, Douglas Robbins and Dr. John Leong

Figure 6. IRTKS binding by $EspF_U$ is dependent upon critical residues in the proline-rich region of an $EspF_U$ repeat.
Brian Skehan, Loranne Magoun and Dr. John Leong

Figure 7. IRTKS-binding by $EspF_U$ is required for pedestal formation due to its role in recruitment of $EspF_U$ to Tir.
Brian Skehan, Douglas Robbins and Dr. John Leong

Figure 8. IRTKS is not required for actin pedestal formation when $EspF_U$ is artificially clustered at the membrane.
Brian Skehan and Dr. John Leong

Figure 9. IRTKS binding by two proline-rich repeats is required for recruitment of $EspF_U$.
Brian Skehan, Douglas Robbins and Dr. John Leong

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a food-borne pathogen that is an important agent of both diarrheal and systemic disease (Kaper, Nataro et al. 2004). Along with the closely related pathogen, enteropathogenic *E. coli* (EPEC), it is a member of the attaching and effacing (AE) family of Gram-negative enteric pathogens, so named because they generate striking histopathological lesions on intestinal epithelia, characterized by a loss of microvilli, intimate attachment of the bacteria to the host cell, and the formation of filamentous F-actin-rich pedestal structures beneath the host cell membrane at sites of bacterial attachment (Kaper, Nataro et al. 2004). The ability to form AE lesions correlates with the ability to colonize the intestine and cause disease in animal models (Donnenberg, Tacket et al. 1993; Ritchie, Thorpe et al. 2003). In addition, the ability to stimulate the localized assembly of F-actin in the host cell has been a model for understanding the control and modification of the mammalian cytoskeleton.

Actin pedestal formation by EHEC and other AE pathogens depends on the delivery of bacterial effector proteins into host cells via a type III secretion system (Hayward, Leong et al. 2006; Frankel and Phillips 2008). One effector required for pedestal formation is the translocated intimin receptor (Tir) (Kenny, DeVinney et al. 1997; Deibel, Kramer et al. 1998). After translocation into host cell, Tir adopts a hairpin loop conformation in the host cell plasma membrane with N- and C-terminal intracellular domains and a central extracellular domain that binds to the bacterial outer membrane protein intimin. Clustering of Tir in the host cell membrane upon intimin binding initiates a signaling cascade, ultimately leading to actin pedestal formation.

EHEC Tir generates pedestals dependent upon the C-terminal cytoplasmic domain harboring a critical Asn-Pro-Tyr458 (NPY458) sequence that is essential for actin signaling (Allen-Vercoe, Waddell et al. 2006; Campellone, Brady et al. 2006; Brady, Campellone et al. 2007). In addition, EHEC translocates into host cells a second effector, $EspF_U$ (aka TccP) that acts in concert with Tir to promote actin pedestal formation (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004). An EHECΔ*espF*$_U$ mutant generates pedestals at approximately one tenth the efficiency of WT on cultured monolayers (Campellone, Robbins et al. 2004) and is impaired at the expansion of an initial infectious niche during infection of infant rabbits (Ritchie, Brady et al. 2008). $EspF_U$ contains multiple 47-aa proline-rich repeats, and a 20-residue amphipathic alpha-helical region of the repeat is capable of binding and activating WASP/N-WASP (Campellone, Robbins et al. 2004; Garmendia, Carlier et al. 2006; Cheng, Skehan et al. 2008; Sallee, Rivera et al. 2008). $EspF_U$ is recruited to sites of bacterial attachment in a manner dependent on the Tir NPY458 sequence (Brady, Campellone et al. 2007), and Tir and $EspF_U$ form a co-immunoprecipitable complex with N-WASP in infected HeLa cells (Campellone, Robbins et al. 2004).

Although N-WASP and $EspF_U$ are in complex with Tir, neither protein appears to directly bind Tir (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004), and no additional bacterial effectors are required for $EspF_U$-mediated actin assembly (Campellone, Cheng et al. 2008), indicating that this putative factor linking Tir, $EspF_U$ and N-WASP is likely of host origin. In addition, given that actin pedestal formation occurs, albeit at low levels, in the absence of $EspF_U$, the putative host factor may itself stimulate low levels of actin assembly. In the current study, we report that the insulin

receptor tyrosine kinase substrate (IRTKS), a homologue of insulin receptor substrate protein of 53 kDa (IRSp53) and thus a member of a protein family that is capable of transducing actin assembly signals in mammalian cells, is targeted by both Tir and $EspF_U$. The interaction of IRTKS with $EspF_U$ is dependent upon specific residues in the proline-rich region of an $EspF_U$ C-terminal repeat that we show are required for recruitment of $EspF_U$ to Tir by IRTKS and therefore, ultimately essential for the formation of a robust actin assembly complex during EHEC pedestal formation. Furthermore, we have delineated the minimum requirement of IRTKS-binding and N-WASP-binding elements expressed by $EspF_U$ for recruitment to Tir and for downstream activation of N-WASP induced actin assembly by these EHEC encoded effectors.

Experimental Procedures

Strains, plasmids and DNA manipulations. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2 respectively. $EspF_U$ derivatives were cloned into pKC425 or pKC689 (Campellone, Cheng et al. 2008; Cheng, Skehan et al. 2008), to generate GFP-fusion proteins and Tir fusion proteins, respectively.

Bacterial and mammalian cell culture. E. coli strains were grown in LB media at 37ºC for routine passage. Culture of bacteria prior to infection of mammalian cells, and culture and transfection of mouse embryonic fibroblasts (MEFs) and HeLa cells were performed as previously described (Campellone, Robbins et al. 2004; Campellone, Brady et al. 2006; Cheng, Skehan et al. 2008) or using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

Yeast Two-hybrid Analyses. The two-hybrid expression vectors pGAD424 and pBTM116, as well as reporter strain L40, were used to define the interaction between IRTKS, IRSp53, $EspF_U$ and EHEC Tir as previously described (Liu, Radhakrishnan et al. 2002; Cheng, Skehan et al. 2008). ONPG assays were performed as previously described (Campellone, Robbins et al. 2004; Cheng, Skehan et al. 2008)

RNAi experiments. siRNA was performed using stealth RNAi (Invitrogen).
The sequences used were as described in Suetsugu et al. (2006):
control #1, 5'-AGUGGCUAACAGAGGCGUCACAGUA-3';
control #2, 5'- ACUAGACGAAGCGGUACUGAGCCAU-3';
IRTKS #1, 5'- CAAUCCUGGGCUGCGAAAUUUAAUA-3';
IRTKS #2, 5'- CGUGAAAUAUAUGAACGCAACUCUA-3'.
Transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Mammalian cell infections and immunofluorescence microscopy. For microscopic analysis, mammalian cells were grown, infected with bacteria and processed as described previously in Chapter II experimental procedures (Campellone, Robbins et al. 2004; Campellone and Leong 2005; Cheng, Skehan et al. 2008). Cells were treated with mouse anti-HA tag mAb HA.11 (1:500; Covance), mouse anti-HA Alexa-488 (Invitrogen), mouse anti-IRSp53 mAb (1:100; Novus Biologicals) or mouse anti-IRTKS mAb (1:100; Novus Biologicals).

Results

IRTKS binds the two EHEC secreted effectors required for actin assembly Tir and $EspF_U$.

Affinity panning of a phage display library containing 296 clones representing the human SH3-domain containing proteome on recombinant GST-$EspF_U$ resulted in the enrichment of an I-BAR containing protein, IRTKS. Indeed, after enrichment, about one-third of the phage encoded IRTKS (Vingadassalom, Kazlauskas et al. 2009). To determine whether, as predicted, the proline-rich region of $EspF_U$ is required for this interaction, we utilized a yeast two-hybrid assay that had previously shown to reasonably define the N-WASP binding domain of $EspF_U$. $EspF_U$ derivatives were fused to the Gal4 activation-domain (Gal4AD) and co-transformed with LexA DNA-binding domain (LexADBD) fused to the IRTKS-SH3 domain into a yeast two-hybrid reporter strain L40. In this assay, if the two fusion proteins interact, a reporter gene, β-galactosidase, is expressed and the activity of the reporter gene can be measured in a colorimetric assay. When compared to the activity of the LexADBD and Gal4AD negative controls, a single repeat of $EspF_U$ (R47) co-expressed with the IRTKS-SH3 domain induced a 35-fold increase in β-galactosidase activity (Figure 1). Conversely, deletion of the carboxy-terminal 14 residues, encompassing much of the proline-rich region of an $EspF_U$ repeat (R33) co-expressed with IRTKS-SH3 showed no induction of reporter gene activity. As a control to ensure that the $EspF_U$ -induced activity was specific to the presence of the IRTKS-SH3 domain, we co-expressed R47 and the IRTKS-IMD (IRTKS I-BAR domain) and saw no evidence of induction of β-galactosidase. These experiments provided

evidence that the proline-rich region of an $EspF_U$ repeat and not the N-WASP binding alpha-helical region were important for interaction between IRTKS and $EspF_U$ *in vitro*.

To test whether IRTKS is a viable candidate as the linker between the EHEC secreted effectors responsible for actin assembly during infection, Tir and $EspF_U$, we determined whether IRTKS could also bind Tir. We utilized the same yeast-two hybrid system by fusing the required Tir sequence for EHEC-mediated actin assembly, TirC, to the Gal4AD and fused both the SH3 and IMD domain of IRTKS to the LexADBD. Co-transformation of the IRTKS-IMD with TirC showed an 8-fold increase in reporter gene activity when compared to background. This interaction was specific to the IMD of IRTKS as IRTKS-SH3 showed no increase in activity (Figure 1). Together, this data indicates that IRTKS, through different domains, has the ability to bind the two EHEC secreted effectors, Tir and $EspF_U$.

IRTKS can be recruited to sites of bacterial attachment independent of $EspF_U$.

In addition to IRTKS, affinity panning the human SH3-proteome phage display over recombinant $EspF_U$ also resulted in an approximately equivalent enrichment of the IRTKS homolog, IRSp53. In yeast two-hybrid assays, however, IRSp53 showed no ability to interact with Tir. While both IRTKS and IRSp53 were recruited to actin pedestals, as predicted due to their ability to bind $EspF_U$ (data not shown), we tested whether either protein was capable of localizing to sites of bacterial attachment independent of $EspF_U$, which in the case of IRTKS could be a reflection of its ability to interact with Tir. To investigate this, we infected HeLa cells with a strain of EHEC that had been engineered to efficiently translocate Tir but did not express $EspF_U$ (EHEC*ΔdamΔespF*$_U$) (Campellone, Roe et al. 2007). Staining with antibodies against

IRTKS and IRSp53 revealed that IRTKS but not IRSp53 was recruited to sites of bacterial attachment in the absence of $EspF_U$ (Figure 2A). As expected, no F-actin accumulation was seen underneath attached bacteria due to the lack of $EspF_U$.

IRTKS-SH3 can induce actin assembly when artificially clustered at the membrane.

To show that $EspF_U$ could be effectively recruited to Tir by the IRTKS-SH3 domain, we utilized a Tir-fusion construct that has been previously shown to be an effective model for artificial clustering of ectopically expressed proteins at the host cell plasma membrane. The entire cytoplasmic C-terminus of Tir (residues 387-558) was replaced with the IRTKS-SH3 domain (model in Figure 2B). For more efficient integration of ectopically expressed Tir into the plasma membrane, the first transmembrane domain of EHEC Tir was replaced with the transmembrane domain of the HN protein from New Castle Disease Virus (NDV) indicated by "HN" in Figure 2B. The Tir-$SH3_{IRTKS}$ fusion construct and a negative control containing no insert (TirΔC) were ectopically expressed in mouse embryonic fibroblasts (MEFs). These cells were then infected with KC14 harboring *$espF_U$* on a plasmid. While Tir was clearly seen clustered beneath bacteria in both cell lines, only the Tir-$SH3_{IRTKS}$ expressing cells were capable of actin assembly, presumably due to recruitment of $EspF_U$ by the SH3-domain (Figure 2B).

Genetic depletion of IRTKS inhibits $EspF_U$-dependent pedestal formation.

To further examine whether IRTKS function is required for EHEC actin assembly, we used an RNAi approach based on previously published siRNA sequences that efficiently and specifically silence expression of IRTKS (Suetsugu, Kurisu et al. 2006). RT-PCR analysis of cells transfected with a combination of 2 IRTKS siRNAs showed an approximately 90% depletion of IRTKS mRNA compared with control

siRNA (data not shown). To assess the role of IRTKS in pedestal formation, IRTKS-depleted and control cells were infected with KC12/pEspF$_U$. As expected, pedestals formed with high efficiency on cells transfected with non-silencing siRNA - visual quantitation revealed that virtually all infected cells displayed pedestals. In contrast, pedestal formation was diminished more than 5-fold on cells treated with a combination of the two IRTKS siRNAs (Figure 3). Importantly, EPEC, which generates pedestals in an EspF$_U$ independent manner, formed pedestals with high efficiency on IRTKS-depleted cells (data not shown). Finally, the two IRTKS siRNAs each had an inhibitory effect on pedestal formation when used individually (data not shown), suggesting that the pedestal phenotype upon IRTKS siRNA treatment was not due to off-target effects. Thus, these RNAi studies indicate that IRTKS is specifically required for EspF$_U$-mediated actin assembly.

A Tir-EspF$_U$ fusion protein harboring a single N-WASP-binding helix can promote actin assembly when flanked by IRTKS binding sequences.

Previous studies have shown that artificial clustering of Tir- EspF$_U$ fusion constructs at the membrane required two N-WASP binding helices of EspF$_U$ to promote robust actin assembly, consistent with the observation that multimerized activated WASP and N-WASP are highly potent activators of the Arp2/3 complex (Campellone, Cheng et al. 2008; Padrick, Cheng et al. 2008). For example, TirΔC-HP (where the entire C-terminus of Tir has been replaced with a derivative of EspF$_U$ that harbors a single alpha-helical domain (H) and a single proline-rich region (P)) is incapable of promoting actin assembly upon clustering by infection with *E. coli* expressing intimin (Campellone, Cheng et al. 2008; Cheng, Skehan et al. 2008), while TirΔC-HPH, which contains a

second alpha-helical N-WASP binding domain was capable of promoting actin assembly at high levels (Padrick, Cheng et al. 2008) (see also Figure 4, top two rows). IRTKS-family members have been shown to modulate membrane deformation and induce actin assembly due to the presence of a BAR-related domain that binds $PI(4,5)P_2$ and a SH3 domain that could recruit NPFs such as N-WASP (Takenawa and Suetsugu 2007). In addition, when *S. aureus* and anti-Tir antibody were used to cluster Tir- $EspF_U$ derivatives harboring only a partial $EspF_U$ repeat, we found that the inclusion of the proline-rich IRTKS binding sequence was associated with higher levels of actin pedestal formation (Chapter 2, Figure 5). Thus we further investigated whether the IRTKS binding domain might enhance actin assembly by clustering various ectopically expressed Tir- $EspF_U$ fusions in MEFs. First, we tested whether an artificial clustering of an $EspF_U$ derivative containing only a single intact N-WASP-binding helix might retain actin assembly function in the presence of multiple IRTKS binding sequences. We incorporated N-WASP binding mutations in the first or second alpha-helical domain of a two repeat construct to generate H*PHP or HPH*P, respectively (where an * indicates a mutated binding domain). Whereas clustering of TirΔC-HPH*P did not promote pedestal formation, TirΔC-H*PHP was indeed capable of inducing actin assembly at a comparable level to constructs expressing two functional N-WASP binding elements (Figure 4, rows 3 and 4). To insure that the actin assembly activity of TirΔC-H*PHP was not due to residual N-WASP binding to the mutated alpha-helical domain, we tested TirΔC-PHP, in which the proximal helical sequence was entirely deleted. This new construct was capable of inducing actin assembly at the same levels as TirΔC-H*PHP and TirΔC-HPH

(Figure 4, row 5). One interpretation of these results is that a single helical region flanked by two IRTKS-binding sequences is capable of promoting actin assembly.

