Enteropathogenic *E. coli* EspG1/G2 Perturb Microtubules and Impede Tight Junction Regulation and Recovery

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THESIS
Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Microbiology and Immunology
in the Graduate College of the University of Illinois at Chicago, 2014
Chicago, Illinois

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This thesis is dedicated to my husband, Thaddeus Glotfelty, for his tireless support and encouragement.
ACKNOWLEDGEMENTS

Many people were instrumental in enabling this project to progress. I would like to thank Dr. Jerrold Turner at the University of Chicago for providing stable cell lines, technical expertise and for invaluable help developing in vivo assays. Dr. Vladimir Gelfand at Northwestern University donated plasmids and provided consultation on assays involving microtubule motors. Dr. Neal Alto at University of Texas – Southwestern provided EspG GTPase-binding mutants. Dr. Mai Nguyen performed the cloning described in this report and facilitated the development of the FITC gavage permeability assay. Most importantly, without the guidance of my advisor Dr. Gail Hecht this body of work would have been impossible to complete.

LGG
Elements of Chapter I and Chapter IV and Figures 4-6 have been previously published in *Annals of the New York Academy of Sciences*, a John Wiley publication. Permission to re-use the entire text of the manuscript if required (limited portions were used), as well as all figures was freely granted to the author (Glotfelty, L) for publication in this thesis, see APPENDIX.
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<tr>
<td>AMP-PNP</td>
<td>Adenylyl-imidodiphosphate</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
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<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<tr>
<td>COPII</td>
<td>Coat protein complex II</td>
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<tr>
<td>DRA</td>
<td>Downregulated-in-adenoma</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EHEC</td>
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<td>EHNA</td>
<td><em>Erythro</em>-9-(2-Hydroxy-3-nonyl)adenine hydrochloride</td>
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<td>EPEC</td>
<td>Enteropathogenic <em>Escherichia coli</em></td>
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<td>Esp</td>
<td>EPEC secreted protein</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>IFN-γ</td>
<td>Interferon-γ</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>JAM-A</td>
<td>Junctional adhesion molecule</td>
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<td>LEE</td>
<td>Locus of Enterocyte Effacement</td>
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<td>MT</td>
<td>Microtubule</td>
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<td>NHE3</td>
<td>Na⁺/H⁺ exchanger 3</td>
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Nle  Non-LEE encoded

PAK  p21-activated kinase

RFP  Red fluorescent protein

SGLT1 Na+/glucose co-transporter 1

siRNA small interfering ribonucleic acid

TER Transepithelial electrical resistance

Tir Translocated Intimin Receptor

TJ Tight junction

TNF Tumor necrosis factor

ZO-1 Zonula occludens protein 1
SUMMARY

The intestinal epithelial tight junction (TJ) is a dynamic structure, with multiple proteins constitutively recycling between the membrane and the cytosol. The mechanisms required for maintaining the TJ in the homeostatic state and for TJ restoration are not well described. Herein we show for the first time that occludin traffics on microtubules. We also demonstrate that these structures are required for maintaining TJ structure and function. Using the calcium switch assay, we establish that microtubules and dynein-mediated trafficking are required for complete TJ restoration after insult. Interruption of homeostatic TJ protein recycling presents as an intuitive way to disrupt barrier function. Enteropathogenic \textit{E. coli} (EPEC) is an enteric pathogen that induces loss of TJ structure and function. TJ restoration has not been studied with respect to EPEC pathogenesis. Our data establish for the first time that EPEC effector proteins stimulate internalization of occludin and impede TJ restoration. Effector proteins EspG1/G2 disrupt microtubules (MTs). We show that intact MTs promote barrier recovery after EPEC infection. We also demonstrate that EspG1/G2-mediated MT disruption leads to TJ dysregulation and inhibition of TJ restoration. EspG was recently shown to interact with ARF and PAK small GTPases. This report shows that EspG's impact on MTs is independent of its modulation of GTPase signaling, suggesting a broader scope of action for EspG. Taken together, these data illuminate a new MT-dependent aspect of TJ regulation, and suggest that EPEC sabotages host cell protein recycling, preventing TJ restoration and perpetuating loss of barrier function.
1. Introduction

a. The intestinal epithelial tight junction is a dynamic structure

The intestinal epithelium must provide a protective role by separating the luminal contents of the intestine from the underlying mucosal layers. However, it cannot be completely occlusive as it must permit the absorption of water, ions and nutrients. The epithelial tight junction (TJ) enables the intestinal epithelium to perform both occlusive and conductive functions as it selectively restricts the passage of molecules from the lumen to the underlying layers on the basis of size and charge.

The TJ is a continuous paracellular structure that separates apical and basolateral membrane domains (Anderson and Van Itallie, 2009; Mitic and Anderson, 1998; Tsukita and Furuse, 2002). More than thirty proteins have been identified, both integral membrane proteins and peripheral, that comprise or are linked to the TJ (Shen et al., 2008). Members of the claudin family are four-pass membrane spanning proteins that restrict passage across the epithelium on the basis of charge (Amasheh et al., 2009; Colegio et al., 2002; Morita et al., 1999). Occludin is also a four-pass membrane spanning protein and has been linked to size-selective passage (Al-Sadi et al., 2011). These proteins, along with junctional adhesion molecule (JAM-A) and the coxsackie adenovirus receptor, form homotypic and heterotypic associations linking epithelial cells and forming a selectively permeable barrier (Denker and Nigam, 1998). ZO-1 was the first TJ-associated protein to be discovered (Stevenson et al., 1986). Together with family members ZO-2 and ZO-3 it directly interacts with claudins, occludin and JAM-A, linking the junctional structure to the actin cytoskeleton (Furuse, 2010).

TJs are disrupted in multiple disease states, emphasizing the importance of maintaining barrier function. In Crohn’s disease, barrier defects were first described over 25 years ago and have been linked to TNF-alpha-induced expression of myosin light chain kinase and caveolin dependent occludin
endocytosis (Blair et al., 2006; Marchiando et al., 2010; Pearson et al., 1982; Ukabam et al., 1983; Ye et al., 2006). Interleukin-13 (IL-13)-induced upregulation of claudin-2 has been identified as the primary cause of barrier dysfunction in ulcerative colitis (Fuss et al., 2004; Heller et al., 2005; Rosen et al., 2011). The intestine is not the only location where barrier defects cause severe clinical consequences. The blood-testis barrier (BTB) is partially maintained via TJs. Differential expression and localization of TJ proteins have been correlated with increased BTB permeability, leading to increased levels of anti-sperm antibody, a possible factor in male infertility (Cheng et al., 2011; Wang et al., 2010; Wong et al., 2010a; Wong et al., 2010b). In lung epithelial tissues, environmental factors may decrease expression of TJ proteins, thus contributing to the development of asthma (Tai et al., 2006; Vermeer et al., 2009; Vinhas et al., 2011).

While the TJ has many well-established protein-protein interactions, it remodels continually under steady state conditions. Using cells stably expressing fluorescent TJ proteins, fluorescence recovery after photobleaching was used to investigate the homeostatic movement of TJ proteins (Shen et al., 2008). Occludin and ZO-1 showed distinct dynamics. Most occludin (71 ± 3%) diffused laterally within the plasma membrane while 69 ± 5% of ZO-1 shifted in and out of cytosolic pools. The authors noted that occludin has been documented in cytoplasmic vesicles and may also exchange with membrane-bound pools (Morimoto et al., 2005; Nishimura and Sasaki, 2008).

A separate study employed a biotinylation/pulldown assay to investigate occludin recycling (Morimoto et al., 2005). In epithelial cells, approximately 70% of endocytosed occludin was recycled from the cytosol back to the membrane within 5 minutes. Claudin-1 constitutively recycles in kidney, colon and
lung epithelial cells in an endosomal sorting complex required for transport (ESCRT) dependent manner (Dukes et al., 2011).
Figure 1. The epithelial tight junction is a dynamic structure. Numerous reports demonstrate that tight junction proteins recycle in the homeostatic state.

These data suggest that TJ proteins are rapidly exchanged and the TJ is constitutively reconstructed in the steady state (Fig. 1). Patch-clamp recordings of single junctions further support this model (Weber, 2012). Minute fluctuations in current may correspond to recycling events. The dynamic nature of the TJ presents an intuitive mechanism by which TJ restoration could be impeded in disease states, thus prolonging barrier defects.
b. The cytoskeletal elements required for tight junction homeostasis and recovery are not well-defined

Currently there is a need for further investigation regarding TJ homeostasis. The cytoskeleton is intimately associated with TJs. Actin disruption using latrunculin A perturbs TJ integrity via a caveolin-dependent mechanism, indicating microfilaments are important for TJ maintenance. The role of actin in recovery and reconstruction of TJs, however, has not been investigated. The contribution of other cytoskeletal elements, such as microtubules, has only been superficially addressed.

Microtubules (MTs) are comprised of polymerized dimers, each one containing an α- and a β-tubulin monomer (Musch, 2004). They are polarized filaments with a slower growing minus-end and a faster growing plus-end. These filaments continuously “treadmill” with units added to one end and subtracted from the other at varying speeds. Polarization enables MTs to act as “molecular highways” along which cargo is pulled by the motor proteins kinesin and dynein. In polarized epithelial cells, MTs are oriented longitudinally with minus-ends facing apically (Fig. 2) (Achler et al., 1989). MTs also form apical and basal networks in polarized epithelia (Musch, 2004).
While it is accepted that MTs are required for moving cargo long distances such as in neurons, the role of MTs in trafficking TJ proteins is not established. One study used occludin tagged with green fluorescent protein to track protein movement in living cells (Subramanian et al., 2007). Approximately 75% of occludin-containing vesicles traveled at least 4 μm during 30 seconds of tracking. Nocodazole treatment, which impedes MT polymerization, reduced that fraction to approximately 25%, suggesting that MTs are required for the efficient trafficking of occludin. Directionality was not determined.
An additional study examined the requirement of MTs in calcium chelation-mediated TJ disruption (Ivanov et al., 2006). Chelated cells exhibited grossly mislocalized TJ proteins. Following MT disruption, chelation failed to relocalize β-catenin, occludin and ZO-1 from the membrane to the cytosol. Additionally, inhibition of the plus-end motor kinesin prevented chelation-induced movement of occludin and e-cadherin from the membrane to sub-apical compartments. These data suggest that MTs are important for TJ protein internalization, but does not address the role of MTs under homeostatic conditions or in TJ recovery.

c. Enteropathogenic. E. coli disrupt tight junction structure and function

Enteropathogenic E. coli (EPEC) infection induces loss of TJ structure and function. EPEC belongs to a family of related “attaching and effacing” pathogens including enterohemorrhagic E. coli (EHEC) and C. rodentium. These pathogens employ a type-three secretion system, a “molecular syringe” that spans the bacterial and host cell membranes to deliver bacterial effector proteins directly into the host cytosol (Fig. 3) (Jarvis et al., 1995; Jarvis and Kaper, 1996; Wolff et al., 1998).
EPEC contains a 35-kb pathogenicity island termed the Locus of Enterocyte Effacement (LEE) (Karaolis et al., 1997; McDaniel et al., 1995). Commensal *E. coli* lack this chromosomally encoded region. Many, but not all, of the transferred bacterial effector proteins are encoded within the LEE. Effectors encoded outside the LEE are termed “non-LEE-encoded effector” or “Nle”. All effectors have varied deleterious effects on the host cell (Dean and Kenny, 2009; Dean et al., 2005). For example,
NleA interferes with anterograde trafficking through the Golgi apparatus and Tir contributes to the formation of actin pedestals underneath attached bacteria (Kim et al., 2007; Tu et al., 2003). Inhibition of absorption due to effector-mediated downregulation of the Na⁺/H⁺ exchanger (NHE3), the Na⁺/glucose co-transporter (SGLT1) and the anion exchanger downregulated-in-adenoma (DRA) are largely responsible for EPEC-induced diarrhea (Fig. 4) (Dean et al., 2006; Gill et al., 2007; Guttman et al., 2007; Hecht et al., 2004; Hodges et al., 2008). Relocalization of tight junction (TJ) proteins to the cytosol and a corresponding drop in transepithelial electrical resistance (TER), disruption of the intestinal barrier, are well-established consequences of EPEC infection (Dean and Kenny, 2004; Muzam-Moons et al., 2004; Simonovic et al., 2000). Taken together, EPEC infection compromises the physiology of the intestinal epithelium, resulting in severe diarrhea.

TJ disruption is the result of multiple effectors, among them EspF, MAP, NleA and EspG1/G2. EspF is required for loss of TJ barrier function and for the relocalization of occludin from the membrane to the cytosol (McNamara et al., 2001; Shifflett et al., 2005). MAP is also required for these phenotypes (Dean and Kenny, 2004; Dean et al., 2006). NleA was shown to contribute to loss of TJ function potentially by inhibiting anterograde protein transport through the Golgi apparatus (Kim et al., 2007; Thanabalasuriar et al., 2010). Pertinent to the current investigation are EspG1/G2, which have been shown to destroy MT networks and also interfere with GTPase signaling. Infection with these EPEC strains lacking NleA and EspG1/G2 leads to attenuated TER loss compared to wild-type infection (Thanabalasuriar et al., 2010; Tomson et al., 2005). Despite considerable data identifying the effectors responsible for loss of TJ integrity the mechanisms remain unclear.
d. EspG disrupts microtubules and mediates GTPase signaling

EspG1 was first described as a type-three secreted EPEC homologue of the *Shigella flexneri* effector VirA (21% identical and 40% similar) (Elliott et al., 2001). It is also highly conserved across other attaching and effacing pathogens such as enterohemorrhagic *E. coli* (EHEC) O157:H7 and *Citrobacter rodentium*. Its homolog EspG2 is not LEE-encoded and is 42% identical and 62% similar to EspG and 20% identical and 38% similar to VirA (Elliott et al., 2001). Functionally EspG1 can complement a S.
Flexneri VirA mutant (Elliott et al., 2001). A double EspG1/G2 mutant can be complemented by either EspG1, EspG2 or VirA indicating a high level of functional conservation (Smollett et al., 2006; Tomson et al., 2005).

VirA disrupts MT networks and there are extensive reports establishing the similar function of EspG1/G2 (Elliott et al., 2001; Yoshida et al., 2006). It was first observed that MT networks were absent under attached EPEC microcolonies and that this phenomenon was dependent on a functional type-three secretion system (Shaw et al., 2005b). Infection with various EPEC mutants established EspG1/G2 as the relevant effector proteins. EspG1 is also spatially associated with MT loss as tagged bacterial protein localizes directly under microcolonies in areas devoid of MTs (Shaw et al., 2005b).

