INVESTIGATING NON-TIGHT JUNCTION FUNCTIONS OF CLAUDIN-7: REGULATING LARGE INTESTINE STEM CELL FUNCTIONS AND NICHE MAINTENANCE, AND JUNCTIONAL PROTEIN NANOARCHITECTURE

BY

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Tight junctions (TJs), the most apical cell-cell junction, seal adjacent epithelial cells and regulate the passage of ions and small molecules through the paracellular pathway. TJs are made up of claudins, occludins, and junctional adhesion molecules. Claudin-7 specifically is highly expressed in the intestine at both the apical and basolateral compartments. Claudin-7 is essential for barrier function and intestinal homeostasis as deletion of claudin-7 in mouse models induces an inflammatory bowel disease (IBD)-like phenotype, exhibiting severe inflammation, intestinal epithelial damage, mucosal ulcerations, weight loss, and bloody-loose stools. Additionally, claudin-7 is involved in many cellular processes including cell proliferation, cell-matrix interactions, and epithelial-to-mesenchymal transition (EMT). We hypothesize that claudin-7 exhibits other roles in addition to its traditional function in barrier function to regulate large intestine stem cell functions, as well as interaction with and alteration of other cell junction proteins as viewed at the nanoscale level.

In this study, we demonstrate the role of TJ protein claudin-7 in the regulation of large intestine stem cell survival, self-renewal, proliferation, and differentiation, vital processes for

intestinal regeneration. Conditional knockout of claudin-7 in the intestines led to altered gene expression profiles, disrupted epithelial cell differentiation, epithelial hyperplasia, downregulated Notch signaling, and loss of the active stem cell pool. Additionally, isolated claudin-7 deficient crypts were unable to survive and form fully closed spheroids in *ex vivo* organoid culture, demonstrating the essential role of claudin-7 in stem cell survival. Stem cell survival was rescued through activation of the Notch signaling cascade, as well as inhibition of Hippo signaling. Delayed knockout of claudin-7 in established organoids also demonstrated disrupted differentiation ability with loss of claudin-7.

Additionally, claudin-7 has been shown to interact with other junctional complexes to regulate barrier function. In this study, we utilized super-resolution Stochastic Optical Reconstruction Microscopy (STORM) to examine the interaction between junctional proteins at the nanoscale level and highlighted the role of claudin-7 in modulating their interactions. Deletion of claudin-7 led to disorganized cell-cell junctions, as well as disorganized nanoarchitecture and decreased interaction of junctional proteins including TJ proteins ZO-1 and claudin-1, adherens junction (AJ) proteins β-catenin and p120^{ctn}, and focal adhesion proteins integrin $\alpha 2$ and focal adhesion kinase. This demonstrated the importance of claudin-7 in maintaining the functional interaction of junctional proteins.

Overall, our study demonstrated additional non-TJ functions of claudin-7 in regulating large intestinal epithelial stem cell functions including survival, self-renewal, and differentiation, as well as modulation of junctional protein localization and nanoarchitecture. Taken together, these results provide novel information that may be used in the study of intestinal regeneration for IBD and colorectal cancer (CRC).

Investigating Non-Tight Junction Functions of Claudin-7: Regulating Large Intestine Stem Cell Functions and Niche Maintenance, and Junctional Protein Nanoarchitecture

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DEDICATION

To my father Dr. Najih Naser,

my fiancé Mustafa Kilic,

my siblings Abdel-Rahman Naser, and Dalya Naser,

my uncle Dr. Saleh Naser, my aunt Dora Naser,

and my cousins Abed Elrahmen Naser, Ebraheem Naser, and Fatima Naser.

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LIST OF SYMBOLS/ABBREVIATIONS

ABC	Avidin-biotin complex
AJs	Adherens junctions
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
cKO	Conditional knockout
cm	Centimeter
CRC	Colorectal cancer
DAB	3, 3-diaminobenzidine
DAPI	4’6-diamidino-2-phenylindole
DCAMKL1	Doublecortin-like kinase 1
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ES	Enrichment score
FABP-1	Fatty acid binding protein 1
FAK	Focal adhesion kinase
FDR	False discovery rate
FISH	Fluorescent in situ hybridization
GJs	Gap junctions

GSEA	Gene set enrichment analysis
gKO	Global knockout
GO	Gene ontology
GO-BP	Gene ontology-biological processes
GO-MF	Gene ontology-molecular functions
Hrs	Hours
IBD	Inflammatory bowel disease
IESCs	Intestinal epithelial stem cells
IPA	Ingenuity pathway analysis
JAMs	Junctional adhesion molecules
KD	Knockdown
KO	Knock out
Lgr5	Leucine-rich repeat-containing G protein coupled receptor 5
MET	Mesenchymal to epithelial transition
MDCK	Madin darby canine kidney cells
MDCKf3	Ras-transformed MDCK cells
MMP	Matrix metalloproteinase
MOM	Mouse on mouse
mRNA	Messenger ribonucleic acid
NES	Normalized enrichment score
OCT	Optimal cutting temperature compound
OLFM4	Olfactomedin 4
OXEA	Oxyrase/β-mercaptoethylamine

PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-tween
PC	Principal component
PC3	Human metastatic prostate carcinoma cells
PFA	Paraformaldehyde
PN3	Postnatal day 3
PZ-HPV-7	Human prostate epithelial cells
$p120^{ctn}$	p120 catenin
qRT-PCR	Quantitative real time polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
S127	Serine 127
SEM	Standard error of the mean
SRM	Super-resolution microscopy
STORM	Stochastic optical reconstruction microscopy
Tcf	Transcription factor
TEM	Tetraspanin-enriched membrane microdomains
TER	Transepithelial electrical resistance
TJs	Tight junctions
TUNEL	Terminal deoxynucleotide transferase dUTP nick end labeling
WB	Western blot
WT	Wild type
YAP	Yes-associated protein

ZO-1	Zonula occludens 1
2D	Two dimensional
3D	Three dimensional
4OH-TAM	4-hydroxytamoxifen

CHAPTER I: INTRODUCTION

This chapter is modified from Naser et al., Non-Tight Junction Functions of Claudin Proteins: Roles in Cell-Matrix Interactions and Stem Cell Regulation. In: Tight Junctions; Springer Nature. 2022 June 05.

A. Large intestine

The large intestine is made up of four parts: the cecum, colon, rectum and anal canal. It is the last portion of the gastrointestinal tract and functions in water reabsorption and feces elimination. Colon and large intestine are often used interchangeably as the colon makes up the majority of the large intestine. This tissue exhibits high cell proliferation as it renews itself every 5-7 days (van der Flier et al. 2009). Unlike the small intestine, the colon only contains crypts, rather than crypts and villi structures. The cells responsible for maintaining this cycle of renewal are the stem cells which reside in the crypt region and will eventually become the mature epithelial cells–colonocytes, goblet cells, enteroendocrine cells, and tuft cells (Sancho et al. 2015). Colonocytes are essential for absorption; goblet cells secrete mucus which protects the epithelium; enteroendocrine cells secrete gastrointestinal hormones; and tuft cells function in immune surveillance and response (Sancho et al. 2015).

In order to protect the intestinal epithelium, this tissue exhibits many defense mechanisms. These include physical barriers, the immune system, and various protective proteins (mucins). Physical barriers such as the tight junctions (TJs) and adherens junctions (AJs) which exist between adjacent epithelial cells, prevent the passage of pathogens into the subepithelial tissue (Fujita et al. 2006; Gowrikumar et al. 2020). These physical barriers are the first line of defense

and are vital for the prevention of gastrointestinal disorders such as inflammatory bowel disease (IBD) and colorectal cancer (CRC).

B. Tight junctions and claudin-7

Cell-cell junctions form connections between adjacent cells and include apical tight junctions (TJs), adherens junctions, desmosomes, and gap junctions, while cell-matrix junctions include focal adhesions and hemidesmosomes (Farquhar and Palade 1963; Claude and Goodenough 1973; Garcia et al. 2017; Shi et al. 2018). TJs form a seal between adjacent cells to regulate the diffusion of solutes and ions through both barrier and fence functions (Fujita et al. 2006; Gowrikumar et al. 2020). The "fence" refers to separation of apical and basolateral compartments; the "barrier" functions to regulate the paracellular pathway (Otani et al. 2019; Li et al. 2019). Strong TJs are characterized by high transepithelial electrical resistance (TER) and low solute permeability (Lu et al., 2013). TJs are made up of three major integral membrane proteins: claudins, occludins, and junctional adhesion molecules (JAMs) and are vital for regulating permeability of epithelial barriers (Van Itallie and Anderson 2013; Shi et al. 2018; Singh et al. 2018).

The claudin family is made up of 24 members in mammals (Ding et al. 2012). Claudins which contain four transmembrane domains, one intracellular domain with N- and C-termini that face the cytoplasm, as well as two extracellular loops (Lu et al. 2013). Claudins form the backbone of the TJ strands and largely determine the epithelial permeability as they function to regulate paracellular penetrability to small molecules and ions (Venugopal et al. 2019; Bhat et al. 2020). Aside from maintaining TJ integrity, claudin proteins have been implicated in many processes such as epithelial to mesenchymal transition (EMT), cell proliferation, signal transduction, cell-

matrix interactions, stem cell functions, and cancer progression (Singh et al. 2010; Sato et al. 2016; Bhat et al. 2020; Xing et al. 2020). Besides traditional localization at the apical TJ region, some claudins also reside in regions outside of the TJs in which they exhibit unique functions regulating various cellular activities (Inai et al. 2007; Ding et al. 2012; Lu et al. 2013). Furthermore, the differential expression of claudins between healthy and cancerous tissues has highlighted claudins as potential prognostic indicators, therapeutic targets and/or cellular markers for cancer therapies (Ikari et al. 2014; Ouban 2018).

Claudin-7 is highly expressed in the intestine and exhibits a unique basolateral localization, in addition to its traditional localization in TJs (Lu et al. 2013). Claudin-7 is essential in maintaining TJ and intestinal epithelial integrity, as loss of claudin-7 leads to leaky tight junctions, decreased TER and increased epithelial permeability (Fan et al. 2019). Structurally, claudin-7 deletion has been shown to cause intracellular gaps between adjacent cells and loosening between the cell and the underlying matrix (Ding et al. 2012). Claudin-7 knockout mice exhibit an IBD-like phenotype experiencing severe intestinal inflammation, epithelial cell sloughing, mucosal ulcerations, hyperplasia, dehydration, salt wasting, increased cytokine levels, up-regulate matrix metalloproteinases (MMPs) among other symptoms (Ding et al. 2012; Li et al. 2018). They typically die within ten days due to severe dehydration and lack of nutrition, highlighting the critical role of claudin-7 in maintaining TJ integrity (Li et al. 2018).

C. Claudin-7 roles outside of the TJ

While claudins are largely localized to the apical TJ structure, claudin proteins often exhibit altered localization in various tissues and interact with cell-adhesion proteins such as integrin molecules and epithelial cell adhesion molecule (EpCAM), among many others (Rahner et al.

2001; Inai et al. 2007; Lu et al. 2013; Van Itallie and Anderson 2013). Multiple claudins, including claudin-1, -2, and -7, have been associated with extracellular matrix regulation through integrins in the focal adhesion structure (Hagen 2017). Singh et al., reviewed this partnering of claudin proteins with integrins in the regulation of "outside-in" and "inside-out" signaling, highlighting the roles of claudin proteins in cell signaling through integrins (Singh et al. 2017).

Although several claudins interact with other cell-cell adhesion proteins, claudin-7 in particular, localizes to the basolateral membrane in intestinal tissues where it colocalizes with integrin molecules and is involved in cell-matrix adhesion (Ding et al. 2012). Our previous study highlighted claudin-7 interaction and colocalization with integrin α2 at the epithelial basolateral compartment of the small intestine (Ding et al. 2012). Though the apical TJs remained largely intact, loss of claudin-7 led to intercellular gaps and cell-matrix loosening in global claudin-7 knockout mice (gKO) as seen by electron microscopy (Figure 1.1), as well as altered integrin α2 distribution and localization where integrin α2 formed clusters and/or moved towards the apical lateral surface (Ding et al. 2012). In addition to changing cell-matrix protein localization, claudin-7 knockout also disrupted the formation of the claudin-7/integrin α2/claudin-1 complex which normally forms in the intestines (Ding et al. 2012).

In the intestines, integrins play important roles in regulating cell proliferation and intestinal inflammation (Jones et al. 2006; Artis et al. 2000). For example, conditional knockout of integrin β1 in the intestines led to the increased epithelial cell proliferation with dysplasia, reduced Hedgehog expression, and mislocalization of Tcf-4, a claudin-7 expression regulator and a transcription factor required for intestinal epithelial stem cell proliferation (Jones et al. 2006; Darido et al., 2008).

Claudin proteins regulate many cellular functions including cell migration, invasion, and proliferation via interactions with EpCAM in the basolateral compartment (Heiler et al. 2015; Singh et al. 2018). Formation of an EpCAM-tetraspanin-claudin-7 complex alters homotypic cell-cell adhesion and supports resistance to apoptosis (Kuhn et al. 2007; Le Naour and Zoller 2008). Screening of primary colorectal cancer tissue and liver metastasis confirmed co-expression and co-immunoprecipitation of EpCAM and claudin-7, as well as colocalization in the basolateral compartment (Ladwein et al. 2005; Kuhn et al. 2007). Additionally, EpCAM and claudin-7 co-expression with the tetraspanin CO-029 and CD44 variant isoform v6 (CD44v6) inversely correlated with disease-free survival and induced complex formation and recruitment into tetraspanin-enriched membrane microdomains (TEM) (Kuhn et al. 2007). Results of this study suggest that claudin-7 is required for EpCAM recruitment into TEMs as the absence of claudin-7 resulted in decreased association of EpCAM with CO-029 and CD44v6 and lack of EpCAM recruitment into TEMs. (Kuhn et al. 2007; Nubel et al. 2009). Formation of the EpCAM/claudin-7 complex was found to support proliferation and promote tumor progression and metastasis by blocking EpCAM oligomerization which is vital for EpCAM-mediated cell-cell adhesion (Nubel et al. 2009). Furthermore, EpCAM-claudin-7 expressing cells exhibit enhanced motility due to claudin-7 association with actin bundles which further promotes tumorigenicity and accelerates tumor growth (Nubel et al. 2009).

Claudins have been strongly implicated in epithelial to mesenchymal transition (EMT), the process by which cells lose their epithelial properties and gain mesenchymal characteristics, therefore enhancing their motility and invasive potential (Venugoapl et al. 2019). A myriad of studies have highlighted altered claudin expression in various epithelial-derived cancers in a stage-, tumor-, and tissue-specific manner (Dhawan et al. 2010; Kwon 2013; Tabaries and Siegel 2017;

Ouban 2018; Wang et al. 2018). The loss of several claudin proteins has been linked to decreased intercellular adhesion and enhanced tumor cell metastasis (Singh et al. 2010; Sato et al. 206; Osanai et al. 2017; Wang et al. 2018). Wang et al. reported that the expression of claudin-7 in colorectal cancer (CRC) is downregulated as differentiation grade decreases. The low expression level of claudin-7 corresponds to the downregulation of E-cadherin and upregulation of vimentin and snail-1, which promotes the invasion and metastasis of CRC through the regulation of EMT (Wang et al. 2019). Claudin-7 knockdown (KD) in colon cancer cells, HT-29 and DLD-1, induced EMT and colony formation *in vitro*, as well as increased xenograft-tumor growth *in vivo* (Bhat et al. 2016). Moreover, forced expression of claudin-7 in highly metastatic SW620 colon cancer cells induced mesenchymal to epithelial transition (MET) in which cell growth in soft agar and tumor growth *in vivo* were both inhibited (Bhat et al. 2016).

Our previous studies demonstrate a tumor suppressive function for claudin-7 due to its role in cell-matrix adhesion and proliferation (Lu et al. 2011; Lu et al. 2015). Claudin-7 colocalizes with and forms a stable complex with integrin β1. The siRNA silencing of claudin-7 in human lung adenocarcinoma HCC827 cells reduced integrin β1 expression and diminished cell-matrix adhesion (Lu et al. 2015; Kim et al. 2019; Li et al. 2019). Claudin-7 KD in these lung cancer cells affected both cell-matrix interactions and cell growth as these cells exhibited impaired cell-matrix adhesion and grew on top of each other forming spheroids (Figure 1.2), as well as resulted in significantly larger tumor formation when inoculated into nude mice, compared to control cells (Lu et al. 2015). Injection of claudin-7 expressing human lung carcinoma NCI-H1299 cells into nude mice resulted in significantly reduced tumor size compared to cells without claudin-7, further suggesting a tumor suppressive role for claudin-7 through the ERK/MAPK signaling pathway (Lu et al. 2011). Claudin-7 KD in human colorectal carcinoma HCT116 cells increased the migration

ability and enhanced the tumor growth (Li et al. 2019). To elucidate if these proteins regulate cell proliferation and invasion independently or synergistically, further investigation into claudin-7 and integrin β1 interactions suggests that claudin-7 regulates cell motility through integrin β1 (Kim et al. 2019). Overexpression of claudin-7 in HCC827 claudin-7 KD cells resulted in reduced cell proliferation, increased expression of focal adhesion proteins and improved ability to attach to cell culture plates, while ectopic expression of integrin β1 in HCC827 claudin-7 KD cells exhibited increased migration and adhesion but not the cell proliferation (Kim et al. 2019). Taken together, these data identified a new function of TJ protein claudin-7 in maintaining epithelial cell-matrix attachment and cell motility via integrin β1 (Ding et al. 2012; Lu et al. 2015; Kim et al. 2019; Li et al. 2019).

D. Intestinal epithelial stem cells (IESCs)

The large intestine functions largely in reabsorbing water, absorbing vitamins and feces elimination. These functions rely on a heterogenous group of specialized epithelial cells including absorptive colonocytes, mucus-secreting goblet cells, immune-sensing tuft cells and hormone-secreting enteroendocrine cells. As the large intestine rapidly self-renews, production of these epithelial cells is regulated by the intestinal epithelial stem cells (IESCs). IESCs are multipotent adult stem cells that reside in the crypt region of the intestines (van der Flier et al. 2009). Crypt-residing intestinal stem cells are categorized into three groups: active, quiescent, and transit amplifying. Active intestinal stem cells have three fates: 1) to self-renew and become another stem cell, or 2) to differentiate and become an epithelial cell type, or 3) to die (Figure 1.3). Quiescent stem cells are not currently dividing and represent the reserve pool of stem cells in the case of injury. The balance between active stem cells and quiescent stem cells is a hallmark of a functional

intestinal niche, as excessive stem cell production and differentiation may result in cancers such as colorectal cancer (Umar, 2010). Transit-amplifying cells are the progenitor cells which give rise to the various epithelial cell types: absorptive enterocytes/colonocytes, hormone-secreting enteroendocrine cells, mucus-secreting goblet cells, and immune-sensing tuft cells (Nefzger et al. 2016; Xing et al. 2020). Loss of these cell types disrupts intestinal homeostasis, impairs epithelial repair, and is implicated in intestinal disorders including inflammatory bowel disease, colorectal cancer, and necrotizing enterocolitis (Bankaitis et al. 2018; Xing et al. 2018). While many studies have highlighted the role of claudin-7 in cancer progression and metastasis, little is known about the mechanisms by which claudin-7 regulates stem cell function in the intestines, which may lead to these pathologic conditions.

