THE ROLE OF ESPH AND HOST CELL PROTEINS IN ENTEROPATHOGENIC ESCHERICHIA COLI-INDUCED CELL DEATH AND VIRULENCE

by

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LIST OF ABBREVIATIONS

EPEC	Enteropathogenic *Escherichia coli*
A/E	Attaching and effacing
EHEC	Enterohemorrhagic *Escherichia coli*
T3SS	Type III secretion system
LEE	Locus of enterocyte effacement
Esp	*E. coli* secreted protein
Nle	Non-LEE encoded
ORS	Oral rehydration solutions
BFP	Bundle-forming pili
pEAF	EPEC adherence factor plasmid
N-WASP	Wiskott-Aldrich syndrome protein
Arp2/3	Actin-related proteins 2/3
DRA	Downregulated-in-adenoma
NHE3	Sodium-hydrogen exchanger 3
SGLT1	Sodium glucose co-transporter 1
TJ	Tight junction
ZOs	Zonula occludens
DJ	Desmosomal junction
TER	Trans Epithelial Resistance
GEF	Guanine exchange factor
IF	Intermediate filament
ER	Endoplasmic reticulum
TNFR	Tumor necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
FADD	Fas-associated protein with death domain
DISC	Death-inducer signaling complex
WT	Wild-type
siRNA	Small interfering RNA
SFM	Serum free media
MFF	Mitochondrial fission factor
PI	Propidium iodide

Abstract

Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of infantile diarrhea, particularly in developing countries. EPEC belongs to the attaching and effacing (A/E) family of pathogens and harbors a type III secretion system (T3SS) that delivers virulence proteins directly into host epithelial cells. These proteins alter host structure and function, likely facilitating pathogenesis. We recently demonstrated that EspH, an EPEC secreted protein, is a critical virulence factor and that mutant strains lacking *espH* are impaired for pathogenesis. EspH induces host cell death through activation of caspases and mitochondrial fission. We hypothesizes that a wide range of host proteins are implicated in this cell death phenotype. Quantitation of host cell death during EPEC infection using siRNA-mediated knockdown of individual host cell proteins supports this hypothesis. A broad group of host protein knockdowns displayed altered host cell death during infection. The goal of my studies is to identify the host pathway(s) altered during EspH-induced epithelial cell death and, eventually, to establish the significance of this pathway in EPEC virulence.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a Gram-negative, non-toxigenic, attaching and effacing (A/E) diarrheal pathogen that is a significant cause of infant morbidity and mortality in the developing world. Studies across multiple countries indicate that in infants between 0- to 6-months of age, 30-40% of cases of diarrhea can be attributed to EPEC infection (1). Additionally, in one study in a developing country, EPEC-infected neonates showed a 30% mortality rate (2). EPEC is closely related to enterohemorrhagic *Escherichia coli* (EHEC), a major cause of foodborne illness in the developed world, which in the United States in 2005 was estimated to cause 73,000 illnesses with 60 deaths and an economic cost of $405 million (3, 4). The EPEC genome encodes multiple virulence factors including a type III secretion system (T3SS); many of the virulence genes are located within a pathogenicity island called the locus of enterocyte effacement (LEE)(5). The T3SS secretes bacterial virulence proteins directly into host epithelial cells, inducing alterations in host cell physiology leading to disease. These virulence factors, also called effector proteins, interact with host cell proteins to induce these changes, with various effectors impacting different host pathways, including actin polymerization, cytokeratin retraction, tight-junction stability, and cell survival (6-9). We have recently shown that one of these effectors, EspH, has a significant impact on host cell junctional stability, epithelial barrier function, and EPEC colonization efficiency in mouse intestines. Additionally, it has been shown that EspH influences the survival of host intestinal epithelial cells (10).

Modulation of host cell survival is a known virulence strategy in EPEC and similar bacterial pathogens. By promoting host cell survival, a pathogen can delay exposure to dendritic cells

and the subsequent immune response, and this delayed cell death can provide an opportunity to replicate on or within the host cell (11). Alternatively, pathogen-induced host cell death can promote shedding of infected host cells, allowing the associated bacteria to reach neighboring cells and propagate the infection (12). Previous work by Wong *et al.* has shown that EspH activates caspase 3 in host cells, suggesting a potential role in host cell death (10). Although it is known that EPEC effectors are capable of altering host cell survival (9, 13), many of the host cell proteins involved in these EPEC-induced alterations are still unidentified.

We hypothesize that EPEC manipulates host cell survival as a virulence strategy. Delineating the mechanisms of this host-pathogen interaction will provide insight into EPEC virulence and suggest novel strategies for better understanding and treatment of the disease.

Overall Goal: To define the interactions between host and EPEC proteins involved in alteration of host cell survival.

Aim 1: Identify host cell factors involved in EPEC-induced cell death.

1. siRNA knockdown of individual host cell factors followed by an EPEC infection tracking cell death.

2. Confirmatory western hybridization for knockdown of target proteins.

Aim 2: Define the host cell death pathway(s) affected by EspH.

1. Infect cells with wild type EPEC and a *ΔespH* mutant and perform western hybridization of proteins predicted to be affected by EspH.

Aim 3: Identify the regions of EspH necessary for host cell death using alanine-scanning mutants.

1. Infect cells with EPEC strains containing alanine-scanning EspH mutants and assay for host cell protein alteration and cell death phenotypes.

These studies will help elucidate the mechanism of EPEC-induced cell death and the role specific regions of EspH play in pathogenesis. The identification of specific host and pathogen effectors required for virulence could reveal novel targets for disease intervention.

Chapter 1: Literature Review

Attaching and Effacing Pathogens and Enteropathogenic *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped bacterial species encompassing many different commensal and virulent strains which are adapted to colonize a wide range of hosts. Commensal *E. coli* is a normal part of the gut flora, and certain non-pathogenic strains are recognized as viable probiotics. However, pathogenic strains contain a wide variety of virulence factors that contribute to a range of diseases including diarrhea, urinary tract infections, meningitis, and hemolytic uremic syndrome(14). These pathogenic strains contribute significantly to both enteric and non-enteric infections, and are a major cause of morbidity and mortality worldwide (1, 2). One diarrheal pathogen included in this group is enteropathogenic *Escherichia coli* (EPEC).

EPEC belongs to a group of pathogens known as attaching and effacing (A/E) pathogens. These A/E pathogens produce a characteristic lesion on gut epithelial cells. The pathogen first attaches intimately to host intestinal epithelial cell surfaces and causes erosion of the brush-border microvilli (14). Concomitant cytoskeletal rearrangements promotes stable colonization of the host cell (15).

EPEC is a leading cause of infant diarrheal disease, especially in the developing world, with studies estimating that 30% to 40% of cases of infant diarrhea can be attributed to the

pathogen in certain countries(1). It causes characteristic watery diarrhea, fever, and vomiting, and can lead to severe dehydration if left untreated(16). Oral rehydration solutions (ORS) are a common treatment for diarrhea, promoting uptake of fluid through manipulation of specific transporter channels: however, EPEC disrupts these channels, rendering ORS ineffective as a treatment for the disease(17). Additionally, no vaccine currently exists for EPEC, and with the rise in antibiotic resistance as a major concern, alternate targets for treatment of this disease are important (18, 19).

EPEC Virulence Factors

Bundle Forming Pili

Bundle forming pili (BFP) are bacterial structures that promote bacteria-bacteria and bacteria-host attachment. This helps promote bacterial microcolony formation during infection. Both the *bfp* operon and the *perABC* operon (a regulator of BFP and Ler) are encoded on the EPEC adherence factor plasmid (pEAF). BFP-harboring EPEC strains were described first, and are known as typical EPEC, while the subsequently discovered BFP-negative strains are called atypical EPEC. The prototype strain E2348/69 used in the studies described in this document is a typical EPEC strain, and *bfp*-deficient derivatives of this strain were shown to have a virulence defect in human volunteer studies (20).

Locus of Enterocyte Effacement

EPEC encodes many of its virulence factors on a 35Kb pathogenicity island known as the locus of enterocyte effacement, or LEE. The LEE encodes the components of a type III secretion

system (T3SS), multiple secreted effectors, and chaperone proteins associated with these effectors. It is divided into five different operons (LEE1 - LEE5) and expression is controlled by the LEE-encoded regulator (Ler)(21).

Type III Secretion System

The T3SS is the mechanism by which the EPEC-secreted virulence proteins enter host cells. The entire system is a syringe-like structure that spans both bacterial membranes and forms a pore in the host cell membrane and enables the translocation of effectors. The basal body is comprised of multiple proteins and spans both EPEC cell membranes. The ATPase activity of the basal body protein EscN drives effector proteins through the system. EspA forms a hollow, filamentous needle connecting the bacterium and the host cell, while EspB and EspD form a pore in the host plasma membrane necessary for the translocation of effectors (21, 22).

Effector Proteins

After the T3SS is established, a time-dependent secretion of various effector proteins into the host begins (23). These proteins contain an N-terminal secretion signal that differs from one effector to another, and is necessary for translocation through the T3SS. Additionally, these proteins contain C-terminal chaperone signals that are important for initial recruitment to the T3SS. Effector proteins are driven through the T3SS by the ATPase activity of EscN. As the effectors enter the host cell, they impact various cell systems and produce a wide variety of dynamic effects including: actin remodeling, increased host-bacterial attachment stability, anti-

apoptotic signals, pro-apoptotic signals, tight- and desmosomal-junctional disruption, and altered host cell barrier function (22).