One study confirmed retention of MT networks following infection with an EspG1/G2 mutant (Tomson et al., 2005). Infection with an EspG1/G2 mutant did not eliminate TER loss, indicating that intestinal barrier function was still compromised in the presence of intact MTs. However, there was delayed loss of TER compared to wild-type infection, suggesting that intact MTs modulate the impact of effectors EspF and MAP via an undetermined mechanism. In a separate study, ectopic expression of EspG1 in mammalian cells did not induce TER loss, but increased paracellular permeability to 4 kDa dextran, indicating that MTs are required for regulating selective permeability (Matsuzawa et al., 2005). The exact mechanism has not been determined.
Reductionist systems have further confirmed the impact of EspG on MTs. Transient expression of EspG depolymerizes MTs in mammalian cells in the absence of EPEC infection (Tomson et al., 2005). Further work demonstrated that EspG1 directly complexes with α-tubulin using purified His-tagged EHEC EspG and tubulin heterodimers (Fig. 5) (Hardwidge et al., 2005). Purified EspG also depolymerizes MTs in solution, underscoring its impact on the cytoskeleton (Matsuzawa et al., 2004; Tomson et al., 2005).

Figure 5. EspG disrupts microtubule networks. EPEC EspG has been demonstrated to bind directly to α-tubulin. The presence of EspG alone in the absence of other effectors induces depolymerization of microtubules into tubulin subunits. From Glotfelty et al., 2012 Jul;1258:149-58. Copyright © 2012 by John Wiley Sons, Inc. Reprinted by permission of John Wiley & Sons, Inc.
More recent investigations identified additional functions for EspG. Selyunin et al. demonstrated that EHEC EspG binds both to ADP-ribosylation factor (ARF) and p21-activated kinase (PAK) proteins using a yeast two-hybrid screen (Fig. 6) (Selyunin et al., 2011). Structural analysis revealed that when bound to EspG, ARF6 adopts a conformation nearly identical to that of the active GTP-bound state, thus disrupting the normal guanine nucleotide cycle. ARF proteins are required for organization of vesicle transport (D'Souza-Schorey et al., 1998; Franco et al., 1999; Kahn, 2009). Microinjected EspG localized to and dispersed the Golgi apparatus suggesting trafficking interference. Golgi dispersal is a well-established indicator of MT disruption but we note here that Selyunin et al. found that microinjected EspG did not disrupt MT networks after 20 minutes (Cole et al., 1996; Ho et al., 1989; Rogalski and Singer, 1984; Thyberg and Moskalewski, 1999; Wehland et al., 1983). This may be due to procedural variation as all prior ectopic expression studies were performed using either stable or transient transfection of espG and MTs were examined at later time points.

In contrast, EspG binds and activates PAK1, inducing a 7.6-fold increase in activity. PAK binding is dependent on the formation of the EspG-ARF<sub>GTP</sub> complex. A separate study confirmed that EspG binds to the Rac/Cdc42-binding site of PAK1 (Germane and Spiller, 2011). The PAK family of proteins transduces signals from Rac1 and Cdc42 GTPases, regulating cytoskeletal dynamics and cell motility (Bokoch, 2003). The authors concluded that by imitating a small GTPase, EspG permits EPEC to bypass native GTPases and enable PAK-dependent actin remodeling regardless of the status of native Rac/Cdc42. These studies identify modulation of GTPase signaling as a function of EspG and suggest that this effector plays multiple roles in EPEC pathogenesis.
Another role for EspG may be linked to its localization at the Golgi apparatus (Selyunin et al., 2011). Clements et al. ectopically expressed EspG from EPEC and EHEC, EspG2 from EPEC and VirA from S. flexneri and found that all proteins induced Golgi disruption and inhibited protein secretion (Clements et al., 2011). A yeast two-hybrid screen identified Golgi matrix protein GM130 as a potential binding partner for all ectopically expressed proteins. Another EPEC effector, NleA, has been described previously as an inhibitor of the secretory pathway (Kim et al., 2007). By binding to SEC24, NleA
inhibits COPII anterograde trafficking. Clements et al. compared NleA’s activity with all EspG constructs and determined that EspG was a more powerful inhibitor of protein secretion.

These findings open new avenues of investigation into the potential roles of EspG1/G2 in EPEC pathogenesis. All of EspG’s functions potentially contribute to EPEC-induced TJ disruption, either by destroying MTs and blocking TJ protein recycling, inhibiting secretion of TJ proteins, or by as-yet undetermined mechanisms related to vesicle trafficking or cytoskeletal remodeling.

e. Goals

The goals of this study are two-fold. The role of MTs in maintaining TJ structure and function is unknown. The need for further investigation into the cellular components required for dynamic TJ maintenance is compelling. Additionally, the requirement for MTs in TJ recovery has not been addressed. We hypothesize that MTs are required for occludin recycling in the homeostatic state and for TJ restoration. Our first aim is to determine the role of MTs in maintaining TJ structure and function and to investigate their contribution to TJ restoration following injury (Fig. 7).

Interruption of TJ protein recycling presents as an intuitive mechanism to impede TJ recovery. The effectors EspG1/G2 have been shown to disrupt MT networks and contribute to loss of barrier function. The mechanism by which EspG1/G2 induce TER loss has not been determined and may be related to their impact on MTs. We hypothesize that EspG1/G2-mediated MT destruction prevents the recycling of TJ proteins and hence impedes TJ restoration, thus perpetuating barrier loss. Our second aim is to determine the role of EspG in EPEC-induced TJ disruption and inhibition of recovery (Fig. 7).
The goals of this study are twofold. Aim 1 is to determine the role of MTs in maintaining TJ structure and function and to investigate their contribution to TJ restoration after insult. Aim 2 is to determine the role of EspG in EPEC-induced TJ disruption and inhibition of recovery.

**f. Approach**

This study requires a technique to disrupt MTs in a synchronized fashion, preferably without the deleterious effects of pharmaceuticals. To achieve this goal, cold exposure was used to depolymerize MTs rapidly and completely (Baas et al., 1994; Banan et al., 2000; Breton and Brown, 1998). Reincubation at 37°C induces complete MT regeneration. Addition of nocodazole prior to rewarming completely prevents polymerization. With its emphasis on TJ recovery, a technique that allows for separation of TJ disruption from the process of restoration is required. Calcium switch assays were
therefore employed to cleanly separate TJ disruption from recovery. Chelation of calcium from monolayers grown on Transwells drives the internalization of TJ proteins and loss of TER. Reintroduction of calcium induces rapid and complete recovery of structure and function (Farshori and Kachar, 1999; Ivanov et al., 2006; Ivanov et al., 2004; Martinez-Palomo et al., 1980; Rothen-Rutishauser et al., 2002; Stuart et al., 1994).

Calcium switch assays performed on monolayers lacking MTs or in the presence of inhibitors of MT motors allowed us to determine the role of these structures in recovery. These are well suited to investigating the roles of MTs and MT motor proteins in TJ recovery (Ivanov et al., 2006). The overall approach to our first goal is to disrupt MTs and determine the impact on TJ homeostasis and restoration.

An ΔespG1/G2 mutant has been successfully used to investigate the role EspG and we have obtained this strain for infection studies (Elliott et al., 2001). To address the role of EspG in the absence of other effectors, the gene was cloned into a mammalian expression vector and ectopically expressed in epithelial cells. This technique has been used successfully to investigate the role of other EPEC effectors (Kim et al., 2007; Weflen et al., 2010). Site-directed binding mutants can be used to investigate the role of EspG’s interaction with ARF and PAK (Selyunin et al., 2011). The overall approach to our second goal is to investigate TJ integrity and TJ recovery after EspG-induced MT loss.
g. Summary of findings

The experimental findings of this project advance our understanding of how TJs are maintained in the homeostatic state. Further data also contribute to our knowledge of how EPEC perturbs TJ integrity. Living cells expressing RFP-occludin and GFP-tubulin were imaged in real-time. Occludin was demonstrated to traffic on MTs, establishing definitively for the first time that these structures are required for occludin transport. The average velocity and total distance traveled by occludin-containing vesicles were significantly decreased after MT disruption. Perturbation of MTs in intestinal epithelial cells induced occludin to relocalize from the membrane to the cytosol in a dose- and time-dependent manner. TJ function was correspondingly compromised. These data establish that MTs play a vital role in the maintenance of TJ structure and function.

To address the role of MTs in TJ restoration, MTs were perturbed and calcium switch assays were performed. In the absence of MTs, TJs recovered more slowly and to a significantly less degree compared to controls, suggesting that occludin is unable to recycle back to the plasma membrane and reconstruct TJs.

The role of MT motors in TJ reconstruction was determined by inhibiting each motor with a pharmaceutical agent or siRNA construct. Inhibition of dynein by either method, but not kinesin, significantly impeded TJ recovery from calcium chelation. In the absence of dynein occludin lack the motor it needs to recycle back to the membrane, thus perpetuating barrier loss. Taken together, these data strongly support a role for MTs in maintaining TJ integrity and enabling TJ restoration.

We speculated that EspG-mediated MT loss might have similar effects on TJs as those associated with artificial MT disruption and dynein inhibition. EPEC has a well-established impact on TJs including
relocalization of occludin to the cytosol, but to date internalization has not been established.

Internalization of TJ proteins has, however, been demonstrated after actin depolymerization or TNF treatment (Marchiando et al., 2010; Shen and Turner, 2005). Prior to investigating the impact of EspG-induced MT loss, we wanted to determine how occludin relocalized to the cytosol. A biotinylation assay demonstrated for the first time that EPEC infection induces occludin internalization.

We hypothesized that EPEC effector proteins, including EspG1/G2, impeded TJ restoration during infection in addition to initially disrupting TJs. Effectors were demonstrated to impair TJ recovery after calcium chelation, supporting our hypothesis. In an infection model, EspG1/G2 were shown to participated in impeding TJ restoration, further strengthening our conclusion.

Having determined that MTs promote TJ recovery after EPEC infection, we wanted to investigate the effects of EspG in the absence of other effectors. Ectopic expression of EspG impeded TJ recovery after calcium switch, confirming our previous results using nocodazole and suggesting that EPEC obstructs TJ recovery by MT disruption. EspG also induced MT destruction, tubulin degradation and relocalization of occludin to the cytosol. These phenotypes are hallmarks of wild-type EPEC infection and hitherto had not been shown to be the result of EspG alone. Golgi dispersion, a hallmark of MT disruption, was also induced, a result that was confirmed in an infection model.

A new role for EspG has recently been uncovered, namely as a small GTPase regulator that sequesters active ARF and activates PAK. To investigate the contribution of these protein interactions to EspG’s effects on MTs we obtained EspG ARF- and PAK-binding mutants from our collaborator Dr. Neal Alto (Selyunin et al., 2011). Expression of these constructs also caused MT destruction and Golgi dispersal, indicating that EspG may have disparate roles in EPEC pathogenesis. Bacterial delivery of
EspG and the ARF- and PAK-binding mutants also disrupted MTs, further confirming our previous results.

Taken together, these data demonstrate for the first time the role of MTs in TJ homeostasis and recovery. Although previous reports implied that these structures were important in TJ physiology, it has never been demonstrated conclusively (Ivanov et al., 2006; Subramanian et al., 2007). We speculate that MTs are required for constitutive recycling of TJ proteins. In the absence of MTs, trafficking of TJ proteins is impeded and TJs are unable to reconstruct at the membrane.

EPEC-mediated TJ disruption is the result of multiple effectors, including EspG, but the mechanisms remain largely unknown. This report is the first to link the impact of EspG on MTs to TJ dysregulation and to demonstrate that although EspG has multiple functions, they are divorced from one another. In this respect, our data support a broader role for EspG in EPEC pathogenesis that is similar to that of EspF, an effector that has so many diverse functions it is termed EPEC’s “Swiss army knife”.
a. Rationale

There is evidence demonstrating a role for actin in TJ regulation, but the role of MTs has not been conclusively determined (Shen and Turner, 2005). Evidence suggests that MTs are required for occludin trafficking, as nocodazole treatment significantly reduces total distance traveled by occludin-containing vesicles (Subramanian et al., 2007). A separate study determined that MTs are required to move TJ proteins from the membrane to the cytosol following calcium chelation, but the role of these structures in maintaining TJ homeostasis was not examined, nor was their role in TJ reconstruction (Ivanov et al., 2006).

Both of these studies were performed prior to the definitive demonstration that TJs are dynamic structures (Morimoto et al., 2005; Shen et al., 2008). Since then, additional work has confirmed that TJ proteins recycle constitutively (Dukes et al., 2011; Dukes et al., 2012). The cellular components required for recycling TJ proteins are not known and the mechanisms regulating this process are poorly understood.

Given the number of disease states involving intestinal barrier disruption, determining the cellular processes required for the maintenance and restoration of TJs could lead to the development of recovery-enhancing therapeutics and shorter, less taxing disease courses. The hypothesis of Chapter 2 is that MTs are essential for TJ maintenance and complete recovery of TJ function after insult. The aim of Chapter 2 is to determine whether MTs are required for TJ maintenance and recovery.
b. Occludin traffics on microtubules

There are limited data suggesting that occludin traffics on MTs, but this event has not been demonstrated (Achler et al., 1989; Ivanov et al., 2006; Subramanian et al., 2007). To determine if MTs participate in occludin transport, stably transfected RFP-occludin MDCK cells were transiently transfected with a GFP-tubulin vector in order to visualize MTs. Live-cell streaming movies were captured. Here, occludin-containing vesicles are shown for the first time to traffic on MTs (Fig. 8).

