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# *Salmonella* biases epithelial differentiation through Wnt and Notch signaling, which may contribute to diarrheal pathogenesis

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

Andrew Quach

Committee in charge:

Professor Kim E. Barrett, Chair
Professor Milton Saier, Co-Chair
Professor Soumita Das
Professor Ashley Juavinett

2020

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The Thesis of Andrew Quach is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

University of California San Diego

2020

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ABSTRACT OF THE THESIS

*Salmonella* biases epithelial differentiation through Wnt and Notch signaling, which may contribute to diarrheal pathogenesis

by

Andrew Quach

Master of Science in Biology

University of California, San Diego, 2020

Professor Kim E. Barrett, Chair

Professor Milton Saier, Co-Chair

Non-typhoidal *Salmonella* is one of the most burdensome foodborne diarrheal pathogens. Previous work has shown a decrease in expression of the chloride/bicarbonate exchanger SLC26A3 (Down-Regulated in Adenoma; DRA)

following *Salmonella* infection and it is thought that diarrheal symptoms could stem from the downregulation of DRA. However, despite its prevalence, the mechanism by which *Salmonella* elicits diarrhea is not entirely known. To elucidate this, we have developed an enteroid model to recapitulate both the absorptive and secretory lineages of the intestinal epithelium. Enteroid-derived monolayers (EDMs) were infected with *Salmonella* where relevant protein expression was studied using qRT-PCR and western blot. Infection reduced expression of DRA and Hes1 while upregulating ATOH1 and Muc2. Hes1 is a Notch pathway downstream signaling molecule and thus a precursor to the absorptive epithelial lineage that expresses DRA. ATOH1, in contrast, is a Wnt pathway downstream signaling molecule and a precursor to secretory lineages, including goblet cells that express Muc2. The involvement of Notch was further investigated by inhibiting Notch signaling using a γ-secretase inhibitor, which reproduced the downregulation in Hes1 and DRA and upregulation in ATOH1 and Muc2 seen with infection. Secretory transporters NKCC1 and CLCA1 were also upregulated following infection, which might contribute to the imbalance between absorption and secretion. Our findings suggest that the pathogenesis of diarrheal disease in the setting of *Salmonella* infection may reflect Notch inhibition and an accompanying shift in differentiation from absorptive to secretory cell types and transporters, a decreased capacity for absorption, and thus the accumulation of diarrheal fluid.

INTRODUCTION

## The Pathophysiology of *Salmonella* Infection

Foodborne illnesses, also colloquially referred to as food poisoning, by definition are the result of consumption of food or water sources that have been contaminated by parasites, viruses, or pathogenic bacteria (or toxins produced by microorganisms). These illnesses pose a risk to global health and are especially problematic in areas like developing countries due to the lack of effective sanitation (Lloyd-Evans *et al*., 1984). Particularly, *Salmonella*, a gram-negative and facultative anaerobe, has been classified as an enteric pathogen that contributes to cases of foodborne illness. Those who are infected with this enteric pathogen typically experience a wide array of symptoms, some of which include fever, chills, abdominal cramps, myalgia, and diarrhea. Specifically, it has been estimated that there are 2.8 billion annual cases of diarrheal illnesses, where non-typhoidal *Salmonella* accounts for 3.8% of those incidents, roughly around 93.8 million (Majowicz *et al.,* 2010). Moreover, *Salmonella* is the most burdensome bacterial diarrheal pathogen in terms of lost productivity and mortality (Majowicz *et al*., 2010). However, despite the prevalence of *Salmonella* infection, there is still not a clear understanding as to the mechanism through which *Salmonella* acts upon to elicit diarrhea.

The pathogenesis of *Salmonella* infection rests, in part, on the bacteria's ability to invade the intestinal epithelium and access subepithelial immune cells via various mechanisms. One of the mechanisms includes entry though Microfold cells (M cells). M cells are a subset of intestinal epithelial cells that help transport antigens and bacteria from the lumen into the lamina propria where they can then be targeted by immune cells

such as macrophages (Jepson *et al*., 2001). Another mechanism of invasion is through the type III secretion system (T3SS) that is encoded by *Salmonella* pathogenicity island 1 (SPI-1). The SPI-1 T3SS becomes active when in contact with intestinal epithelial cells, which in turn allow it to secrete effectors that are important for internalization into non-phagocytic enterocytes (Broz *et al*., 2012). Enterocytes are absorptive cells present in the intestinal epithelium and like M cells, they can be used by *Salmonella* as a point of entry. Upon entry via either mechanism, *Salmonella* is engulfed by phagocytic immune cells like macrophages. Within the macrophages, the bacteria may either be degraded by lysosomal enzymes or undergo replication in the cytoplasm to allow for further proliferation (Broz *et al*., 2012). In addition, *Salmonella* invasion provokes an inflammatory response marked by release of various proinflammatory cytokines like IL-8. Cytokines like IL-8 serve as further protection against *Salmonella* as they help induce chemotaxis for recruitment of neutrophils to the site of infection (Zha *et al*., 2019). It had been believed that the inflammatory response occurs simultaneously with the diarrhea; however, studies from our lab have shown that the diarrhea develops before, and is independent of, the onset of inflammation (Marchelletta *et al*., 2015). Instead, diarrhea is postulated to occur due to alterations in intestinal ion transporters expressed by the intestinal epithelium and were the major focus of this study.

**Ion Transporters Expressed by the Intestinal Epithelium**

Ion transporters expressed by the intestinal epithelium primarily play a role in the absorption and secretion of various ions and nutrients out of and into the intestinal lumen, respectively (Figure 1). Diarrheal phenotypes have been ascribed to the

decreased absorption of ions and/or increased secretion of ions via intestinal ion transporters (Barrett, 2016), thus leading to an excess number of ions present in the lumen. This accumulation is paired with the imbalance of water absorption, as there is an outflow of water from the intestines into the intestinal lumen.

An apical chloride/bicarbonate anion exchanger known as SLC26A3 or Down-Regulated in Adenoma (DRA) was found to be downregulated upon *Salmonella* infection in a murine model (Marcheletta *et al*., 2013). DRA is involved in the absorption of chloride ions; therefore, decreased expression of DRA would ultimately lead to a buildup of chloride ions in the lumen. Furthermore, mutations in DRA have been found to lead to Congenital Chloride Diarrhea (CCD), an autosomal recessive disease characterized by high fecal $Cl^-$ concentration and watery content (Holmberg, 1986 and Höglund *et al*., 1996). Accumulation of chloride in stool is a key characteristic of diarrheal symptoms (Kaplan and Vitullo, 1981); therefore, the mechanism contributing to the downregulation of DRA following *Salmonella* infection is worth investigating.

Together with the decrease expression of DRA, it was possible that other transporters, especially transporters that play a role in secretion, might also be involved in the diarrhea caused by *Salmonella*. SLC12A2, also known as Na-K-2Cl cotransporter or NKCC1, is present in the basolateral membrane of crypt epithelial cells and facilitates the uptake of $Na^+$, $K^+$, and $2Cl^-$ ions (Grubb *et al*., 2000, Das *et al*., 2018). The chloride ions that are loaded into the cytosol by NKCC1 can then secreted across the apical membrane by chloride channels. Furthermore, Chloride Channel Accessory 1, also referred to CLCA1, has been identified as a potential calcium dependent chloride channel. It is thought that it may play a role in regulating the chloride conductance

across the cell membrane and possibly a role in chloride secretion (Gruber *et al*., 1998). However, the exact molecular function of CLCA1 in the gut is still quite controversial but may be relevant in a pathogenesis perspective. As a whole, downregulation of DRA following *Salmonella* infection could also be accompanied by changes in other ion transporters like NKCC1 and CLCA, so it was worth investigating whether they have a role in the imbalance of absorption and secretion in associated diarrhea.

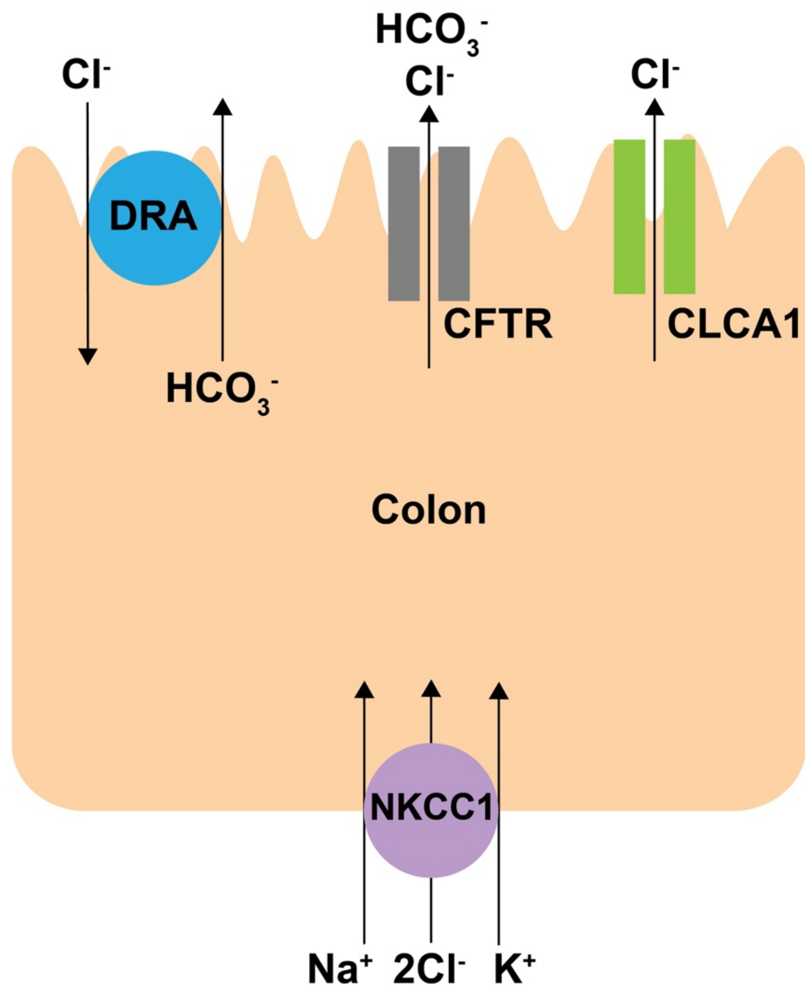



**Figure 1. Ion transporters present throughout the intestinal epithelium.** The large intestine is mostly referred to as the colon. Ion transporters DRA, CFTR, and CLCA1 help with the absorption and/or secretion of various ions as depicted by the arrows. DRA, CFTR, and CLCA1 are localized in the apical membrane of the epithelium, while NKCC1 is present in the basolateral side. These transporters are present in the colon as a whole; however, they are not all expressed by the same cell, as the intestinal epithelium is composed of various cell types with specific ion transport functions.

## The Physiology of the Intestinal Epithelium

The intestinal epithelium is comprised of various cell types (Figure 2). There are stem cells present in the crypt, which can divide into other stem cells and into pluripotent transit amplifying cells (Rangel-Huerta and Maldonado, 2017). Arising from the stem cell progeny, there are four differentiated cell types present in the intestinal epithelium. Specifically, these cell types include: enterocytes, which are absorptive cells that facilitate electrolyte absorption as well as other solutes like sugars and vitamins, goblet cells, which are secretory cells that secrete mucin (mucus), Paneth cells, which are secretory cells that secrete antimicrobial peptides like alpha-defensin for immunity purposes, and enteroendocrine cells, which are secretory cells that secrete hormones (Cheng *et al*., 1974, Ouellette, 2006). These cell types are distributed to different locations of the intestinal epithelium, Paneth cells are typically found in the crypts while enterocytes, goblet cells, and enteroendocrine cells are located predominantly in the villus region. Besides the four differentiated cell types, there are a subset of epithelial cells largely located in the crypt that secrete electrolytes like chloride (Welsh *et* al., 1982, Geibel, 2005). These epithelial cells with chloride secretory capabilities are thought of as immature forms of enterocytes (Matthews *et al*., 1998). However, these cells lose their chloride secretory functions as they fully differentiate into absorptive enterocytes during the course of epithelial cell migration from the crypt to the villus (Matthews *et al*., 1998).

