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Characterizing the Role of Cortactin in Actin Pedestal Assembly by Enterohemorrhagic Escherichia coli (EHEC)

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Characterizing the role of Cortactin in actin pedestal assembly by enterohemorrhagic *Escherichia coli*

An honors thesis by

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May 2013

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TABLE OF CONTENTS

I. Abstract…4

II. Introduction…5-10

III. Materials and Methods…11-14

IV. Results…15-19

V. Discussion…20-22

VI. Figures and Legends…23-32

VII. References…33-35
Enterohemorrhagic *Escherichia coli* (EHEC) is a major foodborne cause of bloody diarrhea and renal failure. During colonization of the intestine, EHEC injects the transmembrane receptor protein Tir and the cytoplasmic effector protein EspF₁ into host cells to reorganize the actin cytoskeleton into adhesion “pedestals.” EspF₁ has been shown to bind and activate the actin nucleation factor N-WASP to drive actin polymerization into pedestals. However, EspF₁ can still assemble pedestals in cells lacking N-WASP, suggesting that this effector protein is able to also trigger N-WASP-independent pathways of actin polymerization during infection. Cortactin is an atypical nucleation factor that localizes to pedestals, but its precise role in pedestal formation in the presence or absence of N-WASP has not been well defined. To test whether Cortactin functions in pedestal assembly, I used RNA interference to silence Cortactin expression in N-WASP wild type (WT) and N-WASP knockout (KO) mouse fibroblasts, infected these cells with an *E. coli* strain expressing myc-tagged EspF₁ and HA-tagged Tir, and examined them for actin pedestals. Consistent with previous observations, N-WASP KO cells contained slightly fewer and less-intensely stained actin pedestals than WT cells when each was treated with control siRNAs. Similarly, Cortactin depletion in WT cells resulted in a modest decrease in the number and intensity of actin pedestals. However, when Cortactin expression was silenced in KO cells, pedestal assembly was virtually abolished even though Tir and EspF₁ were still present. These results suggest that N-WASP and Cortactin are functionally redundant, and that Cortactin plays a previously-unrecognized potent role in N-WASP-independent pedestal assembly.
INTRODUCTION

Epidemiology and Clinical Relevance of Pathogenic *E. coli*

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are closely related bacteria that cause diarrheal illnesses after ingestion of contaminated food and water. EPEC causes infantile diarrhea in underdeveloped countries and has been used as a model organism for studying host-pathogen interactions for many years. In contrast, EHEC - most notably serotype O157:H7 - is a more clinically relevant pathogen in the US, Europe, and Japan, where it causes severe bloody diarrhea and hemolytic uremic syndrome (HUS) (Donnenberg and Whittam 2001). HUS is a life-threatening condition characterized by acute renal failure, and EHEC infection is the leading cause of pediatric kidney failure in the US (Razzaq, 2006). Throughout the 1990s, EHEC frequently appeared in the news due to outbreaks traced back to undercooked ground beef from fast food restaurants. Although the number of outbreaks has decreased over the past 8 years, the diversity of food sources containing the bacteria have increased. In 2011, outbreaks from contaminated lettuce, spinach, bologna, and hazelnuts took place across 18 states (CDC, 2011). There is no effective treatment for EHEC infection, and antibiotics can actually increase the risk of complications and HUS (Serna and Boedecker 2008).

The overall goal of my project is to gain a better understanding of the mechanisms of EHEC pathogenesis so that we may eventually develop new therapies for EHEC-associated disease.

**EHEC Pathogenesis: Attaching and Effacing “Pedestal” Lesions and Shiga Toxins**

After ingestion, EHEC colonizes its human hosts by adhering tightly to intestinal epithelia and generating attaching and effacing lesions, structures characterized by a loss of normal brush border microvilli and cytoskeletal rearrangements into actin “pedestals” beneath bound bacteria
These lesions contribute to disease symptoms by creating a replication niche and causing tissue damage (Hayward et al., 2006). Upon successful colonization, EHEC can release large quantities of Shiga toxins (Stx) into the intestine, which enter endothelial cells, inhibit protein synthesis, and eventually cause cell death (Johannes and Romer, 2011). Since the assembly of pedestal lesions is a requirement for all subsequent aspects of EHEC pathogenesis, it is important to gain a better understanding of the mechanisms of cytoskeletal rearrangements used during colonization.

Functions and Regulation of the Actin Cytoskeleton

The actin cytoskeleton is present in all eukaryotes and is crucial for controlling cell shape, cell movement, and many aspects of membrane transport (Figure 1). Globular (G)-actin monomers continually undergo cycles of polymerization into filamentous (F)-actin followed by periods of depolymerization, and the assembly of actin into filaments is a driving force underlying numerous cellular functions (Campellone and Welch 2010). To nucleate new actin filaments and organize them into branched networks, virtually all cells rely on the Arp2/3 complex, a seven-subunit protein complex. However, the Arp2/3 complex is not a potent nucleator by itself. It requires cooperation with actin nucleation-promoting factors (NPFs) in order to effectively nucleate actin into filaments (Goley and Welch 2006).