E. Notch and Hippo signaling cascades regulate IESC function

Maintenance of the IESC niche is closely regulated by the interplay of several signaling pathways, including the Notch and Hippo cascades. The Notch pathway plays a role in IESC lineage fate, and regulates proliferation, differentiation, and apoptosis while the Hippo signaling pathway is vital for maintaining intestinal homeostasis and regeneration through the regulation of stem cell self-renewal, proliferation, and differentiation (Hageman et al. 2020; Deng et al. 2022). Cell fate and lineage determination is largely determined by the Notch cascade (Jensen et al. 2000; Miyamoto and Rosenberg et al. 2011). The Notch pathway is activated when its ligands (Jagged-1 and Jagged-2) bind to a Notch receptor between two adjacent cells (Kopan et al. 2009; Tyagi et al. 2020). Once activated, Notch is cleaved and the Notch intracellular domain (NICD) is released through a cascade of proteolytic cleavages by tumor necrosis factor-α-converting enzyme (TACE) and γ-secretase (Kopan et al. 2009; Tyagi et al. 2020). The NICD then translocates to the nucleus

and binds transcriptional factors including Hes-1 and mastermind-like-1 (MAML-1) to activate downstream pathways (Kopan et al. 2009; Tyagi et al. 2020). Downregulation of Hes-1 led to increased differentiation of the secretory lineage cell types highlighting Notch regulation of IESC lineage fate determination (Jensen et al. 2000; Miyamoto and Rosenberg et al. 2011). Additionally, deletion of Notch receptor 1 and Notch receptor 2 was associated with impaired proliferation and disruption of the epithelial layer (Okamoto et al 2009; Carulli et al. 2015). Other studies have implicated aberrant activation of the Notch pathway in the pathogenesis of CRC (Zagouras et al. 1995; Reedijk et al. 2008; Tyagi et al. 2020). Overall, Notch plays a key regulatory role in maintaining the stem cell niche, along with the Hippo cascade.

Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) act as transcriptional coactivators in the Hippo cascade to regulate expansion of the crypt stem cell and progenitor populations (Hong et al. 2016; Mo et al. 2014; Imajo et al. 2015; Deng et al. 2022). When the Hippo pathway is activated, activated LATS1/2 kinases phosphorylate YAP/TAZ inducing YAP/TAZ cytoplasmic accumulation and degradation by the ubiquitin ligase β-TrCP, therefore no transcription will occur (Figure 1.4). When Hippo signaling is inactive, inactivated LATS1/2 kinases are unable to phosphorylate YAP/TAZ, allowing YAP/TAZ to translocate to the nucleus and bind with TEAD family transcription factors to coregulate transcription of Hippo target genes (Figure 1.4). In the intestine, YAP plays a critical role in maintaining IESC pluripotency and is predominantly located in Lgr5 positive active stem cells and transit-amplifying cells (Deng et al. 2022). Increased YAP activity leads to expansion of the IESC and progenitor cell population, contributing to intestinal regeneration (Deng et al. 2022). Loss of YAP in dextran sodium sulfate (DSS) colitis models led to significantly impaired intestinal regeneration (Cai et al. 2010). Additionally, YAP overexpression enhanced organoid formation,

while YAP inhibition impaired organoid formation and decreased the number of IESCs (Gregorieff et al. 2015; Yui et al. 2018; Serra et al. 2019). These pathways work to maintain intestinal homeostasis and a functional stem cell niche.

F. Regulation of small intestinal stem cell functions by TJ protein claudin-7

Besides the barrier function, TJs have been found to interact with many associated proteins to modulate cell differentiation and proliferation by regulating signaling pathways, transcription factor expression and gene expression (Gonzalez-Mariscal et al. 2008). When intestinal epithelial differentiation is induced in intestinal enteroids, claudin proteins distribute heterogeneously among the various cell types (Pearce et al. 2018). Claudin-1 is the most highly expressed in goblet cells and Paneth cells in organoids, while claudin-7 is enriched in the differentiated cell types such as enterocytes, goblet cells, and Paneth cells (Pearce et al. 2018). Claudin-2 is detected in both crypt and villus cells of the small intestine but is restricted to the undifferentiated crypt cells in the colon (Rahner et al. 2001). These studies suggest that, in addition to their traditional roles in regulating epithelial barrier function and polarity, claudins also regulate specific cell functions that contribute to cell proliferation and differentiation.

Our previous study demonstrated that claudin-7 is essential in maintaining intestinal epithelial stem cell (IESC) functions and intestinal epithelial self-renewal (Xing et al. 2020). The essential role of claudin-7 was revealed in both global *Cldn7* knockout mice (gKO) and inducible, intestinal epithelial-specific conditional *Cldn7* knockout mice (cKO) as both mice were unable survive for more than two weeks (Ding et al. 2012; Li et al. 2018; Xing et al. 2020). Importantly, deletion of claudin-7 reduced the number of IESCs and disrupted epithelial differentiation and proliferation in both gKO and cKO mice. Loss of claudin-7 led to significantly decreased OLFM4

positive IESCs, as seen in both gKO and cKO mouse intestinal crypt region and claudin-7-deficient enteroids (Figure 1.4) (Xing et al. 2020). Isolated crypts from gKO mice were unable to form budding enteroids and enteroid survival was greatly reduced with claudin-7 deletion, suggesting a critical role for claudin-7 in supporting IESC survival (Xing et al. 2020). More importantly, the Wnt/β-catenin signaling pathway - an essential signaling pathway for IESC survival and self-renewal - was suppressed in claudin-7-deficient intestines (Xing et al. 2020). These exciting findings revealed that claudin-7 is vital in the process of replenishing daily epithelial cell loss at the tips of villi by sustaining the stable pools of IESCs for epithelial self-renewal, and by promoting differentiation into mature epithelial cells (Xing et al. 2020).

Given the critical role of claudin-7 in maintaining IESC functions, it is essential to understand the regulation of claudin-7 in the epithelial self-renewal. HNF-4α directly interacts with the *Cldn7* promoter, which in turn up-regulates the claudin-7 protein during IESC differentiation (Farkas et al. 2015). Additionally, the transcriptional factor *Hopx* was found to stimulate claudin-7 expression in the mouse colonic epithelium (Lili et al. 2016). Tcf-4 also maintained the low expression level of claudin-7 at the bottom of colonic crypts via Sox-9 to suppress the tumor cell polarization (Darido et al. 2008). The receptor and downstream targets of Wnt/β-catenin signaling pathway, *Olfm4 and Lgr5,* were suppressed in claudin-7-deficient small intestines (Xing et al. 2020). Interestingly, they are both active stem cell markers in the mouse small intestine. The Wnt/β-catenin signaling pathway is essential for IESC survival and its defect will lead to the depletion of IESCs (Fevr et al. 2007). It has been reported that claudin-7 overexpression enhances β-catenin/Tcf activity and promotes tumor formation in xenograft mice (Bhat et al. 2015). These studies demonstrate that claudin-7 interacts with the Wnt/β-catenin signaling pathway in controlling intestinal epithelial cell differentiation and proliferation (Xing et

al. 2020). Moreover, EpCAM, the epithelial cell adhesion protein that directly binds to and stabilizes claudin-7, is required for stem cell survival and proliferation (Ladwein 2005; Barth et al. 2018). Deletion of EpCAM resulted in significantly decreased protein expression of claudin-7 and epithelial integrity was severely compromised (Ladwein et al. 2005; Barth et al. 2018). Additionally, enteroid survival and growth were attenuated, suggesting that IESC function was compromised (Ouchi et al. 2021).

G. Summary and Rationale for this study

Claudin-7 has been implicated in many pathologic conditions including inflammatory bowel disease (IBD) and colorectal cancer (CRC) (Darido et al. 2008; Oshima et al. 2008; Bhat et al. 2015; Wang et al. 2019). IBD, which encompasses both Ulcerative colitis (UC) and Crohn's disease (CD) has multifactorial etiology—autoimmune effects, genetic factors, environmental effects, microbiome factors—with no cure currently (Chassaing et al. 2014). Treatments for IBD simply alleviate the symptoms, leaving patients to cope with these chronic conditions. As of 2015, an estimated 3.1 million adults in the United States were diagnosed with IBD, leading to over 1.8 million ambulatory care visits each year (Crohn's and Colitis Foundation, 2019). This led to a total annual financial burden of $14.6-31.6 billion in the US alone (Crohn's and Colitis Foundation, 2019). Additionally, 18% of patients suffering from IBD develop other health complications such as colorectal cancer, which is the second leading cause of cancer death in the United States (Wang et al. 2018; Crohn's and Colitis Foundation, 2019; Siegel et al. 2020). The colon is affected by UC, CD, and CRC making it the main tissue of interest to work towards a cure. Understanding the role of claudin-7 in the large intestine stem cell niche can provide new information for therapeutic targets for these debilitating diseases.

H. SPECIFIC AIMS:

Aim 1. Determine if claudin-7 regulates large intestine stem cell survival, differentiation, and proliferation.

Hypothesis: Stem cells isolated from claudin-7 knockout crypts will lose their ability to self-renew and differentiate, resulting in decreased number of epithelial cell types, loss of the active stem cell pool, and increased proliferation, as shown by in vitro and in vivo models.

Aim 2. Establish signaling pathways claudin-7 acts through to modulate large intestine stem cell activity and methods to rescue aberrant pathways.

Hypothesis: Claudin-7 may regulate stem cell functions in the large intestine through Wnt/β-catenin independent signaling pathways such as the Notch signaling pathway and/or the Hippo pathway.

Aim 3: Examine changes in nanoarchitecture of cell junction proteins with loss of claudin-7.

Hypothesis: Loss of claudin-7 will result in localization and nanoarchitectural changes of other TJ proteins, AJ proteins, and focal adhesion proteins.

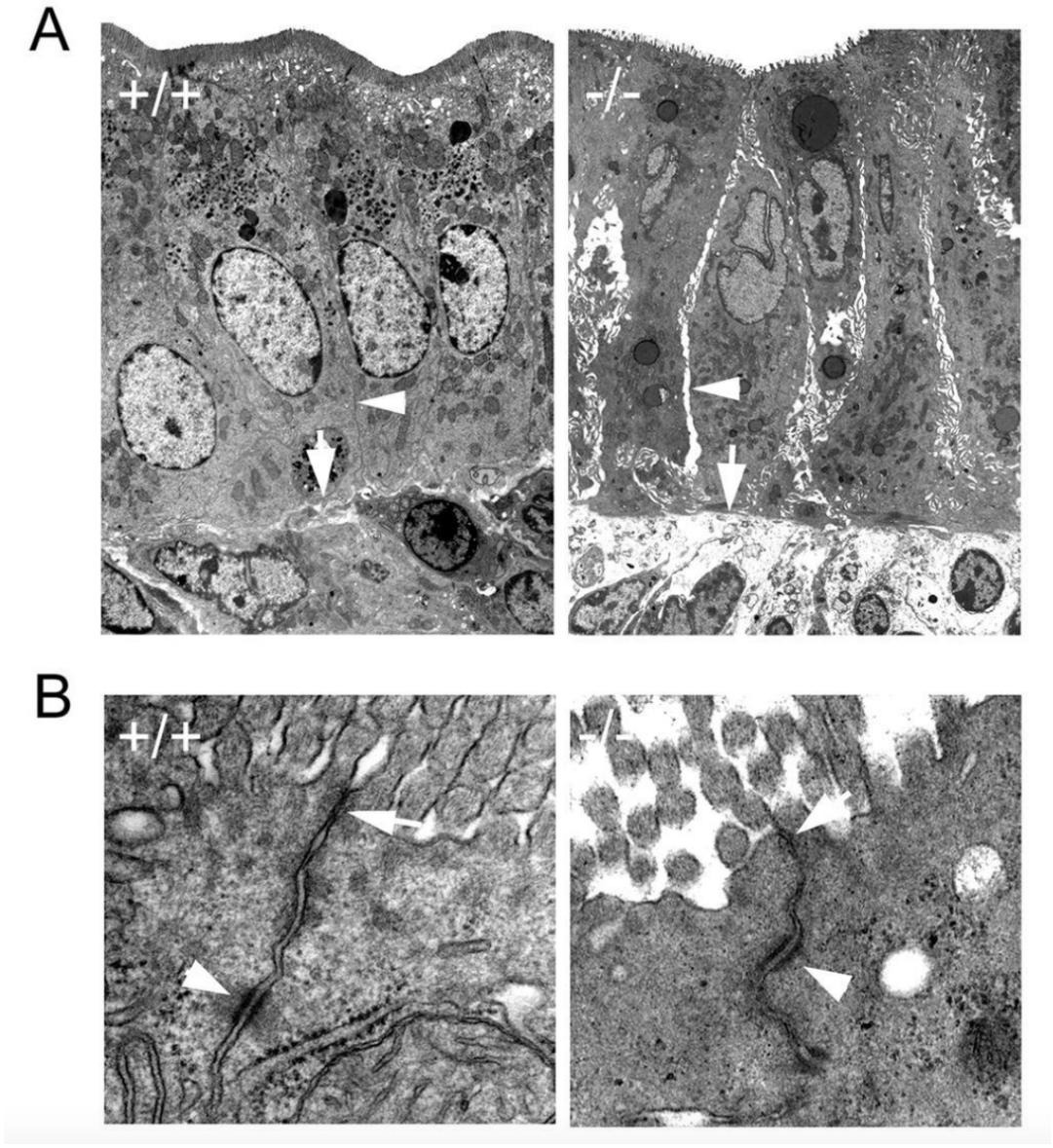

A
+/+
-/-
B
+/+
-/-

Figure 1.1 Disruption of cell adhesion in claudin-7 gKO intestines. Electron micrographs show postnatal day 5 wildtype (WT, +/+) and gKO (-/-) small intestines. (**A)** The arrowhead in (-/-) pointed to the intercellular gap along the gKO lateral membrane compared to that of WT (+/+, arrowhead). The arrow in (-/-) revealed the loosening of cell-matrix connection in gKO versus the close contact between cell and matrix in WT intestines (+/+, arrow). (**B)** The arrows in (+/+) and (−/−) pointed to the apical TJ. The arrowheads indicated the desmosome. Magnifications: **A**, ×5,000; **B**, ×50,000. Reprinted from *Gastroenterology*, 2012, 142(2):305-315**,** with permission from Elseiver.

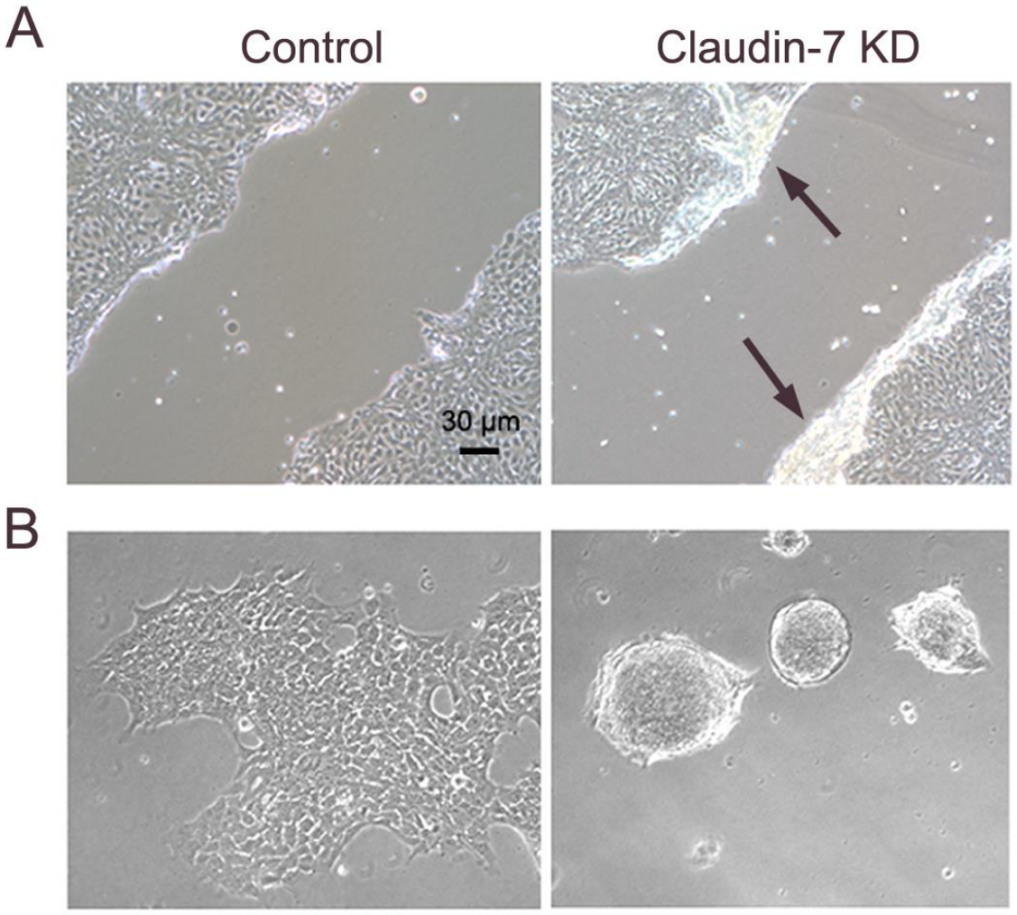

A
Control
Claudin-7 KD
30 μm
B

Figure 1.2 Reduced cell-matrix adhesion in claudin-7 KD cells. (A) Scratches were made on the confluent HCC827 control and claudin-7 KD cell monolayer. Claudin-7 KD cells were easily peeled off along the scratch as shown in arrows, while the control cells were well attached to the plate. (**B)** When cultured on uncoated glass coverslips, HCC827 claudin-7 KD cells formed spheroids while the control cells were able to spread out and formed a monolayer. Reprinted from *Molecular Cancer*, 2015, 14:120, 1-15**,** published as open access by BioMed Central.