A single $EspF_U$ repeat can stimulate actin pedestal formation when fused to Tir residue 505 near the Tir C-terminus.

An alternative interpretation to the result that TirΔC-PHP but not TirΔC-HP was capable of inducing actin assembly is that membrane proximity of the N-WASP binding helical sequence is a critical parameter, and that the role of the N-terminal (and membrane proximal) proline-rich sequence is as a spacer, moving the helical sequence away from the plasma membrane. To test this hypothesis we generated Tir_{505}-HP, a Tir fusion protein in which a single $EspF_U$ HP repeat was fused to Tir residue 505, 53 residues from the Tir C-terminus, rather than to residue 387, as is the case for TirΔC-HP. A 505 residue N-terminal Tir fragment contains the NPY_{458} sequence, which is capable of binding to IRTKS. To obviate the potential complication of a second IRTKS-binding sequence, we substituted three arginines (RRR) for residues NPY_{458}. (For simplicity, the mutation is not noted in the nomenclature and this Tir derivative will be described as Tir_{505}.) Tir_{505} derivatives were ectopically expressed in MEFs and clustered at the membrane by *E. coli* expressing intimin. As expected, Tir_{505}, lacking any $EspF_U$ elements, was unable to promote actin assembly. However, Tir_{505}-HP, which contains only a single repeat of $EspF_U$, was able to induce actin assembly at levels comparable to a Tir_{505}-HPH construct that expresses two N-WASP binding elements (Figure 5 and data not shown). N-WASP binding was critical for actin assembly, because Tir_{505}-H*P, which carries a mutation of the alpha-helical domain was devoid of any actin assembly activity

indicating that the IRTKS-binding region was not sufficient to induce actin assembly in this assay.

Residues in the proline-rich region of an $EspF_U$ repeat that are critical for IRTKS binding.

To further explore what role, if any, the IRTKS-binding region had in actin assembly, we first sought to define the residues of $EspF_U$ required for IRTKS-binding. Thus, we designed a series of mutants to test in the yeast two-hybrid system described in Figure 1. Having previously shown that deletion of the C-terminal 14 residues of an $EspF_U$ repeat blocked binding by IRTKS in yeast two-hybrid assays (Figures 1 and 6), we designed an $EspF_U$ construct that contained only the 23 residue proline-rich region ("PR" in Figure 6) fused to the Gal4AD and co-transformed the reporter strain L40 with LexADBD-IRTKS-SH3. The PR construct closely approximated the reporter activity of a full repeat "HP" (Figure 6). For construction of point mutants, we targeted components of each of the three PxxP domains predicted to be potentially responsible for IRTKS-$EspF_U$ interaction (Figure 1). The previous yeast two-hybrid assays had indicated that the C-terminal 14 residues of $EspF_U$ were critical for binding IRTKS. In addition, as observed in Chapter II, Figure 4B, TirΔC-R30, a Tir-$EspF_U$ fusion that lacks a putative PxxP SH3 binding motif, PNWP (residues 31-34), is defective for actin assembly, indicating a possible role for these residues in actin assembly (Cheng, Skehan et al. 2008). The $PNWP_{34}$ corresponds to a sequence in EspF, $PSRP_{34}$ that has been shown to play a critical role in the binding to the SH3 domain of synexin-9 (SNX9) (Alto, Weflen et al. 2007). Thus, we tested alanine substitution of residues I27, P31, W33, P36, and P39 (Figure 6A) in a yeast two-hybrid assay, and found that each of these residues

significantly diminished binding to IRTKS-SH3 (Figure 6B). P39A had approximately a six-fold defect and I27A, P31A, and W33A completely abrogated any activity in this assay.

IRTKS binding by $EspF_U$ is required for pedestal formation due to its role in recruitment of $EspF_U$ to Tir.

To test the role of IRTKS binding in pedestal formation, we generated HP*HP*, a two-repeat $EspF_U$ construct in which each repeat incorporated the W33A mutation that disrupts IRTKS binding and ectopically expressed it as a GFP-$EspF_U$ fusion in MEFs. When MEFs expressing wild-type GFP-HPHP fusion construct were infected with KC12, an *E. coli* that is capable of translocating Tir but does not express $EspF_U$, the fusion was recruited to sites of bacterial attachment, as demonstrated by myc staining, and induced actin pedestal formation (Figure 7A, top row). As predicted, a two repeat construct defective for binding N-WASP, GFP-H*PH*P, was efficiently recruited to bacteria despite the inability to promote F-actin pedestals (Figure 7A, middle row). Notably, the IRTKS binding mutant GFP-HP*HP* failed to be recruited to bacteria and did not induce any actin assembly, suggesting that IRTKS binding was an essential activity for $EspF_U$ recruitment to sites of clustered Tir.

To test whether the defect of GFP-HP*HP* in actin assembly was due solely to its inability to be recruited to Tir, we tested whether the HP*HP* derivative of $EspF_U$ could stimulate pedestal formation when clustered by translational fusion to Tir. As previously shown, wild-type TirΔC-HPHP, in which the TirC-terminal cytoplasmic domain was replaced by the two repeat HPHP sequence, triggered actin assembly upon clustering by infection with *E. coli* expressing intimin (Figure 7B, top row). The two repeat TirΔC-

H*PH*P that is defective for N-WASP binding and activation was clustered but did not induce actin assembly as predicted (Figure 7B, middle row). Finally, when the two repeat HP*HP* derivative, which lacks the ability to bind IRTKS, was fused to Tir and clustered in the plasma membrane, robust actin pedestals were observed, indicating that the critical actin assembly defect in GFP-HP*HP* was due to its inability to be recruited to Tir by IRTKS.

To determine whether actin assembly could be stimulated upon bacterial clustering of an $EspF_U$ derivative that entirely lacked any IRTKS binding domain, we fused $EspF_U$ derivatives to the near full-length Tir fragment Tir_{505}, which we previously showed could be used to demonstrate pedestal forming activity of a single HP $EspF_U$ repeat (Figure 5). We first confirmed that the W33A mutant that abolishes IRTKS binding had no effect in this assay, because clustering of ectopically expressed Tir_{505}-HP* in MEFs resulted in apparently wild-type levels of actin assembly (data not shown). We then tested Tir_{505}-H30, which lacks 17 residues of the proline-rich segment of a single repeat as well as Tir_{505}-H18 which lacks 29 residues. In fact, both of these Tir_{505} fusions were competent for pedestal formation, indicating that the IRTKS binding domain is entirely dispensable for actin assembly in this assay (Figure 7C).

The ability of the IRTKS-binding defective $EspF_U$ derivative to generate pedestals when translationally fused to Tir suggests that IRTKS is similarly dispensable when pedestal formation is triggered by Tir- $EspF_U$ fusions. To test this, we ectopically expressed HA-tagged TirΔC-HPHP in HeLa cells that were co-transfected with two siRNA duplexes against IRTKS, or two control siRNAs and infected with *E. coli* expressing intimin. IRTKS depleted HeLa cells revealed no quantitative defect in actin

assembly (Figure 8), indicating that IRTKS is not required for assembly when $EspF_U$ and Tir are artificially linked. (The quality of the actin pedestals appeared to differ between IRTKS depleted cells and controls, raising the possibility that IRTKS expression may yet play some role in actin pedestal morphology). Previously, RT-PCR for IRTKS expression in HeLa cells utilizing identical siRNAs indicated that IRTKS mRNA levels were approximately 10% of control (Figure 2b), suggesting that this gene silencing protocol is effective. Consistent with this hypothesis, parallel experiments performed here utilizing HeLa cells expressing GFP-HPHP-myc infected with KC12 which translocates Tir but not $EspF_U$ revealed an actin assembly defect (data not shown).

IRTKS binding by two proline-rich repeats is required for recruitment of $EspF_U$.

Having defined the minimal regions of $EspF_U$ involved in N-WASP mediated actin assembly, we wanted to similarly define the minimum requirements of IRTKS binding to promote efficient recruitment of $EspF_U$. To accomplish this, we generated GFP-$EspF_U$-myc fusion proteins expressing different $EspF_U$ binding domains. GFP-HPHP, a GFP derivative containing two $EspF_U$ repeats, was capable of complementing KC12, an *E. coli* that expresses Tir but not $EspF_U$, for pedestal formation, indicating the derivative contains the information required for both recruitment and potent N-WASP stimulation (Figure 9). GFP-HPH consists of two N-WASP-binding elements and TirΔC-HPH had previously been shown to be capable of inducing actin assembly when clustered at the membrane (Figure 4). However, ectopic expression of GFP-HPH in MEFs followed by infection with KC12, which expresses Tir but not $EspF_U$, revealed a recruitment defect for this construct and correspondingly, a defect in actin assembly (Figure 9). The inability of GFP-HPH to be recruited suggested that efficient recruitment

may require two proline-rich IRTKS-binding sequences. In fact, GFP-PHP was efficiently recruited to sites of bacterial attachment. Furthermore, consistent with the ability of a single N-WASP binding helix to trigger actin assembly when fused to Tir505, GFP-PHP was capable of complementing KC12 for actin pedestal formation (Figure 9). Thus, the minimal functional $EspF_U$ derivative consists of a single N-WASP binding domain flanked by two IRTKS-binding sequences.

Discussion

$EspF_U$ binds and activates WASP-family actin nucleation promoting factors (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004) and artificial fusion of $EspF_U$ to Tir clustered at the plasma membrane is sufficient to trigger actin assembly (Campellone, Cheng et al. 2008; Cheng, Skehan et al. 2008; Sallee, Rivera et al. 2008). However, although Tir, $EspF_U$, and N-WASP are associated in host cells and can be co-immunoprecipitated from infected HeLa cell lysates, neither $EspF_U$ nor N-WASP directly interact with Tir (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004). In fact, antibody clustering of ectopically expressed Tir and $EspF_U$ is sufficient to induce $EspF_U$-mediated actin assembly (Campellone, Cheng et al. 2008), indicating that a third, host-encoded factor, is required for formation of this actin assembly complex. Because each $EspF_U$ repeat contains multiple PxxP sequences, we screened an essentially complete collection of human SH3 domains (Karkkainen, Hiipakka et al. 2006) and identified IRTKS as an avid binding partner of $EspF_U$. Subsequent detection of a ternary complex of $SH3_{IRTKS}$, $EspF_U$, and GBD_{WASP} in vitro supports the model that IRTKS is part of an $EspF_U$/N-WASP-containing complex that potently stimulates Arp2/3 (Vingadassalom, Kazlauskas et al. 2009).

The IRTKS SH3 domain bound to $EspF_U$ and its IMD bound to TirC in yeast two-hybrid assays and was subsequently shown to bind Tir in a manner dependent on the NPY458 sequence, previously shown to be critical for $EspF_U$ recruitment, in infection assays (Vingadassalom, Kazlauskas et al. 2009). Furthermore, IRTKS but not the closely related family member, IRSp53, was recruited to sites of bacterial attachment independent of $EspF_U$ and actin. Ectopic expression of a TirΔC-$SH3_{IRTKS}$ fusion protein recruited bacterially expressed $EspF_U$ and induced actin pedestal formation. Genetic depletion of IRTKS using siRNA inhibited $EspF_U$-mediated actin assembly, a defect that could be rescued by artificially clustering $EspF_U$ at the membrane. Together, these results provide compelling evidence that IRTKS specifically, by interacting with Tir and $EspF_U$, promotes the formation of a complex of bacterial and host factors that trigger robust actin assembly beneath bound bacteria. It should also be noted that in contrast to our results which were obtained using HeLa cells, Stradal and coworkers have implicated IRSp53 in actin pedestal formation using murine cell lines (Weiss, Ladwein et al. 2009), and the relative roles of members of this gene family during pedestal formation in different cell types remains to be fully determined.

IRTKS, as a member of the IRSp53 family, is involved in signal transduction pathways that link deformation of the plasma membrane and remodeling of the actin cytoskeleton (Scita, Confalonieri et al. 2008). IRTKS promotes actin assembly and membrane protrusions when overexpressed in mammalian cells (Millard, Dawson et al. 2007), so it is possible that its role in pedestal formation may extend beyond simply recruiting $EspF_U$ to sites of clustered Tir at the plasma membrane. In fact, an EHECΔ*espF*$_U$ mutant retains the ability to generate low-level Tir-mediated localized

actin assembly in vitro (Campellone, Robbins et al. 2004) and to trigger some AE lesions during infection of the mammalian host (Ritchie, Brady et al. 2008). In addition, mutation of critical residues in the IRTKS-binding site of Tir (NPY_{458}) also blocks the $EspF_U$ independent actin assembly pathway, implicating IRTKS in this low level actin assembly pathway (Brady, Campellone et al. 2007) The IRTKS family member, IRSp53 C-terminal SH3 domain has been shown to interact with cytoskeletal factors such as the Ena/VASP protein Mena, Eps8, and the formin mDia, as well as the Arp2/3 activators WAVE2 (Scita, Confalonieri et al. 2008) and N-WASP (Lim, Bu et al. 2008). The IMD binds F-actin, the GTPase Rac, and lipids, and additionally is structurally reminiscent of a Bin-amphiphysin-Rvs167 (BAR) domain, which binds and deforms membranes, generating invaginations during endocytosis. IMDs, also known as I-BAR (inverse BAR) domains because of their opposite curvature, triggers protrusive membrane deformation (Cory and Cullen 2007; Scita, Confalonieri et al. 2008), and it is tempting to speculate that this activity of the IRTKS IMD might contribute to the morphology of AE lesions. This possibility is underscored by the morphologic difference in $EspF_U$-mediated actin pedestals when IRTKS is genetically depleted (Fig. 9).

Recent work has shown that, within a 47-residue C-terminal $EspF_U$ repeat, a segment consisting of approximately 20 residues forms an amphipathic helix and an extended arm that binds and activates WASP/N-WASP (Cheng, Skehan et al. 2008; Sallee, Rivera et al. 2008). This region has no apparent role in the recruitment of $EspF_U$ to sites of bacterial attachment, because a mutant of the alpha-helical region in $EspF_U$ that disrupts N-WASP binding and actin assembly, appeared fully capable of recruitment.

We show here that a different segment of an $EspF_U$ repeat, rich in prolines, is required for binding to the IRTKS and IRSp53 SH3 domains.

The proline rich segment of an $EspF_U$ repeat contains several PxxP sequences, which are canonical SH3 recognition elements. PxxP sequences are denoted class I or class II depending on their orientation of binding to the SH3 domain. Class I sequences typically carry an essential basic amino acid three residues N-terminal to the PxxP sequence, (i.e. at position -3 relative to P_0xxP_{+3}) (Figure 10A). Prototypic class II sequences carry a basic amino acid two residues C-terminal to P_0xxP_{+3} (i.e. at position +5) (Mayer 2001). In addition, for some SH3 binding sequences, residues considerably distant from the PxxP motif have been shown to be important for recognition (Figure 10). For example, residues -6 and -7 of class I sequences have been shown to contribute to recognition by some SH3 domains (Winters and Pryciak 2005; Kim, Lee et al. 2008). These relatively distant residues are thought to be recognized by a surface of the SH3 domain, i.e. “surface II”, distinct from that recognizing the PxxP sequence, i.e. “surface I” (Figure 10B).

Bockmann et al. examined interaction of the SH3 domain of IRSp53 with several proline rich sequences and derived an 11 amino acid consensus SH3 recogntion sequence (Bockmann, Kreutz et al. 2002) (Figure 10). Interestingly, this sequence contained no basic residue that would identify it as a class I or class II recognition element, however, contrary to most class II sequences, residues C-terminal to the PxxP motif in the consensus sequence had no impact on interaction in these assays, suggesting that the consensus may function as a class I binding site (Mayer 2001; Bockmann, Kreutz et al. 2002). Reinforcing this notion, an IRSp53 recognition element in Eps8 conformed well to

this consensus sequence (Funato, Terabayashi et al. 2004). Finally, probing of a peptide array of overlapping $EspF_U$ peptides with the SH3 domain of IRSp53 resulted in the characterization of binding and nonbinding peptides, and the subsequent identification of a 10 amino acid sequence that was present in all $EspF_U$ peptides competent for IRSp53 recognition and absent in all nonbinding peptides (Weiss, Ladwein et al. 2009).