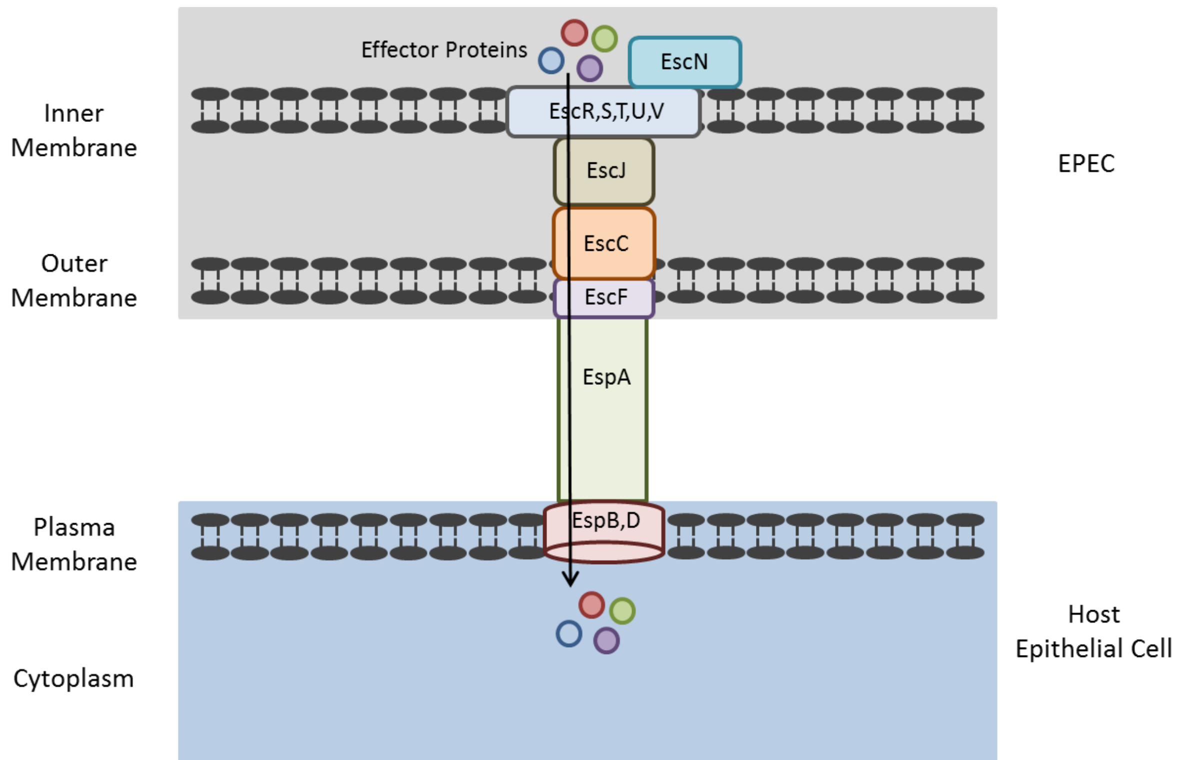

Figure 1 Diagram of the EPEC Type 3 Secretion System (T3SS). The EPEC T3SS spans the bacterial inner and outer membrane, the extracellular space between the bacterial and host cell, and the host plasma membrane. The basal body is comprised of EscR, S, T, U, V, J, and C. EscF forms a needle structure which EspA binds to when forming the filamentous syringe-like extension spanning the gap between the two cells. On the host side, EspB and EspD form a pore allowing the translocation of various effector molecules into the host cytoplasm. This translocation is driven by the ATPase activity of EscN (24).

EPEC Effector Protein Functions

Host Cell Adhesion

EPEC infection begins with attachment to the host cell. Attachment occurs in multiple stages, but the earliest contact is mediated by BFP in typical EPEC strains (20). Pili promote bacteria-host attachment, prior to Tir-dependent intimate adherence.

Effector-Mediated Host Cell Changes

Unlike pathogens that produce toxins as their primary means of causing disease, EPEC causes disease by secreting effector proteins that alter host cell physiology in a dynamic manner. Epithelial cell microvilli effacement, transporter disruption, and junctional disruptions all contribute to the diarrheal phenotype and many of these changes are induced by one or more LEE-encoded effector proteins (14).

Each effector may have pleiotropic effects on host cells, and multiple effectors may impact a particular host cell function. Effector proteins are secreted in a hierarchical fashion and, correspondingly, there is a progressive alteration in host cell physiology (25).

Microvilli Effacement and Pedestal Formation

One characteristic sign of EPEC infection is the loss of microvilli on infected cells and the rearrangement of actin polymers to form a raised pedestal underneath the EPEC microcolony.

This effacement occurs following translocation of Tir, the first effector protein designated for secretion into the host cell once the T3SS is established (14).

Tir integrates into the host plasma membrane where it binds the EPEC-membrane protein intimin. Tir-intimin interaction results in intimate attachment of the bacteria to host cells and causes phosphorylation of the cytosolic region of Tir. This recruits the host protein Nck, which attaches to Tir and activates Wiskott-Aldrich syndrome protein (N-WASP) and actin-related proteins 2/3 (Arp2/3). Together this complex induces actin polymerization beneath the microcolony, causing the formation of an actin pedestal (6, 26). The exact function of this pedestal is unknown, but it is hypothesized to play a role in avoiding host immune responses (27).

Alteration of Transporters

As was previously mentioned, EPEC inactivates certain host ion transport proteins, which is one possible contributor to the diarrheal phenotype (17). As transporters fail to absorb water and electrolytes, diarrhea becomes more likely. The EPEC effectors EspG1 and EspG2 disrupt the microtubule network and stimulate internalization of the chloride transporter DRA (28). Likewise, the sodium transporters NHE3 and SGLT1 are inactivated by multiple effectors (29). A common treatment for diarrheal disease, oral rehydration solution therapy, is dependent on the function of SGLT1. This makes ORS therapy an inefficient treatment for EPEC-induced diarrhea (17). Finally, the effacement of the microvilli during EPEC infection removes much of

the host cell surface area that would normally promote healthy fluid absorption and secretion, reducing the host cells' ability to regulate water transport (6).

Tight Junction Disruption

Tight junctions (TJs) are protein complexes that form a fence between the apical and basolateral plasma membrane domains of epithelial cells. These complexes are comprised of occludins, cluadins, zonula occludens (ZO-1, 2, 3) as well as cytoplasmic scaffolding proteins. TJs regulate the diffusion of water, ions, and neutral molecules through intercellular space in the epithelial layer. TJs also maintain the electrochemical gradient necessary for transcellular transport (30). EPEC infection disrupts many proteins in the TJ complexes, including occludin and zonula occludens 1 (ZO-1), which leads to compromised barrier function and redistribution of apical and basolaterial transmembrane proteins. This disrupts normal transcellular transport, provides additional sites for bacterial attachment, and promotes pro-inflammatory responses and diarrhea (17). The EPEC proteins EspF, Map, EspG, NleA and EspH have all been shown to play a role in barrier disruption (31-33).

Desmosomal Junction Disruption

Desmosomal junctions (DJs) are protein complexes that form between adjacent epithelial cells along the basolateral plasma membrane domain. DJs are important for maintaining cell-cell adhesion and have been shown to be integral to barrier integrity (34). The desmosome is formed from three major gene families: cadherins, armadillo proteins, and plakins. Desmoglein and desmocollin are transmembrane cadherin proteins that bind homologously or heterologously to desmosomal cadherins on adjacent cells. This binding forms the extracellular

junction of the DJ. The cytoplasmic domain of desmosomal cadherins bind to the armadillo proteins, plakoglobin and plakophilin, which form desmosomal plaques. These proteins in turn bind to a plakin, desmoplakin. Desmoplakin anchors the desmosome by binding to intermediate filaments (IFs)(35). We recently demonstrated that EPEC infection results in the loss of the desmosomal transmembrane proteins desmoglein-2 (Dsg2) and desmocollin (Dsc). This effect appears to be predominantly dependent on the effector protein EspH. EspH interacts with RhoGEFs, guanine exchange factors that are necessary for maintaining normal Rho GTPase activity (36). EspH-RhoGEF interaction leads to inactivation of the GTPase RhoA; this results in a decrease in actin polymerization and a retraction of intermediate filaments. IF retraction destabilizes desmosomes, ultimately leading to internalization of the desmosome and degradation in the lysosome. This causes separation of the lateral membranes of adjacent epithelial cells, loss of monolayer integrity, and loss of barrier function (33).

EPEC Alteration of Host Cell Survival

When certain conditions are met, such as those present in a bacterial infection, host cells undergo programmed cell death through a variety of possible mechanisms, including intrinsic and extrinsic apoptosis, necroptosis, and pyroptosis (37). The exact type of cell death depends on the signaling pathways being activated, and each type of death can lead to a wide variety of downstream effects. Over time, many pathogens have evolved mechanisms to evade, manipulate, and alter host cell death responses to improve pathogen replication, survival, and dissemination within the host (37).

Apoptosis

Apoptosis is a process of programed cell death that is a normal part of many mammalian cell lifecycles. Cells undergoing apoptosis usually display characteristic membrane blebbing, cell shrinkage, and chromatic condensation. Two main groups of proteins, the Bcl2 protein family and the cysteine-aspartate specific proteases (caspases), are chiefly involved in the apoptotic pathway (37). Caspases are further categorized as initiator (caspase-2, 8, 9, 10), executioner (caspase-3, 6, 7), and inflammatory (caspase-1, 4, 5) caspases. Initiator caspases are normal present in an inactive, procaspase form that is activated through a dimerization event initiated by an upstream apoptotic signal. Active initiator caspases directly or indirectly induce cleavage of executioner caspases (38). Executioner caspase activity leads to DNA fragmentation and cell death (39). Inflammatory caspases are not involved in apoptotic cell death, but promote pyroptosis (38). Apoptosis can be initiated through two distinct pathways. The intrinsic pathway

is triggered by a signal originating from within the apoptotic cell, while the extrinsic pathway is triggered by external stimuli. Ultimately both pathways activate executioner caspases, leading to cell death (40).

Intrinsic Apoptosis

Intrinsic apoptosis is triggered by various cell states including DNA damage, endoplasmic reticulum (ER) stress, and many viral infections. Under normal conditions, anti-apoptotic Bcl2 proteins bind and inhibit pro-apoptotic Bcl2 proteins like Bak and Bax, maintaining the host cell. Under stress conditions, BH3 proteins activate, inhibit the anti-apoptotic Bcl2 proteins and activate pro-apoptotic Bak and Bax, thus create a pro-apoptotic environment within the cell (41). This causes a reduction in mitochondrial membrane integrity, leading to cytochrome C release. In the cytosol, cytochrome C associates with Apo1 and caspase 9 to form a complex known as the apoptosome, which activates downstream executioner caspases 3, 6, and 7 (40). Active executioner caspases propagate further executioner caspase activation and induce DNA degradation and actin filament cleavage leading to disrupted intracellular transport (39, 40, 42).

Extrinsic Apoptosis

Extrinsic apoptotic signaling begins with the activation of a death receptor belonging to the tumor necrosis-factor receptor (TNFR) family. This includes receptors such as TNFR, TRAIL-R, and Fas (40). These transmembrane proteins contain extracellular ligand-binding domains, which, when bound to their ligand, activate the cytoplasmic death domain responsible for

propagating the death signal to downstream signaling and effector molecules, leading to cell death.