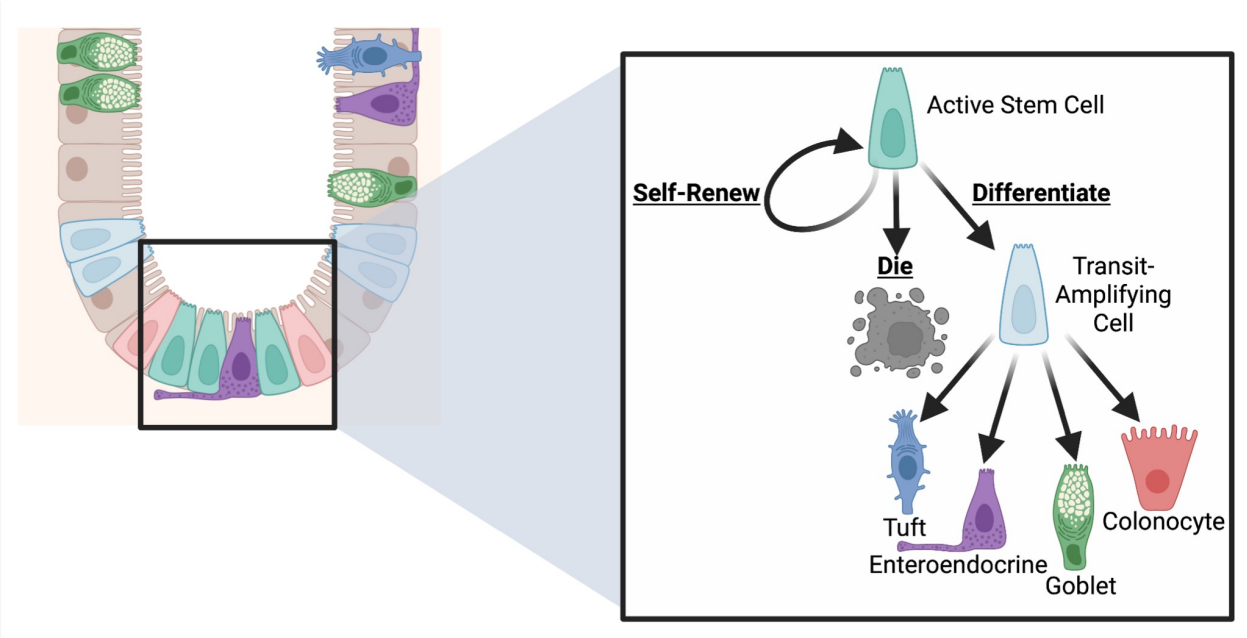

Active Stem Cell
Self-Renew
Differentiate
Die
Transit-
Amplifying
Cell
Tuft
Enteroendocrine
Goblet
Colonocyte

Figure 1.3 Colon intestinal epithelial stem cell (IESC) fate schematic. IESCs are subcategorized into active, quiescent, and transit-amplifying cells. Active stem cells may self-renew or differentiate to become transit-amplifying cells, then eventually further differentiate into the various epithelial cell types.

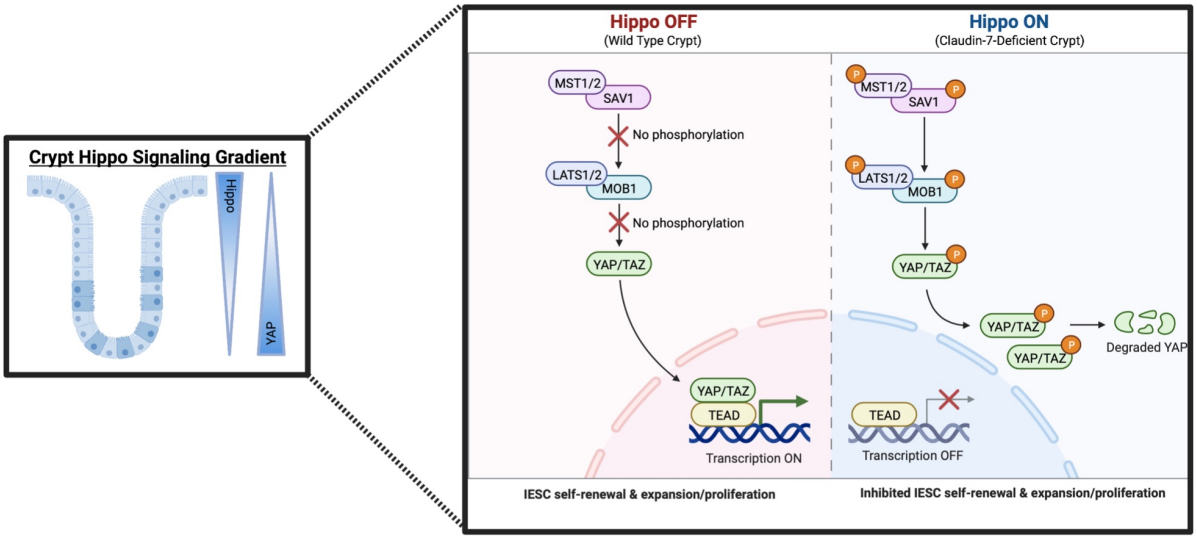

Crypt Hippo Signaling Gradient
Hippo
YAP
Hippo OFF
(Wild Type Crypt)
Hippo ON
(Claudin-7-Deficient Crypt)
MST1/2
SAV1
No phosphorylation
LATS1/2
MOB1
No phosphorylation
YAP/TAZ
YAP/TAZ
TEAD
Transcription ON
TEAD
Transcription OFF
YAP/TAZ
YAP/TAZ
Degraded YAP
IESC self-renewal & expansion/proliferation
Inhibited IESC self-renewal & expansion/proliferation

Figure 1.4 Hippo signaling cascade. In the intestine, the Hippo pathway exhibits a gradient, with increased signaling at the top of the crypts, and decreased towards the bottom. In the normal wild-type crypt, Hippo signaling is off in which YAP translocates to the nucleus and induces transcription to promote IESC self-renewal and growth. Contrastingly, when Hippo is turned on, YAP is phosphorylated and degraded, inhibiting transcription and IESC growth.

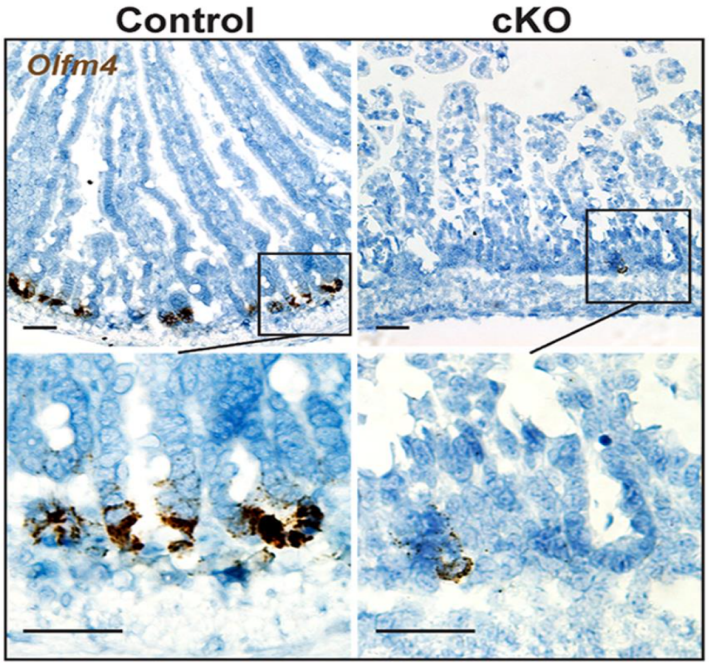

Control
cKO
Olfm4

Figure 1.5 Loss of active crypt stem cells in claudin-7 cKO small intestines. Active intestinal epithelial stem cells (IESCs) were labeled with crypt stem cell marker *Olfm4* mRNA using fluorescence in situ hybridization (FISH) in 3-month-old WT (+/+) and cCldn7$^{fl/fl\text{-}T}$ (-/-) small intestines. Scale bar: 100. Reprinted from *Cellular and Molecular Gastroenterology and Hepatology*, 2020, 9(4):641-659, published as open access by Elsevier on behalf of the AGA Institute.

CHAPTER II: THREE-DIMENSIONAL CULTURE OF MURINE COLONIC CRYPTS TO STUDY INTESTINAL STEM CELL FUNCTION *EX VIVO*

This chapter is modified from Naser et al., Three-Dimensional Culture of Murine Colonic Crypts to Study Intestinal Stem Cell Function Ex Vivo, Journal *of Visualized Experiments; (188):e64534. 2022 October 11. doi:10.3791/64534*

A. Summary

Regeneration of the intestinal epithelium is controlled by the self-renewing and differentiating capabilities of active stem cells located at the bottom of the crypt region. Organoid culture is an important and attractive approach to study signaling molecules and environmental cues that regulate stem cell survival and functions. Here, we present a protocol for the isolation of colonic crypts, embedding of these isolated crypt cells into a three-dimensional Matrigel system, and culturing crypt cells to form colonic organoids capable of self-organization, proliferation, self-renewal, and differentiation. This model allows us to manipulate the environment—knocking out specific proteins such as claudin-7, activating/deactivating signaling pathways, etc.—to study how these effects influence the functioning of the colonic stem cells. Previously, we have reported that claudin-7 is required for intestinal epithelial stem cell functions in the small intestine (Xing et al. 2020). In this protocol, we have established a colonic organoid culture system in order to study the role of claudin-7 in the large intestine. In addition, organoid culture is also a useful system to screen small molecules or potential drugs for the treatment of human intestinal disorders.

B. Introduction

Intestinal organoid culture is a three-dimensional (3D) *ex vivo* system in which stem cells are isolated from the intestinal crypts of primary tissue and plated into a gel matrix (Hughes et al. 2010; Sato et al. 2011). These stem cells are capable of self-renewal, self-organization, and organ functionality (Sato et al. 2011). Organoids mimic the tissue microenvironment and are more similar to *in vivo* models than two-dimensional (2D) *in vitro* cell culture models, although less manipulatable than cells (Wallach and Bayrer, 2017; Shankaran et al. 2021). This model eliminates obstacles encountered in 2D models such as lack of proper cell-cell adhesions and cell-matrix interactions, and homogenous populations, as well as reduces the limitations of animal models including high cost and time (Angus et al. 2020). Intestinal organoids—also referred to as colonoids for those grown from colon-crypt-derived stem cells—are essentially mini-organs which contain an epithelium including all cell types that would be present *in vivo*, as well as a lumen. This model allows manipulation of the system to study many different aspects of the intestine such as the stem cell niche, intestinal physiology, pathophysiology, and gut morphogenesis (Wallach and Bayrer, 2017; Fan et al. 2019; Angus et al. 2020). It also provides a great model for drug discovery, the study of human intestinal disorders such as inflammatory bowel disease (IBD) and colorectal cancer, patient-specific personalized treatment development as well as studying tissue regeneration (Gupta et al. 2016; Yoo and Donowitz, 2019; Shankaran et al. 2021; Qu et al. 2021).. In addition, the organoid system can also be used to study cellular communication, drug metabolism, viability, proliferation, and response to stimuli (Gupta et al. 2016; Yoo and Donowitz, 2019). While animal models may be used to test potential therapeutics for intestinal pathologic conditions, they are quite limited, as studying multiple drugs at once poses a challenge. There are more confounding variables *in vivo*, and associated cost and time are high and long, respectively. On the other hand, the organoid culture system allows for screening of many therapeutics at once

in a shorter time period and allows for personalized treatment through the use of patient-derived organoid culture (Yoo and Donowitz, 2019; Shankaran et al. 2021). The ability of colonic organoids to mimic tissue organization, microenvironment and functionality also makes them an excellent model to study regeneration and tissue repair (Qu et al 2021). Our lab has established a small intestine organoid culture system to study the effect of claudin-7 on small intestine stem cell functions (Xing et al. 2020). In this study, we established a large intestinal organoid culture system to study the stem cell's ability, or lack of ability, to self-renew, differentiate, and proliferate in a conditional claudin-7 knockout (cKO) model.

Claudin-7 is a very important tight junction (TJ) protein that is highly expressed in the intestine and is essential for maintaining TJ function and integrity (Ding et al. 2012). Claudin-7 KO mice suffer from an IBD-like phenotype exhibiting severe inflammation, ulcerations, epithelial cell sloughing, adenomas, and increased cytokine levels (Ding et al. 2012; Lu et al. 2013). While it is widely accepted that claudins are vital for epithelial barrier function, new roles for claudins are emerging. Claudins have been shown to be involved in proliferation, migration, cancer progression and stem cell function (Xing et al. 2020; Ding et al. 2013; Lu et al. 2013; Bhat et al. 2015; Lu et al. 2015; Wang et al. 2018; Wang et al. 2019). It is currently unknown how claudin-7 impacts the stem cell niche and function of colonic stem cells. As the intestine rapidly self-renews approximately every 5-7 days, maintenance of the stem cell niche and proper functioning of the active stem cells is vital (Wang et al. 2013). Here we establish a system to examine the potential regulatory effects of claudin-7 on the colonic stem cell niche.

C. Materials and Methods

C.1 Reagent/equipment preparation

The following reagents/equipment should be cooled prior to the start of their associated experiments:

- Phosphate buffered saline (PBS) should be cold for washing colon tissue during crypt isolation.
- Rocker/rotator should be placed in a 4 °C refrigerator to be used for incubation with epithelial dissociation media.
- Gel matrix should be removed from -20 °C and thawed on ice to be ready for plating.
- Centrifuge should be pre-cooled to 4 °C prior to spinning down crypts for plating.
- 0.1% Sodium Citrate Buffer should be cooled and kept on ice prior to *in situ* cell death detection.

The following reagents/equipment should be warmed prior to the start of their associated experiments:

- 96-well culture plate must be warmed for 24 hours prior to plating.
- L-WRN media should be warmed before adding to plated crypts and before each media change.
- Water bath should be heated to 94 °C prior to beginning staining.

C.2 Murine colonic crypt isolation and plating

All animal experiments and procedures were approved by the East Carolina University (ECU) Animal Care and Use Committee (IACUC) and conducted in compliance with guidelines from the National Institutes of Health and ECU on laboratory animal care and use. Inducible,

intestinal-specific claudin-7 knockout mice were generated by crossing C57BL6 Claudin-7-flox transgenic mice with Villin-CreERT2 mice (Li et al. 2018). Large intestines were isolated from two three-month-old Claudin-7Fl/Fl-T mice and thoroughly cleaned by removing attached adipose tissue and feces, then cut open longitudinally and washed 10-15 times with cold phosphate buffered saline (PBS). Tissue was then cut into 3-5 mm pieces and incubated for 90 mins at 4°C with shaking in 5mM EDTA and 10 µM Y-27632. Following incubation, tissue pieces were washed 10-15 times with cold PBS, crypts were freed by continuous handshaking for 10 minutes in 10 µM Y-27632 and filtered through a 70 µm nylon cell strainer. The crypt containing media was then centrifuged at 200 x g for 10 mins, the supernatant was discarded, and the pellet was resuspended in 3-4 mL of cold PBS. The concentration of crypts was approximated and the appropriate volume of crypts to plate 10 crypts per µL of Matrigel were centrifuged at 200 x g for 5 mins.

Following pelleting of crypts via centrifugation, the supernatant was removed and Matrigel was added to pelleted crypts, then thoroughly mixed. After allowing partial solidification, 10 µL of Matrigel + crypts was added to the center of each well of a pre-warmed 96-well culture plate. The plate was placed in the incubator at 37°C with 5% CO2 for 10-20 mins to allow Matrigel to fully solidify. Crypts were incubated with L-WRN media from Harvard Medical School Gastrointestinal Organoid Derivation and Culture Core supplemented with 1 × penicillin/streptomycin, 10 mmol/L HEPES, 1 × GlutaMAX, 1 × B27, 1 × N2; (all from Life Technologies), and 50 ng/mL murine recombinant epidermal growth factor (Peprotech; catalog number 315-08). Crypts are then incubated at 37 °C with 5% CO2 and media is changed every 2-3 days.

C.3 Claudin-7 knockout in culture

To induce claudin-7 knockout in culture, crypts are left to grow normally in culture for 24 hours. After 24 hours, crypts are treated with 3 μM 4-hydroxytamoxifen (4OH-TAM) (Sigma; catalog number 94873) and cultured for 10 additional days. Culture medium containing fresh 4OH-TAM was changed every 2 days. DMSO was used as a vehicle in control wells. Images of organoids were taken using a Zeiss Axio Imager M2 microscope (Carl Zeiss, Thornwood, NY), equipped with Metamorph Imaging software.

C.4 Harvesting and embedding of colonic organoids

After 11 days of culture, organoids are fixed with 4% paraformaldehyde (PFA) for one hour at room temperature then incubated with 30% sucrose for 24 hours at 4°C. Cryomolds were filled to 90% capacity with optimum cutting temperature (OCT) compound then 30% sucrose was removed from wells via vacuum suction. To remove organoids from the culture plate, 10 μL PBS was added to each well, and the bottom of the well was gently scratched with a pipette tip. The PBS containing dissociated organoids was then carefully loaded into the cryomold containing OCT. This process was continued until organoids have been removed from all wells. Organoids were then flash frozen in a stainless-steel Dewar flask with dry ice and 2-methylbutane and stored at -80°C until ready to section.

C.5 Immunofluorescence

Slides containing 5 μm thick organoid sections were boiled at 94°C in 10mM Sodium Citrate Buffer for 10 mins. Slides were assembled with 0.2% Triton X-100 in Sequenza rack, incubated with 100mM Glycine for 15 minutes, and blocked for 45 minutes with 5% bovine serum

albumin. Primary antibody was incubated overnight at 4°C. Slides were rinsed with PBS, secondary antibody was added for 1 hour in the dark, rinsed with PBS, and mounted with FluoroGel + DAPI. Images were taken using a Nikon ECLIPSE Ni-E microscope (Carl Zeiss, Thornwood, NY), equipped with NIS-Elements software.

C.6 Cell death detection

Organoids are stained utilizing an *in situ* cell death detection kit, TMR Red (Roche, catalog number 12156792910) according to manufacturer instructions. Briefly, organoids were fixed inside the well with 4% PFA for 1 hour at room temperature, rinsed with PBS, and incubated with cold 0.1% Sodium Citrate Buffer for 2 minutes. Organoids are then rinsed with PBS, incubated with TUNEL reaction for 1 hour at 37°C, rinsed again with PBS, stained with Hoechst (1:2500) and rinsed with PBS before visualizing. Images were taken utilizing the Evos FLoid Imaging System (ThermoFisher Scientific, Waltham, MA).