Analysis of particle tracking data revealed that the average velocity of occludin in control cells was 1.59 μm/second ± 0.07 over 15s and that the average distance traveled was 24.3 μm ±1.08 (Fig. 8C, D). Disruption of MTs with cold temperature and nocodazole, significantly impeded the movement of occludin with the average velocity falling to 0.66 μm/second ± 0.13 and the average distance traveled reduced to 13.6 μm ±1.67 total over 15s (Fig. 8B, C, D).
Figure 8. Occludin traffics on microtubules. (A) RFP-occludin stably transfected cells were transiently transfected with GFP-tubulin. Streaming live-cell movies demonstrate that RFP-occludin traffics on GFP-microtubules. (B) MT disruption using cold temperatures/nocodazole impedes occludin trafficking. RFP-occludin does not travel significantly during over a minute of imaging. (C) Particle tracking completed using the Manual Tracking plugin for ImageJ demonstrated the average total distance traveled over 15 seconds by occludin to be 24.3 μm ±1.08. Nocodazole treatment reduced average total distance to 13.6 μm ±1.67 (‡p < 0.001). (D) The average velocity of occludin over 15 seconds was 1.59 μm/second ± 0.07. With nocodazole treatment, this was reduced to 0.66 μm/second ± 0.13 (‡p < 0.001)
c. Microtubules are required for maintenance of tight junction structure and function

Having demonstrated that MTs are required for occludin trafficking, we sought to determine their role in maintaining TJ structure in the steady state. Nocodazole treatment of Caco-2 cells induced progressive disruption of MTs over 4 hrs (Fig. 9A). Cells harboring disrupted MTs were also stained for occludin. A dose- and time-dependent impact of nocodazole on occludin localization was seen with progressive cytosolic accumulation of occludin (Fig. 9B).

Tight junction-associated occludin, but not ZO-1 or claudin-1, correlates with intact barrier function (Shen and Turner, 2005). Since MT disruption perturbed TJ structure, the impact on TJ function was also explored. TER of Caco-2 cells was measured over time following treatment with nocodazole. Twenty and 40 µg/ml, but not 5 µg/ml, significantly decreased TER over time as compared to controls, correlating with the progressive loss of MTs and relocalization of occludin (Fig. 9C). These data suggest that intact MT networks are required to maintain the association of occludin with TJs and to preserve TJ function under steady state conditions.
Figure 9. Intact microtubules are required for maintenance of TJ structure and function. (A) Nocodazole treatment disrupted microtubules in Caco-2 cells in a time dependent manner. Nuclei are shown in blue. (B) At 5 and 8 hours of nocodazole treatment, after complete microtubule disruption, occludin accumulated in the cytosol in a dose- and time-dependent manner. (C) Treatment of Caco-2 cells with 5 μg/ml nocodazole did not significantly perturb TJ function. Twenty and 40 μg/ml nocodazole perturbed barrier function in a dose- and time-dependent manner. * p < 0.05, § p < 0.01
d. Microtubule disruption inhibits tight junction recovery after calcium switch

Since MTs appear to be involved in TJ maintenance, experiments were designed to investigate the role of MTs in TJ restoration. Calcium switch assays are a well-established approach for studying TJ restoration (Cassidy and Tidball, 1967; Ivanov et al., 2004; Muza-Moons et al., 2003; Rothen-Rutishauser et al., 2002; Stuart et al., 1994). Chelation of extracellular calcium drives the internalization of TJ proteins and barrier function is lost (Cereijido et al., 1981). Normalization of extracellular calcium concentration allows for the rapid recovery of TJ structure and function.

Caco-2 cells were treated with 5 μg/ml nocodazole for 1 hr in order to disrupt MTs but preserve TER; calcium switch assays were then performed. TER dropped ~90% from baseline readings within 10 minutes as expected at which point EDTA-containing medium was removed and replaced with complete medium. TER was then tracked in the presence and the absence of MTs. TER recovery of control monolayers was progressive and steady, reaching baseline levels by 4 hours following the replacement of calcium (Fig. 10). In contrast, monolayers harboring disrupted MTs recovered to only 40.8% ± 4.96 of baseline by that time suggesting that these structures are crucial for TJ restoration following injury (Fig. 10).
Figure 10. Microtubule disruption inhibits tight junction recovery after calcium switch. Caco-2 cells pre-treated with nocodazole for 1 hour recovered significantly less TJ function following calcium switch compared to untreated controls. * p < 0.05

e. Temperature-mediated microtubule disruption inhibits tight junction recovery

Low dose nocodazole had little effect on TJ structure or function for short time periods. To circumvent deleterious effects of prolonged exposure to this drug and to synchronize MT disassembly, we took advantage of the well-demonstrated sensitivity of MTs to cold (Baas et al., 1994; Breton and Brown, 1998; Turner and Tartakoff, 1989). Exposure of cells to 4°C for as little as 30 min depolymerizes essentially all MTs which reform rapidly upon return to 37°C. Caco-2 cells were incubated at 4°C for 1 hr, at which time no intact MTs were identified (Fig. 11A). Western blotting demonstrated tubulin was not degraded during depolymerization (Fig. 11B). Nocodazole was added to one group of monolayers prior to returning to 37°C to maintain MT disruption. MTs began to reform as early as 10 min after
return to 37°C in the absence of nocodazole and recovered completely by 1 hr. MTs did not reform in the presence of nocodazole (Fig. 11A).

To investigate the effects of rapid and synchronized MT disruption on TJ structure, occludin staining was performed on monolayers that had undergone temperature-induced MT disruption. After 1 hr of incubation at 4°C, no occludin movement was observed, as anticipated since chilling inhibits protein trafficking (Fig. 11C). Cells that were chilled and returned to 37°C in the absence of nocodazole reformed MTs within minutes and did not exhibit relocalization of occludin. In contrast, occludin localization within the cytosol increased progressively over 5 hrs following maintained MT disruption, suggesting that TJ proteins utilize MTs to maintain localization at the plasma membrane (Fig. 11C).
Figure 11. Temperature-mediated microtubule disruption inhibits TJ recovery. (A) When chilled, MTs rapidly and completely disassemble and when re-warmed they rapidly re-form. If treated with nocodazole, MTs do not reassemble, even after return to 37°C. Nuclei are shown in blue. (B) Chilling does not degrade tubulin. (C) Cells were treated as in (A) and stained for occludin. Prolonged disturbance of MTs using cold/nocodazole induces occludin accumulation in the cytosol. (D) Chilled/nocodazole treated cells recover significantly less TJ function after calcium switch compared to controls. Chelated controls were chilled, but not treated with nocodazole, permitting microtubules to re-form. * p < 0.05, § p < 0.01, ‡ p < 0.001
The role of MTs in TJ restoration was tested using the calcium switch assay. Control monolayers recovered to 80.6% ± 5.29 of baseline TER values by 4 hrs while those harboring disrupted MTs recovered to only 46.6% ± 5.01% of baseline values (Fig. 11D). The delay in TER recovery induced by nocodazole alone and cold/nocodazole was similar, demonstrating that these methods induce comparable levels of MT disruption.

**f. Efficient tight junction restoration requires dynein but not kinesin-mediated trafficking**

Cargo transport along MTs requires the motor proteins kinesin and dynein (Hackney, 1996; Ogawa, 1991). Kinesin is a plus-end directed motor and dynein is a minus-end directed motor (Schnapp and Reese, 1989; Wang et al., 1995). In polarized epithelial cells, MTs are oriented with their minus-ends towards the apical plasma membrane (Fig. 12). Dynein therefore transports cargo in the apical direction and kinesin in the basolateral direction.
To determine the role of dynein in TJ maintenance, Caco-2 cells were treated with erythro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride (EHNA), which inhibits cGMP- stimulated phosphodiesterase, and stained for tubulin or occludin. EHNA had not impact on MT networks over 6 hours as demonstrated by tubulin staining (Fig. 13A). However, EHNA-treated cells revealed significant occludin relocalization to the cytosol compared with controls (Fig. 13B). This suggests that minus-end directed trafficking is required to maintain occludin localization at the plasma membrane in steady state conditions.
Figure 13. Pharmacological dynein inhibition perturbs TJs and impedes TJ restoration. (A) EHNA treated (500 μM) Caco-2 cells were fixed and stained for MTs. EHNA does not affect MT structure. (B) EHNA-treated cells were fixed and stained for occludin. EHNA induces relocalization of occludin to the cytosol in Caco-2 cells. (C) The calcium switch assay was performed on EHNA-treated cells and untreated controls. Dynein inhibition significantly impedes TJ recovery compared to untreated controls. ‡ p < 0.001
The role of dynein in TJ restoration was tested using the calcium switch assay. Caco-2 monolayers were treated with EHNA for 1 hr prior to chelation. Control cells recovered to 76.8% ± 3.29 of baseline TER by 4 hrs following the reintroduction of calcium (Fig. 13C). Inhibition of dynein reduced significantly the recovery of TER, which reached only 47.9% ± 3.94 of baseline TER by 4 hrs, suggesting that minus-end directed trafficking is crucial for efficient TJ recovery (Fig 13C).

To more directly address the role of minus-end directed trafficking on TJ recovery, dynein heavy chain and dynactin were knocked down using siRNA. Maximal inhibition of minus-end directed trafficking is achieved by knocking down both these proteins. Inhibiting minus-end directed trafficking disperses the Golgi apparatus, therefore the number of cells exhibiting Golgi dispersal is a standard measurement of dynein/dynactin siRNA knockdown (Loschi et al., 2009; Palmer et al., 2009). Scrambled siRNA had no impact on the Golgi while dynein/dynactin siRNA transfection induced Golgi dispersal in 50-60% of cells (Fig. 14A). Of note, dynein/dynactin siRNA induced occludin relocalization to the cytosol after 8 hrs, confirming our previous data that minus-end directed trafficking plays a role in maintaining TJ structure (Fig. 14B). Monolayers treated with scrambled siRNA reached baseline 97.2% ± 3.46 TER value 4 hrs following the calcium switch assay (Fig. 14C). Monolayers treated with dynein/dynactin siRNA, however, recovered TER to only 71.5% ± 5.62 of baseline values (Fig. 14C). We note here that a greater degree of barrier function was recovered after dynein/dynactin siRNA transfection and calcium switch compared to EHNA treatment. We attribute this to the variability of transfection. Knockdown efficiency may be variable among cells in the same Transwell whereas a drug treatment affects all cells equally. Additionally, the use of Transwells requires confluent monolayers and transfection of confluent cells significantly reduces transfection efficiency. Despite these technical difficulties, dynein/dynactin
knockdown significantly impairs TJ restoration compared with scrambled siRNA controls. These data support the findings obtained with pharmacologic inhibition of dynein using EHNA.

Figure 14. Dynein knockdown using siRNA impairs TJ recovery. (A) Dynein/dynactin siRNA constructs were transfected into MDCK cells, fixed and stained for giantin. Dynein/dynactin knockdown induces dispersal of the Golgi apparatus. Anti-giantin stain is shown in green, nuclei in blue. (B) MDCK cells were treated as in (A), fixed and stained for occludin. Minus-end directed trafficking is required for maintaining TJ structure. (C) MDCK cells were treated as in (A) and the calcium switch assay was performed. Inhibition of minus-end directed trafficking significantly inhibits TJ recovery.

‡ p < 0.001
In addition to lateral MT networks, there are other networks in epithelial cells that have a random orientation (Musch, 2004). Plus-end directed trafficking along these networks may contribute to TJ recovery. To investigate whether kinesin inhibition affected MTs structurally, Caco-2 cells were treated with the kinesin inhibitor adenylyl-imidodiphosphate (AMP-PNP) and stained for tubulin. MTs in AMP-PNP treated cells were indistinguishable from control cells after 6 hrs (Fig. 15A). The role of plus-end directed trafficking in TJ restoration was investigated using the calcium switch assay. Prior to chelation, Caco-2 monolayers were treated with AMP-PNP for 1 hr. Control monolayers recovered 69.4% ± 6.05 of baseline TER 4 hours after calcium switch and AMP-PNP-treated monolayers recovered 77.0% ± 7.30 of baseline, a non-significant difference (Fig. 15B). We note that control monolayers did not fully recover by 4 hours. While every effort is made to standardize TER assays, a significant amount of variation is expected. Variation in recovery of control monolayers may be attributable to passage number, growth conditions, age of the cells, temperature of the room the TER measurements were taken in, etc. Our data represent at least 3 separate experiments all of which support a non-significant difference between recovery of control monolayers and monolayers with inhibited plus-end directed trafficking.

To address more directly the role of plus-end directed trafficking in TJ recovery, MDCK cells were transfected with either kinesin heavy chain or scrambled siRNA and calcium switch assays were performed. Unlike dynein inhibition, kinesin siRNA did not disrupt TJ structure (Fig. 15C). There was no significant difference in the recovery of TER from calcium switch in monolayers treated with scrambled siRNA and those treated with siRNA to kinesin heavy chain (81.7% ± 5.08 vs. 78.2% ± 4.03 of baseline TER value at 4 hrs), confirming the data using AMP-PNP (Fig. 15D). These findings demonstrate that plus-end directed trafficking does not contribute to TJ restoration.
Figure 15. Kinesin inhibition does not affect TJ restoration. (A) AMP-PNP treatment, 500 μM, does not alter MT structure. (B) Kinesin inhibition mediated by AMP-PNP has no impact on TJ recovery after calcium switch. (C) Kinesin knockdown using siRNA does not affect occludin localization at the cell periphery. (D) Kinesin inhibition using siRNA does not impair TJ recovery after calcium switch.
3. INVESTIGATING THE CONTRIBUTION OF ESPG1/G2 TO EPEC-INDUCED TIGHT JUNCTION DISRUPTION AND INHIBITION OF RECOVERY

a. Rationale

TJ disruption is a well-established phenotype of EPEC infection. While several effectors responsible for the loss of TJ structure and function have been identified, including EspF, MAP, NleA and EspG1/G2, the mechanisms are not well defined. (Dean et al., 2006; McNamara et al., 2001; Thanabalasuriar et al., 2010; Tomson et al., 2005). An exception is NleA which has been shown to mediate its effects via inhibition of protein trafficking.

EspG1/G2 disrupt MT networks (Hardwidge et al., 2005; Matsuzawa et al., 2005; Shaw et al., 2005b; Smollett et al., 2006; Tomson et al., 2005). Mutation of these effectors does not prevent loss of barrier function, but attenuates it (Tomson et al., 2005). Its impact on TJ structure is currently unknown. EspG may mediate its effects on the TJ via its impact on MTs, but this has not been determined. Recently, EspG was shown to bind to ARF and PAK protein family members, but these interactions have not been investigated with respect to destruction of MTs, or loss of TJ integrity.

Our previous data strongly supported a role for MTs in TJ maintenance and restoration. We speculated that EPEC-induced MT destruction could have the same effect on TJ integrity as nocodazole or cold treatment, namely loss of structure and function and inhibition of recovery after insult. The hypothesis of Chapter 3 is that EspG1/G2 destroys MTs, compromising TJs and impeding barrier restoration. The aim of this chapter is to determine the role of EspG in EPEC-induced TJ disruption and inhibition of recovery.
b. EPEC infection induces internalization of occludin

To date, no investigation has established that EPEC-induced cytosolic accumulation of occludin is migration of surface protein internally rather than accumulation of protein moving though the Golgi (Guttman et al., 2006; McNamara et al., 2001; Shifflett et al., 2005; Simonovic et al., 2000). Moreover, constitutive recycling of TJ proteins suggests that a significant portion of occludin may be cytosolic in the homeostatic state. We therefore determined whether the well-established accumulation of occludin in the cytosol induced by EPEC was due to internalization (McNamara et al., 2001; Simonovic et al., 2000).