Fas Signaling

The interaction of Fas ligand with the extracellular domain of Fas receptor leads Fas internalization (43). The Fas death domain then recruits the adaptor protein Fas-associated protein with death domain (FADD), which then recruits procaspase 8 and activates inducer caspase 8 in a complex called the death-inducer signaling complex (DISC) (44). Active caspase 8 is known to then trigger downstream cell death in multiple ways. Caspase 8 can activate the BH3 protein Bid, which activates pro-apoptotic Bcl2 proteins, leading to cytochrome C release and caspase 3 activation as described previously. Caspase 8 can also form a complex with ER-bound Bap31 and mitochondria-bound Fis1. This complex, known as the ARCosome, activates Bap31 which in turn promotes calcium release from the ER. This calcium release then induces mitochondrial cytochrome C release and, ultimately, cell death (45).

TRAIL Signaling

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) binds to TRAIL-R1 and TRAIL-R2 receptors. This then triggers a pathway similar to Fas signaling, resulting in DISC formation and the activation of downstream caspase 3(45).

TNF Signaling

Tumor necrosis factor signaling occurs through the activation of two different complexes. Complex I is formed by receptor-interacting protein kinase 1 (RIPK1), TNFR type 1-associated death domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2), and cellular inhibitor of apoptosis protein 1 and 2 (c-IAP1/2). This complex is endocytosed, and RIPK1, TRADD, and TRAF2 dissociate, followed by the formation of complex II. This complex then acts in a manner analogous to the DISC complex, activating initiator caspase 8 and promoting downstream activation of caspase 3.

Unlike many of the apoptosis pathways which are notable for producing very little immune response, TNFR1 signaling activates pro-inflammatory genes. RIPK1 and c-IAP1/2 recruit and activate TAK1, which in turn activates IKK, leading to the ubiquitination and degradation of IκB, an inhibitor of the transcription factor NF-κB. When IκB is degraded, NF-κB can localize to the nucleus and induce the expression of various pro-inflammatory molecules (45).

Apoptosis is crucial in host cell responses to bacterial infections, often providing a means of clearing away infected cells, promoting an immune response in certain cases, and overall acting to remove the pathogen from the host (37). As such, many bacterial pathogens have developed methods to interfere with the normal host cell death pathways, and in many cases they dynamically regulate when and how host cells undergo apoptosis. By introducing anti-apoptotic factors early in infection, bacteria can better colonize the host. During later stages of infection, bacteria may actively stimulate host cell death as a means to disseminate within the host, or

into the environment (37). EPEC encodes many apoptosis-regulating effector proteins that act to dynamically alter host cell death phenotypes.

Table 1: EPEC T3SS Effectors

Effector	Function	Reference
Tir	Intimin receptor. Promotes actin polymerization, pedestal formation, bacterial attachment, TJ disruption	(6, 26)
Map	Disruption of mitochondrial structure and function. Mimics CDC42 function	(46)
EspB	T3SS pore component. Myosin interaction and inhibition of phagocytosis	(47)
EspG	Microtubule destabilization. Golgi fragmentation	(48)
EspZ	Inhibits apoptosis	(49)
EspF	TJ disruption, mitochondrial dysfunction, induces apoptosis, effaces microvilli	(12)
EspH	Induces apoptosis, desmosomal disruption	(10, 33, 36)
NleA	Inhibits host protein secretion, tight junction disruption	(31, 50)
EspJ	Inhibits phagocytosis	(51)
NleH	Reduce inflammatory response, inhibits apoptosis	(11)
NleE	Inhibits NF-κB activation	(52)
NleB	Blocks host death receptor signaling	(53)
NleC / NleD	Inhibits IL-8. Cleaves NF-κB and JNK	(54, 55)
NleF	Inhibits apoptosis	(56)

EPEC Effectors that inhibit host cell death

NleB1, B2

This effector protein blocks multiple pro-apoptotic effectors within the TNF complex I pathway, including TRADD, FADD, TRAIL, and RIPK1. NleB is a glycosyltransferase that modifies a shared Arg117 residue in the death domain of all of these proteins, thereby preventing homo/heterotypic death domain interactions, and blocking their function (54).

NleF

NleF inhibits both intrinsic and extrinsic apoptosis by inserting itself into the activation site of caspases, preventing them from being cleaved and activated. It inhibits intrinsic apoptosis by binding and inhibiting caspase 9, while extrinsic apoptosis is inhibited by caspase 8 binding (56).

NleH

During infection, EPEC secretes two proteins, NleH1 and NleH2, that suppress host cell death responses by blocking caspase 3 activation. NleH1 also inhibits cell death by activating Bax-inhibitor 1 protein, which prevents Bax-mediated apoptosis (11).

NleD

NleD is a zinc metalloprotease that cleaves c-Jun N-terminal kinase, preventing JNK-mediated apoptosis (55).

EspZ

EspZ is an early secreted effector protein that has two proposed mechanisms for preventing cell death. The first is the potential to limit translocation of pro-apoptotic effectors such as EspF and EspH, which are secreted after EspZ (49). This could delay the shift towards apoptosis in EPEC-infected host cells.

On the other hand, transfected cells expressing EspZ are protected from apoptosis-inducing agents in the absence of infection, suggesting that EspZ may directly engage host cell death pathways (9, 57). EspZ has been demonstrated to interact with host cell proteins including the glycoprotein CD98 and the mitochondrial protein TIM17B, which are both implicated in epithelial cells survival (58, 59).

EPEC Effectors that Promote Host Cell Death

EspF

This multifunctional effector is involved in tight junction disruption, pedestal formation, prevention of phagocytosis, and the activation of apoptotic cell death in host cells (13). EspF localizes to the mitochondria where it interacts with and inactivates the anti-apoptotic protein Abcf2. This leads to mitochondrial destabilization, cytochrome C release, and cell death (12).

Map

Map is another LEE-encoded effector protein that is functionally analogous to EspF, it localizes to the mitochondria, disrupting membrane potential, causing cytochrome C release, and ultimately causing host cell death (46).

EspH

During EPEC infection, EspH is secreted into the host cell, producing a wide array of phenotypic changes. EspH interacts with host RhoGEFs, and at least some of these alterations are due to this interaction, though which RhoGEFs it interacts with, and any other potential interacting partners are still unknown (10).

RhoGEFs are guanine exchange factors that replace inactive GDP with active GTP in Rho GTPases. Rho GTPases are a protein family (RhoA, RhoB, RhoC) that act as molecular switches for a range of cellular functions including actin cytoskeletal rearrangement, gene transcription, and cell cycle and apoptosis regulation. EspH-RhoGEF interaction inhibits the RhoA activating

function of RhoGEF, effectively locking RhoA in its GDP-bound, inactive form (33). RhoA is involved in actin polymerization dynamics (60). EspH-induced RhoA inactivation alters the actin polymerization dynamics of the host cell, leading to many of the downstream effects of EspH. We recently showed that actin cytoskeletal perturbations trigger intermediate filament disruption and desmosomal junction loss. This junctional destabilization in turn perturbs epithelial barrier function and weakens the cell-to-cell adhesion (33). Currently it is unknown which of these effects, or if another as of yet unidentified effect, leads to EspH induced cell death. However, it is known that EspH activates caspase 3, leading to host cell apoptosis, and previous work in our lab suggests that this may be mediated via alteration of endoplasmic reticulum and mitochondrial bound membrane proteins (10).

EPEC pathogenesis involves dynamic manipulation of host cell survival. Early in infection, EPEC prioritizes secretion of anti-apoptotic effectors, promoting cell survival (23, 49). As infection progresses, EPEC secretes pro-apoptotic effectors, inducing cell death (13). We hypothesize that EPEC manipulation of host cell survival pathways is critical to EPEC pathogenesis. The goal of this study was to identify host molecules involved in the survival/death of EPEC-infected epithelial cells, as well as to define the pathways by which EspH affects host cell death. We discuss the implications of these pathways in EPEC pathogenesis.

Chapter 2: Identification of Host Cell Proteins that Modulate EPEC-Induced Death

Background

Many EPEC effector proteins are known or predicted to interact with host cell proteins (12, 36, 57, 58). However, the full range and impact of these interactions is still unclear. In the context of host cell survival, it is unclear exactly what host proteins are involved during EPEC-infection or how significantly those proteins contribute to the overall cell death observed during infection.

It has been shown that EPEC strains with deletions in effectors that regulate host cell survival are less virulent, and display attenuated levels of colonization *in vivo* (52, 57). This suggests that the balance between host cell survival and host cell death is important to the pathogen's persistence in the host.

Recent studies have shown that through siRNA-mediated knockdown of CD98, overall EPEC-induced cell death was reduced, suggesting a role for the protein during EPEC-induced cell death. Likewise, knockdown of other host proteins during EPEC infection results in significant changes in host cell survival (11). This suggests that many host proteins are involved in EPEC-induced cell death.

In this chapter we broadly identify host cell proteins involved in EPEC-induced cell death, as well as the magnitude of their involvement. Specific protein expression was ablated through siRNA-mediated knockdown, followed by EPEC infection and a time course analysis of overall

host cell death. Cell death levels were compared to quantify the impact of a given protein during EPEC-infection.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2. The source strain for all mutant strains used was enteropathogenic *Escherichia coli* (EPEC) O127:H6 strain E2348/69 (VK003). Bacterial cultures were grown in Luria-Bertani (LB) broth at 37°C with appropriate antibiotics (Kanamycin (60ug/mL).

Cell lines and propagation

Caco-2_{BBE} ($C2_{BBE}$; brush-border-expressing human intestinal epithelial cells) were grown in Dubecco's modified Eagle medium (DMEM) high glucose (Corning, media tech, Inc.), supplemented with 10% fetal bovine serum (FBS), and 20mM HEPES. HeLa (human cervical epithelial cells) were grown in DMEM low glucose supplemented with 10% FBS. All cell lines were grown at 37°C in a 5% CO_2 incubator.

siRNA library

The siRNA library used for this screen was the apoptosis library subset of the *Silencer*™ Select Complete Human Genome Library (Ambion). This library subset included 935 genes known to be associated with apoptosis or cell death pathways, with 3 unique siRNA constructs targeting each gene. A non-targeting siRNA (NTC) was used as a control.

Transfection conditions

HeLa cells were grown to 100% confluency, trypsanized, and seeded in a 96-well black-walled clear bottom microplate at a concentration of 7000 cells per well (PerkinElmer). 24 hours after seeding, 2.5pmol of target siRNA was mixed with 0.3µL Lipofectamine® RNAiMAX transfection reagent and brought up to a total volume of 20µL using Opti-MEM Reduced Serum Media (Thermo Fisher). Following a 10-minute incubation, the mixture was added to a single well of the 96-well plate. This process was performed for each siRNA construct involved in the screen. Cells were then incubated at 37°C in a 5% CO_2 incubator for 5 hours. After 5 hours the transfection media was removed and 100µL of HeLa media was added and cells were returned to 37°C overnight. Due to the scale of these transfections, much of this process was automated using a Biomek FX Automated Workstation (Beckman Couler).