Although this sequence contains two PxxP sequences (PPAP and PNWP) in the N-terminal region of the sequence, alignment with the previously derived 11 amino acid $SH3_{IRSp53}$ consensus sequence suggests that this 10 residue $EspF_U$ consensus sequence lacks the last three residues of the putative operative PxxP sequence (Fig. 10). In fact, re-deriving an $EspF_U$ consensus sequence based only on high affinity binding peptides (based on the apparent signal in the peptide microarray assay) resulted in a modification of the putative consensus sequence to encompass three more amino acids, which restores the C-terminal PxxP sequence (Fig. 10). Furthermore, the 13-residue $EspF_U$ consensus sequence bears high similarity to the previous consensus sequence based on several ligands of IRSp53, with conservation of four out of six critical proline residues. Notably, the hypothesis that this $EspF_U$ sequence is a class I SH3-binding sequence is supported by alignment with the homologous sequence of EspF, which has been shown to serve as a class I recognition sequence for SNX9 (Alto, Weflen et al. 2007). EspF residues critical for SNX9 recognition (highlighted in yellow in Fig. 10) are not strictly conserved in $EspF_U$, providing an explanation for their disparate SH3 binding specificities.

We assayed binding of the proline rich sequence of an $EspF_U$ repeat to the SH3 domains of IRTKS or IRSp53, and found binding by both domains to be disrupted by alanine substitution of several residues of the consensus sequence. The residues altered,

I27, P31, W33, P36, and P39, span the entire 13 residue consensus sequence, supporting the notion that the consensus sequence in fact encompasses the amino acids critical for SH3 domain recognition. Note that I27 represents position -9 of the putative class I sequence, suggesting that surface II of the IRTKS/IRSp53 SH3 domain recognizes residues considerably distant from the PxxP motif (Fig. 10). The ability of $EspF_U$ peptides which lack the putative operative P_0xxP_{+3} sequence to maintain SH3 recognition in peptide array experiments, albeit with somewhat reduced efficiency (Weiss, Ladwein et al. 2009) suggests that the presumed interaction of $EspF_U$ with surface II of the SH3 domains of IRTKS/IRSp53 contributes greatly to binding.

We took advantage of our ability to abrogate SH3 recognition to probe the role of this interaction in pedestal formation. $EspF_U$ two-repeat derivative harboring alanine substitution of W33 in both repeats was not recruited to sites of bacterial attachment and did not promote formation of actin pedestals. W33 is not located within the N-WASP-binding helix, and the W33A mutant had no apparent effect on actin assembly when $EspF_U$ was clustered artificially: TirΔC-HP*HP*, carrying the W33A substitution in both proline-rich sequences, was competent for pedestal formation. Thus, the $EspF_U$ W33A IRTKS binding mutant was specific for recruitment to sites of bacterial attachment, indicating that the major role of IRTKS is in physically linking the N-WASP binding and activation activity of $EspF_U$ to Tir. Consistent with this hypothesis, when fused to Tir_{505}, the alpha-helical region alone, in the absence of the proline-rich IRTKS binding sequence (or even the $EspF_U$ "arm sequence" that makes extended contacts with N-WASP (Cheng, Skehan et al. 2008), was sufficient to promote actin assembly in this experimental system. These results are consistent with the observation that antibody-mediated

clustering of the N-WASP-binding alpha helix (or repeats thereof) was sufficient to promote actin assembly in mammalian cells (Sallee, Rivera et al. 2008)

The ability of a single $EspF_U$ repeat (or even the N-WASP binding portion of a repeat) to trigger actin pedestal formation upon clustering by *E. coli* expressing intimin is in contrast to our earlier work indicating that, when fused to TirΔC, which lacks the entire Tir C terminus, two adjacent $EspF_U$ alpha helical N-WASP binding sequences were required for efficient pedestal formation (Campellone, Cheng et al. 2008; Padrick, Cheng et al. 2008). (The hypothesis that TirΔC-HP is not folded in a manner that allows N-WASP binding seems unlikely, given that it can still initiate actin assembly when clustered by anti-Tir antibody (Chapter II, Figure 4B)). A possible explanation is that a single $EspF_U$ repeat is indeed fully functional, and the apparent requirement for multiple $EspF_U$ repeats, determined by fusion to TirΔC, is a reflection of the membrane proximity of the first $EspF_U$ repeat, a location that may sterically diminish its ability to promote the formation of a multicomponent actin assembly complex. In this scenario, the first repeat of TirΔC-HPHP may act as a "spacer" to allow for function of the second repeat; this putative spacer function might be fulfilled by TirC sequence in the Tir_{505}-HP fusion.

In contrast, supporting a bona fide role for multiple repeats in promoting robust actin pedestals is that $EspF_U$ derivatives harboring multiple repeats triggered more efficient actin assembly *in vitro* (Campellone, Cheng et al. 2008; Padrick, Cheng et al. 2008). It is possible that when fused to Tir_{505}, multiple repeats indeed trigger pedestal formation more efficiently than a single repeat, but that our assay, visualization of phalloidin-stained pedestals, is insufficiently quantitative to detect that difference. It is also possible that the robust actin assembly activity of a single $EspF_U$ repeat when fused

to Tir_{505} is in part a result of the extensive TirC sequences present in these constructs (and lacking in TirΔC), sequences that may contribute to efficient actin assembly by interacting with other, as yet unidentified host signaling proteins. Finally, perhaps tandem repeats of $EspF_U$ are required for pedestal formation when Tir and $EspF_U$ are delivered by the type III secretion system, but that when a single repeat derivative is ectopically overexpressed as a Tir_{505} fusion protein, membrane clustering using an intimin-expressing bacteria permits a sufficient density of the N-WASP-binding $EspF_U$ sequence to effectively mimic the presence of multiple repeats of $EspF_U$.

The division of a repeat unit into two functional recognition elements parallels the $EspF_U$-related *E. coli* effector EspF. Like $EspF_U$, EspF consists of an N-terminal translocation domain and several 47-residue C-terminal repeats, each of which contains an N-WASP binding segment and a proline-rich sequence that is recognized by an SH3 domain-containing protein that binds and deforms membranes. In the case of EspF, the SH3-containing protein is SNX9, which recognizes EspF in a manner dependent upon a RxAPxxP sequence that in the EspF- $EspF_U$ alignment corresponds to the WxAPxxP in $EspF_U$, that we have demonstrated, plays a role in recognition by IRTKS-SH3 (Marches, Batchelor et al. 2006; Alto, Weflen et al. 2007). The EspF-ligand SNX9 however, contains a BAR domain that participates in membrane remodeling during endocytosis (Lundmark and Carlsson 2003), as opposed to the I-BAR related domain (IMD) found in IRTKS. Although $EspF_U$ can complement some functions of EspF (Viswanathan, Koutsouris et al. 2004), EspF plays no apparent role in pedestal formation (Campellone, Robbins et al. 2004), presumably because its proline-rich sequences target a different SH3-domain. This difference notwithstanding, both EspF and $EspF_U$ alter membrane and

actin dynamics by acting as modular and repetitive adaptor proteins that link N-WASP to a membrane-deforming protein.

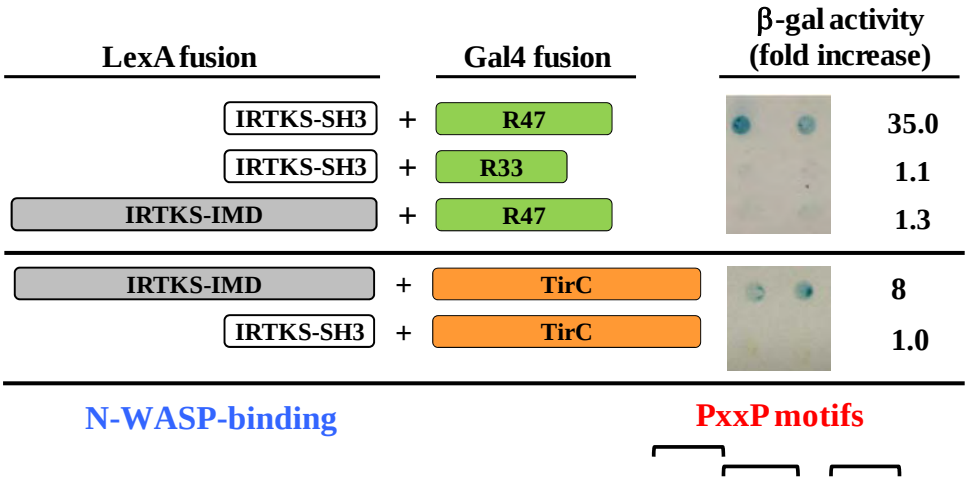
With the identification of IRTKS as an essential link between Tir and $EspF_U$, a striking feature of many components of the actin pedestal signaling cascade is the ability to multimerize (Padrick, Cheng et al. 2008; Sallee, Rivera et al. 2008). The membrane anchoring domain of intimin and the extracellular domain of Tir each encode elements that promote homotypic dimerization (Luo, Frey et al. 2000; Touze, Hayward et al. 2004), leading to the hypothesis that Tir-intimin interactions result in a reticular array-like superstructure of Tir cytoplasmic domains beneath the clustered receptor. This putative array of Tir cytoplasmic domains is recognized by the IRTKS IMD, which, upon dimerization, would be predicted to present physically linked pairs of IRTKS SH3-domains to recruit $EspF_U$. Consistent with this model, we found that although a single proline-rich sequence was capable of binding to IRTKS in a yeast two-hybrid assay, two proline-rich regions are required for efficient recruitment of GFP-$EspF_U$ derivatives to sites of bacterial attachment and for pedestal formation. The repetitive nature of $EspF_U$ also likely contributes to downstream signaling because the tandem N-WASP-binding elements synergistically activate the actin nucleator Arp2/3 (Campellone, Cheng et al. 2008; Padrick, Cheng et al. 2008; Sallee, Rivera et al. 2008). These observations correlate nicely with the finding that all known alleles of $EspF_U$ contain at least two 47-residue C-terminal repeats (Garmendia, Ren et al. 2005). Thus, by targeting distinct domains of Tir and $EspF_U$, IRTKS together with N-WASP, efficiently promotes the formation of a multimeric complex containing bacterial and host factors required to trigger robust EHEC-mediated actin assembly.

Table 1. Strains used in this study.

Strains	Description	Reference
EHECΔ*dam*Δ*espF*$_U$	Derivative of the non toxigenic strain TUV93-0	(Campellone, Roe et al. 2007)
KC14 + pEspF$_U$	EPECΔTir + pKC471	(Campellone, Roe et al. 2007)
KC12	EPECΔ*tir-cesT-eae*::EHEC-HA-*tir-cesT-eae*	(Campellone, Giese et al. 2002)

Table 2. Plasmids used in this study.

Plasmid	Description	Reference
Yeast Two-hybrid plasmids		
pBS50	Derivative of pBTM116 with new multiple cloning site	Present study
pBS51	IRTKS-SH3 domain in pBTM116	(Vingadassalom, Kazlauskas et al. 2009)
pBS53	IRTKS-IMD in pBTM116	(Vingadassalom, Kazlauskas et al. 2009)
pBS58	1R47 in pGAD424	(Cheng, Skehan et al. 2008)
pBS59	1R33 in pGAD424	(Cheng, Skehan et al. 2008)
pBS68	TirC in pGAD424	Present study
pBS102	PR (residues 25-47 of 1R47) in pGAD424	Present study
pBS103	PR_{I27A} in pGAD424	Present study
pBS104	PR_{P31A} in pGAD424	Present study
pBS105	PR_{W33A} in pGAD424	Present study
pBS106	PR_{P36A} in pGAD424	Present study
pBS107	PR_{P39A} in pGAD424	Present study
$EspF_U$ complementation plasmid		
pKC471	$EspF_U$-myc	(Campellone, Robbins et al. 2004)
Transfection plasmids		
pKC689	HA-TirΔC-myc	(Campellone, Cheng et al. 2008)
pKC689-$IRTKS_{SH3}$	HA-TirΔC-IRTKS SH3	(Vingadassalom, Kazlauskas et al. 2009)
pBS65	HA-TirΔC-HPHP	(Cheng, Skehan et al. 2008)
pBS62	HA-TirΔC-HPH	(Padrick, Cheng et al. 2008)
pBS66	HA-TirΔC-HP	(Cheng, Skehan et al. 2008)
pBS64	HA-TirΔC-H*PH*P	Present study
pBS110	HA-TirΔC-HP*HP*	Present study
pBS63	HA-TirΔC-H*PHP	Present study
pBS101	HA-TirΔC-HPH*P	Present study
pBS137	HA-TirΔC-PHP	Present study
pBS144	HA-Tir_{505}	Present study
pBS115	HA-Tir_{505}-HP	Present study
pBS142	HA-Tir_{505}-H*P	Present study
pBS132	HA-Tir_{505}-H_{30}	Present study
pBS134	HA-Tir_{505}-H_{18}	Present study
pBS114	GFP-HPHP	Present study
pBS48	GFP-H*PH*P	Present study
pBS118	GFP-HP*HP*	Present study
pBS123	GFP-HPH	Present study
pBS124	GFP-PHP	Present study



R47 LPDVAQRLMQHLAEHGIQPARNMAEHIPPAPNWPAPTPPVQNEQSRP
R33 LPDVAQRLMQHLAEHGIQPARNMAEHIPPAPNW

Figure 1. IRTKS-SH3 domain binds to a single $EspF_U$ repeat and IRTKS-IMD binds to TirC.

Plasmids encoding the IRTKS-SH3 domain or the IRTKS-IMD were fused to the LexA DNA-binding domain and co-transformed with $EspF_U$ or Tir derivatives fused to the Gal4 transcriptional activation domain into a yeast two-hybrid reporter strain L40. β-galactosidase activity was assessed as an average of three co-transformants and compared with negative controls. Similar results were seen in other transformations.