Mammalian cells express two classes of NPFs that control the assembly of actin monomers into filaments (Rottner et al., 2010). The largest group, Class I, is comprised of the Wiskott-Aldrich Syndrome Protein (WASP) family members: WASP, N-WASP, WAVE1, WAVE2, WAVE3, WASH, WHAMM, and JMY (Figure 2). All of the Class I proteins contain a conserved C-terminal WCA domain which includes one or more WASP homology 2 (WH2)
motifs that bind G-actin and a connector-acidic (CA) peptide that binds the Arp2/3 complex (Campellone and Welch 2010). Each family member appears to have a distinct cellular function (Rottner et al., 2010). For example, WASP and N-WASP are involved in the formation of membrane rearrangements during phagocytosis and endocytosis, the WAVE proteins and JMY drive plasma membrane ruffling and cell migration, WASH controls endosome trafficking, and WHAMM influences ER to Golgi transport (Figure 1). Class II NPFs are structurally distinct from WASP family proteins and include two proteins, Cortactin and HS1 (Figure 2). These factors bind the Arp2/3 complex via N-terminal acidic domains but are thought to be weak nucleators, functioning mostly as accessory proteins and filament branching proteins during Arp2/3-mediated processes (Cai et al., 2008; Selbach and Backert 2005).

Not surprisingly, since actin dynamics is essential for proper cell function, many pathogens reorganize the host actin cytoskeleton during infection (Hayward et al., 2006; Haglund and Welch 2011). EPEC and EHEC are prime examples of extracellular pathogens that use host nucleation factors to polymerize actin. In each case, the bacteria deliver effector proteins into host cells to assemble actin into “pedestals” that protrude from the cell surface and are used for bacterial colonization and motility (Figure 3).

Type III Secretion of EHEC Effector Proteins

When EPEC and EHEC encounter host cells, they use a specialized type 3 secretion system (T3SS) that acts as a molecular syringe to deliver many effector proteins into host cells (Wong et al., 2011). Remarkably, EPEC and EHEC each inject their own translocated intimin receptor (Tir) into the plasma membrane of the host cell (Kenny et al., 1997, DeVinney et al., 1999). Interaction of Tir with the bacterial outer membrane protein intimin results in an “intimate”
adherence of the bacteria to the host cell and is required to trigger the assembly of actin into pedestals (Figure 3). Unlike EPEC Tir, however, EHEC Tir alone cannot effectively form pedestals, because an EPEC strain engineered to express EHEC Tir does not trigger pedestal assembly (Campellone et al., 2002). Therefore, a second effector protein must be injected into the cell in order for the EHEC version of Tir to promote actin polymerization. The second EHEC effector protein that functions in pedestal assembly is called EspF_U (Campellone et al., 2004; Garmendia et al., 2004). EspF_U is crucial for pedestal formation by EHEC because an EHEC EspF_U mutant does not form pedestals and an EPEC strain that expresses EHEC Tir can be induced to form pedestals by EspF_U (Campellone et al., 2004). The importance of intimate adherence and actin pedestal assembly in EHEC pathogenesis is supported by the observations that strains which lack intimin or Tir do not cause any disease in animal models (Ritchie et al., 2003), while an EspF_U knockout strain colonizes a smaller region of the intestine and is attenuated for virulence (Ritchie et al., 2008).

Tir and EspF_U Signaling to N-WASP

The C-terminus of Tir is crucial for the recruitment of EspF_U and subsequent actin nucleation (Campellone et al., 2006). In addition, EspF_U has been shown to co-precipitate with Tir (Campellone et al., 2004), suggesting that pedestal assembly involves the physical interaction between these two effectors. IRTKS and IRSp53 are host proteins that mediate the recruitment of EspF_U to Tir by interacting with both proteins (Weiss et al., 2009; Vingadassalom et al., 2009). Upon association with Tir, EspF_U is thought to bind and activate the Class I nucleation factor N-WASP (Campellone et al., 2008; Cheng et al., 2008; Sallee et al., 2008). In its normal state, N-WASP is auto-inhibited and its WCA domain is sequestered by a central GTPase binding-domain (GBD) (Kim et al., 2000). But EspF_U, which contains six 47-residue proline-rich peptide
repeats, can bind to the N-WASP GBD region (Campellone et al., 2004). Binding of a single EspF\textsubscript{U} repeat can trigger N-WASP-mediated actin assembly \textit{in vitro}, but multiple EspF\textsubscript{U} repeats cooperatively increase actin assembly by synergistically activating N-WASP (Campellone et al., 2008; Cheng et al., 2008; Sallee et al., 2008).