Propidium iodide uptake assay conditions

24 hours before infection, transfected HeLa cells were checked for 100% confluency and culture media was removed. 100µL of serum free DMEM low glucose (SFM) was added to each well and cells were returned to 37°C overnight. Bacterial strains were grown overnight in LB broth with antibiotic selection as needed. 24 hours after switching cells to SFM, bacterial overnight cultures were subcultured at 1:30 in SFM and grown to an optical density (OD_{600}) of 0.4. SFM was removed from epithelial cells and 100µL of bacterial cultures was added, diluted in SFM to achieve a multiplicity of infection (MOI) of 100. Infected cells were incubated at 37°C in a 5% CO_2 for 1 hour at which point SFM containing unattached bacteria was removed and propidium iodide (PI) diluted 1:1000 in SFM was added to each well at a final concentration of 1µg/mL (Abcam, Cambridge, MA). PI uptake by epithelial cells was measured using a microplate reader

(Synergy 2, BioTek Instruments, Winooski, VT). Cells were excited (530/525 nm) and emission (620/640 nm) was recorded for 8 hours at 30 minute or 1 hour intervals.

Calculations and Statistics

siRNA knockdown confirmation experiments were performed with a minimum of four technical replicates. Statistical analysis was performed using Student's t-test and the p-value < 0.05 was considered statistically significant. In large scale siRNA knockdown experiments, each host protein was specifically targeted with three unique, non-overlapping siRNAs. This experiment was carried out only once.

Table 2: Bacterial Strains

Strain	Genotype / Description	Reference
VK003	EPEC WT (O127:H6 strain E2348/69)	(11)
VK008	EPEC Δ*espH* (Kan^R)	(33)
VK053	EPEC Δ*espF* (Kan^R) (aka UMD874)	(61)
VK156	EPEC Δ*espZ* (Kan^R)	(62)
VK638	EPEC Δ*espH*-p*espH* (Kan+Amp^R)	(33)
JLR2	EPEC Δ*espH*-p*espH* w/HA tag (Kan+Amp^R)	(33)
JLR4	EPEC Δ*espH*-p*espH* w/ XhoI silent mutation (Kan+Amp^R)	(33)
M14	EPEC Δ*espH*-p*espH* w/ 5Ala aa88-92 (Kan+Amp^R)	(33)
M16	EPEC Δ*espH*-p*espH* w/ 5Ala aa98-102 (Kan+Amp^R)	(33)
M17	EPEC Δ*espH*-p*espH* w/ 5Ala aa103-107 (Kan+Amp^R)	(33)
M18	EPEC Δ*espH*-p*espH* w/ 5Ala aa108-112 (Kan+Amp^R)	(33)
M19	EPEC Δ*espH*-p*espH* w/ 5Ala aa113-117 (Kan+Amp^R)	(33)
M20	EPEC Δ*espH*-p*espH* w/ 5Ala aa118-122 (Kan+Amp^R)	(33)
M23	EPEC Δ*espH*-p*espH* w/ 5Ala aa132-136 (Kan+Amp^R)	(33)
M24	EPEC Δ*espH*-p*espH* w/ 5Ala aa137-141 (Kan+Amp^R)	(33)
M25	EPEC Δ*espH*-p*espH* w/ 5Ala aa142-146 (Kan+Amp^R)	(33)
M26	EPEC Δ*espH*-p*espH* w/ 5Ala aa147-151 (Kan+Amp^R)	(33)
M27	EPEC Δ*espH*-p*espH* w/ 5Ala aa152-156 (Kan+Amp^R)	(33)
M28	EPEC Δ*espH*-p*espH* w/ 5Ala aa157-161 (Kan+Amp^R)	(33)
M29	EPEC Δ*espH*-p*espH* w/ 5Ala aa162-167 (Kan+Amp^R)	(33)

Results and Discussion

EPEC-induced cell death is affected by the abundance of specific host cell apoptosis proteins

HeLa cells were transfected with siRNA constructs targeting individual apoptosis-related genes to determine the host cell proteins involved in EPEC-induced cell death. Following siRNA-mediated knockdown, cells were infected with EPEC WT and cell death was monitored via propidium iodide uptake assay. Propidium iodide is a DNA-intercalating agent that cannot cross the membrane of live cells and fluoresces when bound to DNA, making it useful in identifying dead cells in a population (63). PI uptake was tracked for 8 hours, and the level of uptake in each knockdown was compared to the level of uptake observed in the NTC transfected cells. Due to the scale of the experiment, only NTC treated wells had replicates in the initial screen. 3 independent siRNA constructs were used for knockdown of each target gene. Fluorescence in each well was measured and compared to the average fluorescence in the NTC-treated wells. This fluorescence was used as an aribitrary measurement of relative host cell death in a well. A construct was considered relevant if its fluorescence was at least 1 standard deviation (SD) above or below the non-targeting control well average fluorescence. If at least 2 of the 3 constructs targeting a given gene caused a relevant change in fluorescence, the gene was considered relevant. Of 935 initial genes ablated, 684 genes showed no change in cell death during infection, 60 genes showed significantly reduced levels of cell death during the infection, while 191 genes showed a significant increase in cell death when ablated.

Multiple gene families caused significant changes in host cell death during EPEC infection

This screen identified multiple gene families that showed a dramatic impact on overall host cell death during infection. siRNA targeting against four Rho guanine exchange factors, members of the ARHGEF family, resulted in increased host cell death. Increase in cell death from the knockdown of TRIO (6-41% increase), VAV2 (24-47% increase), and PLEKHG5 (17-61% increase) suggests a prominent role for these host proteins during EPEC-induced cell death (Figure 2). This supports existing data which shows EPEC inactivation of RhoGEF leads to caspase 3 activation (64). RhoA is a common substrate for all four RhoGEFs identified (65-68). This shared activity suggest that RhoA may also be critical during EPEC-induced host cell death. Additional gene families that appear prominent in EPEC induced cell death include the caspase recruitment domain (CARD), TNF receptor associated factors (TRAF), Bcl2, and proteasome component (PSM) families (data not shown). EPEC-TRAF interaction is known to be anti-apoptotic, and the increased cell death displayed during siRNA targeting against TRAF supports the idea that TRAF is crucial to EPEC-induced cell survival (69).

Mitochondrial fission protein knockdown caused increased host cell death during EPEC infection

Mitochondrial fission is a response to cellular stress, and is a precursor to apoptotic cell death (70). siRNA treatment of various mitochondrial fission associated proteins showed increased levels of cell death during infection, with mitochondrial fission factor (MFF) and optic atrophy 1 (OPA1) targeted cells displaying increased cell death during infection. OPA1 showed an 11-25% increase in cell death in relevant siRNA transfected samples, while MFF targeting constructs

displayed an approximate 26-36% increase in cell death in two of the three constructs (Figure 3). The decrease in cell death in the MFF siRNA 3 transfected cells could be explained by nonspecific binding or reduced binding efficiency in this construct. The increase in cell death from these knockdowns suggests mitochondrial fission and fusion dynamics contribute to host cell survival during infection.

Cells treated with siRNA targeting MFF displayed increased cell death in confirmation assay

One method of confirming the results of the large scale screen was to repeat the transfection and PI uptake assay on a smaller scale, including siRNA constructs replicates to determine significance. Uninfected wells were also transfected to confirm the siRNA alone was not producing significant levels of cell death in the system. At six hours post WT EPEC infection, MFF siRNA1 treated cells (1496AU) show a significant increase ($p<0.05$) in cell death compared to NTC-treated cells (1141AU). This result confirms the cell death increase observed in the full screen for the construct MFF siRNA1. Further analysis of other target proteins is necessary to support the results of the full screen, however this initial confirmation indicates that one target of interest appears to be significant during EPEC-induced host cell death.