Caco-2 intestinal epithelial cells were biotinylated as previously described (Morimoto et al., 2005; Nishimura and Sasaki, 2008). After infection with EPEC, any remaining extracellular biotin was stripped. Infected cells were lysed and internalized, biotinylated proteins were pulled down with avidin beads and blotted for occludin. Compared with uninfected controls, 4 hours of EPEC infection induced a 1.6-fold increase in occludin internalization (Fig. 16). This suggests that relocalization of occludin to the cytosol during EPEC infection is in part due to internalization and not interruption of the secretory pathway. It also suggests that EPEC effectors may override host cell mechanisms that recycle occludin back to the plasma membrane in the steady state.
c. EPEC effector proteins delay tight junction recovery

Having demonstrated that effectors induce internalization of occludin, we wanted to determine the impact of effectors on TJ recovery. T84 cells were infected with wild-type EPEC till TER decreased by ≈40%, indicating that effector proteins were in the host cytosol. A calcium switch assay was performed to wholly disrupt TJs (Cassidy and Tidball, 1967; Cereijido et al., 1981; Denker and Nigam, 1998; Farshori and Kachar, 1999; Stuart et al., 1994). Calcium was reintroduced using media that also contained gentamicin to kill any attached bacteria while allowing internal effector proteins to persist. Recovery of TER was monitored. Chelated monolayers achieved 83.9 ± 6.63% of baseline TER within 4 hours. EPEC-infected cells recovered only 46.1 ± 4.75% of baseline TER within the same time (Fig. 17). These data suggest that effector proteins independently impair TJ restoration after insult.
Figure 17. EPEC effector proteins impair TJ recovery. Pre-infection of T84 cells significantly impaired recovery of TER after calcium switch. * $p < 0.05$

d. Intact microtubule networks promote tight junction recovery after EPEC infection

Our previous investigation suggested that MTs are required for recovery of barrier function. We wanted to determine if EspG1/G2 were responsible for inhibition of recovery induced by EPEC infection. Caco-2 cells were used for this assay instead of T84 cells. Caco-2 cells have a phenotype like that of small bowel enterocytes, while T84s more resemble the colonic crypts (Dharmsathaphorn et al., 1984; Hidalgo et al., 1989). The epithelial cells EPEC is most likely to encounter are more similar to Caco-2...
cells. Both cell lines are polarized intestinal epithelial cells, making the data obtained using both cell lines highly relevant.

Caco-2s were infected with wild-type EPEC and an ΔespG1/G2 strain. After TER decreased by 40%, monolayers were washed, treated with gentamicin and recovery was monitored. Twenty hours after gentamicin, wild-type-infected monolayers attained only 64.1 ± 4.48% of baseline TER values (Fig. 18). ΔespG1/G2- infected cells completely regained barrier function after 17 hours, suggesting that intact MT networks promote TJ recovery after EPEC infection.

Figure 18. Intact microtubules delay loss of TER and promote TER recovery after EPEC infection. Wild-type infection of Caco-2 cells induced a more rapid loss of barrier function compared with ΔespG1/G2 infection. After gentamicin treatment, cells infected with ΔespG1/G2 recovered barrier function more rapidly than cells infected with wild-type EPEC. § p < 0.01
e. Pharmacological microtubule destruction compensates during ΔespG1/G2 infection

EspG was shown to not only affect MTs, but potentially modulate GTPase signaling (Selyunin et al., 2011). To determine if EspG’s effect on TER is mediated via its impact on MTs, these structures were perturbed using cold temperature and nocodazole treatment prior to infection. Wild-type infection rapidly decreased TER as previously reported (Fig. 19) (McNamara et al., 2001; Simonovic et al., 2000). Infection with ΔespG1/G2 induced a gradual drop in TER which has also been previously reported (Tomson et al., 2005). Infection with ΔespG1/G2 in the absence of MTs decreased TER more rapidly than the mutant alone, achieving TER comparable to wild-type infection by 5 hours (Fig. 19). These data demonstrate that pharmacologic MT disruption compensates during ΔespG1/G2 infection and suggest that EspG’s contribution to TER loss is due to its impact on MTs.
Figure 19. Artificial microtubule destruction compensates during ΔespG1/G2-induced TER loss. Wild-type infection of Caco-2 cells induces a precipitous loss of TER. Cold temperature/nocodazole treatment induces a non-significant drop in TER as in Fig. 7. Infection with ΔespG1/G2 induces a gradual loss of barrier function. Destruction of MTs prior to ΔespG1/G2 infection rescues the wild-type phenotype. § $p < 0.01$

**f. EspG-mediated microtubule disruption obstructs tight junction restoration**

Having shown that intact MTs promote barrier recovery after EPEC-infection, we wanted to address the role of EspG in TJ restoration. MDCKs were used because they are polarized epithelial cells, generate resistance, and also readily take up foreign DNA, making them ideal for transfection assays. To investigate the effect of EspG1 in the absence of other effectors, EPEC espG1 was cloned into an RFP-tagged mammalian expression vector and transfected into MDCK cells. A calcium switch assay
was performed and barrier recovery monitored. Three hours after the reintroduction of calcium, control monolayers completely recovered baseline TER. In the same time cells expressing EspG recovered only 85.1 ± 2.81% of baseline TER (Fig. 20). EHEC EspG is 98% identical to EPEC EspG and similarly disrupts MTs. We wanted to determine if it had a similar impact on TJ restoration. An EHEC GFP-EspG construct obtained from our collaborator Dr. Neal Alto recovered 85.1 ± 5.93% of baseline, emphasizing the functional similarity of this protein (Fig. 20). These data confirm our previous results and reinforce the vital role of MTs in TJ recovery.

Figure 20. EspG independently impedes TJ restoration. Data represent TER values 3 hours after calcium switch and are compiled from 3 separate assays. * p < 0.05
g. EspG destroys microtubules, disperses the Golgi and perturbs tight junctions

Despite substantial literature documenting the MT-loss phenotype, there is ongoing debate about the impact of EspG on MTs. One report showed Golgi dispersal 20 minutes after microinjection of EHEC EspG into rat kidney cells, but did not observe MT destruction (Selyunin et al., 2011). A separate study demonstrated Golgi dispersal after transfection of EPEC and EHEC EspG, but did not report a severe effect on MTs. A thickening of MTs was observed rather than depolymerization (Clements et al., 2011). Golgi dispersal is a traditional indicator of MT loss, suggesting that the two studies cited above may not have observed MT destruction due to procedural variation (Cole et al., 1996; Gut et al., 1998; Hestermann and Graf, 2004; Yang and Storrie, 1998). We wanted to reconcile the disparate published effects of EspG on MTs and determine if expression of EspG induced MT loss and concurrent Golgi dispersal. Occludin traffics through the Golgi and is glycosylated before reaching the apical membrane (Gut et al., 1998). Golgi dispersal and subsequent loss of newly synthesized TJ proteins could be another mechanism of TJ disruption. We also wanted to determine the impact of EspG on TJs.

Transient expression of EspG in MDCK cells induced disruption of MT networks after 8 hours (Fig. 21A). Expression of RFP-EspG1 also induced progressive tubulin degradation, a phenotype associated with wild-type EPEC infection (Fig. 21B) (Tomson et al., 2005). In control cells, the Golgi was tightly localized to the nucleus. EspG1 induced a diffuse cytoplasmic anti-giantin stain indicating Golgi dispersal (Fig. 21C). To confirm these results in an infection model, Caco-2 cells were infected with wild-type EPEC and ΔespG1/G2 and stained for giantin. Wild-type infection induced Golgi dispersal, whereas ΔespG1/G2-infected cells retained perinuclear Golgi localization (Fig. 21D).

To determine the impact of EspG on TJs, EPEC EspG-transfected cells were fixed after 8 hours and stained for occludin. EspG induced occludin relocalization from the plasma membrane to the cytosol,
suggesting that homeostatic occludin regulation is perturbed (Fig. 21E). It was previously reported that ectopic expression of EspG did not affect TER but led to increased paracellular permeability to 4 kDa dextran (Matsuzawa et al., 2005). Taken together, these data suggest that EspG-mediated MT destruction has significant effects on MTs, the Golgi and TJs.
Figure 21. EspG acts independently to disrupt microtubules, degrade tubulin, disperse the Golgi and disrupt TJ structure. (A) Transient expression of RFP-EspG1 for 8 hours induces severe loss of MT networks. (B) It also degrades tubulin over time. EspG1 disperses the Golgi after 8 hours after transfection, a phenotype that was replicated using wild-type EPEC expressing DsRed (C). (D) An ΔespG1/G2 mutant expressing DsRed does not induce Golgi dispersal. (E) Eight hours of transient EspG expression also relocalizes occludin to the cytosol.
h. **EspG-mediated microtubule disruption is not due to its role as a GTPase regulator**

The mechanism of EspG-mediated MT destruction is still unknown. Recent work has proposed a new role for EspG as a GTPase regulator, moderating the activities of PAK and ARF (Germane and Spiller, 2011; Selyunin et al., 2011). To determine if these proteins are involved in EspG-mediated destruction of MTs, ARF- and PAK-binding EHEC EspG mutants were obtained from our collaborator (Selyunin et al., 2011). Given the significant structural and functional conservation between EPEC and EHEC EspG, we do not anticipate differences between the two (Clements et al., 2011; Smollett et al., 2006; Tomson et al., 2005).

Transient expression of wild-type EspG, ARF- and PAK-binding mutants in MDCK cells induced MT disruption after 8 hours (Fig. 22A). While these data imply that EspG interactions with ARF and PAK are not required for MT disruption, we considered whether this phenotype might be an expression artifact. We therefore transfected the effector EspF, which has no reported impact on MTs, into epithelial cells. MTs were intact in the presence of EspF, indicating that MT disruption is not an artifact of overexpression and confirming our prior results (Fig. 22B). Expression of all EspG constructs also dispersed the Golgi apparatus, confirming the MT disruption phenotype (Fig. 22C) (Cole et al., 1996; Hestermann and Graf, 2004; Yang and Storrie, 1998).
Figure 22. EspG does not induce microtubule destruction via interactions with ARF or PAK GTPases. (A) Transient expression of wild-type EHEC EspG, and an EspG ARF- or PAK-binding mutant in MDCK cells for 8 hours results in microtubule destruction. (B) Transient expression of GFP-EspF does not alter MTs. (C) Wild-type EspG, the ARF-binding mutant and the PAK-binding mutant all disperse the Golgi after 8 hours of expression in MDCK cells.
To further confirm that ARF and PAK were not involved in EspG-induced MT loss, we cloned the ARF- and PAK-binding mutants into a bacterial expression vector and complemented ΔespG1/G2 with the resulting plasmids. Infection with these strains induced MT disruption, confirming our previous transfection data (Fig. 23). Taken together, these data establish that EspG-induced MT destruction is entirely independent of EspG’s role as a modulator of GTPase signaling.

Figure 23. Bacterial delivery of EspG ARF-and PAK-binding mutants disrupts MTs. Delivery of EspG constructs through the TTSS destroys MTs. Bacteria are shown in green, MTs in red and nuclei in blue.
4. DISCUSSION

a. The role of microtubules in tight junction homeostasis and restoration

The original model of occluding junctions described them as “sealing” off the paracellular pathway in a static fashion (Cereijido et al., 1981). New studies have demonstrated that TJ proteins recycle constitutively, showing that the TJ is dynamic. Our data directly address a need to determine what cellular components are required for these events. To our knowledge this is the first study exploring the role of MTs in TJ reconstruction.

i. Microtubules are conduits for occludin trafficking and are required for tight junction integrity

A previous study examined the impact of nocodazole on the movement of GFP-tagged occludin (Subramanian et al., 2007). Occludin movement was impeded in cells with disrupted MTs, implying that these structures are required for trafficking. Our real-time imaging data demonstrate that occludin-containing vesicles use MTs as “molecular highways.” MT disruption affected significantly the total distance traveled by occludin, reducing it by 44%, supporting the previous finding that in the absence of MTs, occludin movement is severely limited. Together these data suggest that MTs play a vital role in occludin transport.

There is controversy regarding the role of MTs in maintaining TJ structure. One publication reported that treatment of MDCK cells with nocodazole had no impact on TJ structure however only early time points, 60-90 min, were examined (Subramanian et al., 2007). A later study reported that prolonged perturbation of MTs in polarized thyroid epithelium induced the cytosolic accumulation of occludin and a significant drop in TER (Yap et al., 1995). Our results reconcile these controversial data by showing that the effect of MT disruption on TJs is time-dependent as incubation with nocodazole induced dose-
and time-dependent changes in TJ structure and function. The relationship between loss of TJ structure and loss of function after MT disruption supports a vital role for MTs in TJ homeostasis.

We note here that the role of MTs in correctly localizing claudin-1 to the TJ was investigated as well, but only preliminary data was obtained. Nocodazole treatment induced feathering of claudin-1 away from the TJ, but cytosolic accumulation was never observed, even at late time points. We hypothesize that the homeostatic regulation of claudin-1 significantly differs from occludin regulation and the role of MTs may be different. Claudin-1 was not further explored in this study.

The role of recycling in TJ physiology is just beginning to be explored and may provide a framework for interpreting the time-dependent effect of MT disruption on TJs. Cell-surface biotinylation assays demonstrated that occludin is internalized continuously under homeostatic conditions with approximately 70% of internalized occludin being recycled back to the membrane within 5 min (Morimoto et al., 2005). A separate study imaged live cells and used fluorescence recovery after photobleaching and determined that occludin also diffuses laterally within the plasma membrane while ZO-1 recycles from the cytosol (Shen et al., 2008). Claudin-1 was also shown to recycle (Dukes et al., 2011; Dukes et al., 2012).