D. Results

D.1 Claudin-7 is required for spheroid formation

In order to examine the regulatory effects of claudin-7 on colon stem cells, colonic crypts were isolated from murine colon tissue as described above and shown in Figure 2.1A. Once crypts were isolated from the primary tissue, they were plated in a 3D matrix in a 96-well plate to grow for 9 days (Figure 2.1). Normal healthy crypts will close the lumen and become spheroids by day 2 and eventually begin budding and forming the various epithelial cell types at approximately day 5 (Figure 2.1B). Colonoids were allowed to grow until day 11, where they were then harvested for

further experiments (Figure 2.1B). Claudin-7 deficient crypts (claudin-7 KO) failed to form fully closed spheroids and began rapidly dying after one day of 4OH-TAM treatment (Figure 2.1B).

Figure 2.2A highlights the successful growth of colonoids from normal crypts containing claudin-7 (control), as they progress throughout the 9 days of culture. These crypts began to form spheroids by day 2, started budding at day 5, and continued growing and budding until they were harvested at day 9 (Figure 2.2A). In contrast, crypts lacking claudin-7 (claudin-7 KO) deteriorated very quickly (Figure 2.2B). Approximately 2-3 days after treatment with 4OH-TAM, claudin-7 KO crypts had not formed healthy-looking spheroids and appeared only as circular clumps of cells (Figure 2.2B). Crypts isolated from wild type mice were treated with 4OH-TAM to confirm there is no toxic effect due to tamoxifen treatment. These crypts were able to survive and grow normally.

D.2 Claudin-7 deficient crypts rapidly undergo apoptosis

To examine claudin-7 deletion and the survival condition of claudin-7 KO colonoids, we utilized immunostaining method and an *in situ* cell death detection kit (Figure 2.3). Immunofluorescent staining for claudin-7 in harvested control and cKO organoids confirmed successful knockout of claudin-7 in culture (Figure 2.3A). Day 9 control colonoids exhibit very little apoptotic signal (Figure 2.3B). However, claudin-7 KO colonoids display high apoptosis (Figure 2.3B). Without claudin-7, the stem cells were unable to survive, self-renew, or differentiate to form colonoids.

E. Discussion

Organoid culture is an excellent model to study stem cell function, intestinal physiology, drug discovery, modeling human intestinal diseases, as well as studying tissue regeneration and

repair (Xing et al. 2020; Gupta et al. 2016; Almeqdadi et al. 2019; Yoo and Donowitz, 2019; Qu et al. 2020). While it has many advantages, it can be challenging to establish. Care must be taken in all steps throughout the protocol, but most importantly during the plating stage. When mixing the isolated crypts with gel matrix, be sure to thoroughly pipette up and down to break up the crypt pellet that is formed following centrifugation, and to distribute the crypts evenly throughout the matrix. Concurrently, take care to avoid introducing air bubbles into the matrix while pipetting. In order to do this, pipetting should be done slowly and with the pipette tip towards the bottom of the 1.5 mL tube. Additionally, do not allow the gel matrix to fully solidify throughout this process. To prevent premature solidification, mix by carefully pipetting, then place the tube on ice, and repeat this process. Once the isolated crypts and gel matrix are sufficiently mixed, allow the gel matrix to partially solidify. This process may take 1-5 minutes depending on the type/brand of gel matrix used. It should resemble a gel that will move slightly if you tip the tube but should not be too runny that it would spill out if inverted. At this point, you may begin plating 10 µL into the center of each well. The gel matrix should form a 3D dome and should not touch the sides of the well. If the gel matrix spreads and hits the wall of the well, it is not solidified enough; wait until it is sufficiently solidified to form a dome, as the crypts will not survive and grow if the dome is not formed. Once plating is completed properly and crypts are sufficiently supplemented as explained above, the organoids should grow without issue.

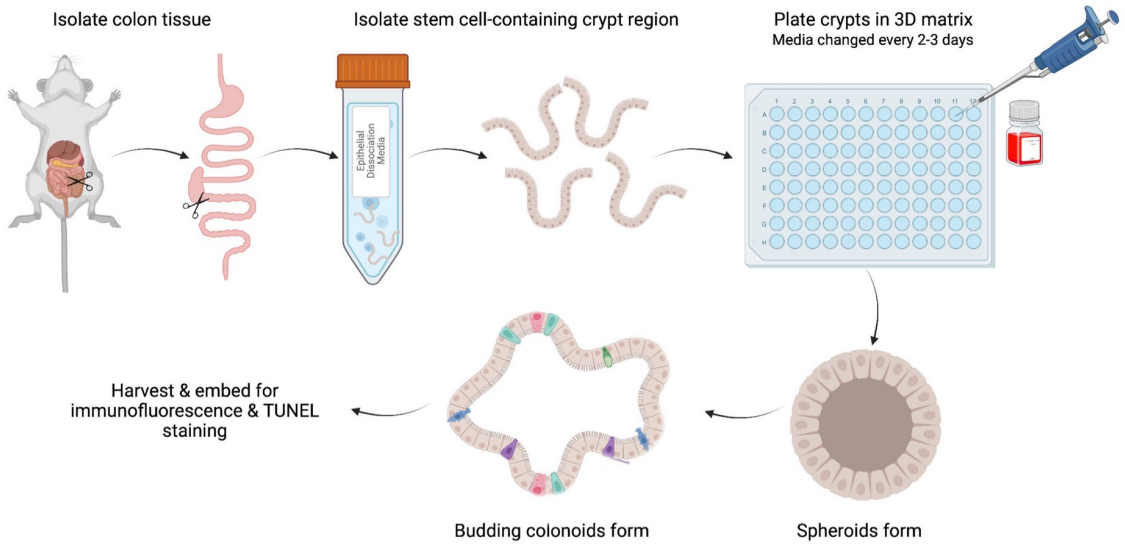
In this protocol, we established a colon organoid system with or without claudin-7 to observe its effects on colonic stem cell survival. While colon organoid culture is an innovative and advantageous system, there are still limitations to the model. Depending on the type of study, lack of immune cells and the microbiota in intestinal organoids may be an advantage or disadvantage (Almeqdadi et al. 2019). For our studies, it is advantageous to investigate claudin-7's regulatory

role on stem cell functions without the immune component. In our case, we can pinpoint that a certain effect is specifically due to claudin-7, rather than other potential variables such as the immune response that would be present in *in vivo* animal models. Conversely, this factor may be a limitation for other types of studies. Establishing colon organoid culture may also be more costly and time intensive than traditional 2D cell lines. However, they can mimic the cellular microenvironment of tissues providing *in vivo* relevance and are much more representative of tissue than 2D cell culture, and still less costly than animal models (Gupta et al. 2016; Shankaran et al. 2021). Given the vast application and enormous potential of intestinal organoid culture, this system is likely to become the ideal model in laboratory research across the world.

F. Acknowledgements

This study was funded by NIH DK10-3166.

A


Isolate colon tissue
Isolate stem cell-containing crypt region
Plate crypts in 3D matrix
Media changed every 2-3 days
Epithelial Differentiation Media
Harvest & embed for immunofluorescence & TUNEL staining
Budding colonoids form
Spheroids form

B

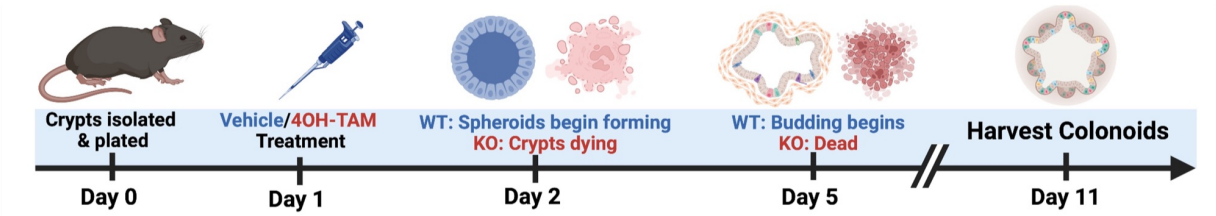

Crypts isolated & plated
Day 0
Vehicle/4OH-TAM Treatment
Day 1
WT: Spheroids begin forming
KO: Crypts dying
Day 2
WT: Budding begins
KO: Dead
Day 5
Harvest Colonoids
Day 11

Figure 2.1 Schematic diagram showing crypt isolation and colonoid growth. **(A)** Graphical depiction of the crypt isolation process, plating in 3D matrix, and growth until harvest. **(B)** Timeline of experiments and colonoid growth in control and claudin-7 KO derived crypts.

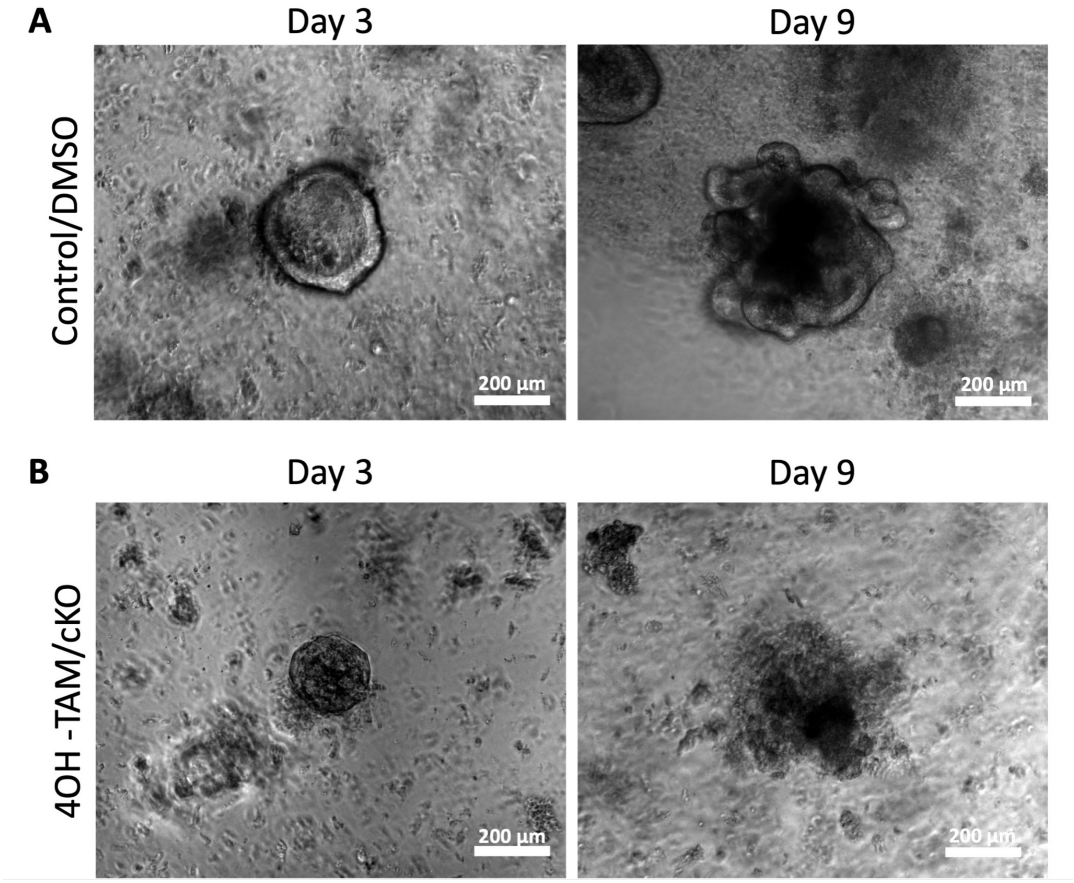

A
Day 3
Day 9
Control/DMSO
200 µm
200 µm
B
Day 3
Day 9
4OH -TAM/cKO
200 µm
200 µm

Figure 2.2 Claudin-7 deficient colonoids are unable to survive and grow. **(A)** Representative images of control/DMSO organoids at day 3 and day 9. **(B)** Representative images of 4OH-TAM/cKO organoids at day 3 and day 9. Scale bars = 200 μm. n = 10.

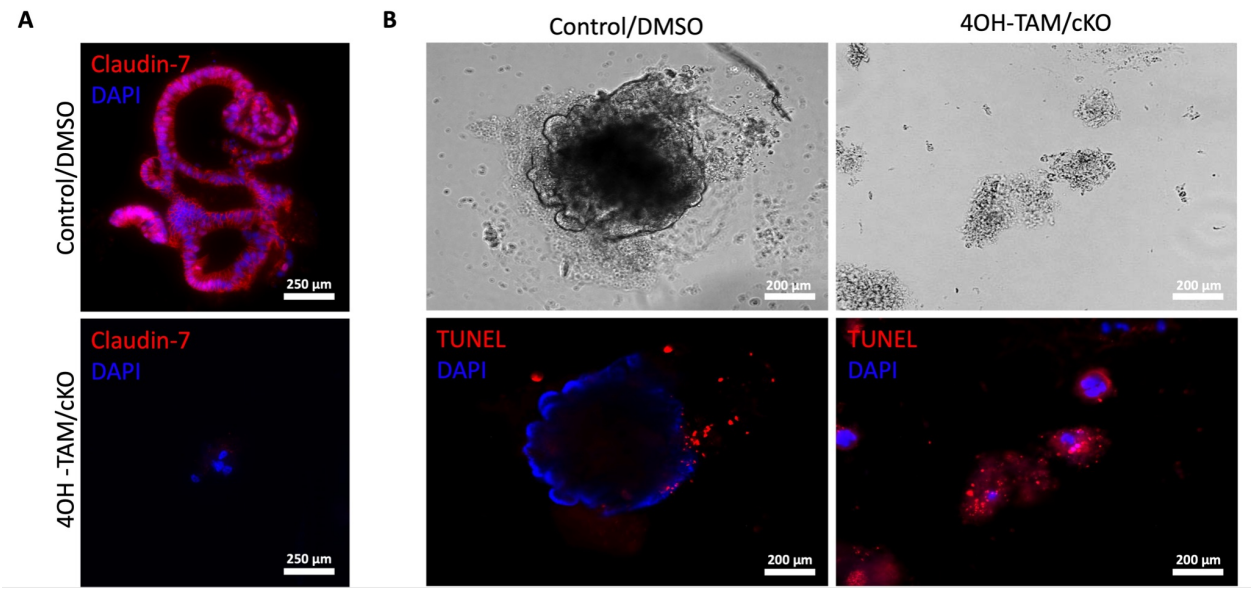

A
B
Control/DMSO
4OH-TAM/cKO
Control/DMSO
4OH -TAM/cKO
Claudin-7
DAPI
250 µm
Claudin-7
DAPI
250 µm
200 µm
200 µm
TUNEL
DAPI
200 µm
TUNEL
DAPI
200 µm

Figure 2.3 Claudin-7 deficient colonoids rapidly undergo apoptosis. **(A)** Claudin-7 staining in day 9 control/DMSO and claudin-7 cKO/4OH-TAM organoids. Scale bars = 250 μm. **(B)** Apoptotic staining in day 9 control and claudin-7 cKO/4OH-TAM colonoids. Scale bars = 200 μm. n = 3.

CHAPTER III: A CRITICAL ROLE OF TIGHT JUNCTION PROTEIN CLAUDIN-7 IN CONTROLLING COLONIC EPITHELIAL STEM CELL SURVIVAL, PROLIFERATION, AND DIFFERENTIATION THROUGH NOTCH AND HIPPO SIGNALING PATHWAYS

A. Summary

Tight junction protein claudin-7 has been implicated in many pathologic conditions of the colon including inflammatory bowel disease, colorectal cancer, and necrotizing enterocolitis. Dysregulation of the stem cell niche and epithelial differentiation is a common driver of these diseases. Here we examine the role of claudin-7 in regulating the colonic epithelial cell differentiation, as well as function and maintenance of the stem cell niche and progenitors. Claudin-7 modulates stem cell survival, proliferation, and differentiation via the Notch and Hippo signaling pathways as demonstrated using an intestinal specific claudin-7 knockout mouse model, and colon organoid culture.

B. Introduction

Colorectal cancer (CRC) is the second most common cause of cancer mortality in the United States with approximately 53,000 individuals dying from CRC annually (Siegel et al. 2020). One of the main causes for the high mortality rate associated with CRC is late detection in which metastasis to secondary tissues has already occurred (Simon et al. 2016; Tepus et al. 2020). Tight junction (TJ) proteins have been shown to be involved in invasion and metastasis, as disruption of the TJs allows malignant tumor cells to detach from the underlying basement

membrane in the primary tissue and travel to secondary tissues–a process known as epithelial to mesenchymal transition (EMT) (Singh et al. 2010; Bhat et al. 2015; Wang et al. 2018).

TJs are made up of three families of proteins: claudins, occludins, and junctional adhesion molecules (JAMs) (Zihni et al. 2016; Suzuki et al. 2020). Claudins are the most crucial component of TJs as they form the backbone of TJ strands and function in regulating ion and small molecule flux between epithelial cells (Ding et al. 2012; Günzel et al. 2012; Wang et al. 2018; Otani et al. 2019; Tsukita et al. 2019; Venugopal et al. 2019). Dysregulation of claudins has been associated with many pathologic conditions including inflammatory bowel disease (IBD), celiac disease, irritable bowel syndrome, enteropathy, colorectal cancer, kidney stone disease, etc… (Günzel et al. 2012; Barmeyer et al. 2015; Curry et al. 2020; González-Mariscal et al. 2020; Das et al. 2021). González-Mariscal et al. discussed the relationship between junctional proteins and cell transformation, migration, and metastasis to highlight their potential use in therapeutic intervention for cancer (González-Mariscal et al. 2020).

Claudin-7 is highly expressed in the intestines and exhibits a unique basolateral localization in these tissues (Ding et al. 2012). Our group previously demonstrated the role of claudin-7 in metastasis and invasion in multiple tissues through the regulation of cell-matrix interactions (Ding et al. 2012; Lu et al. 2015). Deletion of claudin-7 in the intestines led to severe intestinal defects, intercellular gaps, and cell-matrix loosening (Ding et al. 2012). Additionally, knockdown of claudin-7 in HCC827 human lung cancer cells induced epithelial cell detachment, cell-matrix defects, as well as increased tumor size (Lu et al. 2015). Claudin-7 has been shown to be involved in cancer cell metastasis and invasion in CRC as downregulation of claudin-7 promotes EMT and tumor cell growth (Bhat et al. 2015; Philip et al. 2015; Wang et al. 2019). CRC prognosis and recurrence are also correlated with claudin-7 expression levels; expression of claudin-7 is

significantly reduced in cancerous tissues, with an inverse correlation between expression level and disease grade (Singh et al. 2010; Bhat et al. 2015; Quan et al. 2020; Gowrikumar et al. 2021). Individuals with lower claudin-7 expression have a poorer prognosis and shorter disease-free survival (Bhat et al. 2015; Quan et al. 2020; Gowrikumar et al. 2021).