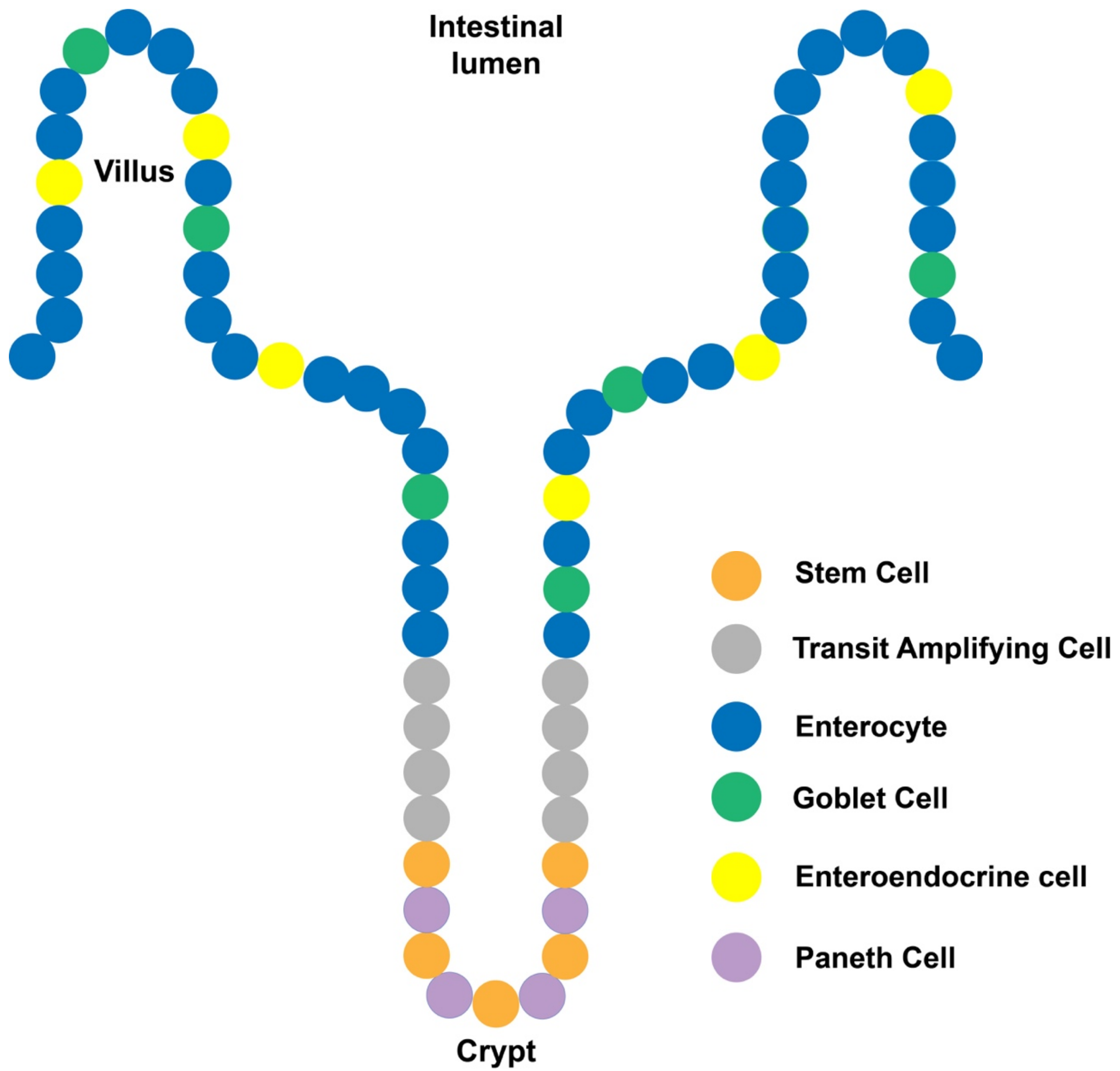



**Figure 2. The various cell types that are present in the crypt-villus axis of the intestinal epithelium.** Stem cells present in the crypts give rise to their progeny for differentiation into four main cell types present in the epithelium, enterocytes, goblet cells, enteroendocrine cells, and Paneth cells.

**Enteroid Model: In Vitro Model to Study *Salmonella* Infection**

Various cell lines have been used to model the intestinal epithelium to study enteric infections. One of these lines are T84 cells. T84 cells are derived from human colon cancer cells and can be polarized. When made into monolayers, they express several brush border properties like ion transporters and various brush border enzymes present in the intestinal epithelium (Pearce *et al*., 2018). However, the disadvantage of using T84 cells is that they are of cancerous origin. Being a cancer line means that it likely lacks normal control over proliferation and differentiation. Another line known as the Caco-2 (heterogeneous human epithelial colorectal adenocarcinoma cells) has also been commonly used to study various enteric infections on the intestinal epithelium. However, a prominent limitation of this particular cell line is that it does not entirely recapitulate the intestinal physiology. Specifically, when Caco-2 cells are differentiated into monolayers, they only differentiate into enterocytes (Delie and Rubas, 1997). Enterocytes only represent one of the four epithelial cell types that make up the intestinal epithelium; therefore, this model has various shortcomings. Other limitations include a lack of representation of various transporters (especially absorptive transporters) and a lack of a mucus layer (Lea, 2015). Also, like the T84 cells, Caco-2 cells are of cancerous origins as well. With these limitations, investigating the effects of enteric pathogens in the T84 cells and Caco-2 cell line may not be as illustrative as other *in vitro* models such as the enteroid model.

Enteroids, also known as organoids, serve as a good model to study enteric infections because they can mimic the crypt-villus axis of the intestine as well as the four different cell types that are present in the intestine. They are cultivated by isolating

stem cells from intestinal tissues from biopsies or surgical specimens, specifically from mice in this study, and can retain pathophysiological features of the intestinal segment from which the enteroids were derived from (Middendorp *et al*., 2014, Suzuki *et al*., 2018). The stem cells are derived from crypts recovered from tissue samples and are plated in Matrigel, comprised of extracellular matrix proteins like laminin, collagen, and more, which helps to create 3D clusters of stem cells and their progeny (Sato *et a*l., 2011). Enteroid cultures can be established quite quickly, typically in less than a week, and can be maintained in a proliferative state through serial passages (Miyoshi and Stappenbeck, 2013). However, enteroids themselves are not useful to study infection because the apical pole of the cells, which is the physiologically relevant surface for infection, cannot readily be accessed by bacteria or other pathogens. To better represent the intestinal epithelium physiology, enteroid-derived monolayers (EDM) are better suited, as they contain the various differentiated cell types. Enteroid-derived monolayers (EDM) can be made on transwell inserts in order to model apical and basolateral polarization. Polarization allows for processes like ion absorption and secretion to be studied as well as allowing physiologically relevant apical access by pathogens (In *et al*., 2016, Yoo and Donowitz, 2019). Consequently, these advantages allow EDMs to function as a great model to investigate the effects of pathogen-epithelial interactions like *Salmonella* infection.

**The Role of Wnt and Notch Signaling in Epithelial Differentiation**

The intestinal epithelium is constantly turning over. Stem cells are proliferating regularly, while their progeny are differentiating into the different cell types. How

intestinal epithelial differentiation occurs is regulated by two opposing pathways known as the Wnt and Notch signaling pathways (Figure 3). These two opposing pathways control the fate of differentiation, specifically into the absorptive versus the secretive lineages. The Notch pathway promotes differentiation into the absorptive cell fate while Wnt signaling biases towards the secretory lineage (Fre *et al*., 2005, Stanger *et al*., 2005, van Es *et al*., 2005, van Es *et al*, 2005). Typically, Wnt and Notch signaling mechanisms are tightly regulated in order to promote intestinal homeostasis for proper absorption and secretion activity levels. However, issues like diarrhea pose an imbalance between absorption and secretion, particularly resulting from a decreased capacity for absorption.

Notch signaling is initiated when an apposing signal-sending cell with a Notch ligand, typically referred to as Jagged or Delta, is able to bind to the Notch receptor on the intestinal stem cell progeny (Hori *et al*., 2013). When the Notch ligand binds, a cascade of proteolytic cleavages is triggered by various enzymes like α-secretase (ADAM 10 and ADAM 17) and γ-secretase (Hori *et al*., 2013). These cleavages break down the Notch protein and release the Notch Intracellular Domain (NICD) into the cytoplasm (Kopan, 2012). Previous work in our lab has shown that NICD is downregulated upon *Salmonella* infection, thus confirming there is an interaction between *Salmonella* and the Notch signaling pathway (in preparation). NICD is able to then translocate into the nucleus where it can interact with a CSL (CBF1, Suppressor of Hairless, Lag-1) to convert it from a repressor to an activating complex. It does so by recruiting proteins like SKI-interacting protein (SKIP) and Mastermind-like 1-3 (MAML) (Kopan, 2012). SKIP is involved in recruiting transforming growth factor beta (TGF-β),

which aids in differentiation, while MAML helps recruit histone acetyltransferases (HATs) along with its cofactor p300. This complex of NICD, CSL, SKIP, MAML, HAT, and p300 all help activate transcription of Notch signaling target genes like the Hairy and Enhancer of Split (Hes) family (Demitrack, 2016). Hes1 in particular is a transcription factor that has been found to control enterocyte fate and is thus a marker of the Notch signaling-driven absorptive lineage (Jensen *et al*., 2000).

The Wnt signaling pathway begins when Wnt binds to the Frizzled (FZD) receptor and the low density lipoprotein receptor related protein (LRP) 5/6 (Mah *et al*., 2016). Binding of Wnt to these receptors causes recruitment of adenomatous polyposis coli (APC), casein kinase 1 (CK1), glycogen synthase kinase-3β (GSK3β), and disheveled (DVL/DSH) to FZD, and Axin to LRP 5/6 (Gao and Chen, 2009). These proteins form a complex near both FZD and LRP 5/6 and ultimately prevent the phosphorylation and ubiquitination of β-catenin. When β-catenin is not phosphorylated and ubiquinated, it will consequently not be degraded (Verheyen and Cottardi, 2011). This allows for the accumulation of active β-catenin in the cytoplasm from where it can then translocate into the nucleus of progenitors derived from stem cells. β-catenin can then bind to lymphoid enhancer factor (LEF) and T-cell factor (TCF), both transcription factors, to activate Wnt signaling target genes like ATOH1/MATH1 (Mah *et al*., 2016). ATOH1 is a marker of the epithelial secretory lineage comprised of goblet, Paneth, and enteroendocrine cells (Yang *et al*., 2001). It is also worth noting that Hes1 negatively regulates ATOH1, which can be quite significant in terms of distinguishing differentiation into which lineage (Zheng *et al*., 2011).

Previous research has discussed interactions of *Salmonella* with either Notch signaling or Wnt signaling, although not both. Xie *et al*. found that cadmium ingestion can worsen *Salmonella* infection by stimulating ROS production, which in turn can stimulate the Notch signaling pathway (Xie *et al*., 2020). Others have found that *Salmonella enterica* serovar typhimurium has an effector known as AvrA, which can be transferred into host intestinal stem cell progeny (Streckel *et al*., 2004, Liu *et al*., 2010). When AvrA is in the cytoplasm, it acts a deubiquitinase to ensure β-catenin is not ubiquitinated where it can then accumulate and activate Wnt targeting genes like ATOH1 (Sun *et al*., 2004, Ye *et al*., 2007). With all this information, there appears to be a link between the Wnt and Notch signaling pathways in the diarrheal pathogenesis of *Salmonella* and is something worth exploring.