We speculate that MTs serve as conduits for the continuous recycling of occludin. With regard to the time-dependent effects of MT loss on TJs, in the absence of MTs, intracellular pools of occludin would accumulate in the cytosol as it would be unable to return to the membrane in an efficient manner. The extent of cytosolic occludin accumulation would depend on the recycling rate. At early time points following MT loss, the amount of occludin accumulated in the cytosol may be below detectable limits, thus explaining prior results. At later time points, we and others showed that MT loss resulted in significant cytosolic accumulation and a corresponding loss of barrier function possibly induced by the inhibition of recycling.
ii. MTs are required for TJ restoration

The cytoskeletal elements required for TJ protein recycling are currently not known, however the movement of TJ proteins to the cytosol has been shown to be MT-dependent. In a previous study, the disruption of MTs prevented the movement of occludin and ZO-1 from the membrane to the cytosol in response to calcium chelation (Ivanov et al., 2006). The authors speculated that internalization required MT-based trafficking. The reverse process of TJ restoration, however, had not been investigated. Nonetheless, taken together, several avenues of previously published data suggested this intriguing possibility. *E. coli* cytotoxic necrotizing factor-1 (CNF-1) induced the internalization of occludin that was associated with Rab11-positive endosomes (Hopkins et al., 2003). Rab11 is involved in endosome recycling and utilizes MTs for trafficking (Casanova et al., 1999; Takahashi et al., 2012; Ullrich et al., 1996). These findings suggest that the recycling of internalized occludin to the membrane could hasten recovery of barrier function following insult.

The mechanisms involved in occludin recycling are not well defined (Morimoto et al., 2005; Nishimura and Sasaki, 2008; Shen et al., 2008). The small GTPases Rab13, Rab11 and Rab8 have been identified as regulating occludin turnover (Hopkins et al., 2003; Morimoto et al., 2005; Peranen et al., 1996; Takahashi et al., 2012; Yamamura et al., 2008). Also, occludin-containing endosomes have been associated with multiple markers including myosin II, caveolin, clathrin and syntaxin-4 (Ivanov et al., 2004; Shen and Turner, 2005; Utech et al., 2005). However, most of these studies have focused on occludin endocytosis in response to stimulus like IFN-γ rather than on the homeostatic mechanisms that regulate occludin recycling.

Our study directly addressed the role of occludin trafficking in TJ recovery using the well-described calcium switch assay and monitoring TJ barrier recovery in the absence of MTs and found that the
recovery of barrier function is significantly inhibited. We speculate that in the absence of MTs, occludin cannot recycle back to the membrane thus impeding the structural and functional recovery of TJs. Our data support the concept of a process that requires MTs and drives the reconstruction of TJs.

While these data support a vital role for MTs in TJ restoration, they also suggest the involvement of MT-independent mechanisms. Actin-based systems may account for the observed recovery of 40% of baseline TER as actin is known to participate in maintaining TJ structure and function (Shen and Turner, 2005). Occludin is mobile without MTs, but travels significantly shorter distances (Subramanian et al., 2007). Simple diffusion of occludin-containing vesicles may contribute to TJ recovery in the absence of MTs, but the observed plateau in TER recovery is not consistent with this possibility, as diffusion alone would result in a slow but continuous rate of recovery that could fully repair TJs. MTs, therefore, seem to play a critical role in TJ reconstruction that cannot be compensated for by other mechanisms.

iii. Microtubule motors transport occludin and dynein is required for tight junction restoration

Particle tracking demonstrates that occludin moves along MTs in all directions and suggests that both dynein and kinesin are utilized. The impact of MT disruption on the velocity of occludin is interesting to view from the perspective of what is known about MT motors. Reductionist assays using purified dynein found the velocity of a single motor to be 800 nm/second (Toba et al., 2006). A single kinesin molecule has a velocity of 204 nm/second (Svoboda and Block, 1994). Multiple motors of either type may pull a single cargo, generating speeds up to ten times greater than an individual motor (Driver et al., 2011; Gagliano et al., 2010; Kural et al., 2005; Levi et al., 2006). Our velocity data therefore are consistent with published literature and suggest that occludin trafficking employs multiple MT-based motors.
There are limited data showing that kinesin is required for junctional disassembly, but the role of MT motors in TJ reconstruction has not been explored (Ivanov et al., 2006). In polarized epithelial cells, MTs are oriented longitudinally, with minus-ends pointed apically and plus-ends pointed basolaterally (Musch, 2004). Kinesin depletion inhibits basolateral transport while MT disruption inhibits transport in both directions (Lafont et al., 1994). Our data suggest that trafficking back to the membrane is mediated by dynein. We speculate that without MTs and hence without dynein-mediated trafficking, recycling occludin cannot reach the membrane, leading to prolonged barrier disruption because the TJ cannot reconstruct.

Other studies have demonstrated a link between dynein and Rab11 that supports our model of MT-dependent TJ reconstruction (Horgan et al., 2010a, b). Rab11 mediates its downstream effects via “family of Rab11-interacting proteins” (FIPS). FIP3 was identified as a primary recruiter of dynein to membranous vesicles, implying that a population of recycling proteins requires MTs and dynein for transport (Horgan et al., 2010a). Our data concur with these findings and strongly support a role for MTs in TJ recycling and restoration. We speculate that in the absence of MTs, recycling occludin endosomes may be able to recruit dynein, but are unable to reach the membrane and reconstruct the TJ.
Figure 24. Summary of Aim 1, determining the role of microtubules in TJ homeostasis and restoration. This report has shown that MTs are required for maintenance of TJ structure and function. We have also demonstrated that dynein-mediated MT transport is required for restoration of TJ structure and function after insult.
b. The contribution of EspG1/G2 to EPEC-induced tight junction disruption

EPEC infection is a major cause of diarrhea in the developing world. Infants are primarily affected by this pathogen and may experience repeated infections (Fagundes-Neto et al., 1997; Hill et al., 1991). This leads to prolonged intervals during early childhood development without the benefit of micronutrients, sufficient glucose, and other nutritive factors absorbed via the intestine (Fagundes-Neto et al., 1996). Repeated enteric infections may seriously impact growth and development (Petri et al., 2008). Discovering new therapeutics that abbreviate or thwart intestinal barrier loss is clinically important.

While the effector proteins participating in TJ disruption are known, the mechanisms are not well-defined. Our study addresses the role of EspG1/G2 in compromising TJ integrity and support a role for inhibition of TJ restoration as a new aspect of EPEC pathogenesis.

TJ disruption occurs as a result of many stimuli. IFN-γ induces internalization of TJ proteins that colocalize with actin and myosin II (Utech et al., 2005). IFN-γ-dependent internalization of TJ proteins occurs via macropinocytosis (Bruewer et al., 2005). TNF treatment induces caveolin-dependent endocytosis of TJ proteins and perturbation of barrier function (Marchiando et al., 2010). While EPEC infection also leads to accumulation of occludin in the cytosol and corresponding loss of barrier function, to date it has not been shown that internalization is responsible for these phenotypes (McNamara et al., 2001; Shifflett et al., 2005; Simonovic et al., 2000).

Blocking Golgi-based protein secretion also induces intracellular accumulation. NleA and EspG1/G2 disperse the Golgi via different mechanisms (Clements et al., 2011; Kim et al., 2007; Thanabalasuriar et al., 2010; Tomson et al., 2005). These effectors also contribute to loss of TER, implying that inhibiting protein secretion may result in loss of TJ integrity (Thanabalasuriar et al., 2010; Tomson et
al., 2005). However, EspF and MAP are also required for TJ disruption. They do not affect protein secretion, suggesting that alternative mechanisms, such as internalization, may be responsible for loss of TJ integrity (Dean and Kenny, 2004; Dean et al., 2006; McNamara et al., 2001; Shifflett et al., 2005).

To determine whether intracellular occludin associated with EPEC infection originates from the membrane, we used a biotinylation/pull-down assay. Our data establish for the first time that internalization is partially responsible for cytosolic occludin accumulation. While we cannot rule out the possibility that Golgi-dispersal contributes to this phenotype, our findings establish that, like the epithelial response to cytokines, EPEC induces peripheral proteins to traffic to the cytosol. We speculate that EPEC-induced internalization may be due to interruption of constitutive protein recycling.

i. EPEC effectors impede tight junction restoration potentially due to microtubule loss.

Previously published data imply that recycling of TJ proteins may participate in barrier restoration. *E. coli* cytotoxic necrotizing factor-1 (CNF-1) induces occludin association with recycling endosomes, suggesting that internalized protein can traffic back to the membrane and hasten recovery (Hopkins et al., 2003). Termination of EPEC infection permits restoration of TJ structure and function, implying that EPEC effectors interfere with TJ maintenance and thus prevent recovery (Simonovic et al., 2000). To address this possibility, we infected monolayers with EPEC, performed a calcium switch assay and killed any attached bacteria with gentamicin. Our data demonstrate that effectors impede recovery of TJ function after insult. Prior studies have focused on disruption of TJs. Inhibition of restoration may be a hitherto undescribed aspect of EPEC pathogenesis.

Having shown that effectors impede TJ recovery we wanted to determine the contribution of EspG1/G2 to this process. We previously demonstrated that in the absence of MTs TJ restoration was impaired, suggesting that EPEC-induced MT destruction could have a similar impact. To date, the role of MTs in TJ recovery after EPEC infection has not been investigated. We addressed this question by infecting
monolayers with wild-type EPEC or a ΔespG1/G2 strain, allowing TER to drop by ≈40% and terminating the infection with gentamicin. Loss of TER after wild-type infection was more rapid compared with ΔespG1/G2 infection as has been published previously (Tomson et al., 2005). We speculate that intact MTs enable continuous barrier restoration during infection with ΔespG1/G2 and thus forestall rapid TER loss. EspF, MAP and NleA are still expressed by ΔespG1/G2 infection thus barrier function is compromised eventually.

In the infection model, intact MTs also promoted TJ recovery after gentamicin treatment compared to wild-type infected cells. Taken together, these data indicate that efficient TJ restoration requires MTs, confirming our previous conclusions. The role of EspG1/G2 in EPEC pathogenesis is therefore expanded to include inhibition of TJ recovery, via its impact on MTs.

MT disruption by nocodazole resulted in equivalent TER loss by ΔespG1/G2 to wild-type EPEC at 6 hours post-infection. Although earlier time points show only partial compensation, we attribute this to unequal effects induced by nocodazole and EspG protein on MTs. It is unlikely nocodazole and EspG disrupt MTs with similar kinetics or via a similar mechanism therefore earlier times are less representative of true compensation.

Our results support a two-armed model of EPEC-induced TJ disruption. The first arm, disruption of TJs, may be initiated by EspF and MAP. The mechanism has not been described, but may involve internalization of TJ proteins. We speculate that concurrent with disruption, the second arm is prevention of restoration. This process may be initiated by EspG1/G2 and perpetuates loss of TJ integrity for the duration of infection. Our previous data demonstrating that MTs are required for TJ restoration support this model.
ii. The impact of independent EspG on tight junction integrity and reconstruction

In addition to supporting a vital role for MTs in TJ maintenance, our data suggested these structures were required for TJ restoration. These results were confirmed in our infection model. To determine the impact of EspG on TJ recovery in the absence of other effectors, this protein was transiently expressed in MDCK cells and calcium switch assays performed. EspG impaired TJ recovery after calcium switch compared to control cells transfected with the empty vector. This assay involved transfection of confluent cells grown on Transwells, conditions which greatly reduce transfection efficiency. Low numbers of cells expressing EspG may account for the small difference in recovery compared to controls. We speculate that by disrupting MTs and impairing occludin recycling, EspG prevents TJ restoration and prolongs barrier loss.

Our earlier results suggest that dynein inhibition may be secondary to MT disruption in impeding TJ homeostasis and restoration. Although we did not evaluate the impact of EPEC infection on dynein, these motors may be targets of other bacterial effectors. Kinesin-1 was identified as a target of the Salmonella effector protein SifA (Diacovich et al., 2009; Dumont et al.; Dumont et al., 2007) SifA binds to SKIP (SifA and kinesin interacting protein) which links the complex to kinesin-1 light chain. The SifA/SKIP complex promotes kinesin-mediated vesicular transport away from Salmonella-containing vacuoles, potentially contributing to their formation or transport. Other enteric pathogens, such as EPEC and EHEC, may also contain effectors that interact with microtubule motor proteins and thus may inhibit TJ protein trafficking in two separate ways, by destroying MTs and by affecting motor proteins. Such redundancy is common among EPEC effectors, a good example being the 5 proteins required for TJ disruption (Dean and Kenny, 2004; McNamara et al., 2001; Simonovic et al., 2000; Thanabalasuriar et al., 2010).
iii. EspG independently affects multiple organelles

Despite significant data establishing EspG’s effect on MTs, the mechanism of EspG’s contribution to barrier loss has not been conclusively determined. We wanted to investigate the impact of EspG in the absence of other effectors. Disassembly of MT networks and tubulin degradation are associated with EPEC infection (Tomson et al., 2005). Purified EspG protein depolymerizes MTs in solution (Hardwidge et al., 2005; Matsuzawa et al., 2004). Our data concur with EspG acting independently to induce MT depolymerization in the absence of other bacterial proteins. EspG expression induced tubulin degradation. Previous work showed that tubulin degradation associated with wild-type infection was absent after infection with ΔespG1/G2, but other effectors were present (Tomson et al., 2005). To our knowledge this is the first time an EPEC effector has been shown to induce protein degradation. Occludin is also degraded during wild-type infection via an unknown mechanism, suggesting that other effectors may induce degradation of specific proteins. Elimination of tubulin monomers may be another means of impeding TJ restoration as new units must be synthesized de novo after EPEC infection (Tomson et al., 2005). The time spend generating new tubulin would further delay TJ reconstruction as our previous data suggest that MTs are required for TJ protein trafficking as well as restoration.

Our data show that EspG disperses the Golgi, a hallmark of MT disruption, both in transfection and infection models. While MT loss may be responsible for this phenotype, EspG binds two other Golgi-associated proteins. EspG binds and sequesters active ARF1 and ARF6 (Selyunin et al., 2011). The ARF family of GTPases regulate vesicle movement, including transport through the ER-Golgi (Appenzeller-Herzog and Hauri, 2006; Kahn, 2009; Selyunin et al., 2011). Although no mechanism has been demonstrated, EspG interference with ARF-mediated trafficking could potentially induce Golgi dispersal. EspG also binds to Golgi Matrix Protein 130 (GM130) (Clements et al., 2011). The physiological relevance of this interaction is currently not described. Whether Golgi dispersal is secondary to MT disruption or the result of an alternative mechanism, we speculate that it contributes to
inhibition of TJ restoration. New tubulin and possibly occludin must be synthesized for complete barrier recovery and Golgi dispersal forestalls this. Thus EspG may retard TJ recovery in multiple ways.