Conversely, upregulation of claudin-7 plays a protective role as increased expression of claudin-7 induces mesenchymal to epithelial transformation (MET) to inhibit colon tumorigenesis (Bhat et al. 2015). Increased claudin-7 also increases chemosensitivity and interacts with integrin signaling molecules to suppress cancer cell proliferation and detachment (Hoggard et al. 2013; Lu et al. 2015). Taken together, claudin-7 may act as a tumor suppressor and is an essential prognostic biomarker in CRC.

CRC progression is marked by increased proliferation of cancerous stem cells progressing from an adenomatous polyp to adenocarcinoma (Aarons et al. 2014; Simon et al. 2016). Claudin-7 knockout mice exhibit intestinal hyperplasia suggesting a role for claudin-7 in controlling proliferation within the intestine (Ding et al. 2012; Li et al. 2018; Xing et al. 2020). Our group previously identified a novel role for claudin-7 in regulating the intestinal epithelial stem cell (IESC) survival, self-renewal, and differentiation in the small intestine through the Wnt/β-catenin signaling pathway (Xing et al. 2020). As the large intestine is the site of many pathologic conditions including CRC and IBD, and pathogenesis involves cancerous stem cells, we sought to investigate the role of claudin-7 on the colonic stem cell functions.

Colonic IESCs located in the bottom of the crypt proliferate and become transit amplifying cells which migrate up the crypt to differentiate into the various epithelial cell types (van der Flier et al. 2009; Zhang et al. 2013). Stem cells are subcategorized into active and quiescent stem cells (Zhang et al. 2013). Active stem cells may self-renew or become transit-amplifying cells to

differentiate into the epithelial cell types, while quiescent stem cells represent the reserve stem cells in the case of injury (Zhang et al. 2013). This differentiation process has been shown to be regulated by Wnt/β-catenin, Notch, and Hippo signaling cascades to determine cell fate (Radtke et al. 2005; Barry et al. 2013; Hansen et al. 2015; Taniguchi et al. 2015; Demitrack and Samuelson, 2016; Xing et al. 2020; Xu et al. 2021). The balance between these IESC types is a hallmark of a functional niche and dysregulation may result in hyperproliferative crypts leading to CRC (Radtke et al. 2005; van der Flier et al. 2009; Zhang et al. 2013; Xing et al. 2020). While Wnt-secreting Paneth cells in the small intestine activate Wnt/β-catenin as the predominant signaling cascade in the small intestine, the colon houses Reg4+ deep crypt secreting cells that activate the Notch signaling pathway in colonic IESCs (Sasaki et al. 2016). Notch signaling plays a critical role in the balance between proliferation, differentiation, and apoptosis, acting as a "gatekeeper" of the stem cell niche (Miyamoto and Rosenberg et al. 2011; Hageman et al. 2020). Cell fate is largely determined by this signaling cascade; depletion of the Notch signaling target gene Hes-1 led to increased secretory lineage demonstrating Notch signaling regulation of IESC lineage fate determination (Jensen et al. 2000; Miyamoto and Rosenberg et al. 2011). Additionally, inhibition of the Notch cascade, and deletion of Notch 1 and Notch 2 receptors was associated with disruption of the epithelial layer and impaired proliferation (Okamoto et al 2009; Carulli et al. 2015). Overall, Notch signaling is vital for regulating proliferation, IESC differentiation, and maintaining the stem cell niche (Miyamoto and Rosenberg et al. 2011). Other studies have demonstrated that aberrant activation of the Notch signaling cascade is involved in the pathogenesis of CRC as upregulation of both Notch1 and Hes1 has been demonstrated in adenocarcinomas (Zagouras et al. 1995; Reedijk et al. 2008; Tyagi et al. 2020).

Hippo (YAP/TAZ) signaling also plays an important role in intestinal homeostasis and regeneration (Hong et al. 2016; Hageman et al. 2020). YAP1 and TAZ have both been demonstrated to maintain and promote stem cell properties, as well as support IESC and progenitor cell proliferation (Hong et al. 2016; Mo et al. 2014; Imajo et al. 2015). Conditional knockout of YAP–the main transcriptional activator of the Hippo cascade–led to increased epithelial apoptosis, as well as impaired survival and proliferation (Cai et al. 2010). *In vitro* studies utilizing organoid culture highlighted the role of YAP in regulating IESC function and survival as YAP overexpression enhanced organoid formation, while YAP inhibition led to reduced organoid formation and decreased IESCs (Gregorieff et al. 2015; Yui et al. 2018; Serra et al. 2019). Hippo pathway dysregulation is also associated with CRC (Wang et al. 2013; Hong et al. 2016; Zheng et al. 2019). Conditional knockout of *Mst1* and *Mst2*, genes encoding Hippo pathway components, led to dysplastic colonic epithelium, as well as spontaneous adenomas (Zhou et al. 2011). The delicate balance of these signaling cascades is vital for maintaining intestinal homeostasis and maintaining the stem cell niche. The relationship between claudin-7 and these signaling cascades has not yet been explored to examine large intestine stem cell functions. While the Wnt/β-catenin cascade is associated with IESC regulation in the small intestine, other pathways are hypothesized to be involved in the large intestine due to differences between these two tissues (Xing et al. 2020). For example, the large intestine lacks Paneth cells which support the stem cell niche in the small intestine by secreting Wnt. Additionally, there are functional and histological differences between the small and large intestine as the small intestine functions primarily for nutrient absorption and contains crypts and villi, while the large intestine functions in water reabsorption and feces elimination, containing only crypts (van der Flier et al. 2009; Sancho et al. 2015). As the large intestine is also the site of many pathologic conditions including CRC, we sought to uncover the

role of claudin-7 in large intestine stem cell function. Understanding the interactions between Notch signaling, Hippo signaling, and claudin-7 expression may provide new therapeutic targets for debilitating large intestinal disorders.

In this study, conditional knockout of claudin-7 *in vivo* led to severe intestinal inflammation, diarrhea, decreased colon length, reduced IESC differentiation, downregulation of Notch signaling, hyperproliferation, and loss of the Lgr5 positive active stem cell pool. Loss of claudin-7 *ex vivo* was associated with reduced organoid survival and impaired IESC differentiation; these functions were rescued through activation of the Notch signaling cascade and inhibition of the Hippo signaling pathway. We report a new role for claudin-7 in regulating the colonic IESC population, stem cell function, and colonic epithelial proliferation through the Notch and Hippo signaling cascades.

C. Materials and Methods

C.1 Claudin-7 Knockout Mice

All animal experiments and procedures were approved by the East Carolina University Animal Care and Use Committee (IACUC) in compliance with guidelines from the National Institutes of Health and East Carolina University on laboratory animal care and use. Global claudin-7 knockout (gKO) mice were generated as previously described (Ding et al. 2012). Postnatal day 3 (PN3) WT and gKO were used in experiments. Inducible, intestinal specific claudin-7 knockout (cKO) mice were generated as previously described (Ding et al. 2018). Briefly, inducible cCldn7 fl/fl-T mice were generated by crossing Villin-CreERT2 mice with Cldn7-flox mice. Three-month-old cKO mice were induced by injecting 2 mg Tamoxifen (Sigma-Aldrich;

catalog number T5648) dissolved in sunflower oil (Sigma-Aldrich; catalog number S5007) 4 times, every other day. Large intestinal tissue was collected and verified for successful knockout of claudin-7 before being used for further experiments.

C.2 Immunofluorescence

Fresh frozen cross-section large intestine was fixed with 4% paraformaldehyde (PFA) for 10 minutes before rinsing with PBS. Antigen retrieval was completed by boiling slides at 94°C in 10mM Sodium Citrate Buffer for 10 mins. Slides were assembled with 0.2% Triton X-100 in Sequenza rack, incubated with 100mM Glycine for 15 minutes, and blocked for 45 minutes with 5% bovine serum albumin. Primary antibody was incubated overnight at 4°C. Slides were rinsed with PBS, secondary antibody was added for 1 hour in the dark, rinsed with PBS, and mounted with FluoroGel + DAPI. Antibodies used are listed in Table 1. Images were taken using a Nikon ECLIPSE Ni-E microscope (Carl Zeiss, Thornwood, NY), equipped with NIS-Elements software.

C.3 qRT-PCR

Total RNA was isolated from control and claudin-7 KO colon tissue using RNeasy mini kit (Qiagen; catalog number 74104) and concentration was measured via spectrophotometry. High-capacity cDNA reverse transcription kits (Applied Biosystems; catalog number 4374966) was used according to manufacturer instructions for cDNA synthesis. Real time qRT-PCR reactions were performed utilizing RT2 Profiler PCR Array System (Qiagen; catalog number PAMM-143Z). Primers used are listed in Table 2. Samples with a GAPDH value of <30 Ct were considered acceptable for analysis. The relative gene expression changes were analyzed using a $2^{-\Delta\Delta Ct}$ method (Ding et al. 2012).

C.4 Western Blot

Large intestinal tissue was minced and sonicated in Pierce™ RIPA Buffer (Thermo Scientific; catalog number 89900) with cOmplete Mini (Roche Diagnostics; catalog number 11836153001). Samples were incubated in RIPA buffer for 20 minutes and centrifuged at 16000 RPM at 4°C twice for 10 minutes each, collecting the supernatant following each spin. BCA assay was run to calculate protein concentration and samples were prepared with 10X sample buffer. Prepared samples were boiled at 90°C for 5 minutes, centrifuged at 16000 RPM for 5 minutes then loaded into gel (30 mg/well). Gel was run at 150V for 1 hour then transferred to membrane at 0.25Å for 3 hours. Following transfer, the membrane was blocked with 0.1% PBS-T/5% non-fat dry milk for 1 hour, washed with 0.1% PBS-T and incubated in primary antibody overnight at 4°C with shaking. The membrane was then washed with 0.1% PBS-T and incubated with secondary antibody for 1 hour at room temperature with shaking. Following secondary antibody incubation, the membrane was washed with 0.1% PBS-T, carefully dried and visualized on the LI-COR Odyssey FC using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific; catalog number 34577).

C.5 Alcian blue staining

Fresh frozen cross-section large intestine was fixed in formalin for 5 minutes before rinsing with PBS. Slides were placed in 3% acetic acid for 3 minutes, stained with alcian blue for 30 minutes, washed in running water for 10 minutes, rinsed with distilled water for 1 minute, counterstained in filtered nuclear fast red for 5 minutes, and washed in running water for 1 minute.

Slides were dehydrated and mounted with Permount. Images were taken using a Nikon ECLIPSE Ni-E microscope (Carl Zeiss, Thornwood, NY), equipped with NIS-Elements software.

C.6 Immunohistochemistry

Fresh frozen cross-section large intestine was fixed in formalin for 5 minutes before rinsing with PBS. Vectastain ® Elite ® ABC Kit, Peroxidase Rabbit IgG (Vector Laboratories; catalog number PK-6101) was followed according to manufacturer instructions. Briefly, endogenous enzyme activity was blocked with 0.3% hydrogen peroxide in water. Tissue was rinsed with PBS and blocked with 5% normal serum for 10 minutes. Slides were incubated with primary antibody for 1 hour, washed with PBS, incubated with biotinylated secondary antibody for 10 minutes then washed with PBS again, and incubated with ABC reagent. Impact DAB kit (Vector Laboratories; catalog number SK-4105) was prepared according to manufacturer instructions and added to slides for 5 minutes to develop. Slides were rinsed with deionized water, dehydrated, and mounted with Permount. Images were taken using a Nikon ECLIPSE Ni-E microscope (Carl Zeiss, Thornwood, NY), equipped with NIS-Elements software.

C.7 Fluorescent in situ hybridization (FISH)

Fresh frozen large intestine was sectioned at 5 um using RNAse-free techniques. Stellaris® RNA FISH protocol for frozen tissue (Biosearch Technologies) was followed. Briefly, slides were fixed in fixation buffer for 10 minutes, washed with nuclease-free PBS, and immersed in 70% ethanol for 1.5 hours. Slides were then immersed in Wash Buffer A (Biosearch Technologies; catalog number SMF-WA1) for 30 minutes. Stellaris® FISH Mouse Lgr5 probe with CAL Fluor Red 590 Dye (Biosearch Technologies; catalog number SS679971-01-48) was mixed to a

concentration of 1.25 uM in Hybridization Buffer (Biosearch Technologies; catalog number SMF-HB1). This probe containing solution was incubated at 37°C for 18 hours in a humidified chamber. All subsequent steps are completed in a dark, humidified environment at 37°C. Slides were then washed with Wash Buffer A for 1 hour, incubated with 5 ng/mL DAPI (Thermo Scientific; catalog number 62247) for 30 minutes, washed with Wash Buffer B (Biosearch Technologies; catalog number SMF-WB1) for 15 minutes and mounted with Vectashield® PLUS Antifade Mounting Medium (Vector Laboratories; catalog number H-1900). Images were taken using a Nikon ECLIPSE Ni-E microscope (Carl Zeiss, Thornwood, NY), equipped with NIS-Elements software.

C.8 Intestinal Crypt Isolation and In Vitro Organoid Culture

Large intestines were isolated from 3-month-old claudin-7 flox mice, cut open longitudinally, and chopped into 3-5mm pieces. Following 90-minute incubation at 4°C in 5 mM EDTA and 10 μM Y-27632, tissue pieces were washed 10-15x in PBS, and crypts were freed by continuous handshaking for ten minutes in 10 μM Y-27632. The isolated crypt solution was filtered through a 70- μm cell strainer, pelted at 200 × g for 10 minutes, and resuspended in PBS. The isolated crypts were mixed with reduced growth factor basement membrane matrix type 2 (R&D Systems; catalog number 3533-001-02) and seeded at 10 crypts/μL matrix in 96-well plates. The organoid culture medium was overlaid at a volume of 10:1 to matrix in the plates (L-WRN media from Harvard Medical School Gastrointestinal Organoid Derivation and Culture Core supplemented with 1 × penicillin/streptomycin, 10 mmol/L HEPES, 1 × GlutaMAX, 1 × B27, 1 × N2; all from Life Technologies), and 50 ng/mL murine recombinant epidermal growth factor (Peprotech; catalog number 315-08). Media was changed every 2-3 days.

Cldn7 gene deletion was induced in organoid culture as previously described (Xing et al. 2020). Briefly, crypts isolated from cCldn7$^{Fl/Fl\text{-}T}$ mice without tamoxifen injection were cultured for 24 hours, followed by the addition of 3 μmol/L 4-hydroxytamoxifen (4OH-TAM) (Sigma; catalog number 94873) and cultured for 10 additional days. Culture medium containing fresh 4OH-TAM was changed every 2 days. Images of organoids were taken using a Zeiss Axio Imager M2 microscope (Carl Zeiss, Thornwood, NY), equipped with Metamorph Imaging software.

Rescue: After crypts have been treated with 4OH-TAM for 24 hours, organoids were treated with 150 μmol/L Jagged-1 (Eurogentee; catalog number 188-204) or 400 nmol/L XMU-MP-1 (Selleckchem; catalog number S8334), or a combination of both, to rescue stem cell survival. Culture medium containing fresh Jagged-1, XMU-MP-1 and Jagged-1 + XMU-MP-1 was changed every 2 days. Images of organoids were taken using a Zeiss Axio Imager M2 microscope (Carl Zeiss, Thornwood, NY), equipped with Metamorph Imaging software.

C.9 Organoid TUNEL staining

Organoids are stained utilizing an *in situ* cell death detection kit, TMR Red (Roche; catalog number 12156792910) according to manufacturer instructions. Briefly, organoids are fixed inside the well with 4% PFA for one hour at room temperature, rinsed with PBS, and incubated with cold 0.1% Sodium Citrate Buffer for 2 minutes. Organoids are then rinsed with PBS, incubated with TUNEL reaction for one hour at 37°C, rinsed again with PBS, stained with Hoechst (1:2500) and rinsed with PBS before visualizing. Images were taken utilizing the Evos FLoid Imaging System (ThermoFisher Scientific, Waltham, MA).

C.10 Microarray

Large intestinal fragments approximately 2 cm in size were isolated from postnatal day 3 WT and gKO pups and collected in RNAlater solution (ThermoFisher Scientific; catalog number AM7020). Total RNA was isolated using the RNeasy mini kit following manufacturer instructions (Qiagen; catalog number 74104). RNA quality was assessed through electrophoresis utilizing Bioanalyzer. High-integrity RNA samples were used for microarray hybridization and profiling at University of North Carolina Chapel Hill Genomics and Bioinformatics Core using the Agilent SurePrint GE 8 60K mouse whole genome chip (Agilent Technologies, Inc, Santa Clara, CA). Microarray results were background corrected and normalized using R software (version 3.3.0). Differential expression was tested utilizing an equal variance t test. P values were transformed to q values through Benjamini and Hochberg's approach (Glickman et al. 2014). Differentially expressed genes were identified if the q value was less than 0.05 and the absolute fold change was greater than 2. Heatmaps were generated utilizing Treeview software, and two group comparisons were performed using Gene Set Enrichment Analysis (version 3.0) against the molecular signatures database.

C.11 Proteomics

Small and large intestinal tracts were isolated from 2–4-month-old mice. Intestines were cut open longitudinally, villi were removed by physical scratching (small intestine only) and intestines were chopped into 5-mm pieces. After 30-minute incubation in EDTA at 4°C (2 mM for small intestine, 3 mM for large intestine), crypts were freed by continuous handshaking for 2 minutes. Crypt-containing solution was filtered through a 70-micron cell strainer, pelleted at 200 × g for 10 minutes, washed in distilled PBS three times, and pelleted again at 16000 rpm at 4°C for 10 minutes. RIPA buffer (0.5% Triton X-100, 0.1% SDS, 150 mM NaCl, 10 mM HEPES, 2

mM EDTA, cOmplete Protease Inhibitor Cocktail (Sigma; catalog number 11697498001), 10 mM Sodium Pyrophosphate, 20 nM Sodium Fluoride, Sodium Orthovanadate) was added to the pellet and sonicated at 4°C until the pellet dissolved and supernatant was collected after centrifugation at 16000 rpm at 4°C. Protein concentration and global quantitative proteomic analyses were performed at the Proteomics Core Facility at University of North Carolina Chapel Hill. Further analyses on data sets included Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, gene ontology-biological processes, and gene ontology-molecular functions.

C.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8.4.1 for Mac (GraphPad Software, La Jolla, CA). Differences among groups were analyzed using the unpaired Student *t* test. Data was presented as mean ± SEM. A p value less than 0.05 was considered to be statistically significant.