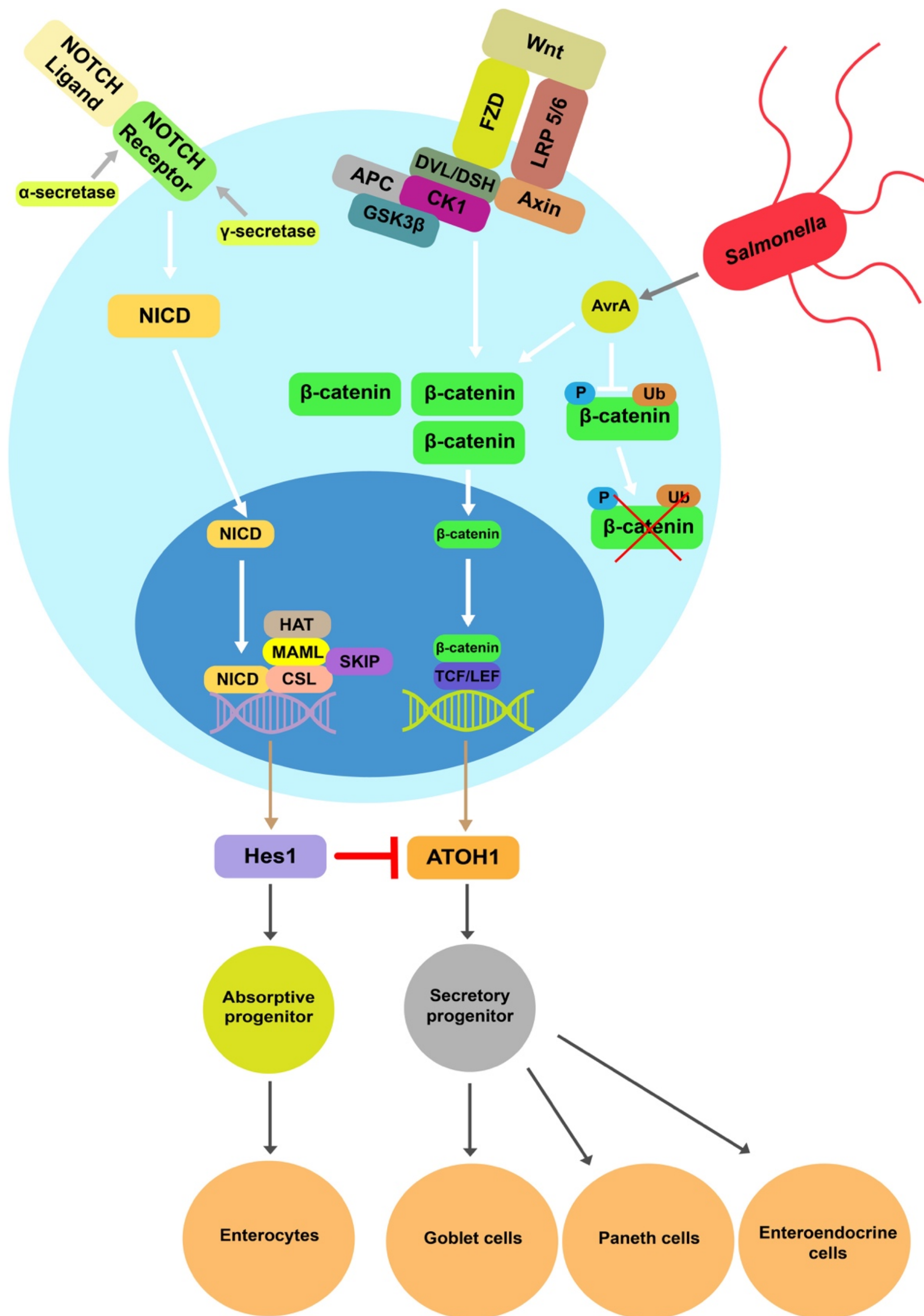



**Figure 3. The Wnt and Notch signaling pathways control intestinal epithelial differentiation.** Notch signaling controls intestinal epithelial cell differentiation into enterocytes through the Hes1 and the absorptive progenitor. On the other hand, Wnt signaling controls epithelial differentiation into the secretory lineage of goblet, Paneth, and enteroendocrine cells through ATOH1 and the secretory progenitor.

## Objectives of Research

One of the main symptoms of *Salmonella* infection is diarrhea. *Salmonella* infection stems from food or water sources that have been contaminated and/or not properly sterilized. This poses a global health issue and is especially problematic in areas like in third world countries. In order to understand the diarrheal pathogenesis of *Salmonella*, the Wnt and Notch signaling pathways are of interest. These pathways control the differentiation fate of epithelial cells between the absorptive and secretory lineages. *Salmonella* infection has been found to downregulate DRA expression in murine models and is a key regulator of the chloride content present in diarrhea. The link between the imbalance in chloride absorption and DRA expression raises additional questions as to whether there also is an imbalance between absorption and secretion processes. Other transporters also like NKCC1 and CLCA1 are of interest as well due to their role in secretory ion transport as well. We hypothesized that *Salmonella* biases towards Wnt signaling as opposed to Notch signaling and that this would lead to a decrease in the absorptive lineage that expresses DRA. The resulting decrease in absorption is hypothesized to be accompanied by an increase in the secretory lineage. To elucidate this mechanism, we used the EDM model to investigate the role *Salmonella* has on Wnt and Notch signaling target genes and various transporters via ~~TEER~~, qRT-PCR, and western blot. In addition, the barrier function of the EDMs was also examined via transepithelial electrical resistance (TEER) in order to examine the effects of *Salmonella* on barrier permeability and integrity.

Investigating the diarrheal pathogenesis of *Salmonella* involving the imbalance between absorption and secretion was the major focus. A small subset of pilot studies

was also conducted to explore the possible beneficial effects of the probiotic strain *Lactobacillus reuteri* on barrier function against *Salmonella* via TEER.

MATERIALS AND METHODS

**Isolation of Enteroids From Murine Intestine**

4 – 7 week old mice of both sexes were briefly exposed to carbon dioxide to promote rapid loss of consciousness, then were euthanized by cervical dislocation. The proximal colon was removed and opened longitudinally. The tissue was rinsed and washed with ice cold phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, California) by repeated vigorous shaking. Using scissors and forceps, the fat and connective tissues were removed before mincing the tissue. 1 mL of collagenase type I solution (2 mg/mL, Invitrogen, Carlsbad, California) was added to the tissue fragments and incubated at 37°C in 5% $CO_2$ for 30 – 60 minutes until most of the epithelial units separated from the tissue fragments. The collagenase solution was resuspended repeatedly during this incubation time until the intestinal crypts were dislodged. Once the crypts were isolated, they were filtered through a 70 μm cell strainer (Thermo Fisher Scientific, Waltham, Massachusetts) and washed with wash buffer composed of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham with HEPES (Sigma St. Louis, Missouri), 10% fetal bovine serum (Sigma), 1% penicillin-streptomycin (Life Technologies), and 1% glutaMAX (Life Technologies). The filtered solution was spun down at 200g for 5 minutes and then resuspended in basement membrane matrix Matrigel (Corning, Corning, New York) before plating and inverting the plate for incubation at 37°C in 5% $CO_2$ for 10 minutes. 50% conditional media (CM) along with 10 μM of TGF-β inhibitor (SB431542) (Selleckchem, Houston, Texas) and 10 μM ROCK inhibitor (Y27632) (Selleckchem) was prepared before adding to the wells by using L-WRN cells (gift from Dr. Thaddeus Stappenbeck [Washington University, St. Louis]).

These L-WRN cells synthesize Wnt3a, R-spondin, and Noggin, which facilitate self-renewal, multipotency, and ultimately organoid formation (Sato *et al*., 2009).

## Culturing and Maintaining Enteroids

Enteroids derived from the proximal colon were scraped into cold 0.5 mM PBS-EDTA (Invitrogen) and spun down at 200g. The resulting cell pellet was trypsinized with 0.025% trypsin (Life Technologies) and incubated for 2 – 3 minutes at 37°C to facilitate the dissociation of the cells from one another. Wash buffer was then added in order to deactivate the trypsin before spinning down at 200g. The resulting cell pellet was resuspended in Matrigel and plated onto a 12 well plate prior to inverting the plate for incubation at 37°C in 5% $CO_2$ for 10 minutes. After 10 minutes of incubation, 50% CM supplemented with 10 μM of TGF-β inhibitor (SB431542) and 10 μM ROCK inhibitor (Y27632) was added. The spheroids were allowed to grow for 48 – 72 hours before preparation of the enteroid-derived monolayers (EDMs).

## Preparation of Enteroid-Derived Monolayers

After 48 – 72 hours of proliferation, the enteroids were trypsinized for 4 minutes with 0.025% trypsin (Life Technologies) and incubated at 37°C in order to generate single cells. $2 \times 10^5$ – $3 \times 10^5$ single cells were seeded onto Matrigel coated (1:40 in PBS, Life Technologies) 6.5 mm Transwells® (Costar, Corning, New York) in 5% conditional media (5% CM). The 5% CM allowed for differentiation as the media was composed of advanced DMEM/F12 media with R-spondin, Noggin from the conditioned media, and 10 μM of ROCK inhibitor (Y27632). The culture medium was changed 24

hours after seeding and allowed to differentiate for 48 hours before infection and measurement of the TEER.

### Growth of Bacteria

*Salmonella enterica* serovar typhimurium strain SL1344 was cultured by inoculating it into Luria-Bertani (LB) broth (Becton Dickinson [BD], Franklin Lakes, New Jersey). *Lactobacillus reuteri* was cultured by inoculating it into De Man, Rogosa and Sharpe (MRS) broth (BD). *Salmonella* was incubated in an orbital shaker at 37°C and 150 RPM for 6 hours under aerobic conditions, while *L. reuteri* was incubated in static conditions at 37°C in 5% $CO_2$ for 6 hours under aerobic conditions. After 6 hours, 100 µL of the bacterial broth cultures were transferred to fresh LB or MRS broth respectively. *Salmonella* was incubated at 37°C for 16 hours, while *L. reuteri* was incubated at 37°C in 5% $CO_2$ for 16 hours before infection. The optical density was taken of the cultures using a spectrophotometer and calculated in order to get $5 \times 10^8$ colony forming units/mL. The bacteria were then resuspended in 1 mL of wash buffer without penicillin-streptomycin before infecting.

### Transepithelial Electrical Resistance

Two days after seeding the EDMs, the transepithelial electrical resistance (TEER) was measured in order to evaluate the integrity of the EDMs. A voltohmeter (EVOM2) with a STX2 chopstick electrode set (World Precision Instruments, Sarasota, Florida) was used. If the TEER value was 200 $\Omega.cm^2$ or greater, then the EDMs were

used for infection.

### *Salmonella* Infection

*Salmonella* was added to the EDMs at a multiplicity of infection (MOI) of 10:1 and incubated at 37°C 1 hour. After 1 hour of bacterial invasion, 50 µg/mL gentamicin (Life Technologies) was added and incubated at 37°C for 1.5 hours in order to kill any extracellular bacteria remaining. After 90 minutes of gentamicin treatment, 5% CM was added to the EDMs and incubation was continued at 37°C for up to 3.5 hours. Following incubation, EDMs were lysed either for qRT-PCR or western blot analysis. Before infection and after each step of the infection, the TEER was measured in order to assess the integrity of the EDMs throughout the experiment.

### Infection with γ-secretase Inhibition

For γ-secretase inhibitor experiments, 100 µM of DAPT (N-[N-(3,5-Difluorophenacetyl-L-alynyl)]-S-phenylglycine *tert*.butyl ester) (AdipoGen, San Diego, California) was maintained in the medium throughout each step of the *Salmonella* infection. After infection, the EDMs were lysed for analysis via qRT-PCR. The TEER was also measured at each step in the infection in order to evaluate the barrier function of the EDMs throughout the treatments.

### *Lactobacillus reuteri* Infection

Lactobacillus reuteri was added to the EDMs at a MOI of 30:1 and incubated at 37°C 1.5 hours. After 1.5 hours, the EDMs were infected with *Salmonella* following the

protocol used for *Salmonella* infection. The TEER was measured throughout the infection in order to assess the barrier function of the EDMs.

**Quantitative Real-Time Polymerase Chain Reaction**

EDMs post infection were lysed using either RNA Lysis Buffer (Zymo Research, Tustin, California) or Lysis Buffer (Qiagen, Hilden, Germany) based on availability, following the manufacturers' instructions. The lysates were placed onto a Zymo-Spin IC Column (Zymo Research) or RNeasy column (Qiagen) where the lysates were washed and purified for proper isolation of the RNA per the manufacturers' instructions. The RNA concentration was measured and checked for purity by verifying the A260/A280 ratio using a nanodrop. The RNA isolated was then converted into cDNA by using qScript cDNA supermix (Quantabio, Beverly, Massachusetts) and stored at -20°C until analysis. The cDNA then was used for quantitative real-time polymerase chain reaction (qRT-PCR) using 2x SYBR Green qPCR Master Mix (Bimake, Houston, Texas), primers (Supplementary Table 1), and the Step-One Plus system (Applied Biosystems, Carlsbad, California). Normalization was done with β-actin or 18S where the Ct values were calculated using delta Ct as $2^{(\beta\text{-actin} - \text{target})}$ or $2^{(18S - \text{target})}$ and expressed as fold change.