Our previous work demonstrated that in the absence of MTs TJ structure and function are compromised, potentially due to inhibition of constitutive occludin recycling. In addition to the effects above, transient expression of EspG induced relocalization of occludin to the cytosol. These data confirm our prior results and underscore the importance of MTs in TJ maintenance. It has been reported previously, that TER is unaffected by EspG though selective permeability may be altered (Matsuzawa et al., 2005).

iv. EspG interactions with ARF and PAK are unrelated to microtubule disruption

It is well-established that EspG1/G2 destroy MTs, but the mechanism is unknown. To determine if EspG’s role as a GTPase mediator was linked to MTs, we transfected EspG ARF-and PAK-binding mutants and stained for tubulin. All constructs induced MT disassembly. These data are not expression artifacts as GFP-tagged EspF had no effect on MTs. Bacterial delivery of EspG and the ARF- and PAK-binding mutants also perturbed MTs, confirming that in the absence of interacting with either small GTPase, EspG still destroys MTs. We note here that two studies reported EspG had no effect on MTs (Clements et al., 2011; Selyunin et al., 2011). We attribute the discrepancy to differences in protocol (protein microinjection vs. gene transfection), cell line used and time of MT assessment. Our results establish that EspG1/G2 has multiple, unrelated functions like EspF. EspF has been implicated in many processes including apoptosis, TJ loss, effacement of microvilli, mitochondrial dysfunction, EPEC invasion, pedestal formation and N-WASP activation (Alto et al., 2007; Crane et al., 2001; Nougayrede and Donnenberg, 2004; Peralta-Ramirez et al., 2008; Shaw et al., 2005a; Weflen et al., 2010). EspG1/G2 may have a similarly broad scope of action.
In the ongoing hunt for therapeutic targets, EPEC effectors with broad capabilities invite further investigation; disabling one potent effector with a potent range of functions could lead to greatly reduced physiological disturbance. The expanding repertoire of EspG1/G2 demonstrates that further investigation is needed to fully understand their role in EPEC-induced loss of epithelial barrier function and inhibition of barrier restoration.
Figure 25. Summary of Aim 2, determining the role of EspG in TJ regulation and restoration. We have demonstrated that EspG destroys MTs, inducing occludin accumulation in the cytosol. We have also shown that intact MTs delay loss of TER during EPEC infection and promote TJ recovery. This may be mediated by the effect of EspG on MTs and/or the Golgi. EspG’s interactions with ARF and PAK are not required for MT destruction.
Conclusion

This report is the first to examine the role of MTs in TJ homeostasis and restoration. Additionally, it is the first to study the role of EPEC effector protein EspG in the context of intestinal barrier loss.

The structure of the TJ has long been known, as well as its occlusive and selectively permeable properties. However, the dynamic nature of the TJ is a relatively new discovery. Until recently, the cellular components required for maintaining the structure did not appear to be worth investigating as the TJ was thought to be static. Recent data has shown actin to be required for maintaining TJ structure and function in the steady state (Shen and Turner, 2005). Occludin has been implied to traffic on MTs, but the relationship to TJ structure and function was not known.

This study is the first to identify MTs as essential for maintaining TJ structure and function in the homeostatic state, revealing a new aspect of TJ regulation. MT-based trafficking is a well-established area of study. By demonstrating the requirement for MTs in TJ homeostasis, we open a new area of investigation, incorporating both TJ proteins and MT physiology.

Additionally, our study of TJ restoration describes a novel aspect of TJ regulation. While many disease states involve epithelial barrier disruption (asthma, inflammatory bowel disease, male infertility, etc) the goal of translational research is to determine how to promote barrier recovery (Heller et al., 2005; Holgate, 2007; Saitou et al., 2000; Tai et al., 2006; Vinhas et al., 2011; Wang et al., 2010; Weber et al., 2010; Wong et al., 2010b). We demonstrate that MTs and dynein are required for complete TJ restoration. To our knowledge this is the first study to definitively identify a cellular component required for this process.

Similarly EPEC infection has been primarily studied in terms of TJ disruption. Effector proteins EspF and MAP are required for loss of TJ structure and function. Their mechanism of action is unknown (Fig.
NleA and EspG1/G2 have also been shown to contribute to barrier loss. NleA acts by inhibiting anterograde transport, preventing newly synthesized proteins from trafficking through the Golgi (Fig. 26C). Although EspG is known to perturb MTs, the mechanism of EspG’s contribution to TJ disruption was unknown.

Having demonstrated that MTs were required for TJ recovery we hypothesized that interruption of a continuous ongoing MT-dependent restoration process could contribute to loss of barrier function. This represents a novel way to approach TJ disruption. In addition to the perturbation mediated by EspF and MAP, we surmised that EspG contributed to barrier loss by inhibiting TJ restoration (Fig. 26B).

Our data establish that EspG prevents TJ restoration during EPEC infection. We also demonstrate that EspG degrades tubulin and induces Golgi dispersal. While we did not definitively determine whether Golgi dispersal was secondary to MT loss, these data suggest that EspG may impede TJ recovery in numerous ways. MT loss may inhibit TJ restoration (as we demonstrated previously), tubulin degradation may contribute to this process, and blocking Golgi trafficking may also impair TJ restoration. Taken together, loss of barrier function may be prolonged during EPEC infection due to EspG.

By illuminating a new role for MTs in TJ restoration, and the role their loss plays in EPEC pathogenesis, many new avenues of investigation are opened. For example, the role of the rab small GTPases have only begun to be investigated with respect to TJs. Rab11 is associated with MTs and might play a role in TJ regulation. Further work is needed to fully explore the relationship between TJs and MTs but in the homeostatic state and during EPEC infection.
Figure 26 – The role of EspG in EPEC pathogenesis in the context of other effectors. (A) EspF and MAP induce TJ disruption via an unknown mechanism. Both TJ structure and function are lost. (B) EspG1/G2 perturb MTs, which may prevent restoration of the barrier during EPEC infection. Currently the contribution of recycled occludin to restoration is not known, nor is the mechanism of recycling well understood. (C) EspG1/G2 also induce Golgi dispersal and may work in concert with NleA to impede anterograde transport of newly synthesized proteins. The importance of new proteins in TJ restoration is currently not known.
c. Limitations

While cell culture models are suitable for establishing proof of principle, working with an enteric pathogen has unique limitations. The intestine undergoes peristalsis, which impacts bacterial attachment and clearing of infection. A mucous layer overlying the epithelium also affects attachment. The gut is also termed “the largest immune organ” due to the presence of gut-associated lymphoid tissue (GALT). The presence of antimicrobials and defensins modulates responses to infection (Masuda et al., 2011). The microbiota also contribute to the host response to enteric pathogens (Bailey, 2012; Collado et al., 2007; Sekirov and Finlay, 2009; Sekirov et al., 2008). None of these are present in cell culture models of infection. Animal models of infection provide a more realistic means of investigating EPEC pathogenesis. We have not demonstrated the role of MTs in TJ homeostasis and restoration in vivo, a significant limitation of this body of work.

EPEC models of infection include a mouse and a rabbit model, as well as a Citrobacter rodentium mouse model of infection. They are not without problems however. Inducing EPEC infection in mice requires significantly more inoculating organisms, $1 \times 10^9$ instead of $1 \times 10^1$ (Savkovic et al., 2005; Shifflett et al., 2005). While infected mice lose significant amounts of weight, diarrhea, the dominant phenotype in humans, is absent. Rabbit models of infection are also not exact parallels of human disease (Marches et al., 2000; Milon et al., 1999).

None of our studies were conducted in animals to confirm our in vitro data, however MT-disrupting drugs are currently used to treat human disease. Colchicine binds to tubulin and inhibits polymerization, a mechanism similar to nocodazole. It is used in the treatment of gout and Familial Mediterranean Fever. Intestinal side effects include diarrhea and increased permeability (Fradkin et al., 1996; Ratnaike and Jones, 1998). While the precise mechanism is not clear, we speculate that MT destruction may be inducing loss of TJ integrity and inhibiting barrier recovery, as we demonstrated in vitro.
Another limitation of this study is that we did not definitively demonstrate that MT disruption inhibits recycling. We infer that MTs are required for recycling based on numerous pieces of evidence from our study and the work of others. It is well-established that occludin recycles constitutively. We definitively showed that occludin traffic on MTs. MTs are required for internalization during calcium chelation and also for recovery of barrier function. Rab11 has been shown to co-localize with vesicles recycling to the membrane and trafficking on MTs. Internalized occludin has been shown to associate with Rab11. Taken together, MTs are required for transport to the cytosol, and transport to the membrane, suggesting that these structures are required for recycling. To definitively demonstrate this we will need to investigate occludin recycling using the previously published biotinylation/pull-down assay in the presence and absence of MTs (Nishimura and Sasaki, 2008). This limitation, however, does not negatively impact our findings that MTs are required for TJ homeostasis and restoration, but opens future avenues of investigation.

**d. Future directions**

**i. Establishing the role of microtubules in tight junction recovery using live-cell imaging of EPEC-infected cells**

Our data provide strong evidence that MTs are required for recovery after EPEC infection, but most convincing would be visual and numerical support of this conclusion. Live-cell movies demonstrating that occludin traffic on MTs established the utility of streaming capture and obtaining numerical tracking data.

To definitively establish the role of MTs in TJ recovery, we will infect RFP-occludin stably transfected cells with GFP-EPEC and GFP-ΔespG1/G2. Our lab has already made these strains. Streaming movies of occludin movement will be captured during infection. To assay TER, infections on Transwells will be performed in parallel. We hypothesize that infection with the ΔespG1/G2 strain will induce less
severe cytosolic occludin accumulation as recycling would be expected to be intact. After treatment with gentamicin, streaming movies of occludin trafficking will be captured. Complete recovery of TER after infection takes up to 24 hours, but with the VivaView enclosed objective system, imaging sessions of up to 5 days can be performed. Numerical data representing the directionality of vesicles (traveling away from or towards the membrane) will be generated from these data and enable objective assessment of occludin is movement. These data will strengthen our conclusion that MTs are required for TJ recovery by providing real-time and quantitative data.

**ii. Determining the role of microtubules in trafficking of other tight junction proteins**

A previous study reported that occludin localization correlates with TER measurements (Shen and Turner, 2005). Live-cell imaging and concurrent real-time TER measurements demonstrated that peripheral occludin corresponded to high TER measurements (intact barrier function) while cytosolic occludin was associated with low TER measurements (disrupted barrier). Localization of TJ proteins claudin-1 and ZO-1 did not correspond with barrier function, yet these proteins are also dynamic. On the basis of this study occludin was selected as the representative TJ protein on which to focus our investigations.

The function of occludin is currently debatable. Occludin expression in fibroblasts induces the formation of TJ-like strands, but in occludin knockout mice intestinal TJs appear normal (Furuse et al., 1996; Saitou et al., 2000; Van Itallie and Anderson, 1997). The occludin-null mice however, have a complex phenotype, suggesting that occludin has a broad role in regulating diverse epithelia (Saitou et al., 2000). The function of the claudins is to regulate the charge-selective permeability of the TJ (Anderson and Van Itallie, 2009; Colegio et al., 2002; Weber et al., 2010). ZO-1 links the integral membrane proteins of the TJ to the actin cytoskeleton (Howarth and Stevenson, 1995; Yu and Turner, 2008).
Given the varied functions of these proteins, we consider that they may respond differently to MT loss. To address this question we will perform initial experiments to determine if other proteins TJ require MTs for correct localization at the membrane. Intestinal epithelial cells will be incubated with nocodazole and stained for claudin-1, claudin-2 and ZO-1. If loss of MTs alters their localization, we will obtain epithelial cells stably transfected with fluorescent TJ proteins from our collaborator Dr. Jerrold Turner. His lab has generated cell lines expressing every TJ protein conjugated to multiple fluorophores. These cells will be transiently transfected with GFP-tubulin as in Fig. 1 and live-cell streaming movies will be captured of claudin-1, claudin-2 and ZO-1 to determine if they too traffic on MTs.

We note here that the biotinylation/pulldown recycling assay is also infinitely applicable to determining the role of MTs in the recycling of the claudins as it is feasible to blot for multiple proteins from one assay as long as they are different sizes. We will perform recycling assays in the presence/absence of MTs, and compare the results for claudin-1, claudin-2, ZO-1 and occludin.

iii. Investigating the recycling of occludin in the absence of microtubules

Our conclusion that MTs play a role in TJ homeostasis is strongly supported by cytosolic accumulation of occludin in the absence of MTs. We interpret these data to suggest that recycling is interrupted and consequently protein collects in the cytosol. While further data from our study also support this conclusion, additional investigation of the role of MTs in occludin recycling is needed to strengthen our interpretation.

Biotinylation/pulldown assays are used to investigate the recycling of membrane-bound proteins (Dukes et al., 2011; Dukes et al., 2012; Morimoto et al., 2005; Nishimura and Sasaki, 2008). Surface proteins are biotinylated, stimulus is introduced, then all remaining surface proteins are stripped of biotin (Fig. 27A). Lysing the cells, pulling down internal, biotinylated proteins and detecting them via Western blot
enables the quantification of internalization. To assay recycling, a second incubation period after the first strip enables further internalization or recycling (Fig. 27B). A second stripping step permits the quantification of internalized protein after a recycling interval. By comparing the first blot (internalization) with the second blot (recycling) rates of recycling are calculated (Morimoto et al., 2005).
Figure 27. The biotin internalization and recycling assay. (A) Biotinylating external surface proteins enables determination of internalization. Instead of EPEC infection, nocodazole treatment or other stimuli may be used. (B) A second incubation permits internalized protein to recycle back to the membrane and be stripped. Comparing the second Western Blot with the blot from (A) enables establishment of recycling.

To determine the role of MTs in this process, we will biotinylate all surface proteins, incubate the cells to allow homeostatic internalization, and then strip external biotin. Some of these cells will be lysed and blotted for TJ proteins. Others will be reincubated with or without nocodazole. These cells will then be stripped a second time and the amount of biotinylated protein remaining internally will be determined via Western blot.

We hypothesize that in the absence of MTs, most internalized occludin will remain internal because it cannot recycling back to the membrane. In the presence of intact MTs, most internalized protein will return to the membrane and be stripped of biotin.
iv. Determining the mechanism of microtubule-dependent recycling

Our real-time, live-cell imaging data definitively demonstrates that occludin traffics on MTs. Additionally, our motor data indicate that dynein is required for transport back to the membrane. We speculate that in the absence of MTs, dynein has no “highway” to transport cargo. Occludin is therefore unable to recycle. Our conclusion assumes a link between dynein and recycling vesicles.