D. Results

D1. Genome-wide microarray analysis highlights the role of claudin-7 in colonic epithelial differentiation, stem cell homeostasis, and pathologic conditions

In order to examine the changes occurring with loss of claudin-7 in an unbiased manner, we utilized genome-wide microarray analysis in which colon from postnatal day 3 (PN3) wild-type (WT) and claudin-7 global knockout (gKO) mice were compared. Approximately 6,000 gene expression patterns were analyzed then grouped into clusters in relation to the WT small intestine for comparison. Out of a total of 29,990 annotated transcripts, 4.1% were downregulated and

0.09% were upregulated in the gKO colon, in comparison to the WT colon (Figure 3.1A). Ingenuity pathway analysis (IPA) demonstrated differences in physiological functions including organ morphology, tissue morphology, tissue development, and immune development, as well as enrichment in diseases and disorders including gastrointestinal disease, inflammatory response, cancer, and organismal injury and abnormalities (Figure 3.1B). Gene set enrichment analysis (GSEA) was then used to examine enriched genes in gKO. Enriched genes were related to epithelial differentiation, colorectal cancer, inflammation, and quiescent stem cells (Figure 3.1C). This is consistent with the literature as loss of claudin-7 has been shown to induce inflammation and epithelial dysplasia, and lead to malignancies (Karabulut et al. 2015; Bhat et al. 2015; Tanaka et al. 2015; Kim et al. 2019; Wang et al. 2019). In WT colon, genes involved in epithelial differentiation were enriched, while in contrast, genes for quiescent stem cells were enriched in the gKO colon, suggesting a shift in crypt stem cell population between WT colonic tissue and claudin-7 KO colon tissue (Figure 3.1C).

GSEA also highlighted poorly enriched Notch signaling in claudin-7 KO colon, prompting our next step to examine Notch target genes (Figure 3.1C). Notch signaling plays a vital role in regulating fate determination in differentiation as well as maintaining intestinal homeostasis and regeneration (VanDussen et al. 2012; Demitrack and Samuelson, 2016; Liang et al. 2019; Das et al. 2021). Downregulation of the Notch signaling pathway has been demonstrated to cause an intestinal epithelial differentiation preference towards the secretory lineage (Quach et al. 2022). Protein levels of activated Notch were significantly decreased in claudin-7 gKO colon compared to WT colon, while total notch remained unchanged (Figure 3.2A). Downregulation of Notch signaling was further investigated by examining Notch ligands and target genes utilizing qRT-PCR (Figure 3.2B). Interestingly, there was significant reduction in the mRNA levels of Dll4,

Hes1, Hes2, and Atoh in claudin-7 gKO colon (Figure 3.2B). Taken together, these data suggest that the Notch signaling pathway is downregulated with loss of claudin-7, potentially leading to disruption in epithelial cell differentiation and homeostasis.

D.2 Claudin-7 conditional knockout mice exhibit an IBD-like phenotype and impaired differentiation

Given the results of our microarray analysis highlighting disruption in epithelial differentiation and dysregulation of the crypt stem cell population with claudin-7 knockout, our inducible-intestinal specific claudin-7 knockout (cKO) mice were utilized to further examine the effect of loss of claudin-7 on epithelial cell differentiation. Claudin-7 cKO mice have mature intestinal epithelium in comparison to the gKO model which houses immature cells that are not fully differentiated.

Claudin-7 cKO mice exhibit an IBD-like phenotype featuring severe intestinal inflammation (Figure 3.3A, red arrow), swollen internal organs, external signs of diarrhea (Figure 3.3A, green arrow), significant weight loss over ten days, reduced cecum size (Figure 3.3A, white arrow) and decreased colon length. Inducible knockout via intraperitoneal Tamoxifen injections, led to rapid weight loss in comparison to WT mice treated with sunflower oil (Figure 3.3B). These mice will die approximately 9-11 days after the first injection. In addition to severe inflammation causing swollen intestines, colon length is also significantly decreased in claudin-7 cKO mice (Figure 3.3C). Histologically, claudin-7 cKO mice exhibit mucosal layer elongation, crypt hyperplasia, inflammatory cell infiltration, abscess formation, submucosal edema, and colonic ulcers (Li et al. 2018).

We sought to evaluate the effects of claudin-7 knockout intestinal epithelial stem cell (IESC) differentiation into the various epithelial cell types - colonocytes, goblet cells, tuft cells, and enteroendocrine cells. Tamoxifen induced knockout of claudin-7 in the large intestine was confirmed via immunofluorescence and western blot (Figure 3.4, A and B). Staining for DCAMKL1 highlighted reduction in immune-sensing tuft cells in claudin-7 cKO mice (Figure 3.5A). This was confirmed via quantification of percent DCAMKL1 positive cells per crypt where tuft cells made up 2.5% of the cell population in WT mice, in comparison to 0.85% in claudin-7 cKO mice (Figure 3.5A). There was significant reduction (5.4% in WT to 0.79% in cKO) in chromogranin A stained cells, marking hormone-secreting enteroendocrine cells (Figure 3.5B). Absorptive colonocytes were also significantly reduced with loss of claudin-7 as seen by a 3.5-fold difference in carbonic anhydrase II positive stained cells (Figure 3.5C). Lastly, alcian blue staining was utilized to examine mucus secreting goblet cells in the large intestine, demonstrating reduction of goblet cells from 49.5% in the WT colon to 21.9% in the claudin-7 cKO colon (Figure 3.5D).

Mirroring the current literature, loss of claudin-7 led to a hyperproliferative epithelium where approximately 87.8% of cells were proliferating as shown by the marker ki67 (Figure 3.6A). Interestingly, localization of proliferative cells differed between WT and cKO colon tissue, as ki67+ cells were only located in the base of the crypt region in WT colon but extended towards the surface of the epithelium in the cKO (Figure 3.6A). Similar results were found utilizing our claudin-7 gKO model (data not shown). Reduction in the epithelial cell types was confirmed via qRT-PCR to evaluate mRNA levels of the associated markers, FABP-1, DCAMKL1, and chromogranin A for absorptive cells, tuft cells and enteroendocrine cells respectively (Figure 3.6B). Overall, claudin-7 cKO mice displayed impaired differentiation ability, as they exhibited

reduced percentage of all epithelial cell types in comparison to WT mice; therefore, claudin-7 plays an essential role in large intestine IESC differentiation (Figure 3.6C).

D.3 Deletion of claudin-7 leads to increased OLFM4 and loss of the active stem cell population

OFLM4 is a robust marker of active stem cells in the small intestine; however, it is associated with inflammation and has been demonstrated as a potential candidate biomarker for gastric cancer and CRC in the large intestine (van der Flier et al. 2009; Yu et al. 2011; Guette et al. 2015). The mRNA levels of OLFM4 in the colon directly opposed small intestine levels, as it was found to be highly expressed in claudin-7 gKO colon via microarray analysis (Figure 3.7A). Additionally, OLFM4 positive cells were significantly increased in the gKO colon, both in the crypt region and the surface epithelium (Figure 3.7B). Contrastingly, WT colon expressed essentially no OLFM4 signal (Figure 3.7B). Furthermore, western blot yielded consistent results as OLFM4 protein level with claudin-7 knockout was significantly increased compared to WT colon (Figure 3.7C). This data aligns with current literature correlating claudin-7 and OLFM4 expression level with CRC progression (Singh et al. 2010; Bhat et al. 2015; Quan et al. 2020; Gowrikumar et al. 2021). Potentially, claudin-7 knockout and associated increased OLFM4 represents a precancerous phenotype.

Contrasting to OLFM4 in the small intestine, Lgr5 is utilized as a robust marker for active stem cells in the colon (van Es et al. 2007; Zhang and Huang, 2013). The intestinal crypt cell types including active stem cells, quiescent stem cells, and transit amplifying cells were examined utilizing qRT-PCR and fluorescent in situ hybridization (FISH). Claudin-7 deletion led to drastically reduced Lgr5 mRNA levels (Figure 3.8A). Bmi1 and Hopx are markers for the quiescent stem cell population, while Prom1 marks transit-amplifying cells. Loss of claudin-7 was

associated with increased mRNA levels of Bmi1, Prom1, and Hopx further emphasizing dysregulation of the crypt cell population in claudin-7 cKO colon (Figure 3.8A). FISH corroborated these findings as there was significantly decreased Lgr5 positive active stem cells in the cKO colon, compared to WT colon (Figure 3.8B; arrowheads: Lgr5 positive active stem cells). These results together suggest loss of the active stem cell population and a potential shift to other cells such as quiescent stem cells and proliferating cells in cKO colon, aligning with the microarray data demonstrating enrichment of quiescent stem cells, rather than active stem cells, in the claudin-7 knockout colon.

D.4 Crypts lacking claudin-7 are unable to form fully closed spheroids and epithelial cell types

We next examined the role of claudin-7 in regulating large intestine stem cell function in the immune-free colonic organoid model system to demonstrate that changes in stem cell function are due to claudin-7, rather than inflammation or immune effects. Our group previously uncovered the role of claudin-7 in regulating intestinal stem cell (IESCs) self-renewal and differentiation in the small intestine through the Wnt/β-catenin signaling pathway (Xing et al. 2020). Paneth cells present in the small intestine are responsible for secreting Wnt to maintain the stem cell niche. When claudin-7 was deleted and differentiation of Paneth cells was negatively affected, the function of the IESCs was disrupted. Contrastingly, in the large intestine, Paneth cells are not present, therefore, differing signaling pathways are thought to be involved in colonic IESC function. We sought to determine what functions claudin-7 may be involved in regarding large intestine IESC functions, as well as the signaling pathways it is modulating.

A colonic organoid system was established utilizing claudin-7 flox mice to examine the role of claudin-7 in large intestine IESC function. The crypt region, which houses the stem cells,

was isolated from inducible-intestinal specific claudin-7 flox mice and plated in a three-dimensional Matrigel system supplemented with L-WRN colonic organoid growth medium. To induce claudin-7 knockout in culture, crypts were treated with 4-hydroxytamoxifen (4OH-TAM) or DMSO after 24 hours of normal culture and cultured for 11 days (Figure 3.9A). Control crypts formed large spheroids within 48 hours of culture and began forming budding structures by day 5 (Figure 3.9B). Day 8 and day 11 showed progressively more buds in control organoids, with some forming more than 16 buds (Figure 3.9B). Contrastingly, by day 2, claudin-7 deficient crypts (4OH-TAM cKO) were not able to form fully closed spheroids and had reduced in number by approximately half, (Figure 3.9, B and C). In the following days, claudin-7 deficient organoids rapidly deteriorated and never formed spheroids or budding structures (Figure 3.9B). The percent of organoids survival over time was quantified, highlighting healthy growth and proliferation of control organoids, compared to significantly reduced survival by day 2 and rapid cell death by day 5 in 4OH-TAM cKO organoids (Figure 3.9C). In situ cell death detection through terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) confirmed that claudin-7 deficient organoids were unable to survive and underwent rapid apoptosis (Figure 3.9D). Given these results, claudin-7 is essential for large intestine IESC survival and growth.

Following 11 days of culture, control and 4OH-TAM cKO organoids were harvested for immunofluorescence and histological staining to examine the epithelial cell types. Healthy organoids rapidly proliferate and formed all intestinal epithelial cell types (Figure 3.10). Claudin-7 expression in control organoids, as well as claudin-7 knockout in 4OH-TAM cKO organoids were confirmed with immunofluorescent staining (Figure 3.10A). The proliferation marker ki67 highlighted rapid proliferation occurring in control organoids (Figure 3.10B). Control organoids differentiated into all epithelial cell types as demonstrated by Alcian blue staining for goblet cells,

as well as staining for the cell specific markers for colonocytes, tuft cells, and enteroendocrine cells (Figure 3.11). Overall, control crypts formed mature organoids which differentiated into all epithelial cell types, while claudin-7-deficient crypts were unable to survive and grow. These results emphasize the vital role of claudin-7 in IESC function, independent of inflammatory or immune responses. Taken together, claudin-7 is essential for survival and proliferation of large intestine organoids.

D.5 Claudin-7 regulates large intestine IESC function through Notch and Hippo signaling pathways

We next sought to uncover which pathway(s) claudin-7 was acting through to modulate large intestine IESC functions. Following the same timeline described above, claudin-7 was knocked out after 24 hours of normal culture. Twenty-four hours following knockout of claudin-7, organoids were treated with various signaling pathway activators and inhibitors, or a combination, for 10 days to rescue IESC function (Figure 3.12A). Based on the data obtained from the microarray analysis, as well as literature highlighting the importance of the Notch and Hippo signaling pathways in regulating IESC expansion and differentiation, activators and inhibitors of these pathways were used to rescue organoid survival, growth, and differentiation (Barry et al. 2013; Hansen et al. 2015; Taniguchi et al. 2015; Demitrack and Samuelson, 2016). Jagged-1 is an activator of the Notch signaling pathway, while XMU-MP-1 is a selective inhibitor of MST1/2, therefore inhibiting the phosphorylation of Yes associated protein (YAP) and allowing translocation of YAP to the nucleus for transcription and activation of stem cell growth and expansion. Interestingly, XMU-MP-1-treated organoids were able to form spheroids which grew in size over time; however, they did not form budding structures (Figure 3.12B). Given this data,

claudin-7 may modulate the Hippo pathway to regulate the survival and growth of the IESCs; however, Hippo inhibition was not able to rescue differentiation ability. Treatment with Jagged-1 restored survival, growth, and differentiation, as these organoids formed healthy spheroids and some budding structures after 11 total days of culture, though there were fewer buds in these organoids compared to control claudin-7-sufficient organoids (Figure 3.12B). These results highlight the importance of the Notch signaling cascade in IESC survival, growth, and differentiation. Lastly, the combination of Jagged-1 and XMU-MP-1 demonstrated a synergistic effect, restoring organoid growth, survival, and number of budding structures to levels comparable to control claudin-7 sufficient organoids (Figure 3.12B). Treatment of 4OH-TAM cKO organoids with Jagged-1, XMU-MP-1, and Jagged-1 + XMU-MP-1 all yielded similar results in terms of organoid survival (Figure 3.12C). While all conditions rescued organoid survival at similar levels, the combination of Jagged-1 and XMU-MP-1 produced significantly more buds than either agent alone (Figure 3.12, B and D). Immunofluorescent staining for claudin-7 confirmed knockout of claudin-7 in rescued organoids (Figure 3.13A). Restoration of differentiation ability was confirmed through immunofluorescent staining (Figure 3.13B). Jagged-1 + XMU-MP-1-treated organoids were highly proliferative and successfully differentiated into all the epithelial cell types (Figure 3.13, A and B). Taken together, these results suggest that both the Notch and Hippo signaling pathways are involved in claudin-7 regulation of IESC function, with Notch being the essential pathway as activation of the Notch pathway restored all IESC functions.

As the Hippo pathway was identified to be important in the IESC survival and growth in colon organoid culture and has been implicated in stem cell self-renewal and regulation of tissue homeostasis, this pathway was further examined using our claudin-7 knockout mouse model (Gregorieff et al. 2015; Hansen et al. 2015). YAP is a transcription coregulator of the Hippo

signaling pathway which is mainly expressed in the active Lgr5 positive stem cells (Avruch et al. 2012; Barry et al. 2013). Immunofluorescent staining was utilized to examine YAP expression and localization in WT and claudin-7 cKO colon (Figure 3.14A). YAP positive cells were largely located in the crypt region of the WT colon, whereas loss of claudin-7 was associated with no YAP expression, demonstrating potential activation of the Hippo signaling cascade, leading to phosphorylation and degradation of YAP (Figure 3.14A). Examination of protein level yielded similar results (Figure 3.14, B and C). The protein level of YAP in the cKO colon was significantly decreased compared to the WT colon, while phosphorylated YAP at serine 127 was significantly increased (Figure 3.14, B and C). Phosphorylation of YAP at S127 leads to destabilization and degradation of YAP (Hansen et al. 2015). This reduction in YAP expression further corroborates our previous results suggesting reduction in the active stem cell population with loss of claudin-7 as active stem cells are the main group expressing YAP.

D.6 Loss of claudin-7 disrupts IESC differentiation ability

In order to further examine IESC differentiation in culture, a delayed claudin-7 knockout model was developed. Claudin-7-deficient organoids were unable to survive beyond 3 days, therefore, they do not have a chance to differentiate, making it difficult to examine changes in differentiation utilizing this timeline. Here, organoids were allowed to grow into mature differentiated organoids until day 6 and day 8, at which times claudin-7 knockout was induced with 4OH-TAM (Figure 3.15A). TUNEL staining was utilized after 48 hours to examine apoptosis (Figure 3.15A). Organoids were harvested 24 hours later to stain for proliferation and epithelial cell specific markers (Figure 3.15A). Within 48 hours of claudin-7 knockout, organoids were highly apoptotic (Figure 3.15B). Proliferation was greatly reduced in the day 7 knockout organoids

compared to day 7 control organoids, highlighting the notion that organoid growth and stem cell expansion is negatively affected by loss of claudin-7 (Figure 3.16A). This finding was confirmed via quantification of the percent of proliferating cells (Figure 3.16C). Immunofluorescent staining for carbonic anhydrase II demonstrated a decrease in the percent of differentiated colonocytes in day 7 knockout organoids, compared to day 7 control organoids (Figure 3.16, A and C). Similar results were found for tuft cells, goblet cells, and enteroendocrine cells at both day 7 and day 9 (Figure 3.16, A-D). This demonstrates reduction in differentiation ability after loss of claudin-7 in culture, mirroring *in vivo* findings. Taken together, these results establish the role of claudin-7 in regulating colonic IESC differentiation, as well as survival and proliferation, highlighting the critical function of TJ protein claudin-7 in large intestine stem cell functions.

D.7 Use of proteomics highlights altered protein expression in claudin-7 cKO large intestine

To examine what other biological processes may be affected by loss of claudin-7, we utilized the unbiased proteomics approach. Isolated crypt lysates from 3-month-old control and claudin-7 knockout mice were sent to the Proteomics Core Facility at University of North Carolina Chapel Hill. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis as well as gene ontology-biological processes (GO-BP) and gene ontology-molecular function (GO-MF) were used to analyze proteomic datasets. KEGG pathway analysis highlighted which proteins were significantly altered in cKO large intestine in comparison to WT large intestine. An adjusted p value less than 0.01 was used as the cutoff to select differentially expressed genes (approximately 500 differentially expressed were found). The principal component analysis (PCA) plot highlights differential protein expression between WT and cKO groups, as WT samples form a cluster separate from cKO samples (Figure 3.17A). Additionally, the claudin-7 cKO group exhibits two

clusters – male and female (Figure 3.17A). Gene ontology analysis of biological processes demonstrated differential protein expression in monocarboxylic acid metabolic processes, small molecule catabolic processes, fatty acid beta-oxidation and cell-cell junction organization (Figure 3.17B). To our surprise, the process with the highest differential expression was monocarboxylic acid metabolic processes (Figure 3.17B). These processes and the most highly differentially expressed proteins associated with each process were then used to create a cnet plot showing the interconnected network of these processes and their altered proteins (Figure 3.17C). This dataset provides new avenues to explore in the future for further involvement of claudin-7 in biological processes outside of the TJ, specifically in metabolism.