**N-WASP-independent Pedestal Assembly**

For several years, EPEC and EHEC were thought to absolutely require N-WASP for actin pedestal formation (Lommel et al., 2001; 2004). However, it was recently shown that EHEC cannot translocate Tir and EspF\textsubscript{U} into N-WASP-knockout cells (Vingadassalom et al., 2010). Remarkably, when introduced into N-WASP-knockout cells by EHEC-independent means, Tir and EspF\textsubscript{U} can still generate pedestals (Vingadassalom et al., 2010). For example, whereas neither EPEC Tir nor EHEC Tir can form pedestals on cells lacking N-WASP, an EPEC strain engineered to express both EHEC Tir and EspF\textsubscript{U} assembles pedestals on N-WASP-knockout cells very efficiently (Vingadassalom et al., 2010). Thus, although EspF\textsubscript{U} is a potent activator of N-WASP \textit{in vitro}, EspF\textsubscript{U} is entirely capable of forming pedestals in cells lacking N-WASP. During N-WASP-independent pedestal assembly, the Arp2/3 complex is still recruited to sites of bacterial attachment, but none of the other WASP-family NPFs localize to pedestals (Vingadassalom et al., 2010). This suggests that EspF\textsubscript{U} must recruit one or more additional host nucleation factors in order to recruit Arp2/3 and polymerize actin into pedestals.

**Cortactin: a Factor that may Function in N-WASP-independent Actin Pedestal Assembly**

While most NPFs do not localize to actin pedestals, the Class II member Cortactin does localize to pedestals (Cantarelli et al., 2002, 2006). Cortactin is often exploited by pathogenic bacteria during infection, but is also considered to be a weak actin nucleator (Selbach and Beckert 2005).
It has been previously shown that RNAi-mediated knockdown of Cortactin or disruption of its SH3 domain negatively affects pedestal assembly (Cantarelli et al., 2006), implying that Cortactin contributes to pedestal formation. It has also been suggested that Cortactin can interact with Tir and/or EspF_U (Cantarelli et al., 2007). However, the precise role of Cortactin in actin pedestal formation in the presence or absence of N-WASP is not clear. Since Cortactin is known to localize to pedestals, I hypothesized that this atypical nucleation factor is involved in N-WASP-independent pedestal assembly by EHEC. The goal of my project was to determine the role of Cortactin in pedestal assembly in cells containing N-WASP and during N-WASP-independent pedestal formation.
MATERIALS AND METHODS

Bacteria and Cell Lines

For routine culturing, all bacteria were grown in LB media at 37°C. The pathogenic strain used for infection was derived from KC12, an EPEC strain in which EPEC Tir was replaced with HA-tagged EHEC Tir (Campellone et al., 2002). The strain used in the current study also harbors a plasmid for expressing EspF_U-myc (Campellone et al., 2004). Before infection, KC12+EspF_U-myc were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) + 100mM HEPES pH 7.4 in 5% CO₂ for 12-18h to increase type III secretion. N-WASP wild-type (WT) and N-WASP knockout (KO) fibroblast-like cells (FLCs) (Snapper et al., 2001) isolated from WT and KO mouse embryos were cultured in DMEM + 10% fetal bovine serum (FBS) + antibiotic-antimycotic and grown at 37°C in 5% CO₂.

Transfections and Infections

Prior to transfection, FLCs at 100% confluence were seeded into 6 well culture plates to achieve 60-80% confluence within 24h. Each well was then transfected using RNAiMAX (Invitrogen) and Silencer Select negative control siRNA, Silencer Select siRNA against GAPDH (Ambion) or Stealth siRNAs (Invitrogen) against Cortactin at 20nM in 1mL DMEM. After a 4 h incubation, 1mL of DMEM + 10% FBS + antibiotic-antimycotic was added to each well and cells were grown for 18-24h. Cells were then reseeded onto glass coverslips in 24-well culture plates to be used for infection after an additional 18-24h, and in 6-well plates to be collected and analyzed by Western blotting. Approximately 48h after the initial treatment of cells with siRNAs, KC12+pEspF_U-myc was diluted 1:1000 into DMEM + 3.5% FBS + 20 mM HEPES pH 7.4, and
cells were infected with 500µL per well. N-WASP WT cells were infected for 3.5h, whereas N-WASP KO cells were infected for 5h.