Rab11 is a small GTPase that utilizes MTs for transport (Ullrich et al., 1996). Previous work has identified it as a marker of recycling vesicles (Horgan et al., 2010b; Takahashi et al., 2012). Interestingly, a link between Rab11 and dynein has been shown. Rab11 mediates its downstream effects via “family of Rab11-interacting proteins” (FIPS). FIP3 was identified as a primary recruiter of dynein to membranous vesicles, implying that a population of recycling proteins require microtubules and dynein for transport (Horgan et al., 2010a). Recycling occludin may be part of this population. One study demonstrated that in response to *E. coli* cytotoxic necrotizing factor-1 (CNF-1) occludin was internalized to Rab11-positive endosomes (Hopkins et al., 2003).

To test this hypothesis, we will request a dominant negative Rab11 construct from a group that has successfully used it to investigate recycling (Ren et al., 1998). Transfection of this construct into cells stably expressing fluorescent TJ proteins and GFP-tubulin will enable us to track protein movement in the absence of Rab11. Alternatively we will knockdown protein expression using siRNA. We will also perform a biotinylation/pulldown recycling assay to determine if TJ proteins recycle after homeostatic internalization in the absence of Rab11.

Rab13 and Rab8 have also been implicated in occludin recycling (Peranen et al., 1996; Terai et al., 2006; Yamamura et al., 2008). Dominant negative constructs will be used to investigate their involvement as well.
v. Determining the role of microtubules in animal models of TJ homeostasis and recovery

The long-term goal of our investigation is to uncover new aspects of EPEC pathogenesis, which give us insight into host epithelial biology. Without confirming our in vitro data in an in vivo model this is impossible. We have developed a mouse model of EPEC infection and our collaborator, Dr. Jerrold Turner, has developed an in vivo perfusion assay that we will use to strengthen our conclusions (Clayburgh et al., 2006; Marchiando et al., 2010; Savkovic et al., 2005; Shifflett et al., 2005).

To determine the role of MTs in TJ homeostasis, mice will be anesthetized and a loop of bowel cannulated at each end. Using a perfusion pump, a solution of nocodazole in buffered saline will be circulated through the bowel while the mouse is under anesthesia. Dr. Turner's lab has successfully used this procedure to determine the impact of various cytokines on the intestinal epithelial TJs (Clayburgh et al., 2005; Marchiando et al., 2010). The perfused tissue will be mounted in Ussing chambers to determine if barrier function is compromised. Alternatively, a solution of ferrocyanide that is neither absorbed nor secreted will be perfused through the bowel to determine mouse water loss (Clayburgh et al., 2006; Marchiando et al., 2010). Tissue will also be snap-frozen and stained for microtubules and TJ proteins. Both have been successfully detected in mouse intestine in our lab.

To determine the role of MTs in TJ recovery after EPEC infection, mice will be treated with streptomycin and orally gavaged with either wild-type EPEC or ΔespG1/G2. Mice will be sacrificed at 24 hour intervals and sections of tissues mounted in Ussing chambers to assay loss of barrier function. Intestinal tissues will snap-frozen and stained for occludin and MTs. Previous data from our lab establish that mice clear wild-type infection within 5-7 days (Royan et al., 2010). We hypothesize that mice infected with ΔespG1/G2 will not experience as severe a loss of barrier function as wild-type infected mice and that they will recover TJ function more rapidly.
This assay determines the impact of MTs in EPEC pathogenesis over several days. To obtain data regarding shorter courses of infection, the perfusion assay described above will be adapted. Rather than perfusing nocodazole through the cannulated bowel, wild-type EPEC and ΔespG1/G2 grown to logarithmic growth phase will be perfused. We have already begun to optimize this assay and we have demonstrated bacterial attachment after 15 min of perfusion with bacteria, followed by a 60 stable incubation and finally a 15 min wash (Fig. 28A). The next step will be to perfuse ferrocyanide through the infected bowel and measure the degree of water loss.

Our lab has also adapted a FITC-based assay to determine intestinal permeability without the technical challenges of using Ussing chambers. Mice infected as previously described are gavaged with free FITC and the fluorophore is allowed to pass through the intestine. If barrier function is compromised, FITC will migrate past the TJs and into the bloodstream. Blood is collected via tail vein or cardiac puncture and the amount of FITC is quantified. Tail vein collection enables repeated assessments of a single mouse and permits monitoring of intestinal permeability without sacrificing new animals each day. Using dextran sodium sulphate-induced colitis as a positive control, we have optimized this assay for use with EPEC-infected animals (Fig. 28B). We hypothesize that, similar to our in vitro studies, mice infected with ΔespG1/G2 will exhibit attenuated loss of barrier function and will recovery TJ function more rapidly compared with wild-type-infected mice.
Figure 28. In vivo studies – preliminary data. (A) Bacterial perfusion of a cannulated bowel loop in a living mouse enables the attachment of EPEC to the epithelium. EPEC are expressing DsRed, actin is green and nuclei are blue. (B) Two days after EPEC infection, mice were gavaged with free FITC and the fluorophore collected via cardiac puncture. Dextran sodium sulphate, which induces colitis, was used as a positive control. EPEC-infected mice display compromised intestinal TJs compared to controls.
5. METHODS

Cell culture

Caco-2 cells (ATCC, Manassas, VA) were maintained and propagated according to ATCC guidelines. Briefly, cells were passaged every 4 days in Dulbecco’s Modified Eagle’s Media (Invitrogen, Carlsbad, CA) supplemented with 15 mM Na₂CO₃ and 20% fetal bovine serum. MDCK I cells and stably transfected MDCK I cells (RFP-occludin) were the generous gift of Dr. Jerrold Turner (University of Chicago). MDCK cells were passaged every 4 days in Dulbecco’s Modified Eagle’s Media (Invitrogen, Carlsbad, CA) supplemented with 15 mM Na₂CO₃, 15 mM HEPES and 10% fetal bovine serum. T84 cells (ATCC, Manassas, VA) were passaged every 10-14 days in DMEM supplemented with 30 mM HEPES, F-12 nutrients and 5% fetal bovine serum. All cell lines were used between passages 10 and 40.

Reagents and plasmids

GFP-tubulin was the generous gift of Dr. Vladimir Gelfand (Northwestern University). Nocodazole (Sigma, St. Louis, MO) was used at a concentration of 5 μg/ml in electrophysiology studies. Erythro-9-(2-Hydroxy-3-nonyl)adenine hydrochloride (Tocris Bioscience, Minneapolis, MN) and adenylylimidodiphosphate (Roche, Indianapolis, IN) were used at concentrations of 500 uM. (Ivanov et al., 2006) Dynein/dynactin, kinesin heavy chain and scrambled siRNA constructs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GFP-EspG constructs were the generous gift of Dr. Neal Alto (University of Texas-Southwestern). The ADP-ribosylation factor binding mutant is a Glu-Arg mutation at position 392. The p21-activated kinase binding mutant is an Asn-Ala mutation at position 212. Both of these constructs have been published and extensively verified. (Selyunin et al., 2011) GFP-EspF was made by Dr. Andrew Weflen, a former member of the Hecht lab, and has also been verified and published. (Weflen et al., 2010)
**Bacterial culture**

The EPEC strain E2348/69 was used in all experiments. Wild-type and ΔespG1/G2 were inoculated into Luria-Bertani broth and grown overnight with shaking at 37°C. Cultures were inoculated 1:33 into cold, serum-free, antibiotic-free cell culture medium (see “Cell Culture”) and grown with shaking at 37°C to the logarithmic growth phase, or OD<sub>600</sub> 0.4. Cultures were spun down and resuspended in an equal volume of serum-free medium. Caco-2 cells were infected at an MOI of 40, T84 cells at an MOI of 800.

**Cloning**

All GFP-EspG plasmids from Dr. Alto were created using EcoRI/BAMHI cloning sites. Inserts were cloned using PCR primers 5' ATA AAT GGA CTT AAT AAT GAC TCC GC (forward) and 5' AGT GTT TTG TAA GTA CGT TTC (reverse). PCR products were double digested using EcoRI and BAMHI (New England Biolabs, Ipswich, MA) as was the bacterial vector pTrc2His (Invitrogen, Carlsbad, CA). Digested inserts and vector were ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA), transformed into OneShot TOP10 competent cells (Invitrogen, Carlsbad, CA) and spread on LB-agar plus 100 μg/ml ampicillin. Single colonies were picked, grown overnight with shaking at 37°C in LB broth plus 100 μg/ml ampicillin and DNA was miniprepped. Correct insertion of espG and GTPase binding mutant sequences were verified using double digestion of plasmids. Plasmid stock was made using the Qiagen Plasmid-Midi prep kit (Qiagen, Valencia, CA), eluted in DNA- and RNAase-free water and stored at -20°C.

**Creation of competent cells and transformation**

Competent ΔespG1/G2 was made by growing the strain in LB broth plus 50 μg/ml kanamycin (Sigma, St. Louis, MO) to OD<sub>600</sub> 0.6. Bacteria were pelleted by spinning 15 min at 5k RPM at 4°C. Pellets were resuspended in 3 volumes of ice-cold 15% glycerol (Sigma, St. Louis, MO) in 0.1M CaCl<sub>2</sub> and spun 15
min at 5k RPM at 4°C. Washing and spinning was repeated 3 times. Final pellets were resuspended in 600 μl of the glycerol wash buffer and frozen immediately in liquid nitrogen. Cells were stored at -80°C.

For transformation, 100 μl cells were thawed on ice and mixed with 20 μg plasmid prep in an ice-cold GenePulser electroporation cuvette (Bio-rad, Hercules, CA). Mixture was electroporated using the GenePulser XCell (Bio-rad, Hercules, CA) and immediately 900 ul SOC media was added. Transformed bacteria were grown with shaking at 37°C for 1 hour and spread on LB-agar plates plus 100 μg/ml ampicillin and 50 μg/ml kanamycin. Colonies were selected after 18 hours of incubation at 37°C.

**Live-cell microscopy and analysis**

RFP-occludin stable MDCK I transfectants were plated to 50% confluence on 35 mm glass bottom microwell dishes (MatTek Corporation, Ashland, MA). Cells were transfected with GFP tubulin (see “Transfection”). Prior to imaging, transfection medium was removed and 3 mls of sterile-filtered phenol red-free, low glucose DMEM (Invitrogen, Carlsbad, CA) plus 5% fetal bovine serum was added. Cells were imaged in this media. Images were captured using the Olympus VivaView microscope at 40X magnification and MetaMorph software. All imaging was performed using “streaming” capture to achieve the highest degree of accuracy and smoothness. Image stacks were generated using MetaMorph and analyzed using the Manual Particle Tracking macro for ImageJ developed by Fabrice Cordeli, Institut Curie, Orsay, France. A minimum of 75 images per particle and 15 particles per condition were tracked (15 seconds of real-time tracking).

**Immunofluorescence**

Occludin staining: cells were fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature. Fixed cells were washed in 50 mM ammonium chloride for 25 min, permeabilized for 3 x 10 minutes in 0.05% saponin (Sigma, St. Louis, MO) in Membrane Blocking Solution (Invitrogen, Carlsbad, CA) and
blocked for 1 hour in Membrane Blocking Solution. Cells were stained using mouse anti-occludin antibody (product T5168, Sigma, St. Louis, MO) at a concentration of 1:200 overnight at 4°C and fluorophore-conjugated goat anti-mouse secondary antibody at a concentration of 1:500 for 1 hour (Molecular Probes) at room temperature. Cells were washed 5 x 5 min in PBS, washed in distilled water and mounted using Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA).

**Tubulin staining:** cells were fixed and stained following our previously published protocol. (Tomson et al., 2005) Three solutions were prepared: microtubule stabilizing buffer or MTSB (1mM EGTA, 4% PEG8000, 100mM PIPES, pH 6.9), TSP was prepared by dissolving 0.5% Triton X-100 (Invitrogen, Carlsbad, CA) in MTSB and 20 mg of dithiobis succinimidylpropionate or DSP (Pierce Biotechnology, Rockford, IL) was dissolved in 1 ml sterile DMSO. Half (500 μl) the DSP was dissolved in 50 ml HBSS (Invitrogen, Carlsbad, CA), and the remaining 500 μl DSP dissolved in TSB. 4% paraformaldehyde was prepared by dissolving 800 mg PFA (Fisher Scientific, Waltham, MA) in 20 ml MTSB at 60°C overnight. PFA was 0.2μm filtered and stored at -20°C. Cells were incubated in HBSS/DSP for 10 min, TSP/DSP for 10 min, TSP for 10 min and PFA for 15 minutes. Fixed cells were blocked for 30 min in Membrane Blocking Solution (Invitrogen, Carlsbad, CA), and incubated with mouse anti-tubulin (product 3526, clone B-5-1-2, Sigma, St. Louis, MO) at a concentration of 1:200 overnight at 4°C. Cells were washed 5 x 5 min in PBS and incubated in fluorophore-conjugated goat anti-mouse secondary antibody at a concentration of 1:500 for 1 hour (Molecular Probes) at room temperature. Cells were washed 5 x 5 min in PBS plus DAPI (product D9542, Sigma, St. Louis, MO), rinsed in distilled water and mounted using Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA).

For microtubule studies performed at 4°C, fixation was performed using pre-chilled solutions in a cold room.

**Giantin staining:** cells were fixed in 3% paraformaldehyde in phosphate buffered saline (Invitrogen, Carlsbad, CA) for 10 min at room temperature, permeabilized in 0.5% Triton X-100 in PBS, blocked in
3% bovine serum albumin (Invitrogen, Carlsbad, CA) for 10 min, stained using rabbit anti-giantin antibody (ab80864, Abcam) at 1:100 for 1 hour and washed 5 x 5 min in PBS. Cells were incubated with fluorophore-conjugated anti-rabbit secondary antibody at 1:400 (Molecular Probes, Carlsbad, CA) for 1 hour and washed 5 x 5 min in PBS. Slides were rinsed in distilled water and mounted using Prolong Gold (Invitrogen, Carlsbad, CA).

For all fixed cells, images were captured using a Leica DM4000B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) and a Retiga Exi CCD camera (Qimaging, Surrey, BC, Canada). Images were captured using SlideBook 4.2 software (Intelligent Imaging Innovations, Denver, CO) and processed using ImageJ.