E. Discussion

CRC is associated with decreased claudin-7 expression, hyperproliferation leading to the formation of adenomas, and loss of stem cell function. In this study, we demonstrate disruption in the large intestine crypt stem cell population, as loss of claudin-7 led to decreased Lgr5 positive active stem cells accompanied with an increase in quiescent and transit-amplifying cells. Without claudin-7, crypts were unable to survive and form functioning spheroids highlighting the vital role of claudin-7 in IESC survival and proliferation. These functions were rescued through modulation of prominent IESC regulatory signaling pathways, Notch and Hippo signaling. Interestingly, both the Notch and Hippo signaling cascades have been implicated in CRC initiation and progression, as aberrant signaling induces hyperproliferation. Regulation of these signaling pathways by TJ protein claudin-7 may play a role in the induction of CRC, as well as in further invasion and metastasis. This idea is further corroborated by increased OLFM4 expression with loss of claudin-7, suggesting our claudin-7 knockout mice as a pre-cancerous model.

Nearly 50% of patients with CRC exhibit chemoresistance and tumor recurrence involving colon cancer stem cells (CSCs) (Gupta et al. 2019). CSCs are capable of self-renewing and differentiating into differentiated cancer cells which cause tumor growth and uncontrolled proliferation (Munro et al. 2017). In our model, we demonstrated a loss in the active stem cell population, and shift towards the progenitor cell population. While there is increased transit-amplifying cells, claudin-7 knockout mice exhibit a decrease in all epithelial cell types, indicating that some other mechanism must be occurring where the progenitor cells become another cell type. Interestingly, CSCs are believed to originate from non-malignant stem or progenitor cells (Clara et al. 2020). Potentially, this shift in crypt stem cell population may induce the rise of a CSC population which originate from these progenitor cells, and hyperproliferate eventually leading to colon tumorigenesis. There are currently ongoing trials in which regulation of Notch, Wnt, Hippo, and Hedgehog signaling pathways is utilized to target CSCs to control stem-cell proliferation, survival, and differentiation (Takebe et al. 2015; Clara et al. 2020). Taken together, claudin-7 plays a vital role in large intestine stem cell functions including survival, proliferation, and differentiation through the Notch and Hippo pathways. This shift in crypt cell population may induce formation of CSCs leading to increased tumorigenicity. Understanding the function of claudin-7 in stem cell regulation and regeneration may serve as a new avenue for CRC treatment and preventive measures to prevent IBD from progressing to CRC.

F. Acknowledgements

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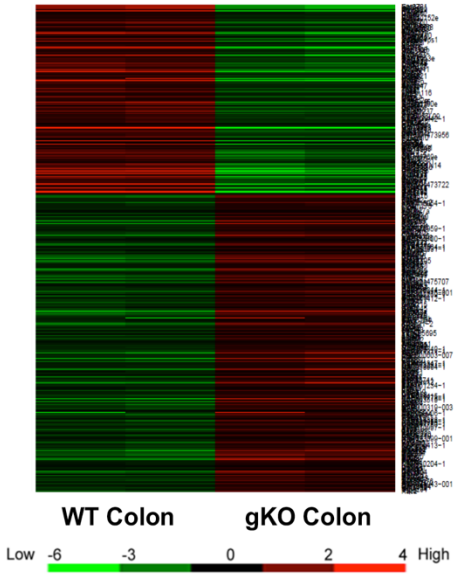
The authors also thank Joani Zary Oswald and Rodney Tatum for their technical assistance. This study was funded by NIH DK103166.

A



B

Physiological Functions

Name	p-value	#Molecules
Organ Morphology	2.92E-04 - 3.73E-02	29
Immune Development	3.64E-04 - 3.39E-02	22
Tissue Morphology	3.64E-04 - 3.73E-02	48
Tissue Development	5.06E-04 - 3.62E-02	45

Diseases and Disorders

Name	p-value	#Molecules
Inflammatory Response	1.62E-04 - 3.73E-02	47
Gastrointestinal Disease	1.66E-04 - 3.39E-02	29
Cancer	6.82E-04 - 3.55E-02	42
Organismal Injury and Abnormalities	7.38E-04 - 3.39E-02	24

C

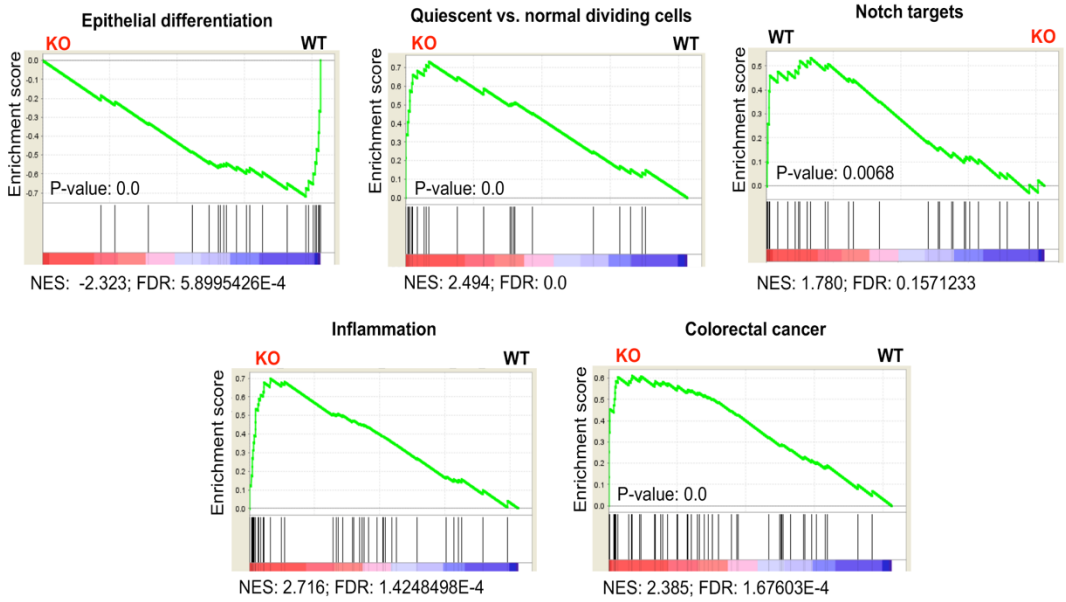


Figure 3.1 Genome-wide microarray analysis highlights differential gene expression between WT and gKO large intestines. **(A)** Heatmap depicting gene expression profile of two independent sets of PN3 WT and gKO large intestines obtained from microarray analysis. **(B)** Ingenuity pathway analysis (IPA) highlights gene expression changes related to physiological functions and diseases and disorders. **(C)** Gene set enrichment analysis (GSEA) of WT versus gKO large intestine. A positive enrichment score indicates gene set enrichment at the top of the ranked list, while a negative ES indicates gene set enrichment at the bottom of the ranked list. The leading-edge subset of a gene set is the subset of members that contribute most to the enrichment score. For a positive enrichment score, the leading-edge subset is the set of members that appear in the ranked list prior to the peak score. For a negative enrichment score, the leading-edge subset is the set of members that appear subsequent to the peak score. $P<0.01$; False discovery rate (FDR) $q<25\%$; Normalized enrichment score (NES). $n = 3$.

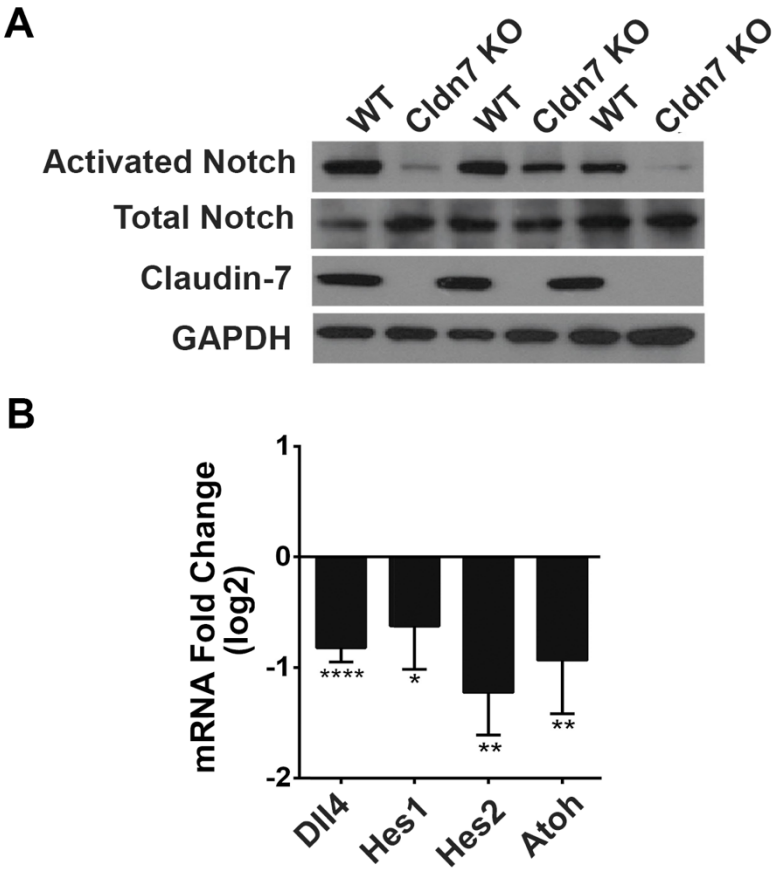
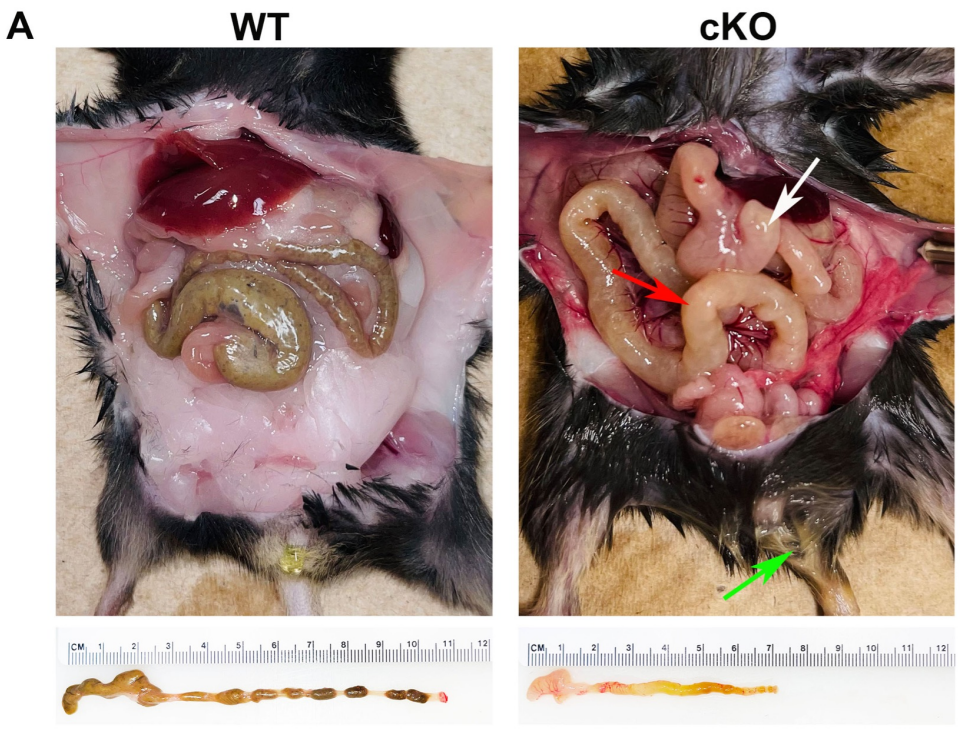

A
WT
Cldn7 KO
WT
Cldn7 KO
WT
Cldn7 KO
Activated Notch
Total Notch
Claudin-7
GAPDH
B
mRNA Fold Change (log2)
1
0
-1
-2
****
*
**
**
Dll4
Hes1
Hes2
Atoh

Figure 3.2 Notch signaling is downregulated in claudin-7 KO large intestine. (A) WB examining activated notch, total notch and claudin-7 protein levels in control/WT and claudin-7 gKO large intestine. **(B)** qRT-PCR for Notch ligands and target genes. n = 3. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$.


A
WT
cKO

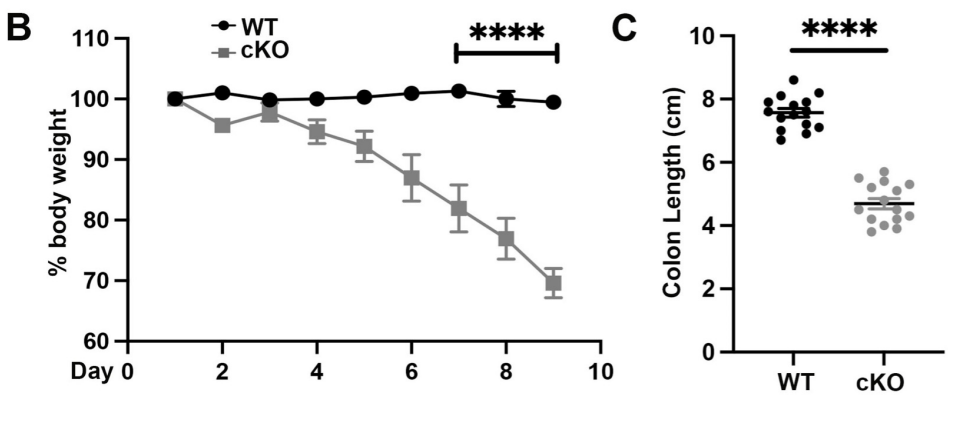

B
WT
cKO
****
% body weight
110
100
90
80
70
60
Day 0
2
4
6
8
10
C
****
Colon Length (cm)
10
8
6
4
2
0
WT
cKO

Figure 3.2 Claudin-7 inducible, intestinal-specific conditional knockout mouse (cKO). **(A)** Claudin-7 cKO mice exhibit severe intestinal inflammation (red arrow), swollen internal organs, small cecum (white arrow), signs of diarrhea (green arrow), weight loss, and decreased colon length. **(B)** Quantification of percent body weight over IP injection timeline. **(C)** Quantification of colon length in centimeters. Values were compared by unpaired t test and plotted as mean ± SEM. n = 15. ****$p \leq 0.0001$.

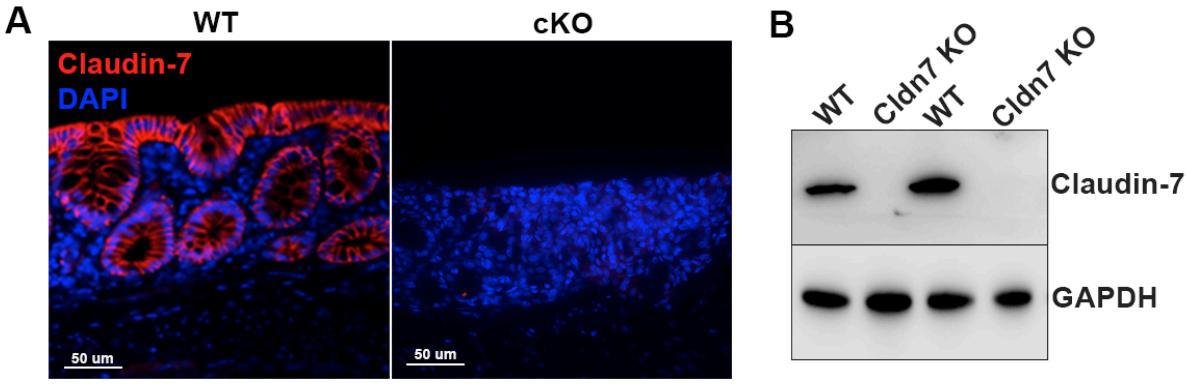

A
WT
cKO
Claudin-7
DAPI
50 um
50 um
B
WT
Cldn7 KO
WT
Cldn7 KO
Claudin-7
GAPDH

Figure 3.4 Claudin-7 is successfully deleted from large intestinal tissue. **(A)** IF staining for claudin-7 in WT and claudin-7 cKO colon tissue. **(B)** WB probing for claudin-7 in WT and cKO colon. *Scale bars* = 50 μm. n = 15.