**Western Blot**

After infection, the EDMs were lysed using cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, and 1% NP40) with protease inhibitor cocktail (Sigma). Multiple EDMs were pooled for each condition in order to yield

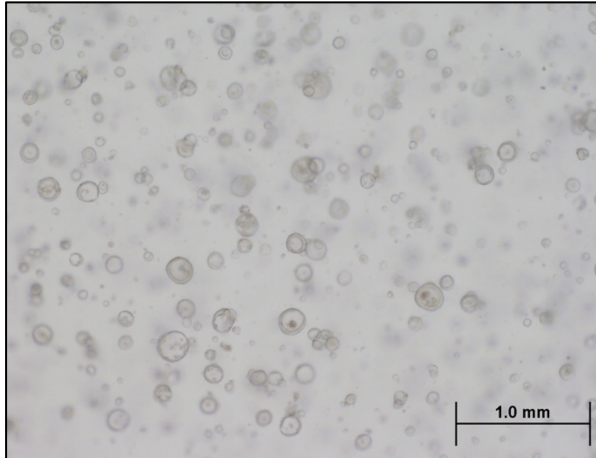
enough protein for detection. The lysate was kept at 4°C and mixed via constant rotation. After 30 minutes, the samples were spun down at 13,000 RPM for 15 minutes at 4°C where the supernatant was collected. Protein concentration was quantified using Pierce BCA Protien Assay Kit (Thermo Fisher Scientific). Equal amounts of protein for each condition were run on a 4% - 20% gel (Bio-Rad, Hercules, California) and ran at 100V for 2 hours. Electrotransfer to a PVDF membrane was done overnight at 30V at 4°C. After the transfer, the blot was blocked using of 5% nonfat dry milk in Tris-Buffered Saline with 0.1% Tween-20 (TBST) for 1 hour at room temperature before probing for the genes of interest. The membranes were incubated with rabbit anti-ATOH1 (1:600, Proteintech, Rosemont, Illinois) or rabbit α-Tubulin (1:2000, Cell Signaling Technology, Danvers, Massachusetts) at room temperature for 1.5 hours, or at 4°C overnight, respectively. The secondary antibody, anti-rabbit IgG (1:2000, Cell Signaling Technology) was added for 1.5 hours at room temperature before adding SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific). Exposure in a dark room ranged from 5 seconds – 5 minutes prior to developing. The resulting bands were quantified by densitometry using ImageJ (National Institutes of Health, Bethesda, Maryland).

RESULTS

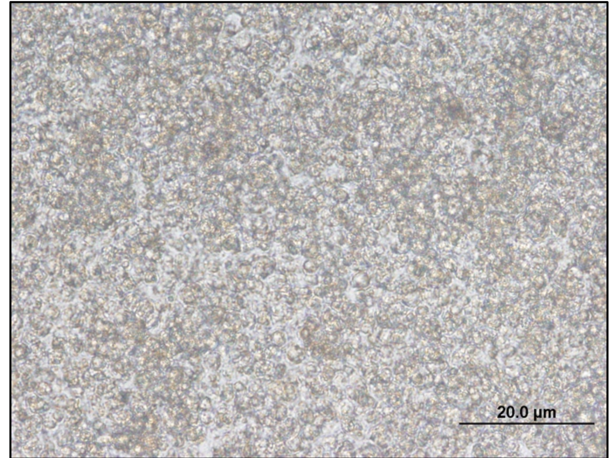
**Differentiating enteroids into enteroid-derived monolayers (EDM)**

Spheroids were maintained through a series of passages and allowed to proliferate in an undifferentiated state until they were ready for proper differentiation (Figure 4A). During differentiation, enteroid-derived monolayers (EDM) were made in order to mimic the different cell types present in the intestinal epithelium (Figure 4B). The EDMs were plated on permeable transwell inserts in order to allow for proper polarization and ultimately to represent intestinal physiology (Figure 4C).

**A**




**B**




**C**

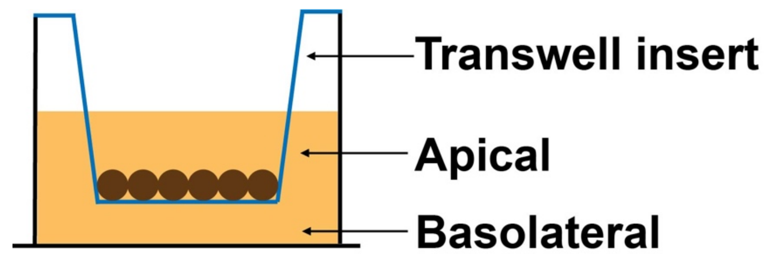



**Figure 4. Culturing and differentiating enteroids into enteroid-derived monolayers (EDMs).** Enteroids derived from murine proximal colon at a proliferative state (A). Enteroids made into EDMs to represent the differentiated cell types (B). EDMs were seeded onto transwell inserts in order to represent the apical and basolateral intestinal physiology where infection and treatment are conducted on the apical membrane (C).

### *Salmonella* disrupts intestinal epithelial barrier integrity

In order to assess the intestinal barrier function of the EDMs, the transepithelial electrical resistance (TEER) was measured throughout each step of the infection (Figure 5). Immediately after the first hour of infection, EDMs challenged with *Salmonella* exhibited a significant decrease in TEER compared to EDMs left untreated. The disruption of the barrier function was observed throughout the entire infection, as seen at the 2.5 hour and 6-hour time points.

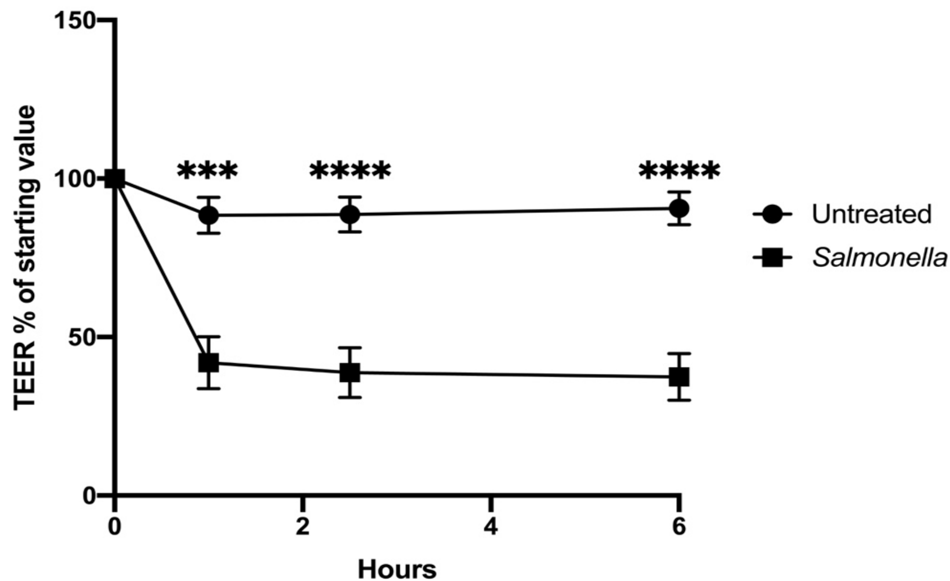



**Figure 5. *Salmonella* disrupts intestinal epithelial barrier function upon infection.** The transepithelial electrical resistance (TEER) was measured at timepoints 1 hour, 2.5, and 6 hours of the infection. Infection with *Salmonella* resulted in barrier dysfunction immediately after the first hour of infection and persisted throughout the entire 6 hours. Data is from 6 independent experiments. Values were represented as mean ± SEM and statistically significant differences were calculated by using a two-tailed t-test (*** = $p < 0.001$, **** = $p < 0.0001$).

***Salmonella* downregulates the absorptive lineage through Notch signaling**

Previous studies in our lab have found that *Salmonella* infection in mice resulted in a decrease in DRA expression (Marchelletta *et al*., 2013). To determine if those results could be recapitulated *in vitro*, the EDMs after infection were lysed for qRT-PCR analysis (Figure 6A). DRA mRNA expression was downregulated following *Salmonella* infection, corresponding to the *in vivo* findings of Marchelletta *et al* (Marchelletta *et al*., 2013*)*. To assess whether decreased expression of DRA could be due to alterations in Notch signaling, Hes1, a marker of absorptive progenitor cells, was investigated to see whether Notch signaling was affected during epithelial infection (Figure 6B). *Salmonella* infection resulted in a decrease in Hes1 compared to EDMs that were untreated, similar to the downregulated expression of DRA post infection.

Hepatocyte Nuclear Factor 1α and 1β (HNF1α and 1β) have been found to play a role in controlling cell fate in the gut, specifically through the Notch signaling pathway (D'Angelo *et al*., 2010). D'Angelo *et al*. found that HNF1α and 1β act upstream of the Notch signaling pathway and can activate expression of DRA (D'Angelo *et al*., 2010). Because of this relationship between the HNF1 proteins and Notch signaling, I tested whether *Salmonella* has an effect on these proteins as well. Indeed, it was found that HNF1α was downregulated following *Salmonella* infection (Figure 6C). Expression of HNF1β was not tested, but could be examined in future studies.

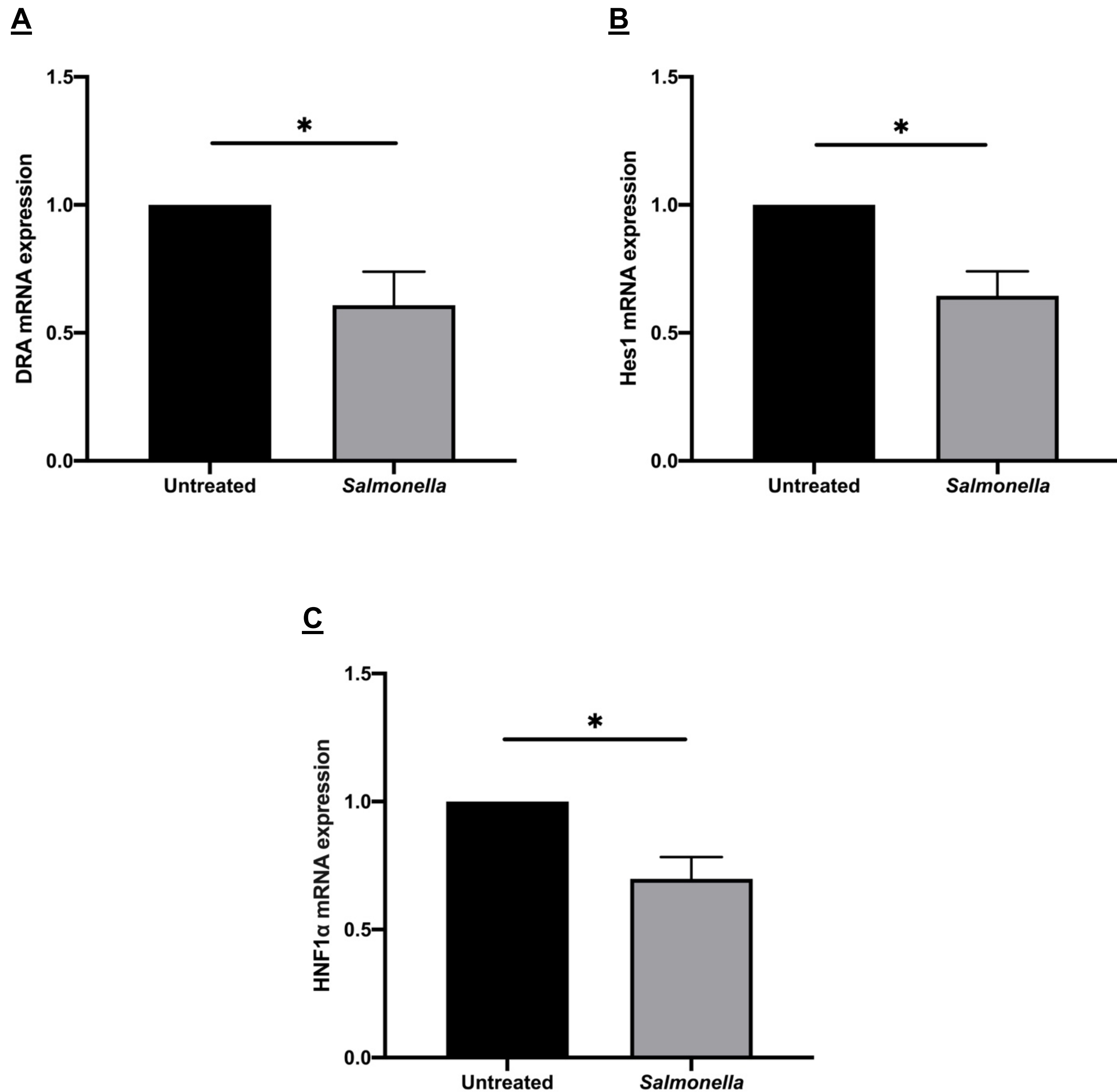



**Figure 6. The absorptive lineage is downregulated following *Salmonella* infection.** EDMs were infected with *Salmonella* strain SL1344 for 1 hour. Following invasion, gentamicin treatment was administered for 90 minutes prior to incubating with 5% conditional media for 3.5 hours. Cell lysates were collected at 6 hours where Hes1 (A), DRA (B), HNF1α (C) mRNA expressions were analyzed via qRT-PCR. Data is from 5 independent experiments for Hes1, 6 independent experiments for DRA, and 4 independent experiments for HNF1α. Values were represented as mean ± SEM and statistically significant differences were calculated by using a two-tailed t-test (* = $p < 0.05$).