**Transfection**

**GFP-tubulin:** 1 μl/cm² Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was mixed with 250 μl Opti-MEM reduced-serum media (Invitrogen, Carlsbad, CA) and incubated at room temperature for 5 min. Separately 4 μg of GFP-tubulin was mixed with 250 μl Opti-MEM. The two mixtures were combined and incubated for 30 min at room temperature and added dropwise to one 35 mm microwell dish. Opti-MEM (1.5 ml) was added and the cells incubated for 12 hours prior to imaging.

**siRNA constructs:** MDCK I cells, grown on either glass coverslips in 2 cm² wells or 0.33 cm² Transwell permeable supports (Corning, Tewksbury, MA), were transfected with siRNA constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). For coverslips, 10 μl each dynein heavy chain and dynactin siRNA was added to 50 μl of Opti-MEM reduced-serum media for each well. Lipofectamine, 2 μl, was added to 50 μl of Opti-MEM for each well and incubated at room temperature for 5 min. The two were combined and incubated for 30 min at room temperature and 100 μl was added to each coverslip. An additional 400 μl of Opti-MEM was added for a total volume of 500 μl. Cells were incubated for 8 hours and fixed/stained (see “Immunofluorescence”). For electrophysiology studies, 3 Transwells were used for each condition. For 3 Transwells, 10 μl each dynein heavy chain and dynactin siRNA was
added to 75 μl of Opti-MEM reduced-serum media. Lipofectamine, 1.5 μl, was added to 75 μl of Opti-MEM and incubated at room temperature for 5 min. The two were combined and incubated for 30 min at room temperature. 50 μl was added to each of 3 Transwells and an additional 50 μl of Opti-MEM was added for a total volume of 100 μl. For calcium switch assays, Transwells were incubated for 24 hours after transfection. Scrambled siRNA constructs were used as controls in all assays.

EspG and EspF constructs: For immunofluorescence, MDCK cells were plated on glass coverslips in 2 cm² wells at 50-60% confluence. For each well, 0.8 μg of each construct was added to 50 μl of Opti-MEM reduced-serum media. For each well, 2 μl Lipofectamine was added to 50 μl of Opti-MEM and incubated at room temperature for 5 min. The two were combined and incubated for 30 min at room temperature and 100 μl was added to each well. An additional 400 μl of Opti-MEM was added for a total volume of 500 μl. Cells were incubated for 8 hours and fixed/stained (see “Immunofluorescence”). For electrophysiology studies, 0.4 μg of each construct was added to 25 μl of Opti-MEM reduced-serum media per Transwell. Lipofectamine, 0.5 μl, was added to 25 μl of Opti-MEM per well and incubated at room temperature for 5 min. The two were combined and incubated for 30 min at room temperature. 50 μl was added to each Transwell and an additional 50 μl of Opti-MEM was added for a total volume of 100 μl. For calcium switch assays, Transwells were incubated for 12 hours after transfection.

**Electrophysiology studies**

Caco-2 or MDCK I cells were grown to confluence (minimum of 7 days for Caco-2 and 5 days for MDCK) on 0.33 cm² Transwell permeable supports of 0.4 um pore size. T84 cells were grown for 7-14 days. Transepithelial electrical resistance was measured using an epithelial voltohmmeter and STX2 manual electrodes (World Precision Instruments, Sarasota, FL). Average resistance for Caco-2 cells was 300 Ω·cm², for MDCK I cells 800-1000 Ω·cm² and for T84 cells 1-1500 Ω·cm². Numerical baseline resistance of each Transwell was set to 0 and all percent changes in resistance were calculated based on initial readings.
For assays done at 4°C, all Transwells were incubated for 30 min, then kept on ice and ice-cold medium with or without 5 μg/ml nocodazole was added to both the interior and exterior of the wells. Transwells were returned to 4°C for a further 30 min and removed to room temperature for calcium switch assays.

For drug treatments (EHNA, AMP-PNP), medium was aspirated from the inside and outside of Transwells and new medium containing the drug of interest was added. Drug-free medium was replaced on control monolayers.

For infection studies, bacteria were added directly to Transwells at the indicated MOI without aspiration. For the tight junction recovery assay, Caco-2 cells were grown as previously indicated and infected with either wild-type EPEC or ΔespG1/G2. TER was followed and when approximately 40% of initial TER was lost, cells were washed in warm medium and fresh medium containing 200 μg/ml gentamicin was added. Recovery of TER was monitored.

For pharmacological “complementation” of ΔespG1/G2, Caco-2 cells were grown as previously indicated on Transwells and treated with cold/nocodazole as described above. After 1h of recovery at 37°C selected monolayers were infected with either EPEC strain grown to logarithmic growth phase (see “Bacterial culture”). TER was followed.

Statistical analyses were performed using Student's t-test (unpaired).

**Calcium switch assay**

Cells grown on Transwell permeable supports were incubated in 100 mM EDTA in Ca²⁺/Mg²⁺-free PBS (Sigma, St. Louis, MO) for 10-20 minutes at 37°C. TER was measured at baseline and followed after chelation. Once resistance had fallen by ≈85-90%, monolayers were washed with warm cell culture medium and medium was replaced with calcium-containing medium. Cells were incubated at 37°C and TER was measured every hour.
For Caco-2 monolayers that had been pre-treated with nocodazole, AMP-PNP or EHNA 1 hour prior to calcium switch, reagents were also added to the recovery medium.

For T84 monolayers that were pre-infected with EPEC, 200 μg/ml gentamicin (Invitrogen, Carlsbad, CA) was added to recovery medium.

**Western Blotting**

**Anti-tubulin:** 35 cm² of Caco-2 cells were harvested in 300 μl Laemmli buffer (Bio-rad, Hercules, CA) plus a 1:100 dilution of protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL). Lysates were sonicated on ice and boiled for 10 minutes. Samples, 10 μl each, were separated on a hand-cast 12% SDS-PAGE gel and transferred to nitrocellulose using a Mini-Protean Tetra Cell apparatus (Bio-rad, Hercules, CA). Membranes were blocked for 1 hour in 5% milk and blotted overnight using anti-tubulin antibody (product T5168, Sigma, St. Louis, MO) at a 1:1000 concentration. Membranes were washed 5 x 5 min in Tris-buffered saline tween-20 (50 mM Tris, 150 mM NaCl, 0.05% tween 20). Membranes were incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody (Sigma, St. Louis, MO) at 1:5000 for 2 hours, washed 5 x 5 min in TBST and developed using the ECL Western Blotting Detection Kit (GE Life Sciences, Pittsburgh, PA).

**Anti-occludin:** membranes were blocked in 5% milk in TBST and incubated overnight in 1:1000 rabbit anti-occludin (product #71-1500, Invitrogen, Carlsbad, CA). Membranes were washed 5 x 5 min in TBST and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Sigma, St. Louis, MO) at 1:5000 for 2 hours, washed 5 x 5 min in TBST and developed using the ECL Western Blotting Detection Kit (GE Life Sciences, Pittsburgh, PA).

**Biotin pull-down assay**

All solutions were made in phosphate buffered saline plus 0.9 mM calcium and 0.33 mM magnesium unless otherwise noted.

Caco-2 cells grown to confluence on 10 cm² dishes were incubated with 3 mls 0.5 mg/ml sulfo-NHS-
SS-biotin (Pierce Biotechnology, Rockford, IL) on ice with shaking at 4°C for 1h. To quench biotin cells were washed 3 x 5 min in 50 mM NH₄Cl on ice with shaking at 4°C. Biotinylated cells were inoculated with logarithmic phase bacteria as described in “Bacterial culture” at an MOI of 40. After 4h of incubation at 37°C, cells were washed twice in warm PBS. Biotin was stripped for 3 x 10 min in 2.5 mM CaCl₂, 100 mM NaCl, 100 mM Tris-Cl and 50 mM sodium 2-mercaptoethanesulfonate (MESNA, Sigma, St. Louis, MO) on ice with shaking at 4°C. Strip solution was quenched by incubating cells for 3 x 5 min in 5 mg/ml iodoacetate (Sigma, St. Louis, MO) on ice with shaking at 4°C. Cells were lysed in 400 ul of modified RIPA buffer: 50 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 1.25% Triton X-100, 0.25% SDS plus 1:100 protease and phosphotase inhibitors (Pierce Biotechnology, Rockford, IL). Lysates were sonicated on ice for 1 min and centrifuged at 4500 rpm for 15 min at 4°C. All subsequent steps were performed on the supernatant only.

Protein concentration was assayed using a BCA protein assay reagent (Pierce Biotechnology, Rockford, IL). Lysates were diluted 1:10 in sterile PBS and pipetted in duplicate into a 96-well plate. Assay reagent was diluted 1:3 in sterile water as directed and 100 μl applied to diluted lysates. Absorbance at 562 nm was read using a plate reader and protein concentration extrapolated using a standard curve.

For each sample 800 ug of protein was added to 200 ul of immobilized avidin slurry (Pierce Biotechnology, Rockford, IL) and incubated with shaking at 4°C for 3h. Samples were spun down at 4500 rpm for 1 min to pellet avidin. Supernatant was decanted and 1 ml PBS was added to wash the avidin and any associated biotinylated proteins. Pelleting and washing was repeated 5 times. For each sample, 180 μl Laemmli buffer (Bio-rad, Hercules, CA) plus a 1:100 dilution of protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL) was added to the avidin and captured protein and boiled for 10 min. Supernatant was separated on a 12% hand-cast SDS-PAGE gel. Simultaneously, 20 μg of protein input was also separated and blotted. Proteins were transferred to
nitrocellulose using the Protean XL system (Bio-rad, Hercules, CA) and blotted for occludin (see “Western Blotting: anti-occludin”).

**In vivo perfusion (adapted from Dr. Jerrold Turner)**

Six- to 8-week old C57/BL6 mice ordered from Jackson Labs are used for this assay. Older mice may be used, but may require more anesthetic.

Anesthesia is induced with 200 μl intraperitoneal xylazine/ketamine, and a 4-5 cm loop of jejunum cannulated at the proximal and distal ends with plastic tubing. The abdominal cavity is covered with damp gauze for the duration of the experiment, and body temperature measured via rectal thermometer and maintained at 37°C using a heating lamp. A prewarmed solution (140mM NaCl, 10mM Hepes, pH 7.4) is first perfused through the jejunal loop at 0.5 ml/min for 20 minutes using a buffer recirculation pump (Bio-rad, Hercules, CA). This is followed by perfusion of the test solution (85mM NaCl, 10mM Hepes, 20mM sodium ferrocyanide, 5mM KCl, 5mM CaCl2, 20 mM mannitol, 10nM fluorescein-conjugated 3000 MW dextran, 20nM Texas red-conjugated 10000MW dextran, pH 7.4) in a recirculating manner for 3 hours. Anesthesia will be monitored during this time by repeat (q 15’ or less) checks of palpebral reflex and toe-pinch and anesthesia will be maintained by repeated peritoneal application of ketamine and xylazine, as described above, or by dosing with pentobarbital (50 mg/kg ip). In some studies luminal dextrans will be omitted and mice will be injected with 250ul of 1mg/ml Alexa 488 conjugated bovine serum albumin retroorbitally (under anaesthesia) prior to cannulation. 1 ml aliquots of the test solution will be removed at the beginning and end of the perfusion. After the perfusion is complete, the anesthetized animals will be sacrificed by induction of bilateral pneumothorax. The perfused jejunal segment will then be excised and measured.

For infection studies, prior to perfusion of the test solution, bowel loop is perfused with 2x10⁸ organisms in PBS. Pump is turned off and bacteria are allowed to attach to epithelium for 60 min. A 15 min wash in PBS follows.
**FITC gavage permeability assay (adapted from Dr. Jerrold Turner)**

It is recommended to run a fake experiment at least one week prior to the actual experiment to normalize the mice to the protocol. This means fasting the mice (but allowing water) for 3 hours, gavaging with water, and then another 3 hour fast. Then put the mice back into normal housing conditions. Permeability is known to increase when the mice are stressed. Performing a fake experiment introduces the mice to the protocol and will help minimize experimental noise for the real experiment.

**Bacterial infection**

Two days before infection, give mice 5g/L streptomycin (Sigma, St. Louis, MO) in their drinking water. Next day, replace with normal drinking water. Grow bacteria to OD_{600} 0.4 as directed in “Bacterial Culture.” Resuspend 100 ml bacteria in 1 ml sterile water. Gavage each mouse with 200 μl bacteria using a 22G stainless steel gavage needle (VWR, Radnor, PA).

One day 2 after infection, fast mice without food but allowing water for 3 hours. Transfer the mice to Pur-O-Cell bedding here, and keep them here for the duration of the experiment. Gavage mice with 250 μl of 1 mg/ml FITC solution (Invitrogen, Carlsbad, CA) or (150 μl 80 mg/ml 4 kDa FITC-dextran). Remember to keep the unused FITC to make a standard curve after serum collection, and to record the order in which the mice are gavaged so that the blood is collected in that order 3 hours later.

When gavaging, weigh the mice – this will be used later when calculating the permeability.

After 3 hours (continuing the food fast but allowing water), collect blood.

To collect blood, anesthetize the mice. While the mice are going down, turn on the heat block at a setting where it is warm to the touch, but you can hold you hand there without having to remove it. Place the mice on heat block for 5 minutes.
With a razor blade, clip a 2 cm piece of tail and collect blood from the tail into serum collection tubes as the mice are draped over the heat block. 100 µl is ideal.

Back in lab, spin the collected blood at 10,000 g for 10 min at RT.

Dilute serum 1:4 in water. Add 100 µl/well. Use black plates for fluorescence.

Make a standard curve in water. The dilutions are 1:300, 1:1,000, 1:3,000, 1:10,000, 1:30,000, 1:100,000, 1:300,000, 1:1,000,000, and 1:3,000,000. The easiest way to do this is to start with 1:300 and 1:1,000 and make 10 fold dilutions from those. 30 µl into 270 µl is convenient. Do the standard curve in duplicate.

Read fluorescence in a plate reader with 485 excitation/528 emission.

Calculate permeability values based on the standard curve. Remember to multiply by 4 to correct for the dilution. Normalize your values by dividing by the weight of the mouse (this helps regulate the difference in FITC delivery if mice are sick and have lost weight and are being compared to control mice). A good way to present the data is to call it “serum FITC recovery” and give it units of: (mg/ml/g)x10^-4. It is helpful to multiply by a factor of 10^4 so you are not presenting extremely small numbers.
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