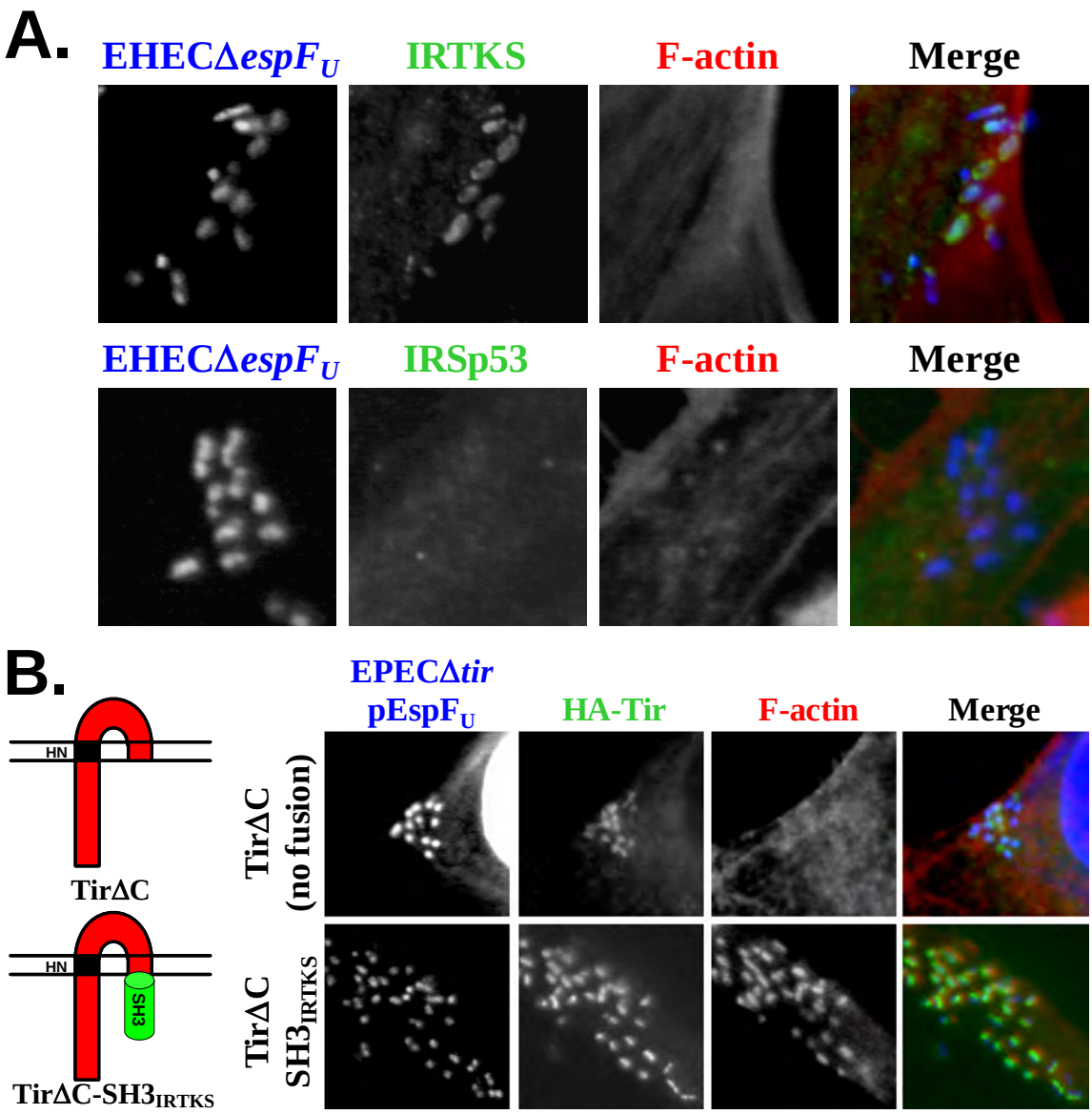


Figure 2. IRTKS is recruited to sites of bacterial attachment in an $EspF_U$-independent manner, and clustering of its SH3 domain is sufficient to trigger pedestal formation. **A.** HeLa cells were infected with EHECΔ*dam*Δ$EspF_U$ and examined after staining with anti-IRSp53 or anti-IRTKS antibody (green), DAPI to localize attached bacteria (blue), and Alexa568-phalloidin (red). **B.** HeLa cells ectopically expressing HA-tagged Tir derivatives in which the C-terminal cytoplasmic domain was deleted (TirΔC) or replaced with the IRTKS SH3 domain (TirΔC-$SH3_{IRTKS}$) were challenged with KC14/p$EspF_U$, a strain that expresses intimin and $EspF_U$ but not Tir, and stained with DAPI (blue) to detect bound bacteria, anti-HA antibody (green) to detect HA-Tir, and Alexa568-phalloidin (red) to detect F-actin.

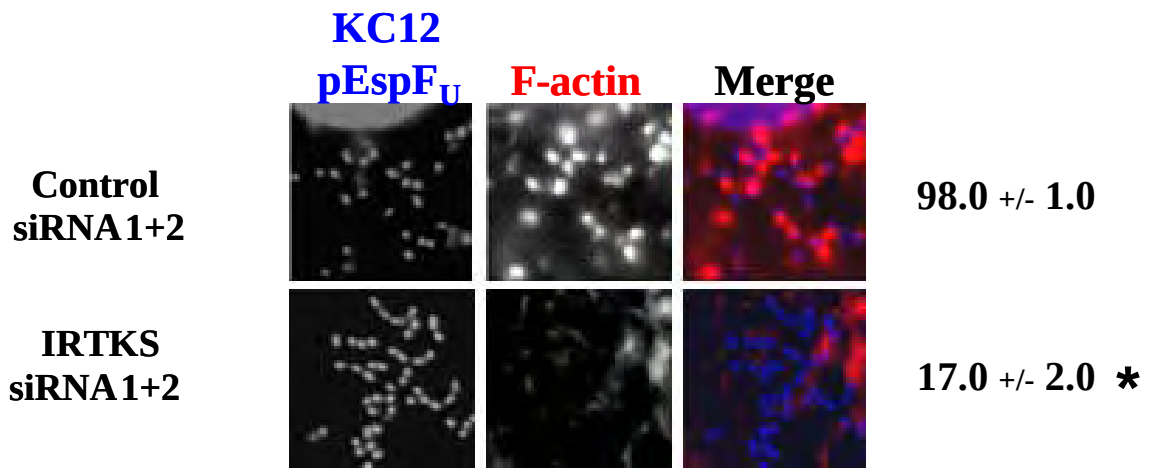


Figure 3. Genetic depletion of IRTKS inhibits $EspF_U$-dependent pedestal formation. HeLa cells transfected with pairs of control or IRTKS siRNAs were infected with KC12/pEspF$_U$. Monolayers were examined after staining with DAPI (blue) to detect bound bacteria and Alexa568-phalloidin (red) to detect F-actin. The percentage of cells competent for actin pedestal formation after infection is shown (*Right*). Shown is the mean +/- SD of at least 3 experiments; *P=0.0001.

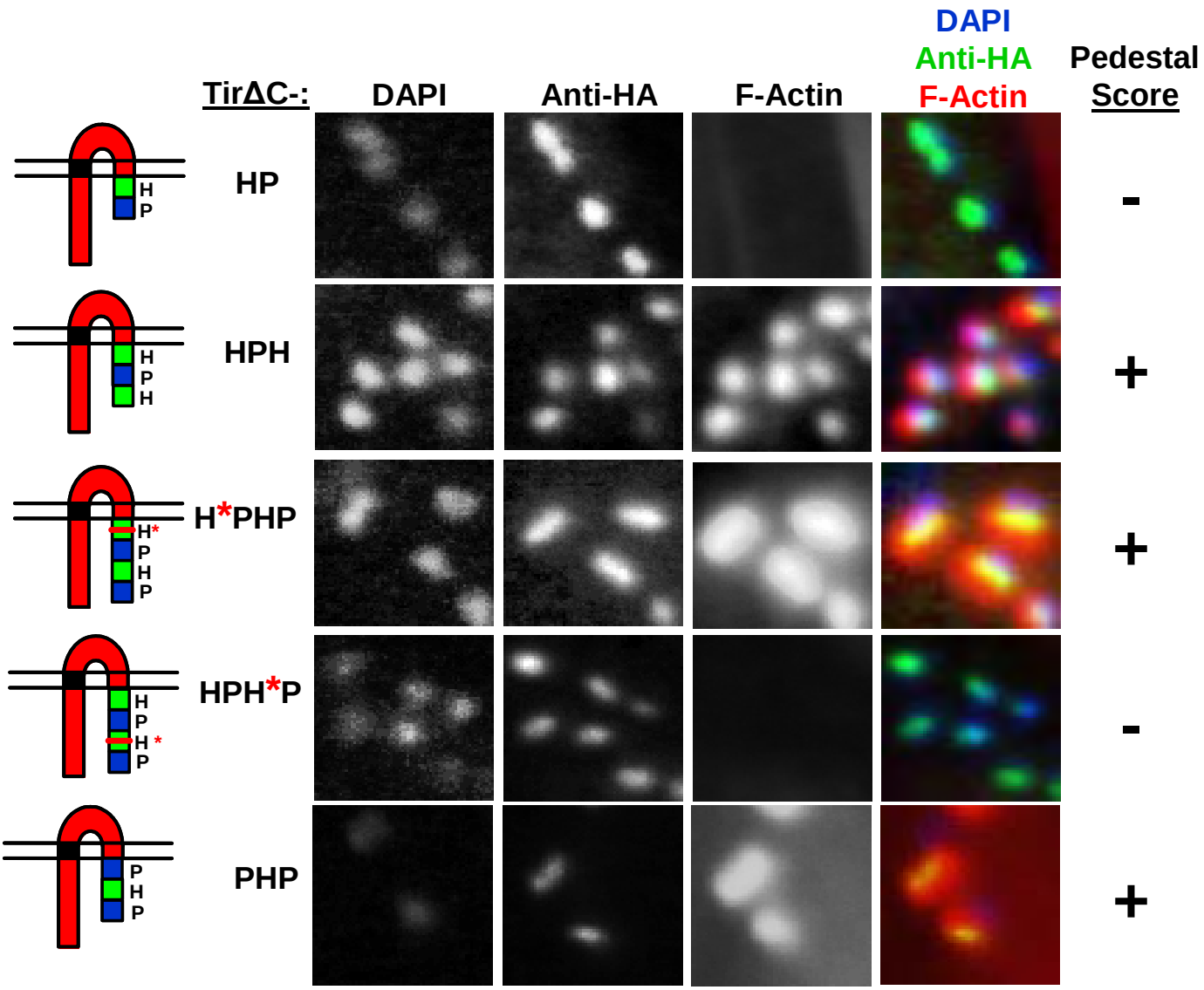


Figure 4. A Tir-EspF$_U$ fusion protein harboring a single N-WASP-binding helix flanked by IRTKS-binding sequences can promote actin assembly.
Mouse fibroblast-like cells (MEFs) ectopically expressing HA-tagged Tir derivatives in which the entire C-terminal cytoplasmic domain (residues 387-558) was replaced with the indicated EspF$_U$ derivatives were challenged with intimin expressing *E.coli* and stained with DAPI (blue) to detect bound bacteria, anti-HA antibody (green) to detect HA-Tir, and Alexa568-phalloidin (red) to detect F-actin. Models of each transfected construct (left) indicate EspF$_U$ derivatives that have been mutated to block the N-WASP binding domain (H) with a red line in the domain and an *.

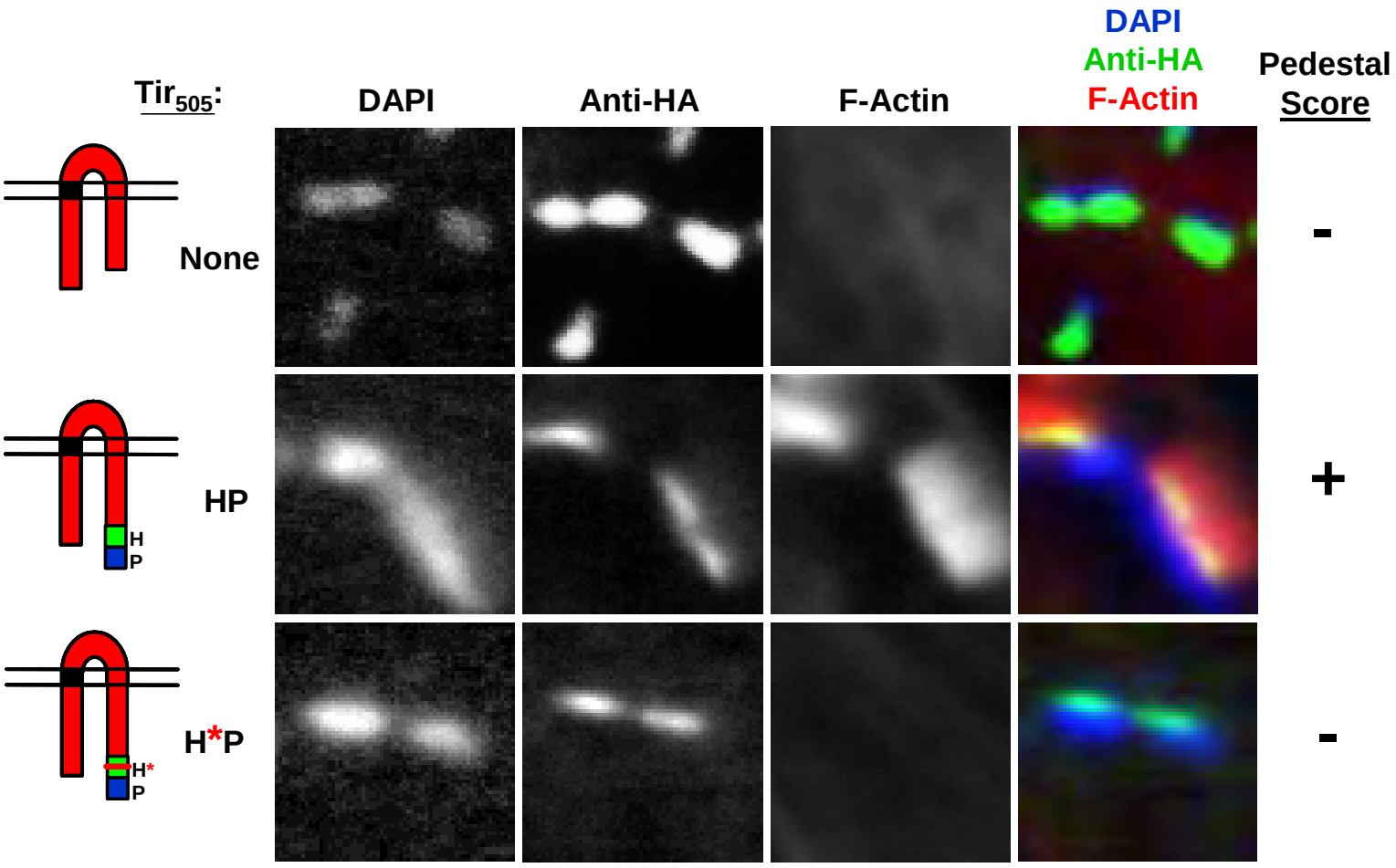


Figure 5. A single $EspF_U$ repeat can stimulate actin pedestal formation when fused to Tir residue 505 near the Tir C-terminus.
Tir_{505}, a nearly full length HA-tagged EHEC Tir derivative (harboring an NPY->RRR substitution to abolish IRTKS-binding), was fused to various derivatives of $EspF_U$, and ectopically expressed in mouse MEFs. Cells were then infected with *E. coli* expressing intimin and stained with DAPI (blue), anti-HA antibody (green), and Alexa568-phalloidin (red).