**Immunofluorescence Microscopy**

Infected cells were washed 2-4 times with phosphate-buffered saline (PBS) at 37°C and fixed in 3.7% paraformaldehyde at 37°C for 35 min. All subsequent manipulations were performed at room temperature. Cells were washed with PBS and then permeabilized using 0.1% Triton X-100 in PBS for 2min, and washed 3 additional times with PBS. Bacteria were detected using 1µg/mL DAPI (Sigma) and F-actin was detected with 0.4U/mL Alexa568-phalloidin (Invitrogen). To visualize Cortactin, the cells were first probed with a Cortactin antibody (Millipore) at 0.5µg/mL. To visualize HA-Tir, cells were probed with a monoclonal antibody against HA (Covance) at 1µg/mL. To visualize EspFU-myc, cells were probed with a monoclonal antibody against myc (Sigma) at 4µg/mL. Alexa488 goat anti-mouse secondary antibodies were used at 4µg/mL (Invitrogen). All antibodies and stains were prepared in 1% BSA + 1% FBS + 0.02% NaN₃ in PBS. Cells were imaged using a 60×/1.42NA or a 100×/1.40NA oil immersion Olympus objective on a Personal DV microscope (Applied Precision) (Chen et al., 2012). Images were captured using Softworx software and processed using ImageJ.

**Immunoblotting**

To prepare cell lysates, transfected cells were washed with PBS, collected in PBS + 2mM EDTA, and lysed in 20mM Tris pH 7.5 + 100mM NaCl + 1% Triton X-100 and 10µg/mL each of aprotinin, leupeptin, pepstatin, and chymostatin, and 100µM PMSF. Cell lysates were then mixed with 2X SDS-PAGE loading buffer + 200mM DTT. Samples were boiled for 10min, centrifuged for 1min and analyzed using 10% SDS-PAGE. The samples were transferred onto
0.2μm nitrocellulose membranes in 24mM Tris + 194mM glycine + 0.375% SDS + 20% methanol at 12V for 24 minutes. Membranes were blocked in PBS + 5% milk (PBSM) for at least 30min prior to probing with primary antibodies. The antibody against Cortactin was used at 0.5μg/mL, the E7 tubulin antibody (Iowa State Hybridoma Bank) was used at 2.9μg/mL, and the GAPDH antibody (Ambion) was used at 0.8μg/mL. Membranes were treated with secondary anti-mouse antibodies conjugated to horseradish peroxidase at 1:5000 and developed using enhanced chemiluminescence (GE Healthcare).

Quantification

To determine the percent depletion of Cortactin triggered by each of the siRNAs, densitometry was performed in ImageJ by measuring the mean pixel intensity of Cortactin bands normalized to tubulin bands and in comparison to background. Images obtained from immunofluorescence experiments were quantified in several ways. First, the number of cells containing >5 phalloidin-stained actin pedestals (Campellone et al., 2004) was counted for WT and KO cells treated with each of the siRNAs. The number of cell-bound bacteria was also quantified by counting the number of visible DAPI-stained bacteria per cell. The % of bacteria containing Tir was quantified by counting how many bound bacteria had HA-Tir localized beneath them, and then the % of HA-Tir foci associated with pedestals was quantified to determine the pedestal formation efficiency in WT and KO cells when Tir was present. Analogous measurements were performed on cells stained for EspFU-myc. Quantification of the mean fluorescence intensity of F-actin staining beneath bound bacteria was also performed using ImageJ. A background measurement was taken in an area of the cell not containing bound bacteria. This value was set to 1, and the relative intensities of F-actin pedestals or 1-micron areas beneath bound bacteria were measured accordingly. The average fluorescence values were calculated using Excel and Prism.
software. Standard deviations and standard errors were also calculated using Prism and significant differences were assessed using ANOVA, Tukey’s Multiple Comparison tests, or Kruskal-Wallis tests.

Expression and Purification of Cortactin, EspF\textsubscript{U}, and N-WASP

Plasmids encoding His- and GST-tagged Cortactin were described previously (Yarar et al., 2002). Expression of His- and GST-tagged Cortactin was induced in \textit{E. coli} Rosetta at 37°C for 4 hours using 0.2mM IPTG. Bacteria expressing His-tagged Cortactin were lysed in 50mM Tris pH8.0 + 250mM NaCl + 100mM KCl + 5% glycerol + 20mM imidazole + 0.1% Triton X-100. Bacteria expressing GST-tagged Cortactin were lysed in PBS + 200mM KCl + 5% glycerol + 0.1% Triton X-100. Each bacterial suspension was mixed with 1mg/mL lysozyme and sonicated six times for 30s at 60% power on a Sonic Dismembrator Model 300 sonicator (Fisher) and centrifuged at 17,000g for 20min at 4°C in an SS34 rotor (Sorvall) to remove debris. His-tagged Cortactin was purified using HisPur Ni-NTA resin (Thermo Scientific) and eluted in His lysis buffer containing 250mM imidazole and lacking Triton X-100. GST-tagged Cortactin was purified using Gluthathione agarose (Pierce) and was eluted in 50mM Tris pH 8.0 + 10mM reduced glutathione in GST lysis buffer lacking Triton X-100. Expression and isolation of purified proteins was verified using SDS-PAGE and Coomassie Blue staining. His-tagged EspF\textsubscript{U} and N-WASP proteins were purified as described previously (Campellone et al., 2008).
RESULTS