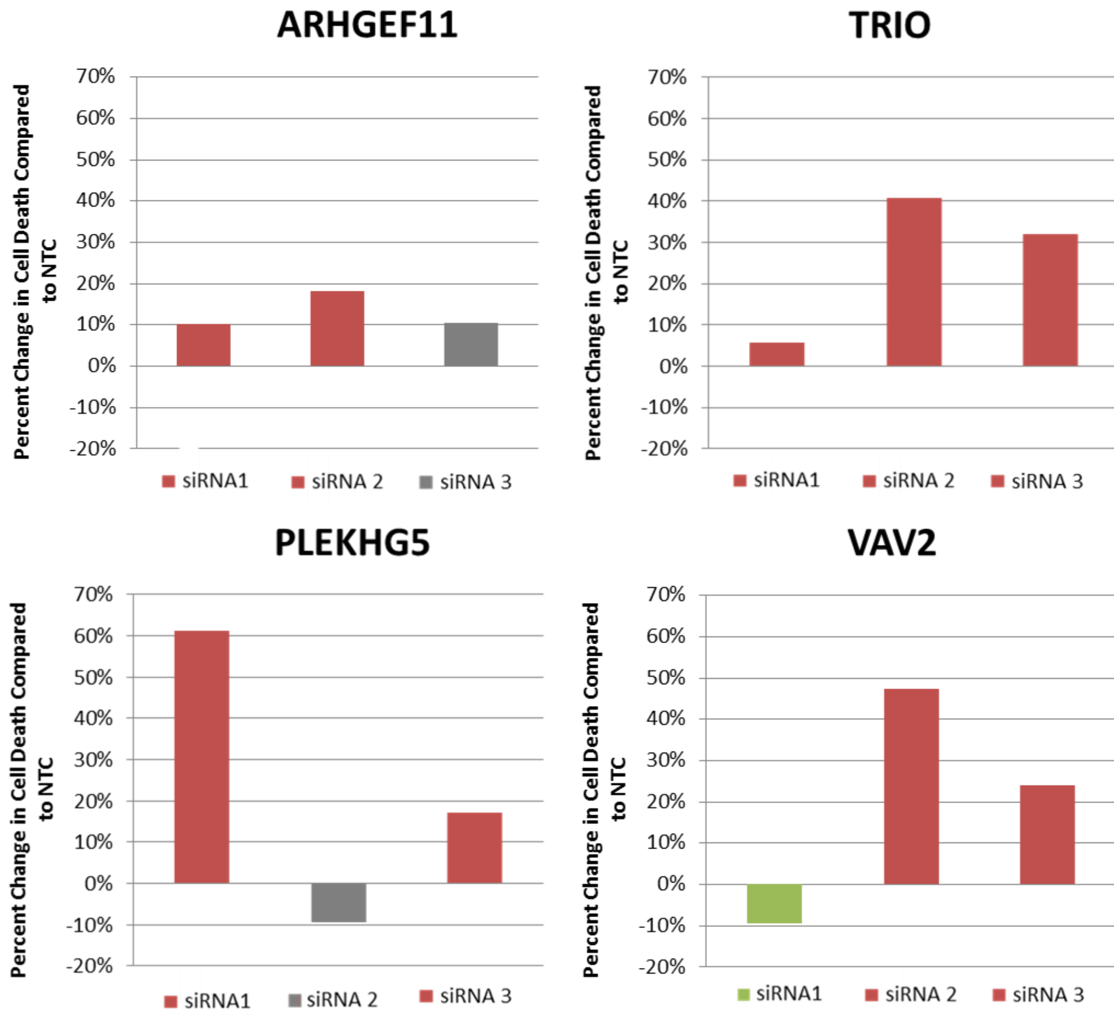

Figure 2 Increased cell death during EPEC infection with ablation of RhoGEFs. HeLa cells were transfected with siRNA constructs specific to ARHGEF11, TRIO, PLEKHG5, and VAV2, followed by an infection with EPEC WT at an MOI of 100 for 8 hours. Data represents the percent change in total cell death at 6 hours post-infection compared to an EPEC-infected well transfected with a NTC siRNA. Red bars indicate an increase in cell death compared to the NTC-treated cells (siRNA-treated well fluorescence was at least 1SD above the NTC-treated well fluorescence). Green bars indicate a significant decrease in cell death (siRNA-treated well fluorescence was at least 1SD below the NTC-treated well fluorescence). Gray bars indicate no change in cell death compared to the NTC-treated cells.

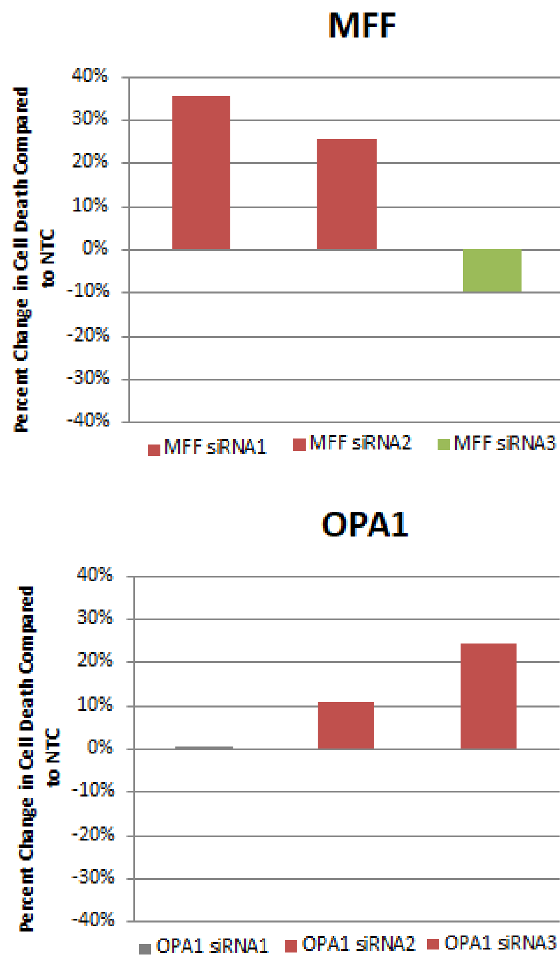

Figure 3 Increased cell death during EPEC infection with ablation of mitochondrial proteins. HeLa cells were transfected with siRNA constructs specific to MFF and OPA1, followed by an infection with EPEC WT at an MOI of 100 for eight hours. Data represents the percent change in total cell death at 6 hours post-infection compared to an EPEC-infected well transfected with a non-targeting control siRNA. Red bars indicate an increase in cell death compared to the NTC-treated cells (siRNA-treated well fluorescence was at least 1SD above the NTC-treated well fluorescence). Green bars indicate a significant decrease in cell death (siRNA-treated well fluorescence was at least 1SD below the NTC-treated well fluorescence). Gray bars indicate no change in cell death compared to the NTC-treated cells.

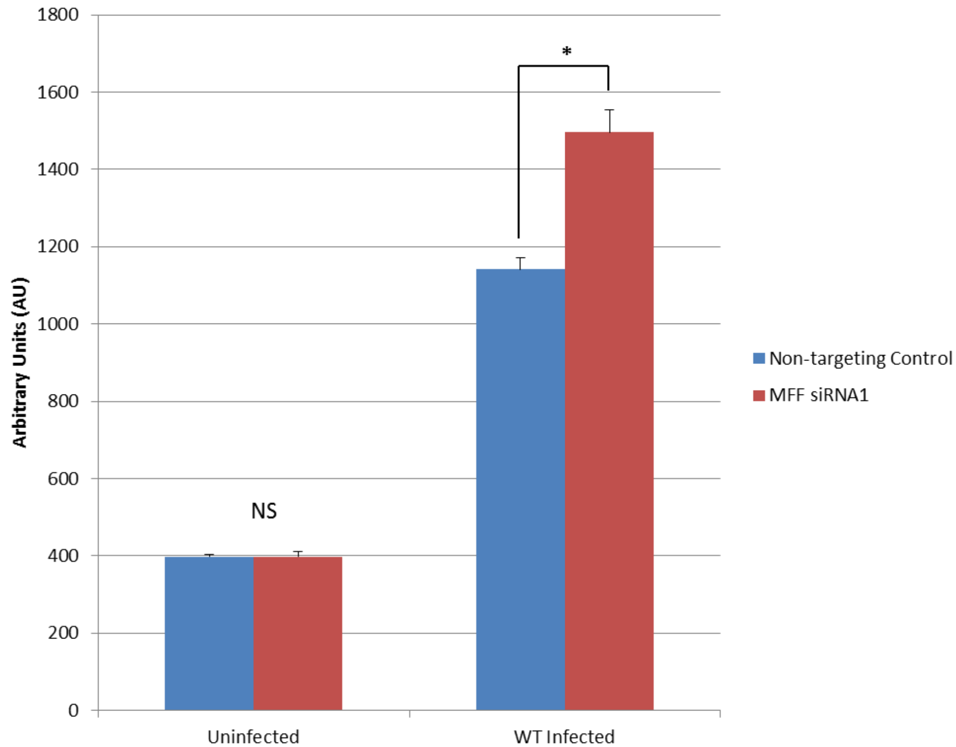

Figure 4 Repeat transfection with siRNA targeting MFF shows a significant increase in host cell death. HeLa cells were transfected with siRNA construct MFF siRNA1 specific to MFF, followed by an infection with EPEC WT at an MOI of 100 or a mock infection with sterile SFM (uninfected) for eight hours. Data represents the arbitrary level of total cell death at 6 hours post-infection compared to an EPEC-infected well transfected with a non-targeting control siRNA. Error bars represent the standard deviation of the mean. *p<0.05; student's T-test.

Chapter 2 Summary

EPEC pathogenesis is dependent on the dynamic regulation of host cell survival through anti- and pro-apoptotic effector proteins and their interactions with normal host pathways. Various studies have identified individual host proteins that influence host survival during EPEC infection. However, the full range and impact of individual host proteins on EPEC-induced host cell death is still largely unknown. To address this gap, this screen aimed to identify the scope and impact of genes in the human apoptosis genome involved in EPEC-induced cell death. This study found that EPEC-regulated host cell death is significantly affected by the ablation of a wide range of host proteins. In some cases, two different siRNA constructs targeting the same gene produced opposite results. This discrepancy could be the result of nonspecific binding in certain constructs resulting in knockdown of unintentional targets. Similarly, differences in cell death levels could be a result of a variable binding efficiency in certain constructs. siRNA targeting of various mitochondria- and RhoGEF proteins caused significant increases in host cell death. Both RhoGEFs and mitochondrial associations have been seen in EPEC infection in previous studies (10, 12), and this data supports the conclusion that EPEC-mitochondria and EPEC-RhoGEF interactions are important during host cell death. In addition to supporting existing findings, this screen provides a broad range of specific targets to be further examined to elucidate their impact on EPEC-induced changes in host cell survival dynamics and overall EPEC pathogenesis. One caveat to this data is that the siRNA-induced knockdown of the target proteins has not been confirmed. This will be addressed via western hybridization analysis of cells transfected under identical conditions to cells in the initial screen.

Chapter 3: The T3SS Effector Protein EspH Dramatically Alters Host Cell Death during EPEC Infection

Background

EPEC effector proteins are important for successful colonization and infection of the host, and while some of these effectors have been well characterized (EspZ and EspF) less is known about other molecules like EspH, notably the mechanisms by which they promote host cell death.

EspH is a 168 amino acid protein encoded by the LEE and secreted by the EPEC T3SS. Previous studies have shown that EspH interacts with the DH-PH domain of RhoGEFs, inhibiting Rho-binding activity and altering host cell actin dynamics (36). Further studies have recently identified specific regions of EspH necessary for EspH-RhoGEF interaction (33). Additionally, EspH induces caspase 3 activation when transfected into host cells, however the underlying mechanism(s) have not been explored (10). Recent *in vitro* work in our lab has identified a wide range of EspH-dependent host cell phenotypic changes, including desmosomal junction disruption, reduced barrier function (33), Bap31 and hFis1 alterations (Wilbur, Roxas, and Viswanathan, unpublished observations), and mitochondrial morphology alterations (Roxas and Viswanathan, unpublished observations). These *in vitro* alterations translate to a colonization defect and reduced pathogenesis *in vivo* (33).

Given the current information on the role of EspH in EPEC pathogenesis, we propose the following model for EspH-induced host cell death. EspH is transfected in host epithelial cells where it binds to RhoGEFs and inhibits RhoGEF activity. Through an unknown mechanism, potentially independent of RhoGEF activity, EspH induces formation of the ARCosome, an early

promotor of host cell apoptosis. Formation of the ARCosome leads to calcium release from the ER, which in turn promotes mitochondrial fission, cytochrome C release, executioner caspase activation, and ultimately, host cell death.

In this chapter we detail the impact of EspH on epithelial cell survival and identify potential pathways through which EspH is affecting host cell death. Additionally, we use a library of EspH site-specific mutants to identify the regions of EspH necessary for host cell death, with the eventual goal of defining the role of this effector in EPEC pathogenesis.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2. The source strain for all mutant strains used was enteropathogenic *Escherichia coli* (EPEC) O127:H6 strain E2348/69 (VK003). Bacterial cultures were grown in Luria-Bertani (LB) broth at 37°C with appropriate antibiotics (Kanamycin (60µg/mL)).