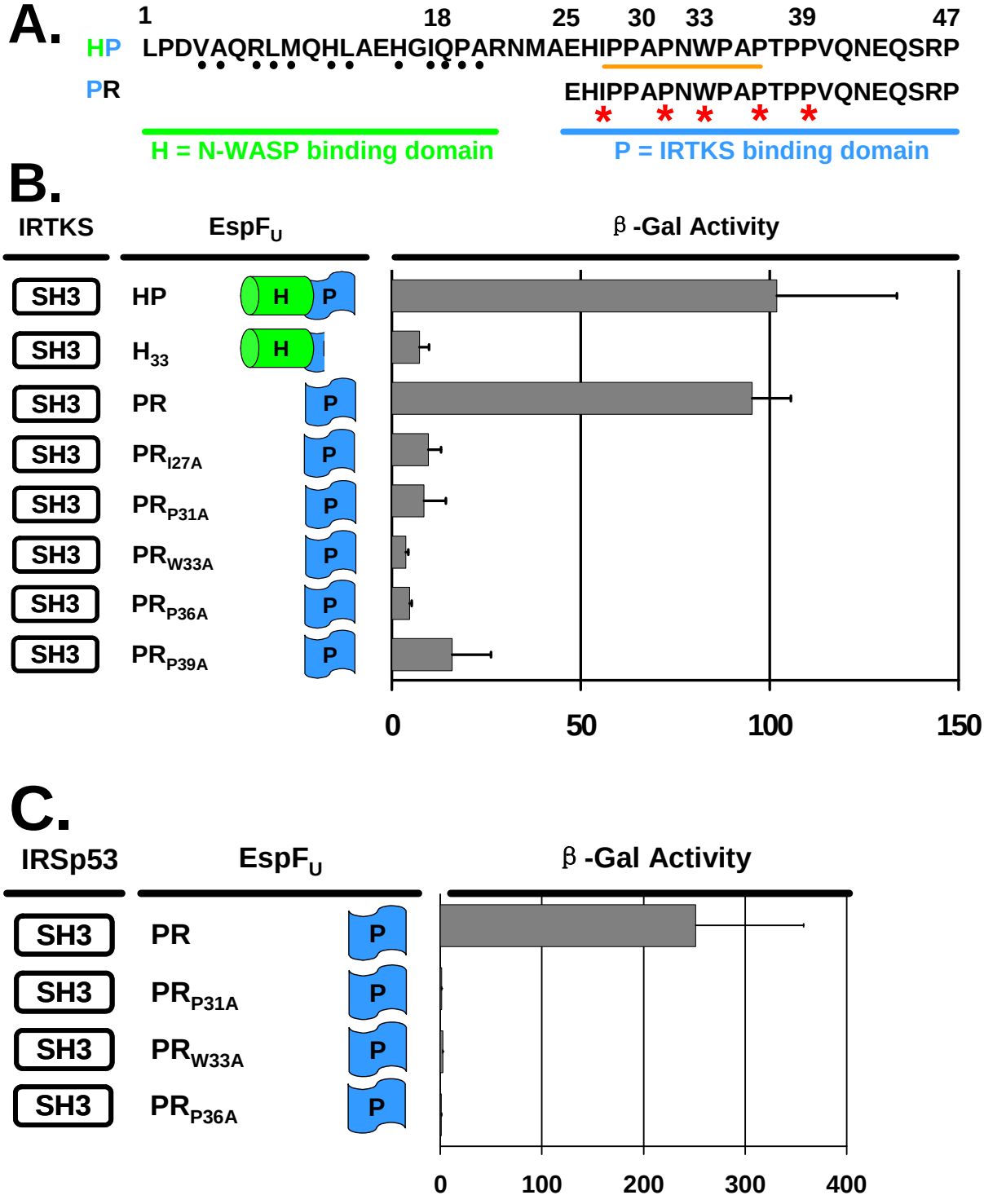
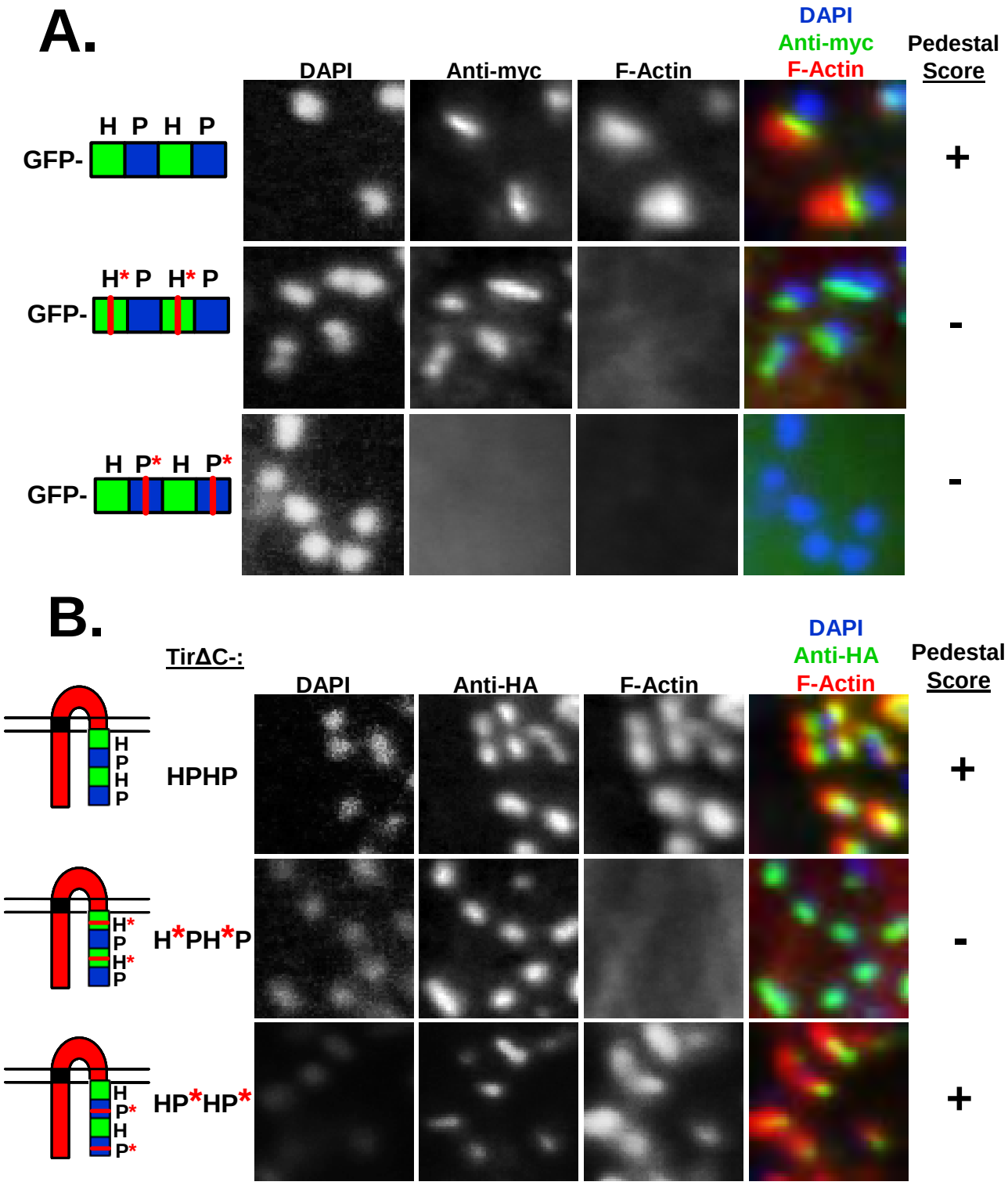


Figure 6. IRTKS binding by EspF$_U$ is dependent upon residues in the proline-rich region of an EspF$_U$ repeat.

A. The sequences of EspF$_U$ derivatives used in yeast two-hybrid analysis. The N-WASP binding domain (Cheng et al., 2008) and IRTKS binding domain are indicated. Residues of EspF$_U$ that are predicted to contact the GTPase binding domain (GBD) of N-WASP

are indicated with ●. Asterisks represent mutations made in Gal4- $EspF_U$ fragments. "PR" describes the proline-rich residues of $EspF_U$ that are thought to interact with IRTKS-SH3 and represents the positive control used to identify IRTKS-binding deficient mutants. The orange line indicates a consensus sequence for $EspF_U$-IRSp53 interaction identified by Weiss et al. **B.** Plasmids encoding the IRTKS SH3-domain fused to the LexA DNA-binding domain and $EspF_U$ derivatives fused to the Gal4 transcriptional activation-domain were co-transformed into a yeast two-hybrid reporter strain L40. β-galactosidase activity was assessed as an average of three co-transformants in Miller Units (MU) with error bars indicating the standard deviation between the three. Similar results were seen in other transformations. **C.** Plasmids encoding the IRSp53SH3 domain fused to the LexA DNA-binding domain and $EspF_U$ derivatives fused to the Gal4 transcriptional activation domain were co-transformed into a yeast two-hybrid reporter strain L40. β-galactosidase activity was assessed as in panel B.


A.
DAPI
Anti-myc
F-Actin
DAPI
Anti-myc
F-Actin
Pedestal Score
H P H P
GFP-
+
H* P H* P
GFP-
-
H P* H P*
GFP-
-
B.
TirΔC-:
DAPI
Anti-HA
F-Actin
DAPI
Anti-HA
F-Actin
Pedestal Score
HPHP
+
H*PH*P
-
HP*HP*
+

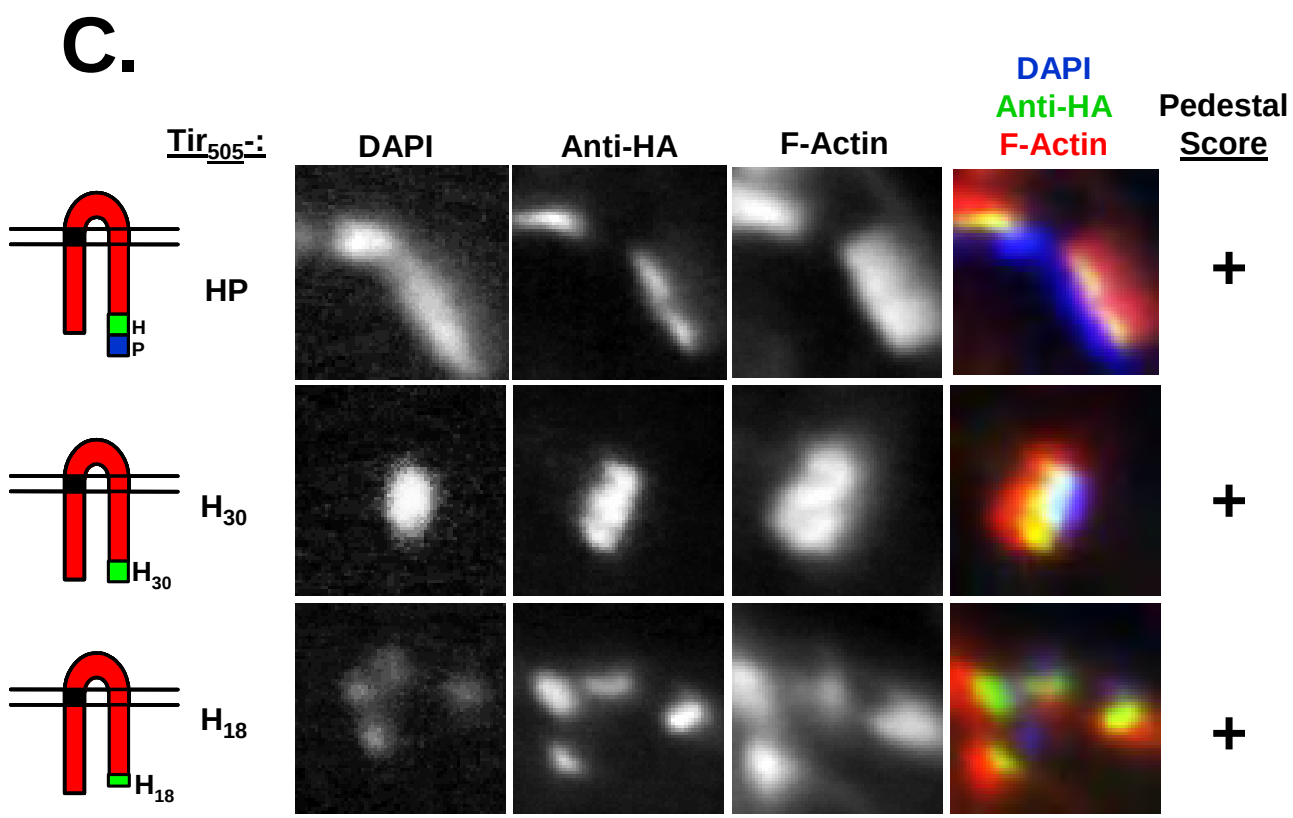


Figure 7. IRTKS-binding by $EspF_U$ is required for pedestal formation due to its role in recruitment of $EspF_U$ to Tir.

A. GFP-$EspF_U$-myc fusion constructs were ectopically expressed in mouse MEFs. The cells were then infected with KC12 and stained with DAPI (blue) to detect bound bacteria, anti-myc antibody (green) to detect $EspF_U$ -myc, and Alexa568-phalloidin (red). The merged images indicate co-localization of $EspF_U$ and actin in yellow. **B.** HA-tagged Tir Δ C fused to the indicated derivatives of $EspF_U$ were ectopically expressed in mouse MEFs and clustered using intimin expressing *E. coli*. Cells were then stained with DAPI (blue), anti-HA antibody (green), and Alexa568-phalloidin (red). **C**. Murine MEF's ectopically expressing HA-tagged EHEC Tir_{505} fused to indicated fragments of the alpha-helical domain (H) of $EspF_U$ were challenged with intimin-expressing *E. coli*.

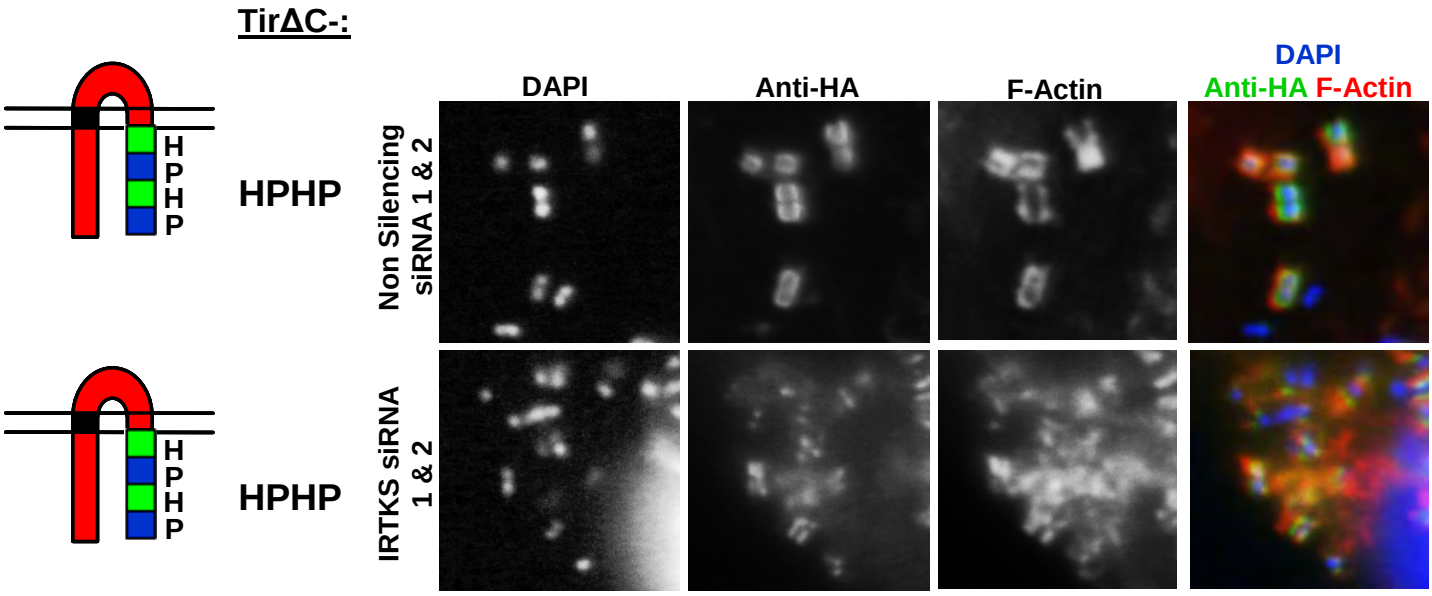


Figure 8. IRTKS is not required for actin pedestal formation when $EspF_U$ is artificially clustered at the membrane.

HA-tagged Tir Δ C-HPHP was ectopically expressed in HeLa cells that had been transfected with either control non-silencing siRNA duplexes or siRNA duplexes that genetically deplete IRTKS. Cells were subsequently infected with *E. coli* expressing intimin and stained with DAPI (blue) to detect bound bacteria, anti-HA antibody (green) to detect HA-Tir, and Alexa568-phalloidin (red) to detect F-actin.