Cortactin expression can be silenced using 3 independent siRNAs

Since Cortactin is known to localize to pedestals and is also capable of binding and activating the Arp2/3 complex to drive actin polymerization, I wanted to further characterize its role in pedestal assembly. To test whether Cortactin has a role in pedestal assembly in the presence or absence of N-WASP, Cortactin expression was silenced using RNA interference. N-WASP-WT and KO cells were treated with a non-specific negative control siRNA, a control siRNA against GAPDH, or three different siRNAs against Cortactin. To determine if Cortactin protein levels were effectively depleted in N-WASP-WT and N-WASP-KO FLCs, cell extracts were examined by Western blotting using a monoclonal antibody against Cortactin and control antibodies against tubulin and GAPDH. Impressively, each of the 3 Cortactin siRNAs dramatically reduced its expression in both WT and KO cells (Figure 4A). Densitometry of the Cortactin immunoblots indicated that cells treated with each of the three siRNAs against Cortactin reduced its expression by 90% compared to the levels in cells treated with either of the control siRNAs (Figure 4B). These results show that each of the Cortactin siRNAs is effective at depleting its expression and can be used for testing the function of Cortactin in actin pedestal assembly.

Cortactin contributes to actin pedestal assembly in the presence of N-WASP

It has been previously shown that the EHEC effector protein EspF_U can bind N-WASP to drive pedestal assembly (Campellone et al., 2004). But because Cortactin also localizes to pedestals and its knockdown is known to have negative effects on F-actin fluorescence in pedestals in HeLa cells (Cantarelli et al., 2002, 2006), I wanted to examine the extent of its contribution to pedestal assembly in the presence and absence of N-WASP. To first determine a role for
Cortactin in pedestal assembly in N-WASP-proficient cells, WT FLCs were infected with KC12+pEspF\textsubscript{U}-myc, fixed, and analyzed for actin pedestal formation using immunofluorescence microscopy. Bacteria were identified with DAPI and F-actin with fluorescent phalloidin (Figure 5A). When WT cells were treated with either of the control siRNAs, about 80% of WT cells had >5 pedestals (Figure 5B), consistent with previous observations (Vingadassalom et al., 2010). Moreover, Cortactin localized to pedestals as expected, in agreement with previous observations (Cantarelli et al., 2002, 2006). When WT cells were treated with any of the siRNAs against Cortactin, antibody staining for Cortactin was dramatically reduced and there was an appreciable decrease in the number of actin pedestals, as only about 50% of infected cells contained >5 pedestals (Figure 5B). These results suggest that Cortactin plays a role in the initiation of F-actin pedestal assembly in the presence of N-WASP.

To further analyze the effects of Cortactin depletion on pedestal assembly, I measured the intensity of F-actin staining beneath bound bacteria using ImageJ software. Background levels of F-actin intensity in the areas of the cell with no bound bacteria were standardized to a value of 1 and WT cells treated with either of the control siRNAs had an average F-actin intensity beneath bound bacteria that was approximately 2.5-fold greater than background levels (Figure 6). WT cells treated with siRNAs against Cortactin had a significant decrease in actin staining intensity to about 1.5-fold greater than background levels (Figure 6). These results indicate that pedestals formed in Cortactin-depleted cells contain fewer F-actin filaments, further suggesting that Cortactin plays a role in pedestal assembly when N-WASP is present.

**Cortactin is crucial for N-WASP independent pedestal assembly**

The observations that EspF\textsubscript{U} is still capable of triggering pedestal formation when delivered into
N-WASP-KO cells using the EPEC type III secretion system or when expressed directly by transfection (Campellone et al., 2008) demonstrated that EspFU can use alternate host nucleation factor(s) to activate the Arp2/3 complex and drive pedestal assembly. To determine if Cortactin could be one such NPF used to form pedestals in an N-WASP-independent pathway, N-WASP-KO cells were infected with KC12+pEspFU-myc, fixed and analyzed for pedestal formation. In KO cells treated with either of the control siRNAs, about 50% of cells had >5 pedestals (Figure 5A-B). Interestingly, this value is virtually the same efficiency with which WT cells treated with siRNAs against Cortactin generated pedestals. Quantification of F-actin fluorescence in KO cells treated with control siRNAs also revealed a phenotype resembling that of WT cells treated with siRNAs against Cortactin in that the average F-actin intensity beneath bound bacteria was only about 1.5-fold greater than background levels (Figure 6). Taken together, these results imply that N-WASP and Cortactin make similar contributions to actin assembly within pedestals.