Cell lines and propagation

Caco-2_{BBE} ($C2_{BBE}$; brush-border-expressing human intestinal epithelial cells) were grown in Dubecco's modified Eagle medium (DMEM) high glucose (Corning, media tech, Inc.), supplemented with 10% fetal bovine serum (FBS), and 20mM HEPES. HeLa (human cervical epithelial cells) were grown in DMEM low glucose supplemented with 10% FBS. All cell lines were grown at 37°C in a 5% CO_2 incubator.

Propidium iodide uptake assay conditions

24 hours before infection, epithelial cells grown in a 96-well black-walled clear bottom microplate were checked for 100% confluency and culture media was removed (PerkinElmer). 100uL of serum free DMEM low glucose (SFM) was added to each well and cells were returned to 37°C. Bacterial strains were grown overnight in LB broth with antibiotic selection as needed. 24 hours after switching cells to SFM, bacterial overnight cultures were subcultured at 1:30 in SFM and grown to an optical density (OD_{600}) of 0.4. SFM was removed from epithelial cells and 100µL of bacterial cultures was added, diluted in SFM to achieve an MOI (multiplicity of infection) of 100. Infected cells were incubated at 37°C in a 5% CO_2 for 1 hour at which point SFM containing unattached bacteria was removed and propidium iodide (PI) diluted to 1:1000 in SFM was added to each well at a final concentration of 1µg/mL (Abcam, Cambridge, MA). PI uptake by epithelial cells was measured using a microplate reader (Synergy 2, BioTek Instruments, Winooski, VT). Cells were excited at (530/525 nm) and emission (620/640 nm) was recorded for 8 hours at 30 minute or 1 hour intervals.

Inhibitor panel screen

Cells were grown to confluence in a 96-well plate and changed to SFM 24-hours prior to infection. 1 hour before infection, the overnight SFM was removed. Cells were then treated with a panel of various cell death pathway inhibitors at concentrations based on manufacturer's recommendations (Table 3). All inhibitors were dissolved in nanopure water or DMSO. DMSO in SFM at a concentration of 2.5% (v/v) was used as a control, reflecting the highest DMSO concentration present in any of the inhibitor wells. Cells were left to incubate for 1 hour.

Immediately prior to infection, cells were washed again 3 times to remove extracellular inhibitor. Cells were then infected with 100µL of bacteria in SFM at an MOI of 100 or mock-infected with 100µL of SFM. Cells were left to incubate for 1 hour before undergoing a Propidium iodide uptake assay to measure cell death.

Western blot

Epithelial cells were grown to confluence in 6-well plates and infected as described in the propidium iodide assay conditions. Lysates were obtained by washing cells on ice with 1x cold PBS. Cells were scraped into 1.7mL microfuge tubes and centrifuged at 4°C at 0.4rcf for 10 minutes. Supernatant was removed and cells were resuspended in 10µL urea buffer (2M thiourea, 7M urea, 4% CHAPS, 1% DTT). Cells were lysed using a combination of urea buffer and 1mm glass beads using the Bead Ruptor 24 Elite (Omni International). This procedure consisted of 1 minute of lysis using the Bead Ruptor at power 5, followed by one minute of cooling the samples on ice. This process was then repeated 5 times to ensure a thorough and homogenous lysate. Samples were then centrifuged at 4°C at 16.1rcf for 30 minutes.

Lysate was the extracted and quantitated using a Pierce 660 quantitation assay. After the final protein quantitation was determined, 25-50µg of total protein was loaded onto a 4-20% SDS-PAGE gel and run for 4-6 hours at 40V. The PAGE gel was then transferred to a PVDF membrane using a Trans Blot Turbo Transfer System (Bio-Rad).

This membrane was then blocked for 1 hour in 5% milk in TBST, followed by overnight incubation in the relevant primary antibody. Primary antibodies used in this study include anti-Caspase8 (CellSignal) 1:1000 raised in mouse and anti-Actin (Sigma) 1:10,000 raised in rabbits.

The following day the membrane was washed 4x for 5 minutes each in TBST and incubated for 1 hour in secondary antibody at a concentration of 1:10,000, followed by another wash 4x for 5 minutes in TBST and treatment with a SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific) a chemiluminescent substrate. Images were acquired using the ChemiDoc (Bio-Rad) imaging system.

Calcium release assay

Cells were grown to confluence in a 96-well plate and changed to SFM 24-hours prior to infection. 1 hour before infection, the overnight SFM was removed and cells were washed with fresh SFM 3 times to remove excess extracellular calcium. Cells were then treated with 10µM Fluo4 AM (Thermo) a calcium binding fluorescent reporter. Cells were left to incubate for 1 hour. Immediately prior to infection, cells were washed again 3 times to remove extracellular Fluo4 AM. Cells were then infected with 100µL of bacteria in SFM at an MOI of 100. The 96-well plate was then immediately read in a microplate reader. Cells were excited (494nm) and emission (506nm) was recorded for 8 hours at 15 minute intervals.

Calculations and Statistics

Statistical analysis was performed using Student's t-test and the $p\text{-value} < 0.05$ was considered statistically significant. Where statistical analysis was performed, each experiment contained at least 4 technical replicates.

Table 3: Inhibitor Panel

Inhibitor	Function	Concentration	Manufacturer
Necrosulfanomide	MLKL necroptosis inhibitor	2µM	EMD Millipore
PARP Inhibitor VIII	Inhibits ATP depletion and mitochondrial release of AIF	20nM	EMD Millipore
Dantrolene	Inhibits intracellular calcium release	120µM	Sigma-Aldrich
Cyclosporin A	Inhibits mitochondrial cytochrome C release	25µM	Sigma-Aldrich
Ferrostatin	Inhibits RIP1 dependent necroptosis	60nM	Sigma-Aldrich
CAS-BIND PRO	High potency pan-caspase inhibitor	10µM	Vergent Bioscience

Results and Discussion

EPEC-induced cell death is dramatically affected by the presence of EspH

While EspH has been shown to induce caspase 3 activation under transfection conditions, it is still unknown how EspH impacts host cell death under EPEC-infection conditions. To determine the level of cell death caused by the presence of EspH, Caco-2_{BBE} cells were infected with various EPEC-effector mutants (Δ*espZ,* Δ*espF,* and Δ*espH*) and cell death was quantitated via propidium iodide uptake assay (Fig 5). Δ*espZ* and Δ*espF* were chosen for this assay because of their established and opposite impact on host cell death. The Δ*espZ* mutant causes higher levels of host cell death, while Δ*espF* causes reduced cell death (12, 57). This would provide a context for any change observed in the Δ*espH* strain. At 4 hours post-infection Δ*espH*-infected and uninfected cells showed similar levels of cell death, however Δ*espH*- and WT-infected cells displayed high levels of cell death approaching the level of death observed in the methanol-treated positive control. The p*espH* complement strain restored cell death to WT EPEC levels. At

no point in the infection did Δ*espH* display the level of cell death observed in the methanol control, while all other mutants eventually reached this point. This change indicates that EspH is a key contributor to EPEC-induced epithelial cell death, with relatively greater cytotoxic impacts than EspF.

Delineation of EspH domains/regions relevant for its cytotoxic activity

One aim of this study was to identify those regions of EspH required for its host cell death phenotype. A library of EspH alanine scan mutants was used to identify those regions through another propidium iodide uptake assay. The alanine scan mutants sequentially replaced every 5 amino acids in the protein with alanine residues, and the T3SS-dependent secretion of corresponding mutant EspH proteins was confirmed (data not shown). While mutants M19, 24, 25, and 28 showed some restoration of EspH-dependent cell death, the majority of mutants tested showed no increase in cell death compared to the EspH-deletion strain (Fig. 6). This impaired cell death phenotype could be due to attenuated ability to engage host cell death pathways, or a broader disrupted folding of the protein or impaired translocation of the mutant protein into host cells. Two mutants of interest were amino acid 88-92 (M14) and 98-102 (M16) mutations. These mutants were both unable to restore the wild type cell death phenotype, and they were both impaired for RhoGEF binding activity (33), suggesting that Rho inhibition is a partial contributor to host cell death. This preliminary screen suggests that specific regions of EspH are important contributors to its overall cytotoxic function, however more detailed analysis will be required to definitively identify the domains required for EspH-induced cell death and the mechanism(s) behind its cytotoxicity.

EPEC-induced cell death is reduced through caspase inhibition

This study aimed to identify the host cell pathways that EPEC, and specifically EspH, altered as part of their pathogenicity. To broadly identify the components involved in EPEC-induced cell death, epithelial cells were treated with a panel of host cell death pathway inhibitors followed by infection with WT EPEC and a PI uptake assay for cell death. The results indicated that out of the inhibitor panel, the pan-caspase inhibitor Cas-BIND Pro (Vergent Bioscience) displayed the greatest reduction in host cell death during infection, indicating that caspases are a critical component of EPEC-mediated host cell death (Fig. 7). This supports existing data (10) and prompted further investigation into the specific role of caspases in EspH-dependent epithelial cell death. The lack of protection from the other inhibitors could be a result of multiple cell death pathways activating concurrently due to various EPEC effectors impacting diverse host cell death pathways. While the significance of the caspase inhibition is interesting, the lack of protection in the other inhibitors warrants further exploration.

Caspase 8 cleavage is EspH dependent during EPEC infection

To further elucidate the pathway behind EspH dependent cell death, we analyzed various disparate published and unpublished data. Previous work has demonstrated EspH-dependent alterations in host proteins hFis1 and Bap31. hFis1 is a mitochondrial fission protein that is upregulated in the presence of EspH (Roxas and Viswanathan, unpublished observations). Bap31 is an endoplasmic reticulum protein that is cleaved during EPEC-infection in an EspH-dependent manner (Wilbur and Viswanathan, unpublished observations). Both hFis1 and Bap31 along with caspase 8 form a pro-apoptotic complex called the ARCosome (71). Additionally, in

the siRNA screen, caspase 8 associated protein 2 (CASP8AP2) displayed an overall reduction in host cell death. CASP8AP2 is known to activate caspase 8 as part of Fas-mediated apoptosis (72). Given the prominent impact of EspH on ARCosome components and the importance of caspase activity in EPEC-induced host cell death, caspase 8 was identified as a protein of interest. Western hybridization revealed that caspase 8 cleavage is EspH dependent, and that the M14 and M16 regions of EspH are necessary for this cleavage, as shown by the reduced level of cleavage in those mutants (Fig. 8). In contrast, mutants M19, 24, and 28 showed a restoration of cell death during the PI uptake assay and also induced caspase 8 cleavage at near WT levels. This data suggests an additional connection between EspH-RhoGEF interaction and EspH-dependent host cell death.