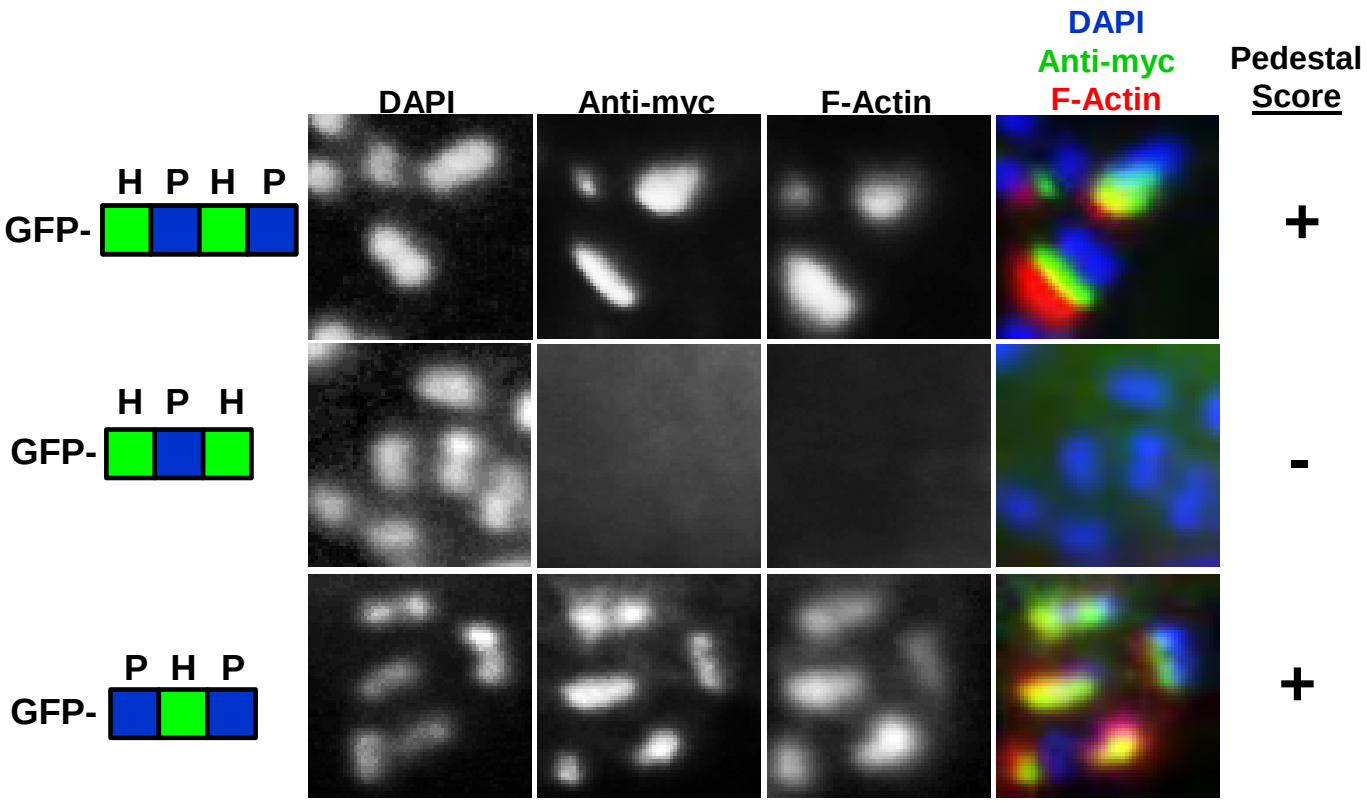


Figure 9. IRTKS binding by two proline-rich repeats is required for recruitment of EspF$_U$.
GFP-EspF$_U$-myc derivatives were ectopically expressed in mouse MEFs. Cells were subsequently infected with KC12, which translocates Tir but not EspF$_U$, and stained with DAPI (blue), anti-myc antibody (green), and Alexa568-phalloidin (red).

A.

BAI1	PPPQQPLPPPP	(Bockmann, Kreutz et al. 2002)
Mena	PLPSTGPPPPP	(Bockmann, Kreutz et al. 2002)
WAVE1	PPPPVPPPPPP	(Bockmann, Kreutz et al. 2002)
Shank 3 (P1)	PPPQTAPPPPP	(Bockmann, Kreutz et al. 2002)
Shank 3 (P2)	PPPTFSPPPPP	(Bockmann, Kreutz et al. 2002)
Shank 1	PPPTTYPPEPP	(Bockmann, Kreutz et al. 2002)
Shank 2	PPPQSVPPPPP	(Bockmann, Kreutz et al. 2002)
Consensus #1:	PpPxxxppxPP	(Bockmann, Kreutz et al. 2002)
Eps8	PGIPPPPRAPAPVPP	(Funato, Terabayashi et al. 2004)
$EspF_U$	IPPAPNWPAP	(Weiss, Ladwein et al. 2009) present in binders; absent in non-binders
	IPPAPNWPAPPLP	(Weiss, Ladwein et al. 2009) present in high affinity binders; absent in non-binders
EspF	LGFKPARPAPPPP	(Alto, Weflen et al. 2007) class I: +XΨPXΨP; "Ψ"=hydrophobic, "+"=basic)
	-9 -6 -3 0 +3	

B.

	-9 -6 -3 0 +3	
$EspF_U$	27-IPPAPNWPAPPLP-39	
	A A A A A	

Figure 10. SH3 domain recognition .
(A) Alignment of proline-rich sequences in ligands known to bind to the SH3 domain of IRSp53 Residues boxed in green are conserved among these sequences and residues boxed in yellow represent amino acids key for recognition of EspF by its ligand, SNX9. (B) Substitutions in $EspF_U$ that abrogated binding to $IRTKS_{SH3}$ in yeast two-hybrid assays.

CHAPTER IV

DISCUSSION

The observation that the EHEC secreted effector $EspF_U$, required for robust actin assembly, co-localized and bound to the actin nucleation promoting factor, N-WASP provided a clue as to how these bacteria manipulate the actin cytoskeleton during infection (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004). In fact, N-WASP is required for EHEC to induce actin assembly during an infection, similar to other Type III secreting pathogens such as EPEC and *Shigella* (Egile, Loisel et al. 1999; Gruenheid, DeVinney et al. 2001; Lommel, Benesch et al. 2004). $EspF_U$ has been shown to bind and activate WASP family nucleation promoting factors and artificial clustering of $EspF_U$ at the plasma membrane is sufficient to induce actin assembly (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004; Cheng, Skehan et al. 2008). Given the multiple modalities that are present in the host cell to regulate WASP family members, the specific mechanism used by $EspF_U$ to activate N-WASP and thus induce F-actin pedestal formation was of particular interest. Perhaps the most widely studied mechanism of N-WASP activation is through the small Rho-GTPase Cdc42 which binds to the GBD of N-WASP at the CRIB (Cdc42/Rac interactive binding domain) inducing a conformational change that globally destabilizes the autoinhibited conformation (Kim, Kakalis et al. 2000; Buck, Xu et al. 2001; Leung and Rosen 2005) and can be further destabilized by interaction with other ligands like Toca-1 (Leung, Ally et al. 2008). This frees the VCA domain to interact with Arp2/3 complex resulting in the formation of a branched actin network. Previous studies have shown that $EspF_U$ binds N-WASP in the

GBD at a location distal to the CRIB domain (Garmendia, Carlier et al. 2006), indicating that it activates N-WASP by some other means.

In work presented here, NMR resolution of the structure of $EspF_U$ in complex with the GBD revealed that $EspF_U$ forms an amphipathic alpha-helix, similar to the C region of N-WASP when bound to the GBD in an autoinhibited conformation (Cheng, Skehan et al. 2008). Functional i*n vitro* and *in vivo* studies validated the derived structure of the complex. Truncation of the helix, or mutation of key residues in the helix, resulted in decreased N-WASP binding, and in diminished actin assembly both *in vitro* and in mammalian cells (Cheng, Skehan et al. 2008; Sallee, Rivera et al. 2008). The structural and functional data together, indicate that $EspF_U$ binds and activates N-WASP through structural competition, displacing the VCA domain from the GBD. Thus, inherent in the binding and recruitment of N-WASP, by $EspF_U$ is the displacement of the N-WASP VCA domain, with the resultant activation of N-WASP and stimulation of Arp 2/3. Both recruitment and activation of N-WASP by $EspF_U$ are likely required for pedestal formation: plasma membrane expression of a Tir-N-$WASP_{VCA}$ fusion protein, which expresses a constitutively active VCA domain, does not induce actin pedestal formation until membrane-clustered, a step analogous to recruitment (Campellone, Cheng et al. 2008). Conversely, expression of a truncated N-WASP derivative lacking the VCA domain (and thus incapable of activation) is recruited to sites of bacterial attachment but not only does not promote actin assembly, but also acts as a dominant negative inhibitor of pedestal formation.

EHEC requires two secreted effectors to induce efficient actin pedestal formation, Tir and $EspF_U$. While Tir, $EspF_U$, and N-WASP can be immunoprecipitated in a complex

from infected cell lysates, no direct interaction between Tir and $EspF_U$ or Tir and N-WASP has been shown (Campellone, Robbins et al. 2004; Garmendia, Phillips et al. 2004). Ectopic expression of $EspF_U$ and Tir in host cells followed by Tir clustering is sufficient to recruit $EspF_U$ and induce actin assembly (Campellone, Cheng et al. 2008). These observations indicate that recruitment of $EspF_U$ to Tir is mediated by an additional host factor.

Panning of a phage display library, expressing the human SH3 proteome, on recombinant $EspF_U$ enriched for binding of the SH3-domain containing proteins IRTKS (Vingadassalom, Kazlauskas et al. 2009). IRTKS contains an IMD that bound to Tir *in vitro* and IRTKS localized to sites of bacterial attachment independent of $EspF_U$. Ectopic expression of the IMD or the SH3 domains of IRTKS blocked pedestal formation, as did genetic silencing of IRTKS. Also enriched from the SH3 phage display library by affinity panning on $EspF_U$ was the SH3 domain of IRSp53, a homolog of IRTKS. In pulldown experiments, full length IRSp53 and its IMD directly interacted with Tir in a manner dependent upon Tir NPY_{458} (D. Vingadassalom, pers. comm.). However, unlike IRTKS ,IRSp53 did not localize to sites of bacterial attachment independent of $EspF_U$ (Vingadassalom, Kazlauskas et al. 2009), and ectopic expression of the $IRSp53_{IMD}$ only marginally diminished pedestal formation. Genetic depletion of IRSp53 had no discernible effect. Weiss et al., have found that IRSp53 can similarly bind to Tir, and in contrast to our findings, that IRSp53 localized to sites of bacterial independent of $EspF_U$ (Weiss, Ladwein et al. 2009). Finally, pedestal formation was significantly diminished on a cell line deficient in IRSp53, a defect that was rescued by ectopic expression of IRSp53 or IRTKS. The source of these apparently discrepant results is not clear.

Notably, while Stradal and coworkers utilized MEFs, we predominantly used HeLa cells, raising the possibility that the relative importance of IRSp53 or IRTKS in pedestal formation could vary with the specific cell type. However, utilizing MEFs, we have not found IRSp53 to be recruited to sites of bacterial attachment in the absence of $EspF_U$, indicating that the divergent findings are not simply a reflection of cell species.

IRTKS might be responsible for low-level actin assembly induced by EHEC in the absence of $EspF_U$ as this activity is abrogated by disruption of the IRTKS-binding site. It is conceivable that an ancestral *E. coli* strain lacking $EspF_U$ triggered this low level actin assembly pathway, which presumably provided some selective advantage. Given that more robust actin assembly associated with $EspF_U$ results in expansion of bacterial numbers during mammalian infection (Ritchie, Brady et al. 2008), it is tempting to speculate that acquisition of the means to promote efficient actin assembly was acquired. In the case of EPEC, a Nck-binding site could have evolved at Tir Y474 which permitted tyrosine phosphorylation and subsequent recruitment Nck and N-WASP resulting in robust actin assembly. EHEC may have diverged by acquiring $EspF_U$, an effector expressed on a bacteriophage, presumably by horizontal transfer. $EspF_U$ is homologous to the LEE-encoded effector EspF, which also contains multiple C-terminal repeats harboring an N-WASP-binding alpha helical domain that activates N-WASP,and is capable of inducing actin assembly when artificially clustered at the membrane (Vingadassalom, unpublished results), but plays no role in actin pedestal formation in the course of a physiological infection (Campellone, Robbins et al. 2004). The variation in repeat sequences of EspF and $EspF_U$ is predominantly in the proline rich region (Figure 1), which for EspF mediates binding to the SH3 domain of SNX9. Thus, it is tempting to

speculate that EspF and $EspF_U$ have a common origin, and that divergence of only a few amino acids in the proline rich segment resulted in disparate SH3 domain binding specificity and distinct function.

N-WASP-binding mutants of $EspF_U$, incapable of actin assembly were efficiently recruited to Tir, indicating that the N-WASP-binding domain is not required for recruitment. In contrast, GFP-$EspF_U$ derivatives harboring a point mutant that interferes with IRTKS binding were incapable of recruitment to Tir and correspondingly, $EspF_U$-mediated, actin assembly. Artificial clustering of these IRTKS-binding deficient derivatives at the plasma membrane was sufficient to rescue the actin assembly defect. These data indicate that $EspF_U$ contains two distinct functional elements in each C-terminal repeat, an N-WASP-binding alpha-helix, and an IRTKS-binding proline-rich region.

Having defined the key residues involved in each distinct functional domain of an $EspF_U$ repeat, perhaps the most intriguing question remaining is the apparent necessity for multiple repeats. To date, no $EspF_U$ expressing *E. coli* found in nature has expressed fewer than two repeats, and single repeat derivatives cannot complement an EHECΔ*espF*$_U$ mutant (Garmendia, Ren et al. 2005; Campellone, Cheng et al. 2008). Here we found that a minimum of two IRTKS-binding elements are required for recruitment to EHEC Tir, indicating that an actin assembly defect in smaller $EspF_U$ derivatives is likely due at least in part, to inefficient recruitment. Consistent with this hypothesis, ectopic expression of HPH, which can activate robust actin assembly when artificially clustered at the membrane in TirΔC-HPH, was insufficient for recruitment to Tir and thus unable to induce pedestal formation. It is possible that $EspF_U$ binding by N-

WASP or IRTKS results in steric interference in binding of the reciprocal protein to an adjacent binding site. For example, N-WASP binding to GFP-HPH may prevent efficient binding by IRTKS, and account for the recruitment defect of this a derivative. Gel filtration chromatography has indicated that the $WASP_{GBD}$ and $IRTKS_{SH3}$ can form a ternary complex with a five repeat derivative of $EspF_U$ (Vingadassalom, Kazlauskas et al. 2009), and this approach could be taken to test whether two $EspF_U$ repeats are required for complex formation. Another way to address the requirements of two proline-rich regions in a functional assay is to independently mutate each P region in the GFP-PHP construct. Experiments examining the function of P*HP and PHP* should reveal further information. Alternatively, there may be an absolute requirement for two proline-rich regions for IRTKS binding.

To define the minimum sequence of $EspF_U$ required to activate N-WASP and promote actin pedestal formation, we utilized three different experimental systems. Replacement of TirC with $EspF_U$ derivatives that were clustered in the membrane indicated a minimum requirement of three tandem functional domains, either TirΔC-HPH or TirΔC-PHP. Studies have shown that multimerization of WASP family proteins dramatically increases the kinetics of Arp2/3 activation (Campellone, Cheng et al. 2008; Padrick, Cheng et al. 2008; Sallee, Rivera et al. 2008). Despite lacking a second N-WASP-binding element, TirΔC-PHP could promote clustering of N-WASP that mimics multimerization by other means. First, it has been shown that high efficiency antibody clustering of Tir- $EspF_U$ fusion containing only a single repeat unit could induce actin assembly, whereas intimin-expressing bacteria, which may not cluster the Tir-$EspF_U$ derivative as efficient as antibody, could not (Cheng, Skehan et al. 2008; Sallee, Rivera et

al. 2008). Second, dimerization of IRTKS through its IMD (Suetsugu, Murayama et al. 2006) would permit cross-linking of two proline-rich regions of $EspF_U$ and could lead to functional multimerization of $EspF_U$.