In contrast to cells lacking either Cortactin or N-WASP, KO cells treated with siRNAs against Cortactin, which lack both proteins, exhibited a drastic decrease in pedestal efficiency. Only 5-10% of these cells had >5 pedestals (Figure 5A-B). Moreover, KO cells lacking Cortactin had an average F-actin intensity beneath bound bacteria equal to the background level of staining in the cell (Figure 6). Overall, these results show that KO cells form pedestals less efficiently than WT cells, as expected, but when Cortactin is depleted from the KO cells, pedestal formation is virtually abolished.

Cells lacking N-WASP and Cortactin do not form pedestals even when EspFU is present

Both Tir and EspFU are critical for pedestal formation, but N-WASP-KO cells have been shown to be partially resistant to translocation of effectors by EPEC (Vingadassalom et al., 2010). To
confirm that the lack of pedestal formation in KO cells was not due to improper translocation of Tir and EspF$_U$, WT and KO cells were infected with KC12+pEspF$_U$-myc, fixed, and stained for HA-Tir with an antibody against HA, or for EspF$_U$-myc with an antibody against myc. Consistent with previous results (Vingadassalom et al., 2010), when WT cells were treated with control siRNAs or siRNAs against Cortactin, Tir was present beneath bound bacteria, indicating proper translocation from KC12, and pedestals were formed (Figure 7A). Moreover, Tir was also translocated into N-WASP KO cells, irrespective of the presence or absence of Cortactin and pedestals were assembled (Figure 7A). No significant difference in the number or intensity of HA-Tir staining was detected (Figure 7A-B), indicating that Tir was translocated properly under all conditions. Similarly, EspF$_U$ was still visible beneath bound bacteria on all cell types but pedestals did not form when both N-WASP and Cortactin were absent (Figure 8A-C). These results demonstrate that even though Tir and EspF$_U$ are properly delivered into host cells, they are incapable of triggering actin assembly unless N-WASP, Cortactin, or both are present.

N-WASP, Cortactin, and EspF$_U$ can be purified and used in future in vitro assays

Although we now know that Cortactin plays a role in pedestal assembly, the nature of any physical interactions between Cortactin and EspF$_U$ is still unclear. It has been previously suggested that EspF$_U$ can interact with Cortactin, but the affinity and stoichiometry of this interaction has not been explored. To ultimately characterize how these two proteins to bind one another and stimulate actin assembly in vitro, I purified recombinant forms of each of these proteins. His- and GST-tagged versions of Cortactin were expressed in E.coli and isolated using Ni-NTA affinity beads or GST affinity beads and expression was verified on SDS-PAGE gels (Figure 9A). His-tagged versions of the N-terminal region and C-terminal proline-rich repeats of EspF$_U$ as well as the WCA domain of N-WASP were purified in a similar manner (Figure 9B-C).
These proteins will be used in the future to assess whether EspF_U binds directly to Cortactin. Actin polymerization assays will also be performed to determine whether Cortactin and EspF_U cooperate to stimulate actin assembly \textit{in vitro}. Since my results demonstrate that Cortactin plays a key role in actin pedestal assembly, it is tempting to speculate that binding to EspF_U can turn Cortactin into a potent actin nucleation factor.
DISCUSSION

While some aspects of EHEC pathogenesis are well defined, our understanding of how EHEC effector proteins trigger actin pedestal assembly has remained incomplete for many years. EspF_U is known to bind and activate N-WASP to drive actin assembly, but it is also surprisingly capable of generating pedestals in cells that genetically lack N-WASP. This suggested that EspF_U uses one or more alternative host nucleation factors to engage the Arp2/3 complex and trigger actin polymerization into pedestals. Yet, the N-WASP-independent mechanisms whereby EspF_U generates pedestals had not been characterized before the current study. My results now show that Cortactin contributes to pedestal assembly in the presence of N-WASP, and plays a much more crucial role in pedestal assembly in the absence of N-WASP. These observations demonstrate that N-WASP and Cortactin share functional redundancy (Figure 10), and raise the possibility that Cortactin might be a much more potent actin nucleation factor than was previously thought.

Among the known actin nucleation factors, N-WASP is thought to be the strongest activator of the Arp2/3 complex (Campellone and Welch, 2010). Additionally, EspF_U has been shown to be the most robust activator of N-WASP, since it can accelerate actin assembly even faster than the normal mammalian binding-partners of N-WASP (Cheng et al., 2008; Sallee et al., 2008). Taken together, these results demonstrate that an EspF_U-N-WASP-Arp2/3 pathway for actin assembly should provide a remarkably efficient route for building pedestals. That is why it was so surprising that EspF_U was entirely capable of forming pedestals in N-WASP-KO cells when delivered independent of the EHEC T3SS (Vingadassalom et al., 2010). This indicated that N-WASP is not essential for pedestal assembly by EspF_U and that EspF_U can actually use other host actin nucleators to drive actin polymerization during infection.
The Class I NPFs WAVE 1-3, WASH, WHAMM, and JMY have varying activities, but are all generally considered to be strong activators of the Arp2/3 complex. However, none of these proteins localize to pedestals, and they apparently do not play a role in EspF_U-mediated actin assembly. The only Class I NPFs known to localize to pedestals are WASP and N-WASP, and while N-WASP is expressed ubiquitously, WASP is only found in hematopoietic cells. The only broadly-expressed Class II NPF is Cortactin, and it is important to note that Cortactin was previously shown to localize to actin pedestals (Cantarelli et al., 2002, 2006). Its presence in the pedestal suggested that it might be involved in assembling actin within the pedestal, but its precise function during this process had not been well defined.