EspH infection alters host cell calcium release

All major components of the ARCosome complex previously mentioned are impacted by the presence of EspH. hFis1 levels are elevated, Bap31 is cleaved, and caspase 8 is activated. The increase in hFis1 abundance leads to an increase in mitochondrial fission (73), which is also increased during EPEC infection in an EspH-dependent manner (Roxas and Viswanathan, unpublished observations). EspH is also crucial for EPEC-induced activation of caspase 8, and EPEC-infection shows reduced cell death when treated with a pan-caspase inhibitor, suggesting this cleavage is necessary for EPEC-induced host cell death. And EspH induces Bap31 cleavage. Cleaved Bap31 is involved in calcium release from the ER and this calcium release leads to destabilized mitochondria due to calcium overload (74). This induces mitochondrial fission, cytochrome C release, caspase 3 activation, and host cell death (71). To assay calcium release

during infection, cells were treated with the calcium binding fluorescent indicator Fluo4 AM and infected under various conditions. The result of this showed a decreased rate of calcium release in the EspH mutant compared to the heightened rate of release seen in the EspZ mutant and the wild type strain (Fig. 9).

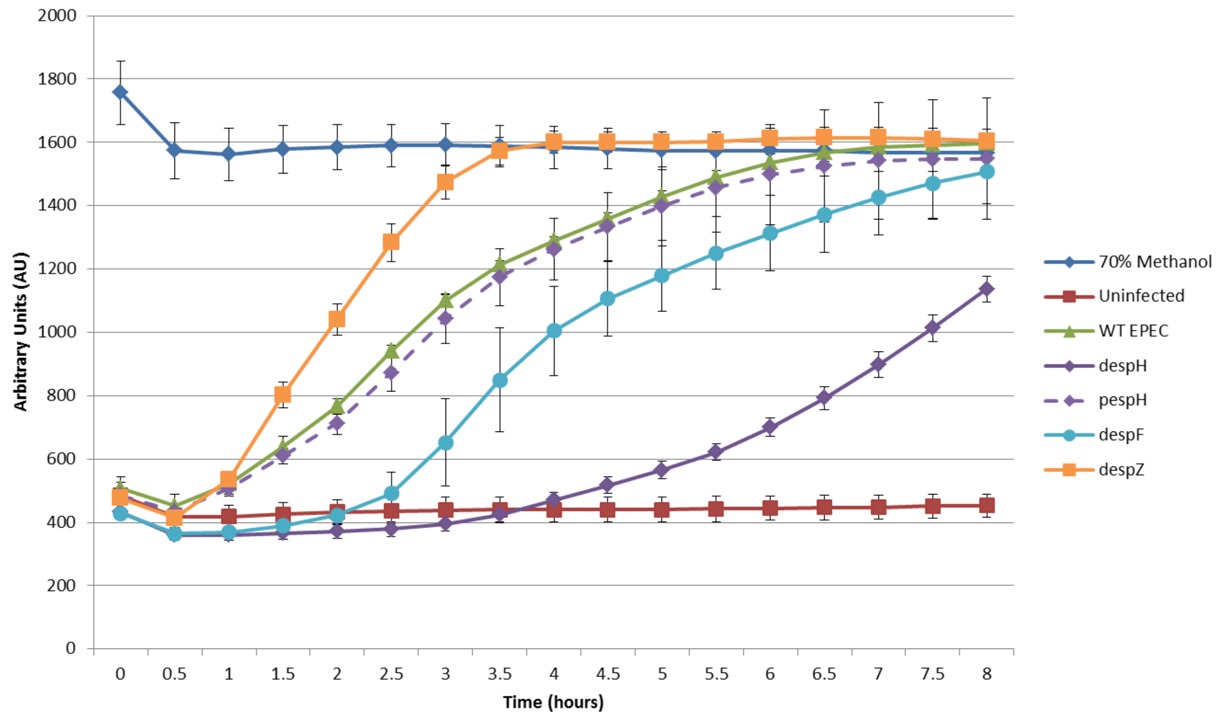

Figure 5 EspH contributes significantly to EPEC-induced host cell death. Caco-2_{BBE} cells were infected with a range of T3SS effector mutants and incubated in a propidium iodide medium for 8 hours, with fluorescence readings taken every 30 minutes. A 70% methanol solution was used as a positive control for cell death, and fresh serum free media was used as a negative control. Error bars represent standard error across 4 technical replicates.

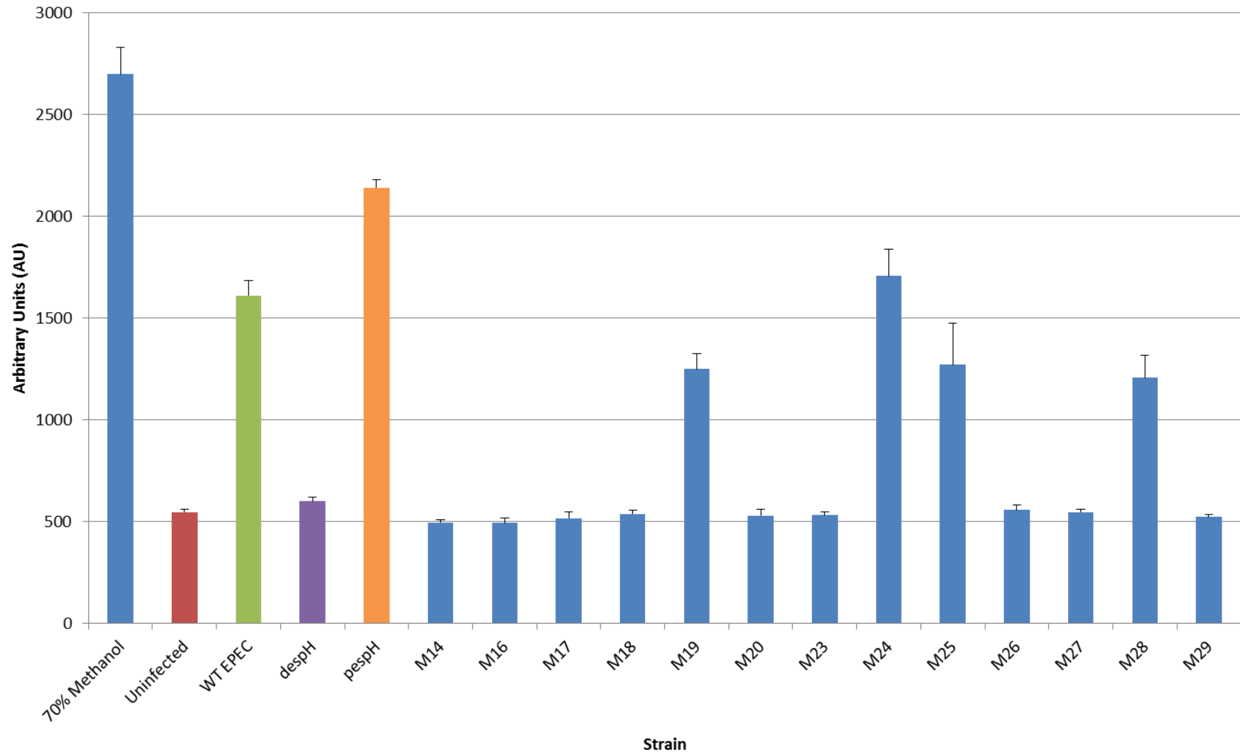

Figure 6 Identification of EspH regions required to promote host cell death. Caco-2_{BBE} cells were infected with a range of Δ*espH* mutants and alanine scan mutant complements and incubated in a propidium iodide medium for 8 hours, with fluorescence readings taken every 30 minutes. A 70% methanol solution was used as a positive control for cell death, and fresh serum free media was used as a negative control. Error bars represent standard error across 4 technical replicates.

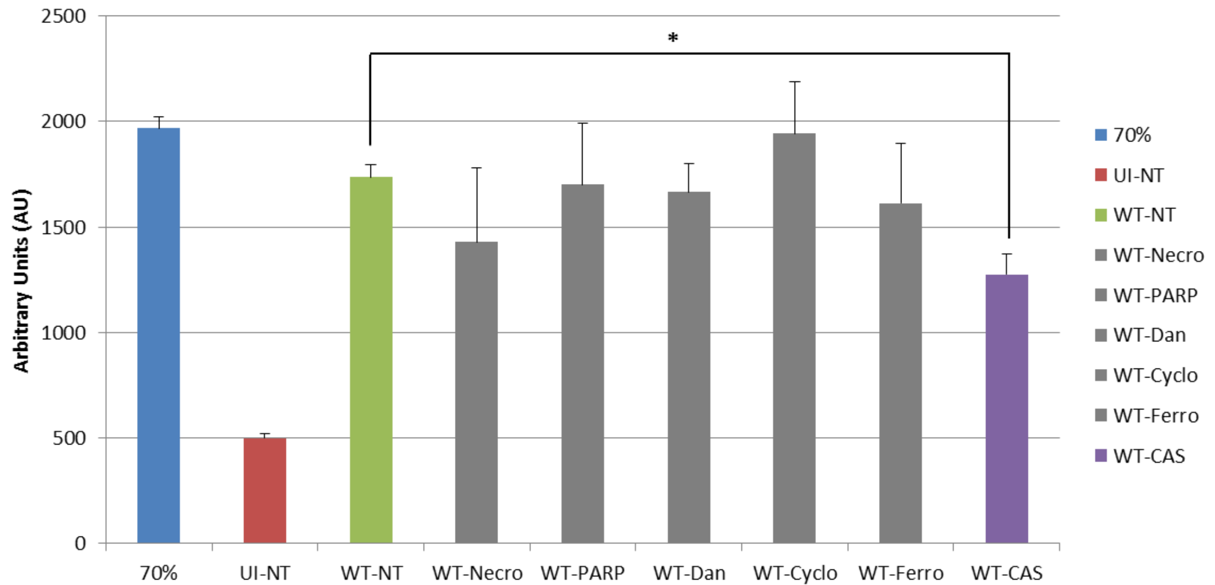

Figure 7 Caspase activity contributes significantly to EPEC-induced host cell death. Caco-2_{BBE} cells were treated with a range of cell death inhibitors: no treatment (NT), necrosulfanomide (Necro), PARP Inhibitor VIII (PARP), Dantrolene (Dan), Cyclosporin A (Cyclo), Ferrostatin (Ferro), and Cas-BIND Pro (CAS), and infected with WT EPEC (WT-) and incubated in a propidium iodide medium for 8 hours, with fluorescence readings taken every 30 minutes. A 70% methanol solution was used as a positive control for cell death, and fresh serum free media (UI) was used as a negative control. Error bars represent the standard deviation of the mean. *$p<0.05$; student's T-test.