### *Salmonella* upregulates the secretory lineage

The downregulation of the absorptive lineage led to the thought that epithelial differentiation might be reciprocally biased towards the secretory lineage following infection. Since both Wnt and Notch signaling control epithelial differentiation, respectively to the secretory lineage and to the absorptive lineage, it was believed that there could be a shift in between these two pathways during the pathogenesis of *Salmonella* diarrhea. To compare, ATOH1 is a Wnt signaling target gene while Hes1 is a Notch signaling target gene; therefore, expression of ATOH1 was analyzed to see if there were any changes post infection (Figure 7A). In fact, ATOH1 mRNA expression was upregulated following infection with *Salmonella*. Protein analysis also showed an increase in ATOH1, but repeated testing is required since the housekeeping gene tubulin unexpectedly did not have constant expression between the untreated and *Salmonella* treated conditions (Figure 7B). Since ATOH1 is a general secretory cell type marker, Mucin 2 (Muc2) was examined in order to see if there was further bias into the secretory lineage (Figure 7C). Muc2 serves as a marker for goblet cells, which are one of the three cell types of the secretory lineage that intestinal stem cell-derived progenitors can differentiate into. Following infection, Muc2 expression was higher in infected EDMs compared to untreated, which suggests an increase in goblet cell production.

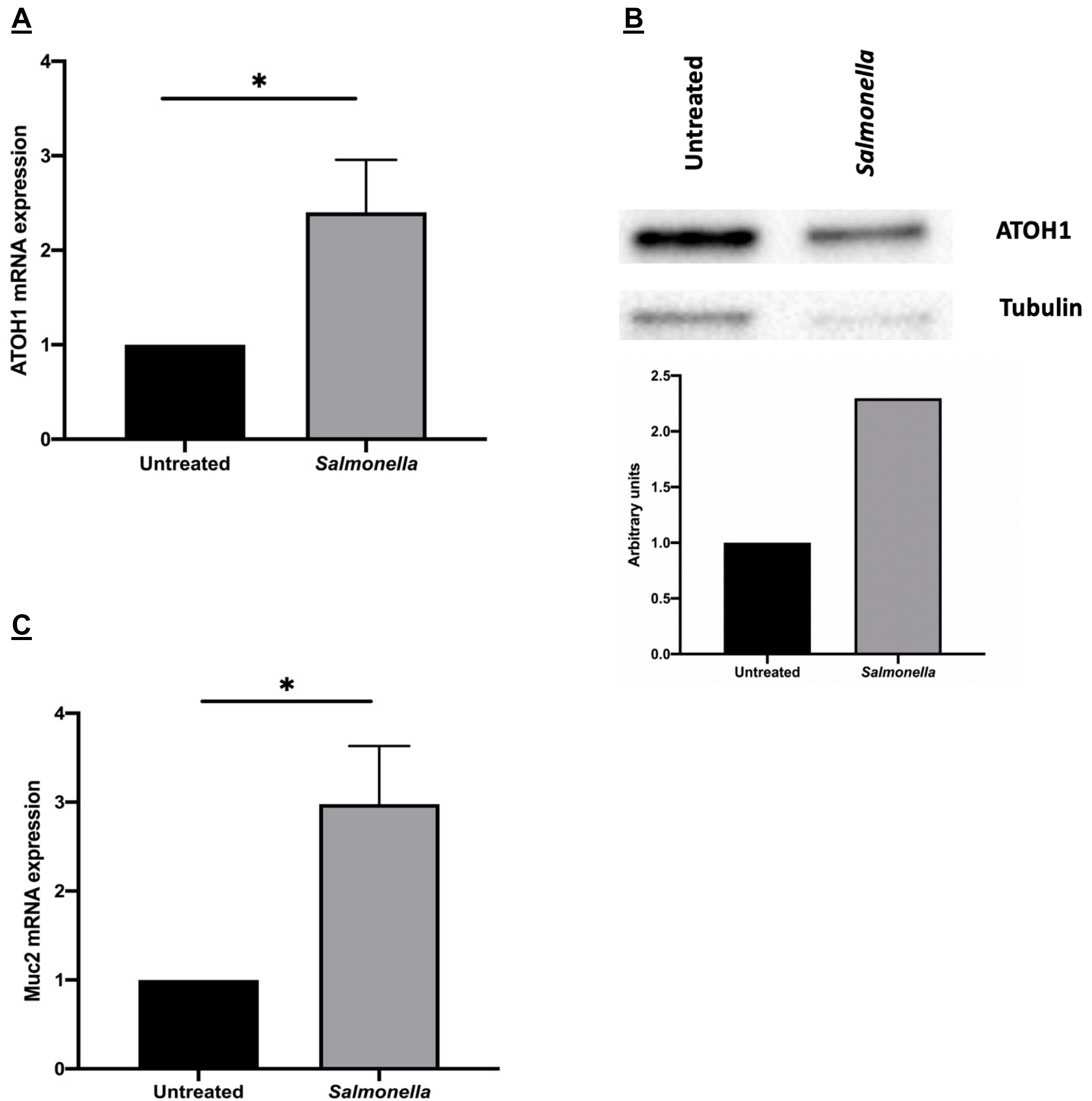



**Figure 7. *Salmonella* biases differentiation into the secretory lineage.** EDMs were infected with *Salmonella* strain SL1344 for 1 hour. Following invasion, gentamicin treatment was administered for 90 minutes prior to incubating with 5% conditional media for 3.5 hours. Cell lysates were collected at 6 hours where ATOH1 (A) and Muc2 (C) mRNA expressions were analyzed via qRT-PCR. Data is from 7 independent experiments for both ATOH1 and Muc2. Values were represented as mean ± SEM and statistically significant differences were calculated by using a two-tailed t-test (* = $p < 0.05$). Following 6 hours, the cell lysates were loaded on SDS-PAGE and immunoblotted with ATOH1 antibody for protein expression analysis (B). Densitometry was done to quantify the size of band and data is from 1 independent experiment.

**γ-secretase inhibition does not affect barrier function**

DAPT, a γ-secretase inhibitor that targets the Notch signaling pathway, was used in order to test the effects of *Salmonella* infection on intestinal epithelial differentiation. Before testing the expression of the Wnt and Notch signaling target genes and their respective lineages, barrier integrity was measured throughout the six-hour infection process with analysis conducted only at the six-hour final time point (Figure 8). Similar to findings mentioned earlier, *Salmonella* significantly decreased the TEER compared to untreated EDMs. However, DAPT had no significant effect on barrier function, nor did it alter the ability of infection to reduce the TEER. These findings suggest that the ability of infection to reduce barrier integrity is unrelated to its effect on Notch signaling.

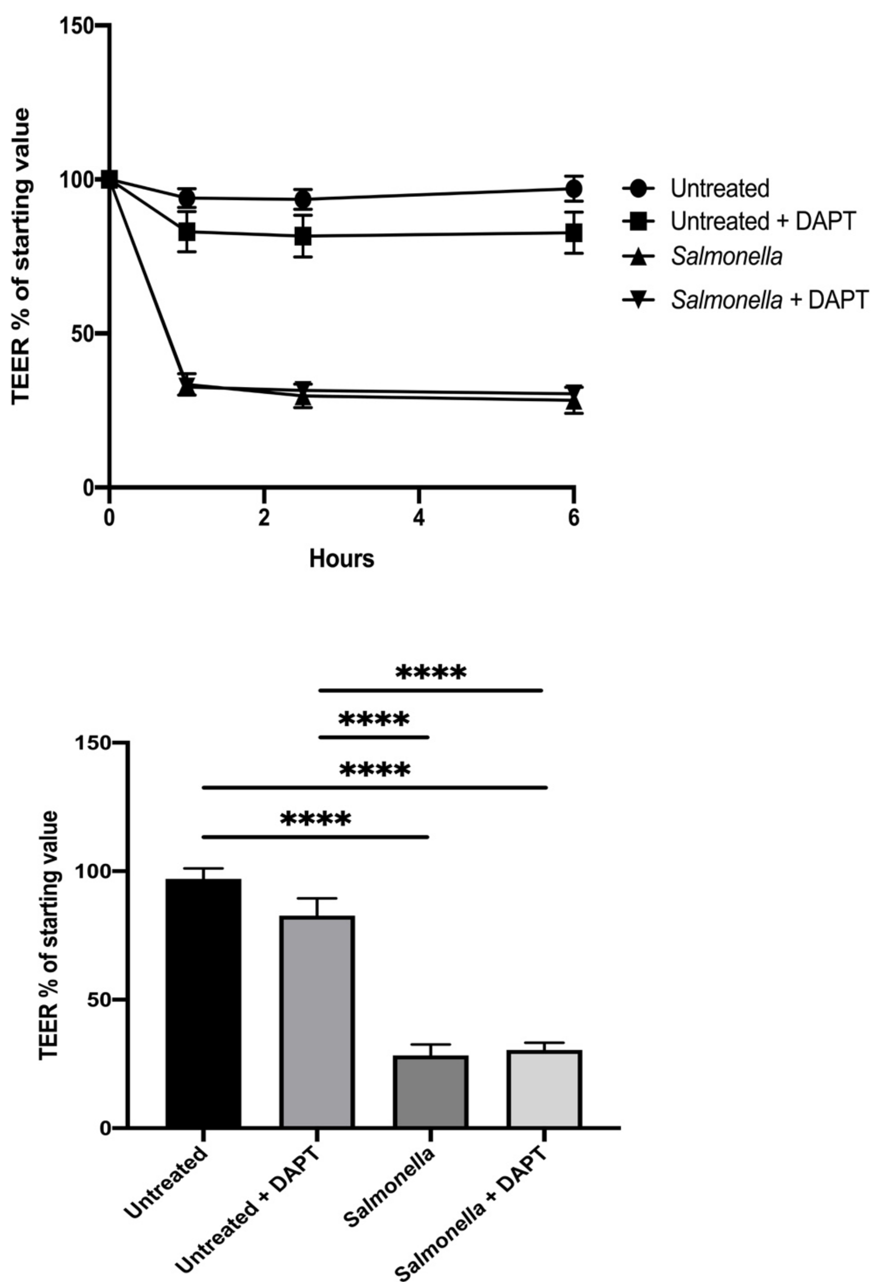


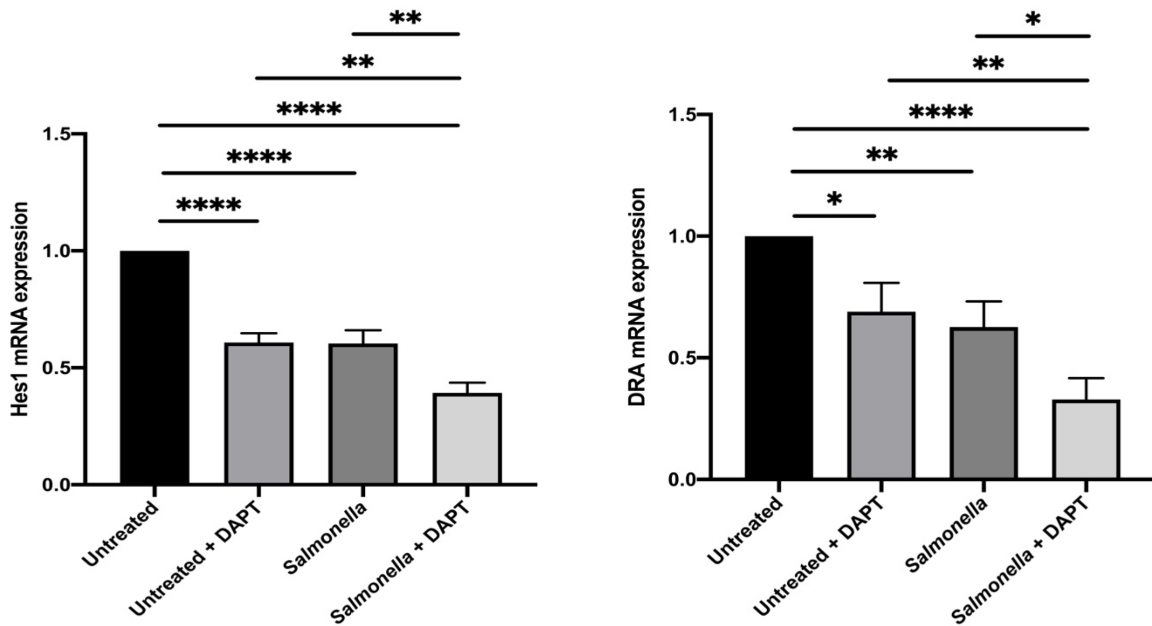

**Figure 8. Inhibition of γ-secretase does not affect the barrier integrity.** EDMs were infected with *Salmonella* strain SL1344 for 1 hour. Following infection, EDMs were treated for gentamicin for 90 minutes prior to incubating with 5% conditional media for 3.5 hours. 100 μM of the γ-secretase inhibitor (DAPT) was maintained in the medium throughout each step of the infection. The TEER was measured at time points 1 hour, 2.5, and 6 hours of the infection. Data is from 5 independent experiments and values were represented as mean ± SEM. Statistically significant differences were assessed only at the 6-hour time point by repeated measures ANOVA with a Tukey's post-test (**** = $p < 0.0001$).