Cortactin has long been characterized as a weak actin nucleator that functions primarily as an accessory protein during actin polymerization by stabilizing F-actin branches. It has been shown that Cortactin is capable of binding the Arp2/3 complex, but with an affinity much lower than that of the WASP-family proteins (Uruno et al., 2001). In contrast to its unimpressive nucleation activity, Cortactin has been demonstrated to be a key player in F-actin branching, because depletion of Cortactin results in a decrease in amount and persistence of branched F-actin filaments (Cai et al., 2008). My results may now cause us to re-evaluate the thinking that Cortactin is more important for branching than for nucleation, because I showed that it is clearly crucial for the initiation of actin pedestal assembly in the absence of N-WASP, and that it even enhances the efficiency of pedestal formation in the presence of N-WASP.

Interestingly, based on results from \textit{in vitro} actin polymerization assays it is thought that Cortactin and N-WASP could cooperatively bind the Arp2/3 complex and work synergistically during actin assembly (Weaver et al., 2002). It has also been suggested that Cortactin binds EspF_U (Cantarelli et al., 2007), but the nature of the interaction has not been well characterized.
Therefore, in the future it will be important to systematically determine how the purified versions of EspF<sub>U</sub>, Cortactin, and N-WASP each contribute to actin assembly.

Overall, my studies have enhanced our understanding of the ways in which EHEC takes over the cytoskeletal nucleation machinery of host cells. Not only does it change our views of Cortactin as a nucleation factor, but it creates new possibilities for investigating how Cortactin and N-WASP cooperate in normal cells and how these cells might control actin dynamics using N-WASP-independent mechanisms. Continued efforts aimed at characterizing the interactions between Cortactin and EspF<sub>U</sub> during pedestal assembly will also undoubtedly reveal new insights into EHEC pathogenesis. In conclusion, we hope that our current results and future work will contribute to the development of better approaches for preventing or controlling EHEC infections.
Figure 1. WASP-family actin nucleation factors are involved in many essential cell functions and are used to reorganize the cytoskeleton during infection with EHEC. WASP-family members control a variety of normal membrane remodeling processes, but during infection with EHEC, effector proteins recruit nucleation factors to reorganize actin into “pedestals” that promote colonization and motility. N-WASP localizes to actin pedestals and cooperates with EHEC effector proteins to activate the Arp2/3 complex and polymerize actin into pedestals. This figure was modified from: https://sites.google.com/site/campellonelab/.
NPFs are grouped into two major classes: WASP-family proteins (Class I) and atypical nucleation factors (Class II). Class I NPFs include 8 WASP-family proteins that drive actin polymerization by binding actin monomers and the Arp2/3 complex via their conserved C-terminal “WCA” domains. WASP and N-WASP can both localize to actin pedestals, but WASP is restricted to hematopoetic cells and N-WASP is expressed ubiquitously. Cortactin is the major Class II NPF. It binds Arp2/3 via an N-terminal acidic “A” domain and is known to localize to actin pedestals. Other domains present in the Class I and Class II NPFs are described in (Campellone and Welch, 2010; Rottner et al., 2010). This figure was modified from (Campellone and Welch, 2010).
Figure 3. EHEC translocates Tir and EspF_U into host cells using its type 3 secretion system. EHEC uses a type 3 secretion system (T3SS) to inject many effector proteins into host cells. Tir and EspF_U are the two effectors that are essential for pedestal formation. The surface adhesin intimin binds to translocated Tir to promote intimate bacterial adherence to the host cell and clustering of Tir. Tir interacts with EspF_U, which then recruits N-WASP to reorganize actin into pedestals beneath bound bacteria. As indicated by the “?” it is not yet known if Cortactin can also stimulate actin nucleation within pedestals. This figure was re-drawn from: https://sites.google.com/site/campellonelab/.
Figure 4. Three independent siRNAs can effectively knockdown expression of Cortactin. 
A. N-WASP-WT and N-WASP-KO cells were treated either with control siRNAs (siControl and siGAPDH) or with three different siRNAs against Cortactin (siCttn 1-3). Protein extracts from transfected cells were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with antibodies against Cortactin, tubulin, and GAPDH to visualize expression levels. B. Densitometry was performed in ImageJ by measuring the mean pixel intensity of Cortactin bands in comparison to tubulin bands. Cortactin expression was reduced by ~90% when treated with any of the three siCttn RNAs compared to the control siRNAs. Data represent the mean ± range from two experiments. AU, arbitrary units. ***, p<0.001.