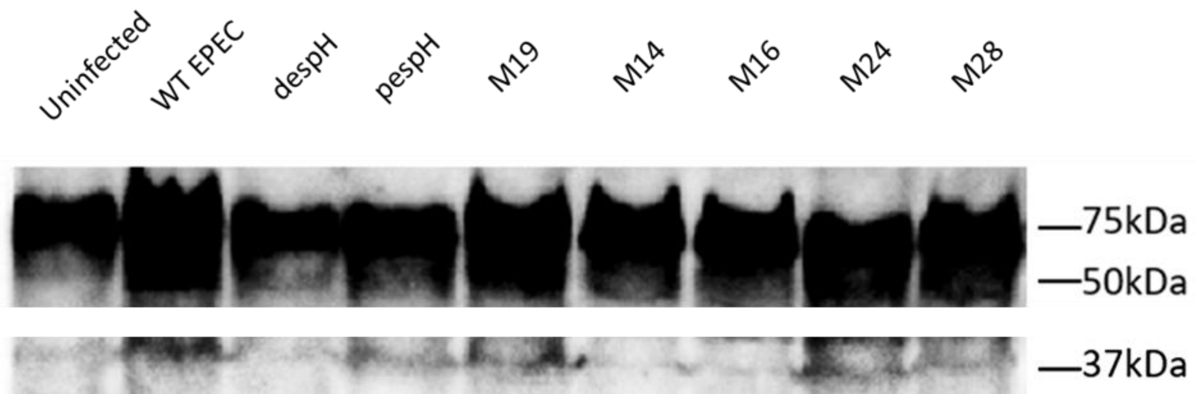

Figure 8 Caspase 8 cleavage is EspH dependent during EPEC infection. 50µg of lysate from infected Caco-2_{BBE} cells. The cleaved portion of caspase 8 is approximately 42kDa, and a matching band appears at this point on the blot. Between WT EPEC and the Δ*espH* strain there is a notable decrease in cleavage that is restored in the complement (pespH). Similarly, the cell death deficient mutants M14 and M16 show very little cleavage product. Strains M19, 24, and 28 partially restored cell death in the PI uptake assay, and also restore caspase 8 cleavage.

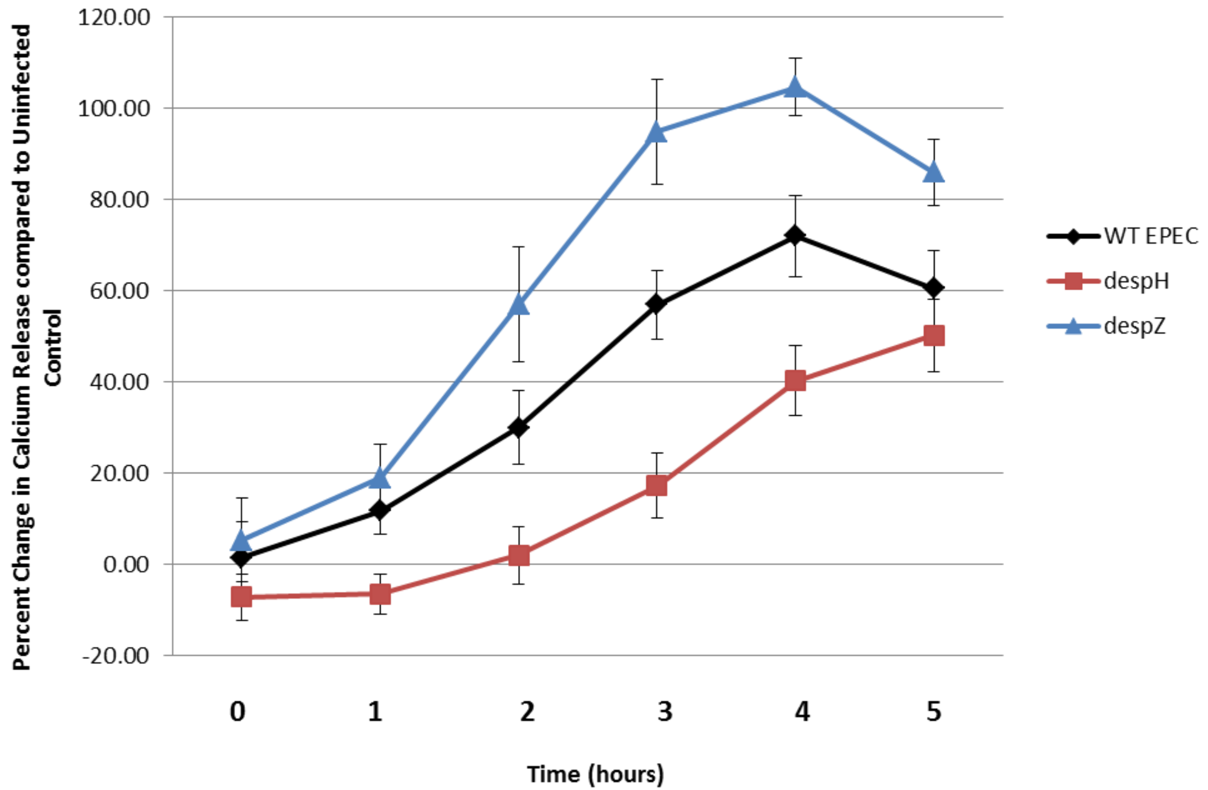

Figure 9 Calcium release during EPEC infection is impacted by EspH. Caco-2_{BBE} cells infected with WT, Δ*espZ,* and Δ*espH* during a calcium release assay over 5 hours. Values are given as a percent change in calcium release compared to an uninfected control. Error bars represent standard error across 4 technical replicates.

Chapter 3 Summary

The effect of EspH on certain aspects of host cell death has been examined in previous studies, however no explanation of the mechanism or pathway behind EspH-induced cell death has been elaborated. The results of this study confirm that EspH is a major component of EPEC-induced host cell death, far more so than the established cell death effector EspF. Preliminary mutant studies have identified some regions of EspH that contribute to its ability to cause host cell death. These regions were broadly identified in groups of 5 amino acid segments, and more specific examination of the individual amino acids in these regions could provide a more nuanced and detailed understanding of their role in EPEC pathogenesis. Two of these regions have been shown to be necessary for RhoGEF interaction and by extension, inhibition of RhoGTPases. The immediate downstream pathways leading to host cell death remain undefined.

Various lines of evidence suggest that EspH is inducing mitochondrial calcium overload. Our model suggests a pathway by which EspH triggers ARCosome formation through upregulation of hFis1, activation of caspase 8, and induction of Bap31 cleavage. This activity then leads to EspH-dependent calcium release, promoting mitochondrial calcium uptake, which in turn destabilizes the mitochondria, causing mitochondrial fission and ultimately host cell death. Further exploration of this pathway is necessary to better elucidate the exact mechanisms behind EspH-induced host cell death, however current data suggests that EPEC-induced host cell death occurs through this pathway in an EspH-dependent manner.

Discussion

EPEC and related pathogens dynamically regulate host cell survival during infection to delay host immune responses, promote bacterial growth, and enable dissemination through the host. Regulation of host cell death represents a significant component of EPEC pathogenesis, and it is primarily influenced by the secretion of pro- and anti-apoptotic effector proteins into the host cell. These effectors then interact with host proteins to influence existing cell pathways, altering the overall survival of the host cell.

Studies have identified a wide range of effector-host protein interaction during EPEC-infection. EspH-RhoGEF and EspF-mitochondria interaction have been previously characterized and are known to significantly alter host cell survival pathways (10, 12, 36). However no comprehensive study has been performed to explore the impact of individual host proteins during EPEC-induced host cell death. This led to the siRNA knockdown and PI uptake screen performed in Chapter 1. In this screen multiple gene families including TRAF, PSM, CARD, and RhoGEF were identified as having an effect on epithelial cell death during EPEC infection. Mitochondrial fission genes were also identified in this screen. The full potential of the screen data has not been explored, but these results provide many novel targets for elucidating EPEC-modulated host cell survival. Further investigation is needed to verify these hits and understand their function during EPEC-infection.

EspH interaction with specific host cell pathways was also explored in this study. Previous studies have implicated EspH in changes in host cell RhoGEF activity, actin cytoskeletal

dynamics, and caspase 3 activation, however the pathways through which EspH is acting have not been fully detailed (10, 36). This study examined the contribution of EspH to host cell death and found that in comparison to another pro-apoptotic effector EspF, EspH showed a greater contribution to cell death *in vitro*. Additionally, alanine-scanning mutagenesis revealed multiple regions of the EspH protein that could be contributing to its pro-apoptotic phenotype. The caspase 8 and calcium release data in this screen are also suggestive of EspH influencing the established Bap31-caspase8 apoptotic pathway (71). While this pathway and EspH's role in it have not been fully explored, it gives a better understanding of how EspH influences the host, and provides a foundation for further investigation into EspH virulence during EPEC infection.

My key contributions to the understanding of EPEC-host interaction during EPEC-modulated host cell survival include:

1. *The role of individual apoptosis proteins during EPEC-induced host cell death:* 251 apoptosis-related genes from multiple gene families were found to be potentially significant during EPEC-induced host cell death.

2. *The host pathway affected by EspH-influenced host cell death:* EspH contributed significantly to host cell death and is acting through the Bap31-caspase8 apoptosis pathway.

Future Studies

Future studies will include,

1. Verification of specific genes from the large scale screen.

a. Transfection of siRNA for target genes will be repeated in biological replicate.

b. Transfected cells will be collected and western hybridization will be used to verify protein knockdown.

c. Transfected cells will be infected and screened via PI uptake assay to confirm initial cell death.

2. EspH mitochondrial impact.

a. EPEC- and Δ*espH*-infected host cells will be collected and western hybridization will be used to characterize their impact on mitochondrial proteins.

b. EPEC- and Δ*espH*-infected host cells will be observed under immunofluorescence microscopy to characterize their impact on mitochondrial morphology.

3. EspH direct interactor identification.

a. EspH will be transfected into host cells and cell lysates will be run through a coimmunoprecipitation column pulling down EspH and the proteins associating directly with it.

b. Proteins bound to EspH will be identified through mass spectrometry analysis.

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