**Inhibition of γ-secretase along with Salmonella biases Wnt signaling**

In order to further confirm our previous findings that *Salmonella* biases Wnt signaling as opposed to Notch signaling, EDMs were treated with *Salmonella* and DAPT. One of the key steps to the Notch signaling pathway includes proteolytic cleavages carried out by γ-secretase; therefore, inhibition of this enzyme allows for further confirmation. On the Notch signaling and absorptive lineage, DAPT treatment alone resulted in a decrease in Hes1 and DRA expression compared to the untreated, which confirm the efficacy of the DAPT to downregulate the Notch signaling pathway (Figure 9A). The downregulation results of the EDMs treated with DAPT were also similar to those treated only with *Salmonella* as no significant differences between the two conditions were observed. In addition, Hes1 and DRA expression in EDMs infected with *Salmonella* and DAPT were further significantly downregulated compared to all the other conditions. To counteract the decrease in the absorptive lineage, Wnt signaling was also examined to see if there was any further bias towards the secretory lineage (Figure 9B). EDMs treated with DAPT showed an upregulation in ATOH1 compared to the untreated, which confirm previous studies that there is crosstalk between the Wnt and Notch signaling pathways (Zheng *et al*., 2011). *Salmonella* also showed an increase in ATOH1 expression compared to the untreated, similar to our earlier findings. EDMs treated with DAPT versus EDMs treated with *Salmonella* did not result in any significant differences. However, there was a further increase in expression when treated with both *Salmonella* and DAPT together versus the other conditions. Increase in Wnt signaling along with decreases in Notch signaling led to an analysis of Muc2 to investigate the effect on goblet cell differentiation. Muc2 expression was increased in

DAPT-treated EDMs compared to untreated. *Salmonella* infected EDMs also resulted in upregulated Muc2 expression compared to the untreated as discussed in previous findings as well. Muc2 expression when treated with *Salmonella* and DAPT was significantly higher than those left untreated; however, there was no significant differences compared to EDMs that were treated solely with DAPT or *Salmonella* alone.

**A**

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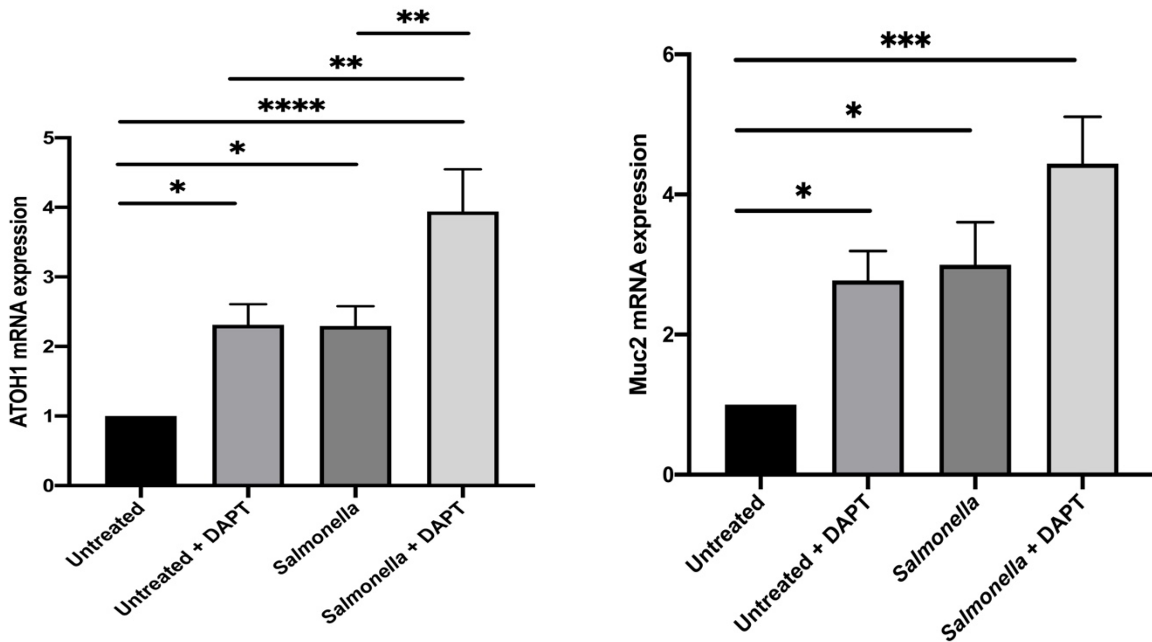


**Figure 9. γ-secretase inhibition and *Salmonella* infection biases Wnt signaling.** EDMs were infected with *Salmonella* strain SL1344 for 1 hour. Following invasion, gentamicin treatment was administered for 90 minutes prior to incubating with 5% conditional media for 3.5 hours. 100 μM DAPT was maintained in the medium throughout each step of the infection. Cell lysates were collected at 6 hours where Notch signaling genes: Hes1 and DRA (A) and Wnt signaling genes: ATOH1 and Muc2 (B) mRNA expressions were analyzed via qRT-PCR. Data is from 7 independent experiments for DRA, ATOH1, and Muc2 and 6 independent experiments for Hes1. Values were represented as mean ± SEM and statistically significant differences were assessed by repeated measures ANOVA with a Tukey's post-test (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

### NKCC1 and CLCA1 secretory transporters are increased after *Salmonella* infection

*Salmonella* downregulates the capacity for intestinal chloride absorption through decreased DRA expression and biases secretory processes like goblet cell differentiation. To supplement these results, secretory transporters like NKCC1 and CLCA1 were examined following infection (Figure 10A and 10B). Following *Salmonella* infection, mRNA for both NKCC1 and CLCA1 were upregulated compared to the untreated conditions. The upregulation of NKCC1 and CLCA1 also offers a further indication that secretion processes may be favored in infection.

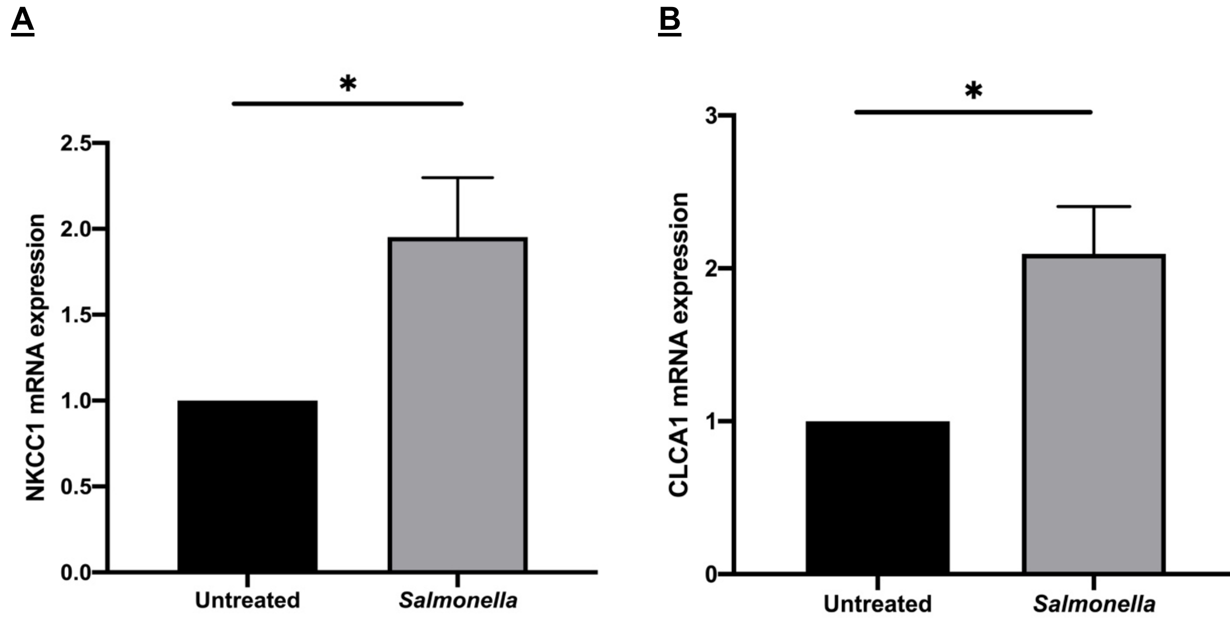


**Figure 10. Other secretory transporters are increased upon *Salmonella* infection.** EDMs were infected with *Salmonella* strain SL1344 for 1 hour. Following invasion, gentamicin treatment was administered for 90 minutes prior to incubating with 5% conditional media for 3.5 hours. Cell lysates were collected at 6 hours where NKCC1 (A) and CLCA1 (B) mRNA expressions were analyzed via qRT-PCR. Data is from 6 independent experiments for NKCC1 and 4 independent experiments for CLCA1. Values were represented as mean ± SEM and statistically significant differences were calculated by using a two-tailed t-test (* = $p < 0.05$).

### *Salmonella* induced IL-8 expression may not depend on Notch signaling

*Salmonella* infection has been known to cause inflammation through the expression of the inflammatory cytokine IL-8 (Eckmann *et al*., 1993, Gewirtz *et al*., 2000). However, the mechanism as to how it induces IL-8 secretion still remains a topic of interest. In the EDM model, IL-8 expression was upregulated after *Salmonella* infection compared to those left untreated, similar to previous literature (Figure 11). Since our previous findings have shown that *Salmonella* downregulates Notch signaling and favors Wnt signaling, DAPT was used to test whether these pathways affect IL-8 secretion since it also downregulates Notch signaling and can upregulate Wnt signaling target genes. DAPT alone did not result in any significant differences in the EDMs left untreated. However, IL-8 expression was significantly higher following *Salmonella* infection compared to DAPT treatment alone. Furthermore, IL-8 expression was similar to EDMs that were treated with *Salmonella* and EDMs treated with both *Salmonella* and DAPT. It may indicate that inhibition of Notch signaling may not have an influence on IL-8 secretion levels seen in infection.