Figure 5. Cortactin contributes to actin pedestal assembly in wild type cells, and plays a crucial role in cells lacking N-WASP. **A.** N-WASP-WT or N-WASP-KO cells were transfected either with control siRNAs or with siRNAs against Cortactin and infected with KC12+pEspF_U-myc. They were then fixed and treated with DAPI to visualize bacteria, antibodies to detect Cortactin, and phalloidin to stain F-actin. When Cortactin is depleted in WT cells, pedestals still form at a relatively high efficiency. When Cortactin is depleted in KO cells, pedestal formation is virtually abolished. **B.** Pedestal formation efficiencies were determined by calculating the % of WT and KO cells with >5 pedestals. Data represent the mean ± S.E. from four experiments in which >50 cells per sample were analyzed. *, p<0.05; ***, p<0.001.
Figure 6. Cortactin contributes to N-WASP-associated pedestal assembly and is crucial for N-WASP-independent pedestal assembly. N-WASP-WT or N-WASP-KO cells were transfected either with control siRNAs or with siRNAs against Cortactin and infected with KC12+pEspFU-myc. They were then fixed and treated with DAPI to visualize bacteria (blue), antibodies to detect Cortactin (green), and phalloidin to stain F-actin (red). The efficiency of actin pedestal assembly was analyzed by measuring the mean fluorescence intensity of F-actin staining beneath bound bacteria. 42-74 bacteria were examined for each sample. NT, not tested; RFU, Relative Fluorescent Units.
Figure 7. N-WASP-knockout cells lack pedestals even though Tir is present. A. N-WASP-WT or N-WASP-KO cells were transfected either with control siRNAs or with siRNAs against Cortactin and infected with KC12+pEspF_U-myc. They were then fixed and treated with DAPI to visualize bacteria (blue), antibodies to detect HA-Tir (green), and phalloidin to stain F-actin (red). B. The efficiency of Tir translocation into host cells was assessed by quantifying the % of adherent bacteria with adjacent HA-Tir staining. 14-151 bacteria were examined for each sample.
Figure 8. N-WASP-knockout cells lack pedestals even though EspF\textsubscript{U} is present. A. N-WASP WT or N-WASP-KO cells were transfected either with control siRNAs or with siRNAs against Cortactin and infected with KC12+pEspF\textsubscript{U}-myc. They were then fixed and treated with DAPI to visualize bacteria (blue), antibodies to detect pEspF\textsubscript{U}-myc (green), and phalloidin to stain F-actin (red). B. The efficiency of EspF\textsubscript{U} translocation into host cells was assessed by quantifying the % of adherent bacteria with adjacent pEspF\textsubscript{U}-myc staining. 13-201 bacteria were examined for each sample. C. The efficiency of pedestal formation was assessed by quantifying the % of EspF\textsubscript{U} foci that were associated with actin pedestals. 12-105 bacteria were examined for each sample.
Figure 9. Purified forms of Cortactin, EspF<sub>U</sub>, and N-WASP can be used in future assays of protein-protein interactions and actin assembly. A. His and GST-tagged Cortactin were purified from E. coli Rosetta using Ni-NTA beads or GST affinity beads. Extracts and elution samples were analyzed by SDS-PAGE and Coomassie blue staining. B. His-tagged versions of the EspF<sub>U</sub> N-terminal region and C-terminal proline-rich repeats were purified using Ni-NTA beads. C. A His-tagged WCA region of N-WASP was also purified using Ni-NTA beads.
Figure 10. A model for the role of Cortactin in actin pedestal assembly. EspF$_U$ contains an N-terminal region required for translocation into host cells via the T3SS and a C-terminus consisting of 6 proline-rich peptide repeats (R1-R6) that can activate the actin assembly machinery. N-WASP and Cortactin have functionally redundant roles in mammalian cells during actin pedestal assembly. Cortactin contributes to pedestal formation in the presence of N-WASP, and plays a particularly crucial role in the absence of N-WASP. Both nucleation factors are known to bind and activate Arp2/3: N-WASP via its WCA domain and Cortactin via its N-terminal acidic domain. When EspF$_U$ is delivered into wild type cells, it is possible that N-WASP and Cortactin can cause a synergistic activation of the actin assembly machinery. EspF$_U$ is known to bind to the central GBD of N-WASP and may interact with the SH3 domain of Cortactin. But the ability of these three proteins to cooperate during actin assembly in vitro remains to be determined. WH1, WASP-homology 1; GBD, GTPase-binding domain; PRD, proline-rich domain; WCA, WH2-connector-acidic; A, acidic; R, F-actin-binding repeat; SH3, Src-homology 3. 
REFERENCES