Finally, the binding capacity of full length $EspF_U$ is still poorly understood. The data has shown that each N-WASP binding element in $EspF_U$ can bind the N-WASP GBD at a 1:1 stoichiometric ratio *in vitro* (Cheng, Skehan et al. 2008), but the ability of each $EspF_U$ repeat to bind multiple ligands including IRTKS, IRSp53, N-WASP, and possibly others in the host cell remains unclear. If each binding element in an $EspF_U$ repeat is capable of binding a ligand, one can imagine that the potential for dramatic multimerization of actin assembly machinery is possible (Figure 2). Tir clustering, IRTKS dimerization, and recruitment of N-WASP by alpha-helical domains in $EspF_U$ could induce large complex formation of actin assembly components at the membrane. The proximity of the host plasma membrane to the alpha helical domain of $EspF_U$ during clustering of the TirΔC-HP fusion could sterically prevent efficient formation of this complex, a limitation that is overcome when a single N-WASP-binding alpha-helix is extended from the membrane as seen with Tir_{505}-H_{18}. An alternative explanation for this finding is that Tir_{505}, while not capable of stimulating detectable actin assembly in artificial clustering experiments, may bind other host factors that increase the efficiency of $EspF_U$ -mediated actin assembly *in vivo*.

Conclusions and Future Work

Modification of the actin cytoskeleton is a requirement for infection and colonization of a variety of pathogens. Further understanding of the mechanisms involved in these host-

pathogen interactions will yield information impacting virulence of these pathogens and provide a model system for studying normal actin dynamics in the host. The use of EHEC as a model system to study actin dynamics has uncovered a novel N-WASP activation mechanism and demonstrated the significance of multimerization of components involved in actin assembly. Finally, the role of IRTKS in EHEC-mediated actin assembly indicates that EHEC has evolved to utilize a host protein to link two secreted effectors that impact colonization. Further understanding of the EHEC-mediated actin assembly pathway will yield new insights into host-pathogen interactions and likely unveil new information regarding the regulation of actin dynamics in other cellular processes.

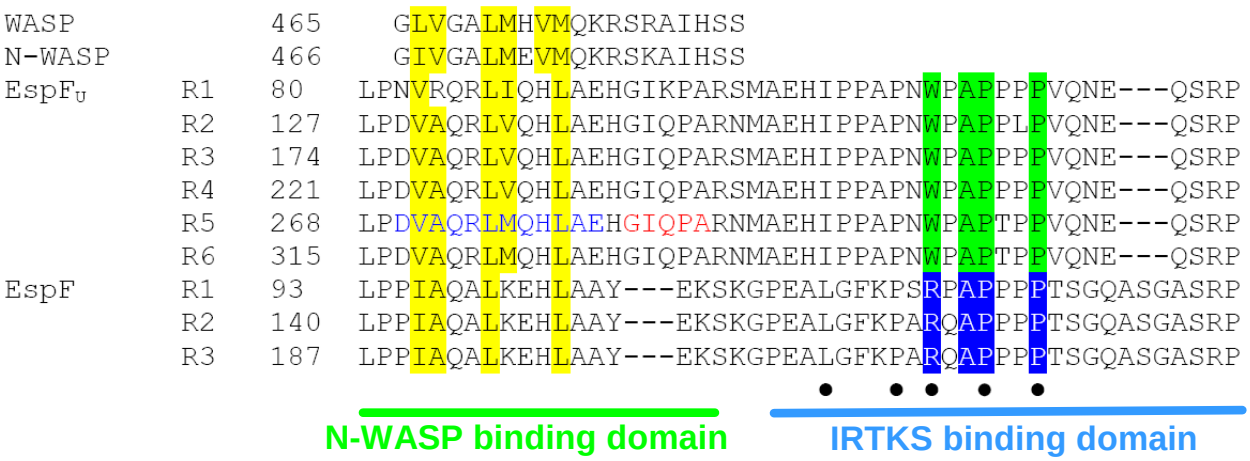


Figure 1. The proline-rich region of $EspF_U$ binds IRTKS SH3 domain.
Sequence alignment of WASP and N-WASP VCA C regions, the fifth repeat element of $EspF_U$ and the first repeat of EspF. Helix residues in the GBD-R33 complex are blue. Residues in the extended $EspF_U$ arm are red. Aligned hydrophobic residues are boxed in yellow. $EspF_U$ residues that are predicted to bind to IRTKS are boxed in green. EspF residues that are predicted to bind SNX9 are boxed in blue. Mutations in $EspF_U$ that blocked interaction in yeast two-hybrid assays are indicated by •.

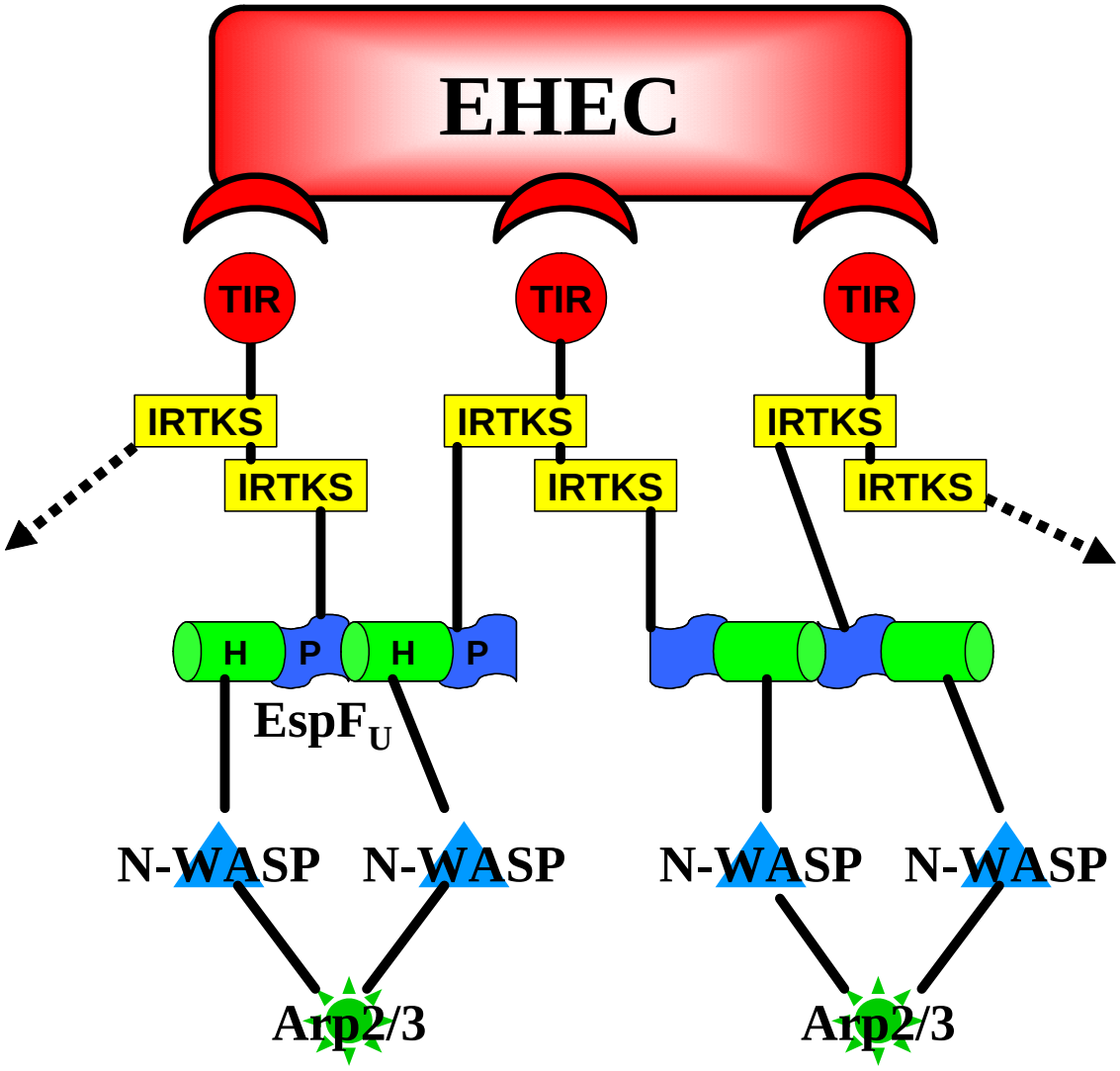


Figure 2. A model demonstrating the significance of multimerization in EHEC-mediated actin assembly.

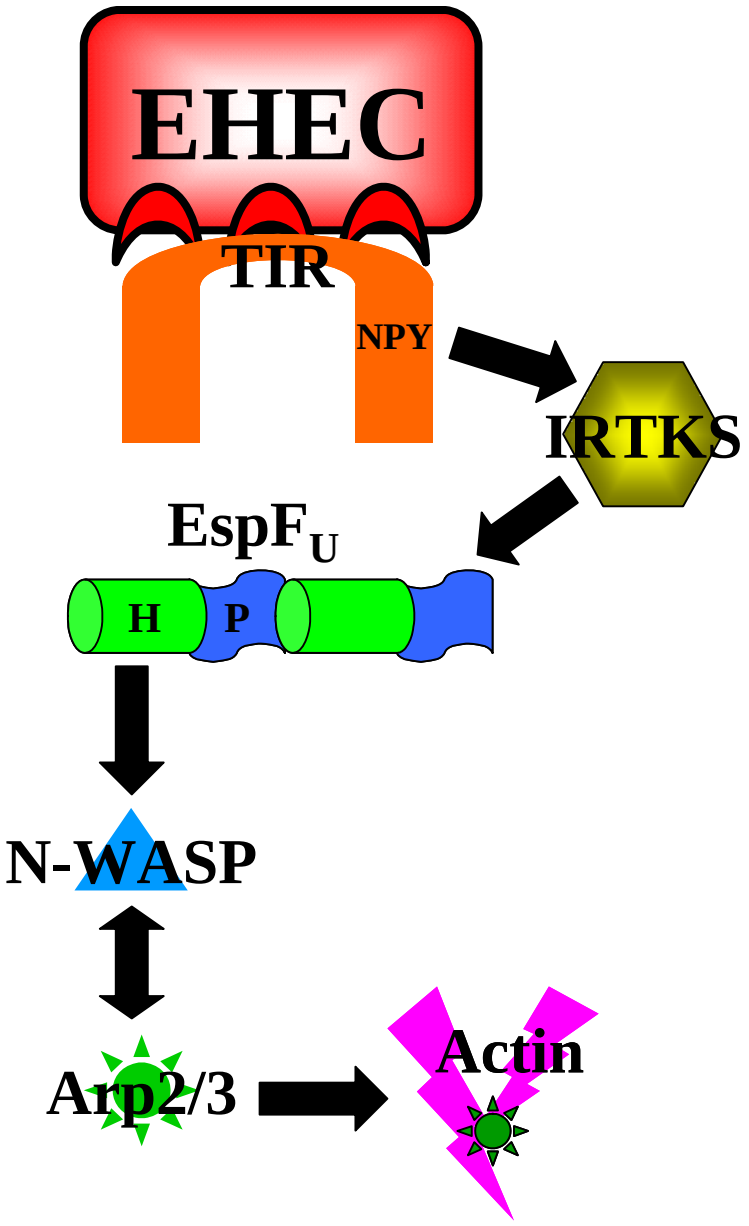


Figure 3. A new working model of Tir-mediated actin assembly by EHEC.

APPENDIX

The following is data from other work that I conducted while in the lab that was not integral to the story presented in my thesis, but may have an impact on future work conducted in the lab.

STM MUTAGENESIS

The use of RAPID (Red Assisted Pathogenicity Island Deletion) was important for identifying the critical Enterohemorrhagic *E. coli* actin assembly effector $EspF_U$ (Campellone, 2004). Another interest of the lab is in identifying other EHEC virulence factors that are critical for colonization of the host. To isolate non-LEE encoded effectors that were critical for colonization, we wanted to expand our library of O-Island deletion mutants first designed to identify $EspF_U$. O-Islands are defined as regions of EHEC serotype O157:H7 that differ in sequence from non-pathogenic E. coli strains as described by Perna et al. A strategy was designed to delete O-Islands that were larger than 1kb that might contain genes important for colonization or other uncharacterized virulence factors. These islands were then replaced with the *cat* gene as previously described (Campellone, 2004) and then tested for actin assembly. All deletion mutants were then tested for actin assembly defects in-vitro with none showing any defect. Tables 1 and 2 list the O-Island Mutants that were successfully made using these techniques in strains EDL933 and the Δstx strain, TUV93-0, respectively. Primers used to create these deletions are in Table 3.

Table 1. O-Island Deletions in EDL933

	O-Island	BP		ORF	Size (kb)
1	OI-1	18,376	24,701	6	6.33
2	OI-7	240,937	275,501	29	34.56
3	OI-8 CP-933H; CP933I	304,283	329,948	34	25.67
4	OI-14-15	367,178	383,667	11	16.49
5	OI-28-29	579,948	609,214	11	29.27
6	OI-30	664,345	675,120	6	10.78
7	OI-35	843,500	856,978	15	13.48
8	OI-44 CP-933M	1,250,521	1,295,459	84	44.94
9	OI-47	1,421,105	1,453,002	29	31.90
10	OI-50 CP-933N	1,628,193	1,673,449	58	45.26
11	OI-51 CP-933C	1,678,756	1,693,687	22	14.93
12	OI-52 CP933X (partial)	1,744,969	1,755,067	13	10.10
13	OI-61	1,974,096	1,980,357	6	6.26
14	OI-62-63	2,021,072	2,024,821	4	3.75
15	OI-66	2,083,901	2,085,489	4	1.59
16	OI-67	2,098,695	2,104,384	3	5.69
17	OI-70	2,227,512	2,235,042	9	7.53
18	OI-76 CP-933T	2,668,389	2,688,820	29	20.43
19	OI-77	2,709,000	2,712,426	4	3.43
20	OI-79 CP-933U	2,743,081	2,782,196	46	39.12
21	OI-80	2,788,634	2,799,118	4	10.48
22	OI-102 P22	3,264,482	3,277,230	14	12.75
23	OI-115-116	3,784,516	3,805,333	27	20.82
24	OI-122	3,920,980	3,942,164	25	21.18
25	OI-133	4,343,953	4,346,981	3	3.03
26	OI-138	4,399,815	4,415,332	19	15.52
27	OI-139	4,420,464	4,427,255	10	6.79
28	OI-141	4,519,832	4,525,226	5	5.39
29	OI-148A LEE (partial)	4,677,931	4,686,861	15	8.93
30	OI-149-50	4,697,340	4,709,959	14	12.62
31	OI-153	4,755,317	4,761,966	4	6.65
32	OI-154	4,767,476	4,773,916	7	6.44
33	OI-156	4,865,772	4,868,268	5	2.50
34	OI-172 (partial)	5,384,041	5,403,508	13	19.47
35	OI-173-5	5,444,873	5,467,575	6	22.70
			Total:	594	546.74

Table 2. O-Island Deletions in TUV93-0

	O-Island	BP		ORF	Size (kb)
1	OI-1	18,376	24,701	6	6.33
2	OI-28-29	579,948	609,214	11	29.27
3	OI-30	664,345	675,120	6	10.78
4	OI-35	843,500	856,978	15	13.48
5	OI-47	1,421,105	1,453,002	29	31.90
6	OI-51 CP-933C	1,678,756	1,693,687	22	14.93
7	OI-61	1,974,096	1,980,357	6	6.26
8	OI-62-63	2,021,072	2,024,821	4	3.75
9	OI-66	2,083,901	2,085,489	4	1.59
10	OI-67	2,098,695	2,104,384	3	5.69
11	OI-70	2,227,512	2,235,042	9	7.53
12	OI-76 CP-933T	2,668,389	2,688,820	29	20.43
13	OI-102 P22	3,264,482	3,277,230	14	12.75
14	OI-112-13	3,692,855	3,696,230	4	3.38
15	OI-133	4,343,953	4,346,981	3	3.03
16	OI-138	4,399,815	4,415,332	19	15.52
17	OI-139	4,420,464	4,427,255	10	6.79
18	OI-141	4,519,832	4,525,226	5	5.39
19	OI-149-50	4,697,340	4,709,959	14	12.62
20	OI-153	4,755,317	4,761,966	4	6.65
21	OI-154	4,767,476	4,773,916	7	6.44
22	OI-156	4,865,772	4,868,268	5	2.50
23	OI-173-5	5,444,873	5,467,575	6	22.70
			Total:	235	249.68

Table 1. Primers used in this study.