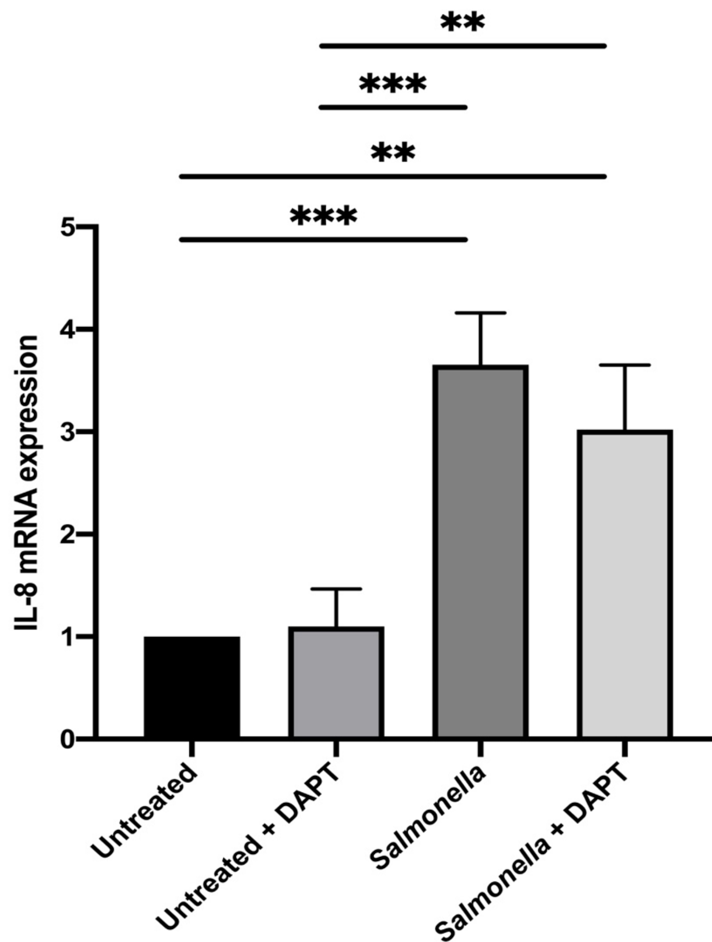


**Figure 11. IL-8 expression may not depend on Notch signaling.** EDMs were infected with *Salmonella* strain SL1344 for 1 hour. Following infection, EDMs were treated for gentamicin for 90 minutes prior to incubating with 5% conditional media for 3.5 hours. 100 μM DAPT was maintained in the medium throughout each step of the infection Cell lysates were collected at 6 hours where IL-8 mRNA expression was analyzed via qRT-PCR. Data is from 5 independent experiments. Values were represented as mean ± SEM and statistically significant differences were assessed by repeated measures ANOVA with a Tukey's post-test (** = $p < 0.01$, *** = $p < 0.001$).

***Lactobacillus reuteri* can restore barrier function against *Salmonella***

Probiotics are of interest because of their possible efficacy against gastrointestinal disorders. Such beneficial effects include the ability to alleviate epithelial dysfunction resulting from invasive pathogens (Resta-Lenert and Barrett, 2009). Therefore, EDMs were pretreated with *Lactobacillus reuteri* to see if there was any positive effects on barrier function (Figure 12). As previously found, EDMs challenged with *Salmonella* resulted in a decrease in barrier function. *L. reuteri* alone had no significant effects on the TEER compared to the untreated condition. However, pretreatment of *L. reuteri* before infection with *Salmonella* led to the restoration of barrier function. The rescue of barrier function may indicate the positive effect of *L. reuteri* against *Salmonella* infection.

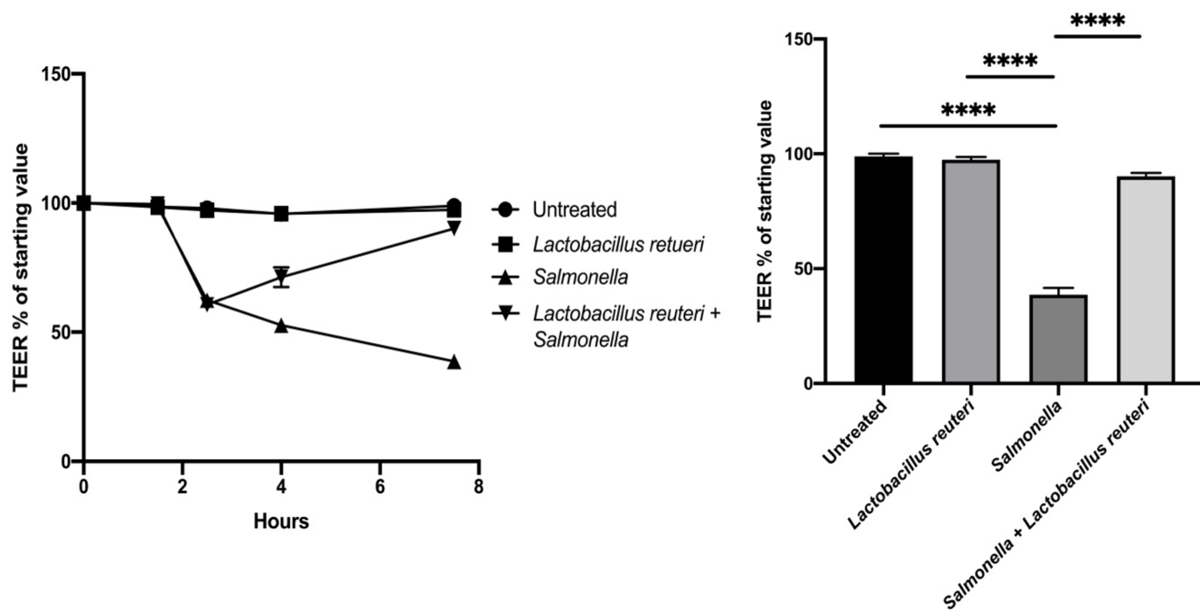


**Figure 12. *Lactobacillius reuteri* restores barrier function against *Salmonella* infection.** EDMs were pretreated with *Lactobacillus reuteri* for 1.5 hours. Following pretreatment, *Salmonella* strain SL1344 was infected for 1 hour. Gentamicin was then added for 90 minutes prior to incubating with 5% conditional media for 3.5 hours. The TEER was measured at time points 1.5, 2.5, 4, and 7.5 hours of the infection. Data is from 4 independent experiments and values were represented as mean ± SEM. Statistically significant differences were assessed only at the 7.5-hour time point by repeated measures ANOVA with a Tukey's post-test (**** = $p < 0.0001$).

## Acknowledgements

Results are being prepared for submission for publication of the material. Quach, Andrew; Jayaratne, Rashini R.; Ibeawuchi, Stella-Rita; Das, Soumita; Barrett, Kim E. The thesis author will be a co-author of this material.

## DISCUSSION

One of the main symptoms of *Salmonella* infection is diarrhea, which may be life-threatening, particularly in vulnerable individuals. However, the mechanism as to how *Salmonella* elicits diarrhea is still not well understood. Previous studies have found that Congenital Chloride Diarrhea (CCD), high chloride content diarrhea, is due to mutations in a chloride/bicarbonate exchanger known as DRA (Höglund *et al*., 1996, Holmberg, 1986). The primary role of DRA is to facilitate the proper absorption of chloride in the intestine; therefore, this mutation coincides with improper absorption and thus a buildup of chloride into the lumen. Studies from our lab have shown that *Salmonella* infection in mice results in downregulated expression of DRA (Marchelletta *et al*., 2013). We have also found that DRA protein expression is decreased following *Salmonella* infection in the enteroid model as well (in preparation). This downregulation of DRA therefore is of interest since there may be a link between changes in DRA expression and the diarrheal symptoms observed in *Salmonella* infection. In our studies, we have focused on elucidating the mechanism behind the downregulation of DRA and have hypothesized that it may be due to changes in the Wnt and Notch signaling pathways that define epithelial cell differentiation.

Initially prior to examining the role of *Salmonella* on epithelial differentiation through Wnt and Notch signaling, the barrier function of the monolayers was examined. The intestinal epithelium acts as a barrier to facilitate the absorption of nutrients, electrolytes, water, and a defense against invasive pathogens (Groschwitz *et al*., 2014). Transepithelial electrical resistance (TEER) was used assess the barrier function of the epithelial cells seeded on the porous transwell inserts. The TEER measurements are mostly reflective of the ionic conductance of the paracellular pathway (Srinivasan *et al*.,

2015). Paracellular permeability is related to the transport through the intercellular spaces between the epithelial cells and is largely influenced by the permeability of tight junction proteins (Groschwitz *et al*., 2014). Tight junctions are apically located intercellular junctions that help maintain the polarity of cells and regulate movement of ions and water. Previous studies have found that *Salmonella* disrupts the tight junction protein Zonula Occludens-1 (ZO-1) (Boyle *et al*., 2006), which coincides with the decrease in barrier integrity observed. It is thought that the disruption of tight junctions can contribute to microbe-associated diarrhea in which the control of ion and water movement is disrupted (Guttman and Finlay, 2009). The decrease in TEER observed in infection may in turn exacerbate *Salmonella*-induced diarrhea.

Notch signaling has been found to play a role in determining the cell fate of intestinal stem cell progeny (Jensen *et al*., 2000). Specifically, it has been found to play a role in differentiation into the absorptive lineage, particularly the enterocytes (Marsh, 1971). Enterocytes, one of the four differentiated cells present in the intestinal epithelium, express DRA, which raised questions as to whether the Notch signaling pathway is affected during *Salmonella* infection and whether this pathway plays a role in the downregulation of DRA. Previous studies in our lab have found that the Notch Intracellular Domain (NICD), a key factor in Notch signaling, is downregulated upon *Salmonella* infection (in preparation). Since the role of NICD is to translocate from the cytoplasm and into the nucleus where it aids in activating Notch signaling genes like the Hes family, this finding led us to assess Hes1 in order to further verify that Notch signaling may indeed be downregulated. Protein analysis of Hes1 revealed that its expression was decreased after infection (not published). This coincides with the

decreased mRNA expression of Hes1 observed here and further contributes to the notion that Notch signaling may be altered. Even more so, Hes1 serves as a transcription factor for the absorptive progenitor and is a crucial marker in intestinal epithelial differentiation into enterocytes (Fre *et al*., 2005, Stanger *et al*., 2005, van Es *et al*., 2005). Since DRA is characteristic of enterocytes, the downregulation of DRA in the EDM model coincided with the decrease in NICD and Hes1 following infection. Furthermore, transcription factors Hepatocyte Nuclear Factor 1α and 1β (HNF1α and HNF1β) have both been found to play a role in the Notch Signaling pathway (D'Angelo *et al*., 2010). D'Angelo *et al.* found that both HNF1α knockout mice as well as HNF1α and HNF1β knockouts resulted in a significant downregulation in Dpp4, specifically a 60% and 80% decrease respectively (D'Angelo *et al*., 2010). Dpp4 is an aminopeptidase that is found in the brush border of enterocytes, which signifies the role of Hnf1 on enterocyte differentiation. With further investigation, it was also found that HNF1α and HNF1β knockout mice downregulate Hes1 and DRA expression as well, further implicating their effect on Notch signaling (D'Angelo *et al*., 2010). With this link, we hypothesized that HNF1 proteins would also be affected during *Salmonella* infection. Following *Salmonella* infection, infected EDMs showed a decrease in HNF1α expression (HNF1β expression was not investigated). These findings as a whole suggest that the Notch signaling pathway may be downregulated upon *Salmonella* infection and could explain, at least in part, why there is a disruption in the absorption of chloride ions that could in turn lead to diarrhea.

The decrease in the absorptive lineage and the Notch signaling pathway shifted the attention towards a potential bias towards the Wnt signaling pathway. Wnt signaling

is responsible for controlling the differentiation of intestinal epithelial cells into the secretory lineage. ATOH1, in particular, is a transcription factor present in this pathway that plays a role in controlling differentiation into goblet, Paneth, and/or enteroendocrine cells (Yang *et al*., 2001). It was found that post *Salmonella* infection, ATOH1 was upregulated, thus suggesting that the Wnt signaling pathway may be activated. It is worth noting that the protein expression of ATOH1 requires repeated testing since tubulin did not show similar expression between the untreated and *Salmonella* infected as the housekeeping gene is expected to do so. Further testing is needed to confirm protein upregulation after *Salmonella* infection, but initial data suggests it corresponds to the increased mRNA expression levels detected. Previous studies have found that Hes1, the transcription factor for the absorptive lineage in Notch signaling, negatively attenuates ATOH1 expression (Zheng *et al*., 2011). Thus, the findings of a decrease in Hes1 and an upregulation in ATOH1 further confirm the crosstalk between the Notch and Wnt signaling pathways. Additionally, since ATOH1 serves as a precursor to the secretory lineage, goblet cells were investigated. Goblet cells are of interest because they play a role in mucus secretion. Mucus polymerization and secretion is imperative against invasive pathogens like *Salmonella* because they form a dense barrier that can protect the surface of the intestinal epithelium (Johansson *et al*., 2008). Specifically, the gel-forming mucin 2 (Muc2) is responsible for forming the structural framework of the intestinal mucus and thus serves a marker to measure goblet cell differentiation. Muc2 expression increased in the presence of *Salmonella*. Ultimately, the increase in differentiation into goblet cells along with an upregulation in ATOH1 and a downregulation of Hes1 may mean *Salmonella* biases towards Wnt signaling.

To further confirm an imbalance between the two signaling pathways, DAPT, a γ-secretase inhibitor, was used. γ-secretase cleaves the Notch receptor when bound to the Notch ligand in order to release NICD; therefore, it plays a vital role in the activation of Notch signaling genes like Hes1 and differentiation into enterocytes. The idea behind inhibiting this enzyme was to see as to whether the downregulation effects of the Notch signaling pathway could be reproduced with direct inhibition of γ-secretase *vs.* *Salmonella* infection. Prior to investigating the effects on Hes1 and DRA, the TEER was measured at various time points during the infection *Salmonella* plus or minus DAPT. It was found that the DAPT did not have any significant effect on barrier function compared to the untreated condition. Furthermore, EDMs treated with *Salmonella* compared to groups treated with both *Salmonella* and DAPT together did not display any significant differences in barrier function. Therefore, this suggested that the decrease in barrier integrity was solely due to *Salmonella* as *Salmonella* has been known to cause epithelial barrier dysfunction. Following infection, the expression of Hes1 was measured and DAPT decreased Hes1 expression similar to the effects found in EDMs treated with *Salmonella*. Even more so, when EDMs were treated with both *Salmonella* and DAPT, Hes1 expression was significantly reduced even more compared to EDMs treated solely with *Salmonella* or DAPT alone. This suggested that *Salmonella* could possibly reducing the Notch signaling pathway, but to further confirm our findings DRA expression was studied. In a similar trend to the Hes1 results, DAPT alone resulted in a decrease in DRA compared to the untreated condition. Treatment of EDMs with both *Salmonella* and DAPT also led to further reduction in DRA expression compared to the other conditions. This coincides with findings that Hes1 is a precursor

to DRA expression, so the downregulation in DRA expression following the decrease in Hes1 expression is not surprising. Ultimately, these findings propose that the decreased expression of DRA following *Salmonella* infection is due to downregulation of both Notch signaling and enterocyte differentiation, likely accounting for diarrheal pathogenesis.

Since *Salmonella* with DAPT caused further downregulation in the Notch signaling pathway, Wnt signaling was also investigated to see whether there would be any additional upregulation effects. ATOH1 was upregulated in EDMs treated solely with DAPT, which confirmed findings that Hes1 negatively regulates ATOH1. ATOH1 expression was similar in EDMs treated with DAPT alone and those with only *Salmonella*. When infected with both *Salmonella* and DAPT, ATOH1 expression was significantly upregulated compared to all the other conditions. It could be that ATOH1 was significantly upregulated due to the fact that *Salmonella* is impairing the Notch signaling pathway and preferentially biasing Wnt signaling. Since ATOH1 is a transcription factor for the secretory progenitor and can lead to goblet cell differentiation, Muc2 expression was investigated to see whether *Salmonella* biases epithelial differentiation into the secretory lineage. Muc2 expression was increased in *Salmonella* infected EDMs as found earlier. It was also increased in a similar way with EDMs treated with DAPT alone. However, Muc2 expression was not significant upregulated when pairing *Salmonella* and DAPT compared to EDMs treated either with *Salmonella* or DAPT alone. This could indicate that there could be epithelial differentiation into the other cell types that are present the intestinal epithelium. Wnt signaling plays a role in differentiation into the three secretory cell types, goblet cells, Paneth cells, and

enteroendocrine cells. Therefore, with the upregulation in ATOH1, Wnt signaling appears to be biased by *Salmonella* as opposed to Notch signaling, but further investigation of Paneth cells and enteroendocrine cells may be necessary.

This theory that there is a disproportion between absorption and secretion through Wnt and Notch signaling also led to the investigation of various secretory transporters. SLC12A2, also known as NKCC1, resides in the basolateral membrane and assists with the uptake of $Na^+$, $K^+$, and $2Cl^-$ ions (Grubb *et al*., 2000, Das *et al*., 2018). It is thought that NKCC1 functions to load chloride into the cytosol of the cell from where it can exit through the apical membrane through various chloride channels like Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (Frizzell and Hanrahan, 2012). More importantly, chloride uptake across the basolateral membrane and apical chloride exit are regulated to maintain the balance of chloride concentration (Matthews *et al*., 1998). Studies in human intestinal epithelial cell lines and human intestinal xenografts have shown an increase in expression of NKCC1 and CFTR after infection with *Salmonella dublin* (Resta-Lenert *et al*., 2002). However, more recent studies in murine models have found that *Salmonella* diarrhea does not increase CFTR expression, but rather redistributes it (Marchelletta *et al*., 2013). Marchelletta *et al*. also found that the NKCC1 compartment was expanded following infection (Marchelletta *et al*., 2013). In the EDM model, challenge of EDMs with *Salmonella* resulted in an increase in NKCC1 expression, which raises questions and warrants further investigation about the interactions NKCC1 may have with other transporters involved in secreting chloride in relation to *Salmonella* induced diarrhea. Additionally, another transporter known as Chloride Channel Accessory 1 (CLCA1) has been quite

controversial in its function in the intestine. It has been hypothesized that it is a calcium dependent chloride channel that helps regulate the chloride conductance across the cell membrane. In the EDM model following *Salmonella* infection, the expression of CLCA1 was upregulated compared to the untreated condition. Increased expression of CLCA1 could contribute to the increased amount of chloride that is being secreted into the diarrhea. The upregulated expression of the secretory transporters NKCC1 and CLCA1 further shed light on the resulting secretory functions of *Salmonella* infection, as previously seen in the effect on the Wnt and Notch signaling pathways.

*Salmonella* has also been known to cause inflammation and neutrophil recruitment to the site of infection in addition to diarrhea. However, it has been found that diarrhea develops prior to, and independent of, the onset of inflammation and neutrophil recruitment (Marchelletta *et al*., 2015). Nonetheless, inflammation is still a key indicator of *Salmonella* pathogenesis and so we examined whether the effects of infection we showed for epithelial differentiation were also required for the inflammatory response, which may exacerbate diarrheal disease at later stages. Previous findings have found that *Salmonella* infection results in inflammation, particularly resulting in increased IL-8 cytokine expression through the NF-κB pathway as an innate immune response (Gewirtz *et al*., 2000). It has been thought that Wnt signaling and the NF-κB pathway may be connected, which could explain the mechanism behind the inflammatory response. Specifically, studies have found that the AvrA effector released by *Salmonella* activates β-catenin, a key player in the Wnt signaling pathway, which in turn inhibits the NF-κB pathway and IL-8 expression (Collier-Hyams *et al*., 2002, Sun *et al*., 2005, Duan *et al*., 2007). Since the Wnt and Notch signaling pathways are

connected in epithelial differentiation, it was thought that there also could be some tie of the Notch signaling pathway in the role of the production of IL-8 during *Salmonella* pathogenesis. In the enteroid model, *Salmonella* alone significantly resulted in the upregulation of IL-8 compared to the untreated condition, similar to findings of Gewrtz *et al*. However, treatment with DAPT did not alter IL-8 expression. The results may indicate that targeting Notch signaling may not have an effect on IL-8 expression. In addition, DAPT downregulates the Notch signaling pathway and upregulates the Wnt signaling pathway through Hes1 and ATOH1 according to our findings. This interaction is downstream of β-catenin, which is needed to bind to the NF-κB pathway for reduction of IL-8 expression. There may not be an interaction with β-catenin when using DAPT; however, additional testing of β-catenin expression following DAPT treatment should be studied. In regard to our previous results indicating that *Salmonella* biases Wnt signaling, this may raise questions as to why an upregulation in IL-8 was still observed in *Salmonella* infected conditions despite previous studies saying Wnt/β-catenin negatively attenuates it. Previous data in our lab has found that β-catenin expression was similar between untreated and *Salmonella* infected EDMs (in preparation). This may mean that there is not enough β-catenin to be able to downregulate the high inflammatory levels of IL-8 present during *Salmonella* infection. Further investigation like stimulating IL-8 expression and amplifying the Wnt signaling pathway in presence of *Salmonella* infection may help elucidate the crosstalk between the inflammatory response observed and the pathogenesis of *Salmonella*.

To combat the effects of diarrhea and inflammation, probiotics have been identified as possible contenders. Probiotics have been found to have positive effects

on the intestine like enhancing the intestinal epithelial function and stimulating mucus secretion against pathogens (Ng *et al*., 2009). Previous studies in our lab have found that there is a beneficial interaction between probiotic strains *Streptococcus thermophilus* and *Lactobacillus acidophilus* and enteroinvasive *Escherichia coli* (EIEC) (Resta-Lenert and Barrett, 2003). Pretreatment of *S. thermophilus* and *L. acidophilus* prevented EIEC induced decrease in TEER and ultimately protected intestinal epithelial cells from EIEC pathogen adhesion and invasion (Resta-Lenert and Barrett, 2003). Moreover, probiotics have also been found to normal epithelial ion transport function (Resta-Lenert and Barrett, 2009). *Lactobacillus acidophilus* has been found to stimulate the expression of DRA (Raheja *et al*., 2010). This could be worth investigating since *Salmonella* induced diarrhea may stem from the downregulation of DRA through Notch signaling and a bias towards Wnt signaling. It would be interesting to see the effects of *Lactobacillus acidophilus* and whether it can also counteract this bias towards the Wnt signaling pathway in the presence of *Salmonella* infection. Initial studies in our lab have looked at the effect of probiotics like *Lactobacillus reuteri* on barrier function. It was found that *L. reuteri* can restore the barrier function when EDMs were infected with *Salmonella*. The restoration of barrier function could be a prelude to other positive cascading effects like a possible rescue of DRA expression against *Salmonella* infection. Decrease in barrier function can worsen diarrhea, meaning the positive effects of *L. reuteri* on barrier function against *Salmonella* contribute to the potential idea of probiotics as potential treatments against diarrheal pathogens. However, this requires further exploration, but ultimately highlights interactions between probiotics and invasive bacteria like *Salmonella*.

In conclusion, *Salmonella* infection in the enteroid model was accompanied by a dysfunction in barrier integrity as well as changes in the Wnt and Notch signaling pathways. Particularly the absorptive lineage regulated by Notch signaling was downregulated while it appeared that *Salmonella* biased towards secretory lineages via Wnt signaling. This was seen in the downregulation in the Notch signaling target genes like Hes1 and the chloride/bicarbonate exchanger DRA. In exchange an upregulation in Wnt signaling target genes like ATOH1 and the goblet cell marker Muc2 was observed. The bias towards secretory lineages was further accompanied by an upregulation of secretory ion transporters NKCC1 and CLCA1 following infection. With this study, it may provide more insight into the mechanism of *Salmonella* diarrheal pathogenesis and how it affects absorptive versus secretory pathways of epithelial differentiation. Our findings suggest that the imbalance of absorption and secretion may account for the decreased capacity for absorption, ultimately leading to the buildup of diarrheal fluid observed in *Salmonella* infection.

**Supplementary Table 1. Primer sequences used for analysis of mRNA expression.**

| | |
|---|---|
| Hes1 Forward: | CAC CGG ACA AAC CAA AGA CG |
| Hes1 Reverse: | CGG AGG TGC TTC ACA GTC AT |
| DRA Forward: | AGG GAA TGC TGA TGC AGT TTG CTG |
| DRA Reverse: | AGT TGA AAT GCT ACA CTT GCC GCC |
| HNF1α Forward: | TGA CCG AGT TGC CTA ATG GC |
| HNF1α Reverse: | ACG ACT TGA CCA TCT TCG CC |
| ATOH1 Forward: | GTT GCG CTC ACT CAC AAA TAA G |
| ATOH1 Reverse: | ACA CAA TAG TCC GTG TTC AGT AA |
| Muc2 Forward: | AAT GAC TTC ACC ACT CGG GAC |
| Muc2 Reverse: | GGG TCT GGG TTG TGG CTT AC |
| NKCC1 Forward: | TCA CAC CTC CAA GGT CAG GA |
| NKCC1 Reverse: | GCT GAG TTG GAG TCT TGC CA |
| CLCA1 Forward: | GTG GAC CAG CCT TTC TAC ATG TCT AG |
| CLCA1 Reverse: | TGT GAC ACA GTT GCC TCT CTC A |
| CXCL1 (IL-8) Forward: | CGC TTC TCT GTG CAG CGC TGC TGC T |
| CXCL1 (IL-8) Reverse: | AAG CCT CGC GAC CAT TCT TGA GTC |
| β-actin Forward: | GAC GGC CAG GTC ATC ACT AT |
| β-actin Reverse: | ACA TCT GCT GGA AGG TGG AC |
| 18S Forward: | GTA ACC CGT TGA ACC CCA TT |
| 18S Reverse: | CCA TCC AAT CGG TAG TAG CG |

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