OI-1koF	5’-TTAGTTGTACATTACCACGATTTTGACTCGGCTCATTATTTGCCCGCTTGGCGGCCGCATGAGACGTTGAT-3’
OI-1koR	5’-ATGACAGATGGTATCTCAACTTCGCCACATTGTCTTTATAAATCAAATATGCGGCCGCTTTCGAATTTCTGC-3’
OI-7koF	5’-ATGAATTCAAAAAAGCTTTGTTGCATATGTGTGTTATTCTCGCTGCTTGCGCGGCCGCATGAGACGTTGAT-3’
OI-7koR	5’-ATAATTTGAAGCATCTGAACTCCCTTGCTTTGCTTTTATTGGATTATCTGGCGGCCGCTTTCGAATTTCTGC-3’
OI-8koF	5’-ACCGTAACAAGCAACAGGCAGGCGTGACAGCCAGCAAACCAAAACTCGACGCGGCCGCATGAGACGTTGAT-3’
OI-8koR	5’-AAGCGCACCGGCCTTCTGGCGTGCTGTAACAAAGGTCAGTACGCCCGCGCGCGGCCGCTTTCGAATTTCTGC-3’
OI-14koF	5’-TGGTCCGGTGAAAGCGGTGTATCTGATTATTGAGGATTGCTGAGAGAGGTGCGGCCGCATGAGACGTTGAT-3’
OI-14koR	5’-GAATTGCCACTTAATGCCAACCATTGCAGAGGTGTCATTATATCCCCTATGCGGCCGCTTTCGAATTTCTGC-3’
OI-28koF	5’-ATGGCGCGATTCCAGTTTAAAAACCGTAAAAATAATGGACTTATTTTTTTGCGGCCGCATGAGACGTTGAT-3’
OI-28koR	5’-ATGCTTATCTTTATCCCGATTCTCATTTTTGTCGCGCTGGTCATTGTCGGGCGGCCGCTTTCGAATTTCTGC-3’
OI-30koF	5’-TTACGAAAGCCCGCTCCCCGCAAAGACTGACGCCAGATAGTTTCTGTCCAGCGGCCGCATGAGACGTTGAT-3’
OI-30koR	5’-ATGTCAACCGGATTACGTTTCACACTGGAAGTGGACGGCCTGCCACCGGAGCGGCCGCTTTCGAATTTCTGC-3’
OI-35koF	5’-ATGTTCACCGCTTTTCGTCAAGTCGTGCCATTTGTCAGCCAGGATCGGGTGCGGCCGCATGAGACGTTGAT-3’
OI-35koR	5’-ATGAGCACCGACCGTAAGCCCGTTATGTTATTGTTTCATTAGTAATAATCGCGGCCGCTTTCGAATTTCTGC-3’
OI-44koF	5’-ATGAAAATCAAGCATGAACACATCCGCATGGCGATGAATGCCTGGGCGCAGCGGCCGCATGAGACGTTGAT-3’
OI-44koR	5’-TCAGGCCAGCGGTGGAGTCTTCAGAGAACCCGTAATTATTCCCGGTAGTTGCGGCCGCTTTCGAATTTCTGC-3’
OI-47koF	5’-ATGCTTATCATTGATCAGTCTGCTATCACCCGCTTTTACTGGCAATTGTAGCGGCCGCATGAGACGTTGAT-3’
OI-47koR	5’-TCATTTGAATACCTTCGCATCACTAAATGCTTTACGACAATGCATAACTCGCGGCCGCTTTCGAATTTCTGC-3’
OI-48koF	5’-ATGGCAGTATTGACGGATACGAAAGCAAGACATATCAAACCTGATGACAAGCGGCCGCATGAGACGTTGAT-3’
OI-48koR	5’-CTATACTGCCAGCATATCAGGATAGTGTTTTGCGATAATATCATTCAGTCGCGGCCGCTTTCGAATTTCTGC-3’
OI-50koF	5’-AAAATCAGCCTGACTCGGGATCAGTTTTTGCCAGATAACTGAAACGTATTGCGGCCGCATGAGACGTTGAT-3’
OI-50koR	5’-ATGGAATTTATCAATGCTTCCTACATCGCAATTACGACCGACCGGGACATGCGGCCGCTTTCGAATTTCTGC-3’
OI-51koF	5’-GTGATGACCGGAATCAAAATTATGAGCAGAGCACTTAACAAACTGAGCGAGCGGCCGCATGAGACGTTGAT-3’
OI-51koR	5’-GTGGGTTTTACCTGTTCGGTAGGTGTTATGATTATCGTAATACCTTTCCCGCGGCCGCTTTCGAATTTCTGC-3’
OI-52koF	5’-GTTTTTACTATTTATCTTTGTTTGTGGTTCTCCTTCAGCAAGCTCAGCGCGCGGCCGCATGAGACGTTGAT-3’
OI-52koR	5’-TTTTTTCCAGAGGCGGTCGAACTCCGCCTTCGCAAAATAAGCGGAAGCGTGCGGCCGCTTTCGAATTTCTGC-3’
OI-61koF	5’-ATGAAACTCAAACATGTTGGTATGATTGTCGTTTCTGTGTTGGCGATGTCGCGGCCGCATGAGACGTTGAT-3’
OI-61koR	5’-TTATTCATAGATAAAAGTGACACCAATGACTGACTGGACGGTTCCGGCTGGCGGCCGCTTTCGAATTTCTGC-3’
OI-62koF	5’-ATGAAATTAAAAATAGTTGCGGTGGTTGTAACTGGTTTGTTAGCTGCGAAGCGGCCGCATGAGACGTTGAT-3’
OI-62koR	5’-TTAGTGATAAAAAGGCCATGAGCTGGAGGAAAACGATCTGTCCAGAAAGTGCGGCCGCTTTCGAATTTCTGC-3’
OI-64koF	5’-CAGAACTGATATAATATCTGACCGTCATTTTTTGGCTCTTTCTTTTGTTCGCGGCCGCATGAGACGTTGAT-3’
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OI-67koF	5’-TTAGAACGTTTTCTTAAAGTTCAGCATCACGCCTTTGTCGCTGATGCCGTGCGGCCGCATGAGACGTTGAT-3’
OI-67koR	5’-ATGAATATTTACTACGATTTATCTTCAAATTCTGCATGGCTTGTTACGCAGCGGCCGCTTTCGAATTTCTGC-3’
OI-70koF	5’-TTACTTTTGATTGTCGATTCTGGTGAGAGCAACCAGGAATGCCAGTGTATGCGGCCGCATGAGACGTTGAT-3’
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OI-76koF 5’-TTACCCTCTATTGTCAAAAGGGTTCAATTCAACAGCAGCCTCTAAATGACGCGGCCGCATGAGACGTTGAT-3’
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OI-148AkoF 5’-TTTCTGTTATCATTACTGCCAATATTTGTTGTTATTGGTACTTCATTCCTGAAAGCGGCCGCATGAGACGTTGAT-3’
OI-148AkoR 5’-AAAGCTGTCGAAATATTAATCGCGATAATGATATCCACCACAACTGTTGGTAGTGCGGCCGCTTTCGAATTTCTGC-3’
OI-149koF 5’-GTGTTTAAAACCAAATGGTTTGCAAGGGAAGCCAGGTCTCATGCAATAACGCGGCCGCATGAGACGTTGAT-3’
OI-149koR 5’-GTGAGGTATATTATGGCAAGTCCTATAAACAGCGGAATAATGATGGCAAAGCGGCCGCTTTCGAATTTCTGC-3’
OI-153koF 5’-TTAAGCCTGGGTTATATTTTGTACAATATCGGGATACCGTTCGAACCAATGCGGCCGCATGAGACGTTGAT-3’
OI-153koR 5’-TTGTCTGTTGGTAATGAGGAGAAGTATTCAATAACCCATAAAGATATAACGCGGCCGCTTTCGAATTTCTGC-3’
OI-154koF 5’-TTAGTTTACAAATTCAATGGTTAAGGTCGCGGTAGCGTTAAAAACGCCGGGCGGCCGCATGAGACGTTGAT-3’
OI-154koR 5’-ATGTATAGTCTTTCCACAAATATAGGCAAAAAATATAATTTGAAAGTGTGGCGGCCGCTTTCGAATTTCTGC-3’
OI-156koF 5’-CTAAAAATTGTCTTTTTTAAAGTTATGCGGATTGAAAACTTGCTCTATTTGCGGCCGCATGAGACGTTGAT-3’
OI-156koR 5’-TTATCTTTTTTTGAAAAAAATTAAGATAAGAAAAAGAACAATATTTGTTAGCGGCCGCTTTCGAATTTCTGC-3’
OI-172koF 5’-TTGATGCCAGCCAGGTTGGTCATTCTCAAATACCTCAGCCTCGGGGAATAGCGGCCGCATGAGACGTTGAT-3’
OI-172koR 5’-TCCAGCGATAATATAACTCCTAAGGTCATTACCCGGGGCAACAGCTCGCGGCGGCCGCTTTCGAATTTCTGC-3’
OI-173koF 5’-TCATGCTTGCGCTCCTTGCGTTGGGTTGCGACGTTCTTCGAAGTTAAGCCGCGGCCGCATGAGACGTTGAT-3’
OI-173koR 5’-ATGACTGATACGCATTCTATTGCACAACCGTTCGAAGCAGAAGTCTCCCCGCGGCCGCTTTCGAATTTCTGC-3’

HOST FACTORS REQUIRED FOR N-WASP INDEPENDENT ACTIN ASSEMBLY

Previous work done by our lab has indicated that while N-WASP can be bound and potently activated by $EspF_U$, it is not necessary for $EspF_U$ -mediated actin assembly if both Tir and $EspF_U$ are ectopically expressed. This has been shown through ectopic expression of Tir and $EspF_U$ and subsequent antibody or *E.coli* expressing intimin induced clustering in N-WASP deficient FLCs. Further examination done by Brady et al, has indicated that N-WASP is important for efficient translocation of effectors by EHEC into host cells, indicating that the defect observed during infection assays on N-WASP deficient cells is due to inadequate translocation of the critical effectors, Tir and $EspF_U$. These results indicate that if these two effectors can be efficiently delivered to an N-WASP deficient cell, $EspF_U$-mediated actin assembly can still occur. In fact, a genetically modified EPEC strain, KC12 + p$EspF_U$ which expresses EHEC *tir-eae* and is complemented with $EspF_U$ on a plasmid, is capable of infecting N-WASP deficient cells and inducing actin assembly presumably due to the enhanced ability to translocate effectors when compared to $EspF_U$. This actin assembly seems to be dependent on the Arp2/3 pathway as overexpression of the N-WASP VCA domain can inhibit this phenotype, indicating that there is a second NPF, other than N-WASP, that can interact with $EspF_U$ to induce actin assembly. To investigate this pathway we attempted to identify potential candidates through immunofluorescent staining in F-actin pedestals or by depletion through RNAi expression that would inhibit actin assembly. Preliminary assessment of the candidates listed in Table 4 by immunofluorescence staining or RNAi depletion revealed no phenotype in $EspF_U$-mediated N-WASP-independent actin

assembly, although the affinity of the antibodies used to verify their presence in pedestals or efficient depletion remains in doubt.

Table 4. Candidates for NPF involved in N-WASP independent actin assembly pathway.

Protein of Interest	Tested by IF	Tested by Depletion
Abi1	yes	yes
Cortactin	no	yes
Pak1	no	yes
VASP	yes	yes
WAVE2	yes	yes

In order to take a less biased approach to identify potential candidates for the N-WASP-independent actin assembly NPF, we utilized a variety of in-vitro actin assembly assays to enrich for potential candidates using a cell-free extract assay. Cell-free extracts were made from wild-type FLCs and N-WASP deficient FLCs. Recombinant GST-EspFU-His and Tir-His were added to extracts and allowed to incubate for 30 minutes. Gultathione beads were then added to the extract to pulldown GST-EspF_U and any associated proteins. Mass spectrometry analysis of three bands isolated on polyacrylamide gel was performed by the University of Massachusetts Medical School Core Facility and those results are attached in Table 5.

Table 5. Mass spectrometry analysis of bands isolated fromGST-EspF_U pulldowns.

Searched against SwissProt using Sequest, Mascot and X!Tandem

			Number of Peptides Identified		
Protein name	**Accession numbers**	**Protein MW**	**Gel 1, Top**	**Gel 2, middle**	**Gel 3, bottom**
Myosin heavy chain, nonmuscle type A (Cellular myo	MYH9_RAT	226326	114	1	1
Myosin heavy chain, nonmuscle type B (Cellular myo	MYHA_RAT	228953	61	1	
Glutathione S-transferase 26 kDa (EC 2.5.1.18) (GS	GT26_SCHJA	25482	1	31	12
Trypsinogen, cationic precursor (EC 3.4.21.4) (Bet	TRY1_BOVIN	25407	1	1	2
RNA-binding protein cabeza (Sarcoma-associated RNA	CAZ_DROME	39124	1		
Glutathione S-transferase Mu 1 (EC 2.5.1.18) (GST	GTM1_MOUSE	25823		8	
30S ribosomal protein S3.	RS3_ECOLI	25834		7	1
Heat shock 27 kDa protein (HSP 27) (Growth-related	HS27_MOUSE	22996		7	
Glutathione S-transferase Mu 2 (EC 2.5.1.18) (GST	GTM2_MOUSE	25569		3	
Glutathione S-transferase P 2 (EC 2.5.1.18) (GST Y	GTP2_MOUSE	23461		2	12
Glutathione S-transferase Mu 5 (EC 2.5.1.18) (GST	GTM5_MOUSE	26619		2	
Ras-related protein Rab-14.	RB14_HUMAN	23910			2

Leong, Sample report created on 05/17/2007

Searched against IPI Mouse using Sequest and X!Tandem

			Number of Peptides Identified		
MS/MS View:Identified Proteins (10)	**Accession Number**	**Protein MW**	**Gel 1, Top**	**Gel 2, middle**	**Gel 3, bottom**
Gene_Symbol=Myh9 Myosin-9	IPI00123181	226 kDa	125	6	
Gene_Symbol=Myh10 Myosin, heavy polypeptide 10, non-muscle	IPI00338604 (+1)	233 kDa	60	1	
Gene_Symbol=Gstp1 Glutathione S-transferase P 1	IPI00555023	24 kDa		2	12
Gene_Symbol=Gstm1 Glutathione S-transferase Mu 1	IPI00230212	26 kDa		9	
Gene_Symbol=Hspb1 Isoform A of Heat-shock protein beta-1	IPI00128522	23 kDa		7	
Gene_Symbol=Myh9 15 days pregnant adult female amnion cDNA, RIKI	IPI00788324	226 kDa	2	1	
Gene_Symbol=Myh10 Myosin-10	IPI00515398	229 kDa	1	1	
Gene_Symbol=Gstm2 Glutathione S-transferase Mu 2	IPI00228820 (+1)	26 kDa		3	
Gene_Symbol=Gstm5 Glutathione S-transferase Mu 5	IPI00114380	27 kDa		2	
Gene_Symbol=Gsta4 Glutathione S-transferase A4	IPI00323911	26 kDa			1

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