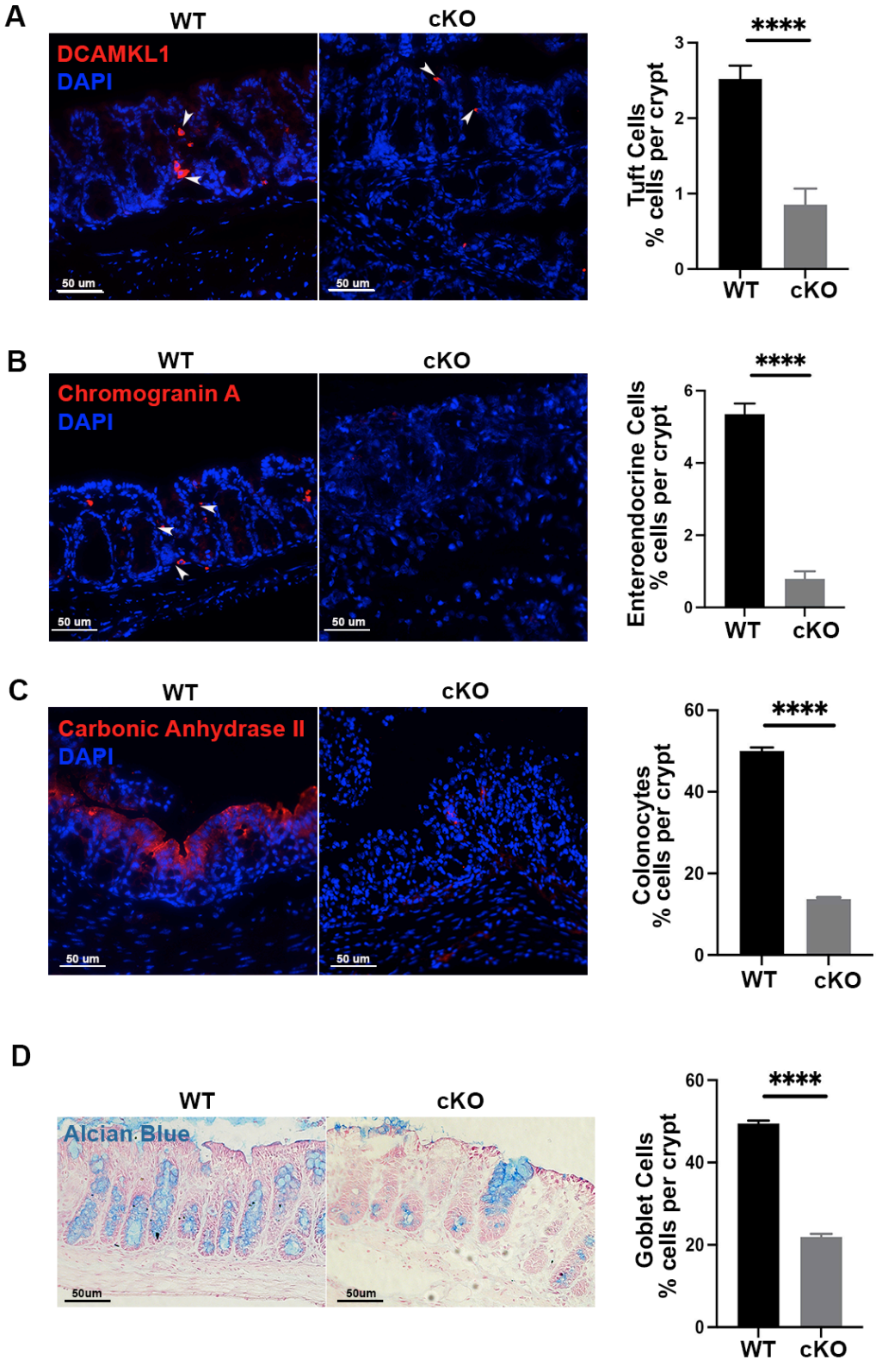

A
WT
cKO
DCAMKL1
DAPI
50 um
50 um
****
Tuft Cells
% cells per crypt
3
2
1
0
WT
cKO
B
WT
cKO
Chromogranin A
DAPI
50 um
50 um
****
Enteroendocrine Cells
% cells per crypt
6
4
2
0
WT
cKO
C
WT
cKO
Carbonic Anhydrase II
DAPI
50 um
50 um
****
Colonocytes
% cells per crypt
60
40
20
0
WT
cKO
D
WT
cKO
Alcian Blue
50um
50um
****
Goblet Cells
% cells per crypt
60
40
20
0
WT
cKO

Figure 3.5 Claudin-7 cKO exhibited reduction in large intestine epithelial cell types. (A) DCAMKL1 staining to mark Tuft cells in WT and cKO colon and quantification of percent tuft cells per crypt. **(B)** Chromogranin A staining to mark enteroendocrine cells in WT and cKO colon and quantification of percent enteroendocrine cells per crypt. **(C)** IF staining for carbonic anhydrase II to mark colonocytes in WT and cKO colon and quantification of percent colonocytes per crypt. **(D)** Alcian blue staining to mark mucus in goblet cells in WT and cKO colon and quantification of percent goblet cells per crypt. *Scale bars* = 50 μm. Values were compared by unpaired t test and plotted as mean ± SEM. n = 15. ****$p \leq 0.0001$.

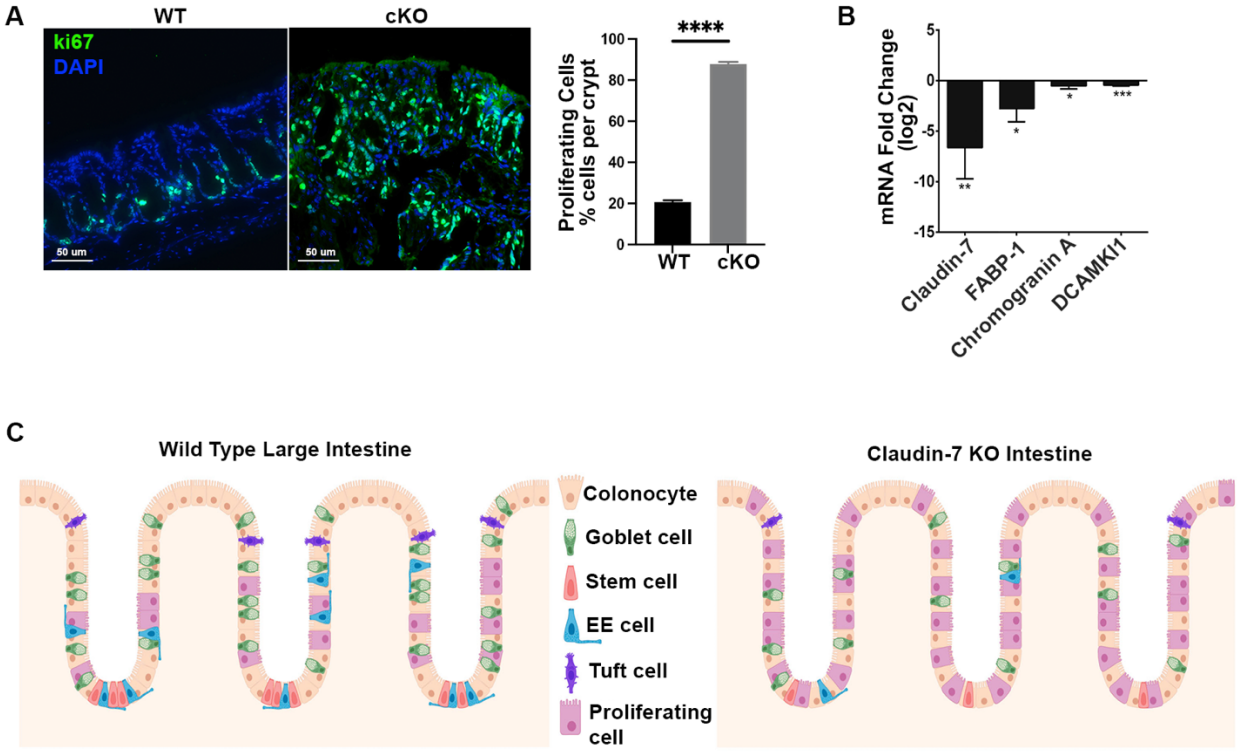

A
WT
cKO
ki67
DAPI
50 um
50 um
****
Proliferating Cells
% cells per crypt
100
80
60
40
20
0
WT
cKO
B
mRNA Fold Change
(log2)
5
0
-5
-10
-15
**
*
*
***
Claudin-7
FABP-1
Chromogranin A
DCAMKI1
C
Wild Type Large Intestine
Colonocyte
Goblet cell
Stem cell
EE cell
Tuft cell
Proliferating
cell
Claudin-7 KO Intestine

Figure 3.6 Loss of claudin-7 leads to a hyperproliferative epithelium. **(A)** Ki67 staining to mark proliferative cells in WT and cKO colon and quantification of percent proliferating cells per crypt. **(B)** qRT-PCR for epithelial cell markers. **(C)** Summary schematic. *Scale bars* = 50 μm. Values were compared by unpaired t test and plotted as mean ± SEM. n = 15. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

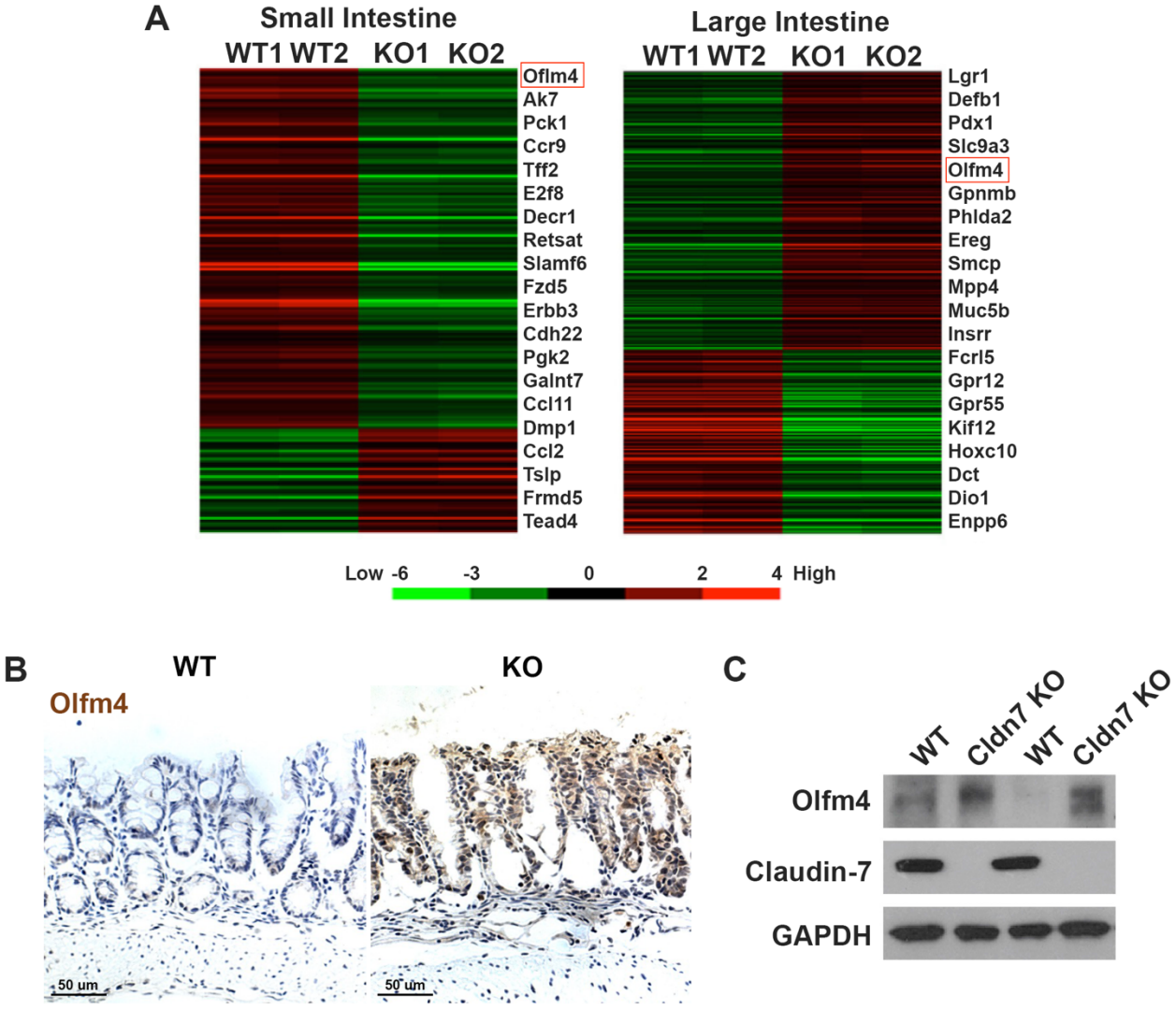

A
Small Intestine
WT1 WT2 KO1 KO2
Oflm4
Ak7
Pck1
Ccr9
Tff2
E2f8
Decr1
Retsat
Slamf6
Fzd5
Erbb3
Cdh22
Pgk2
Galnt7
Ccl11
Dmp1
Ccl2
Tslp
Frmd5
Tead4
Large Intestine
WT1 WT2 KO1 KO2
Lgr1
Defb1
Pdx1
Slc9a3
Olfm4
Gpnmb
Phlda2
Ereg
Smcp
Mpp4
Muc5b
Insrr
Fcrl5
Gpr12
Gpr55
Kif12
Hoxc10
Dct
Dio1
Enpp6
Low -6 -3 0 2 4 High
B
WT
KO
Olfm4
50 um
50 um
C
WT
Cldn7 KO
WT
Cldn7 KO
Olfm4
Claudin-7
GAPDH

Figure 3.7 Claudin-7 KO mice exhibit increased Olfm4. **(A)** Microarray heat map highlighting contrasting Olfm4 expression in WT and claudin-7 gKO colon between small intestine and large intestine. **(B)** Immunohistochemistry staining for Olfm4 in WT and gKO colon. **(C)** WB probing for Olfm4 and claudin-7 in WT and gKO colon. *Scale bars* = 50 μm. n = 3

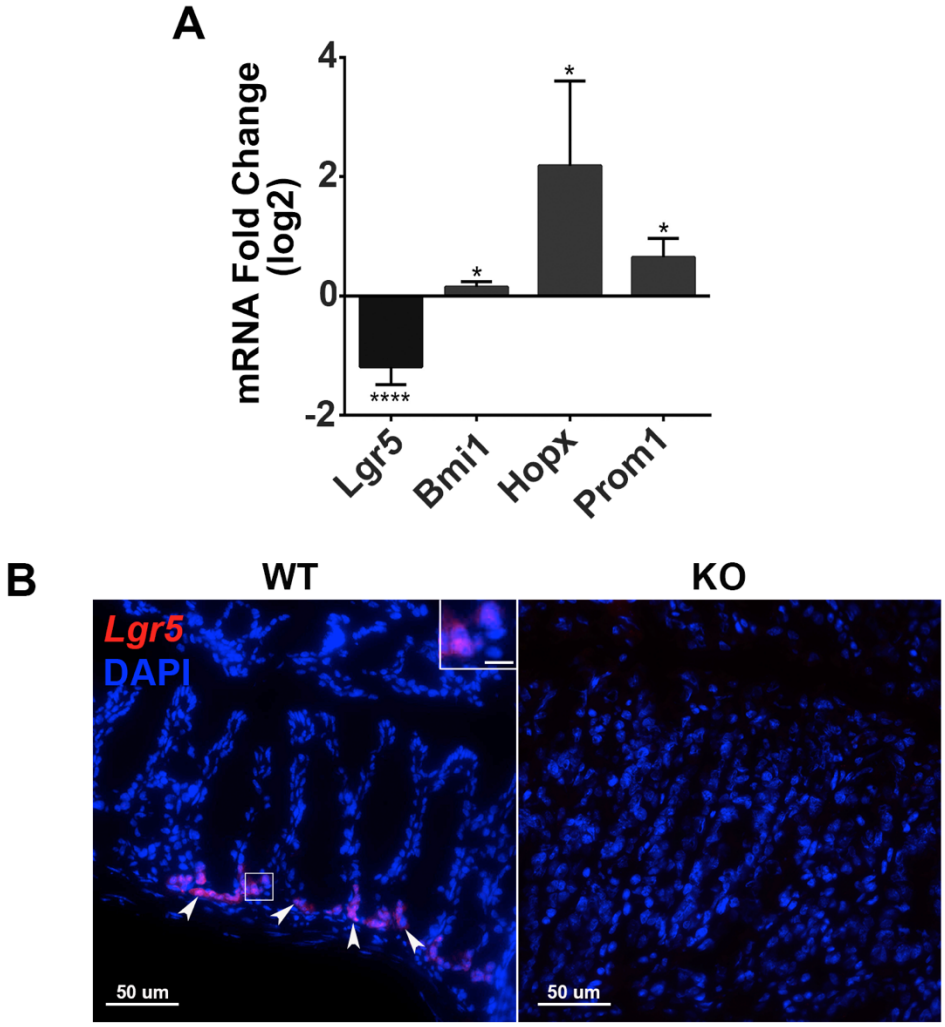

A
mRNA Fold Change (log2)
4
2
0
-2
*
*
*
****
Lgr5
Bmi1
Hopx
Prom1
B
WT
KO
Lgr5
DAPI
50 um
50 um

Figure 3.8 Deletion of claudin-7 leads to a shift in crypt cell population and loss of the active stem cell pool. **(A)** qRT-PCR examination of mRNA levels for Lgr5, Bmi1, Hopx, and Prom1 in WT and cKO colon tissue. **(B)** Fluorescent in situ hybridization for Lgr5 in WT and cKO colon to mark active stem cells. *Scale bars* = 50 μm. *Insert scale bar* = 7.5 μm. n = 3. * $p \leq 0.05$, **** $p \leq 0.0001$.

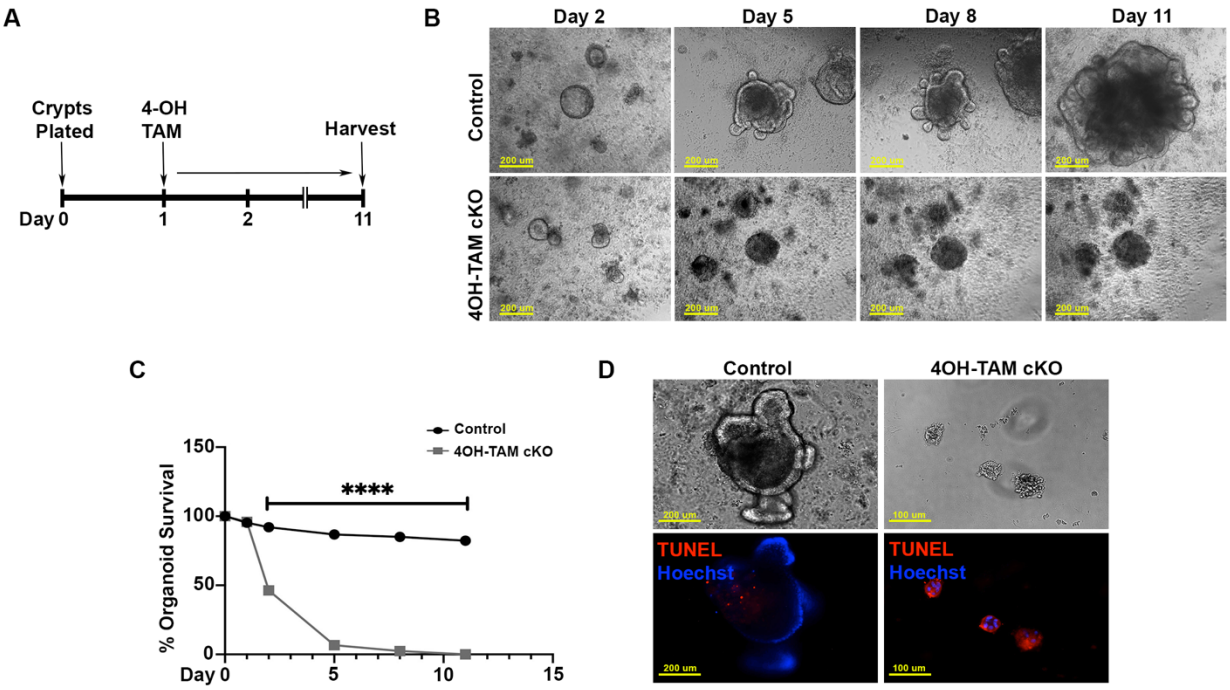

A
Crypts Plated
4-OH TAM
Harvest
Day 0
1
2
11
B
Day 2
Day 5
Day 8
Day 11
Control
4OH-TAM cKO
200 um
C
Control
4OH-TAM cKO
% Organoid Survival
150
100
50
0
Day 0
5
10
15
****
D
Control
4OH-TAM cKO
TUNEL
Hoechst
200 um
100 um

Figure 3.9 Claudin-7 deficient crypts are unable to survive and proliferate. **(A)** Timeline of organoid culture and 4OH-Tamoxifen treatment. **(B)** Control (DMSO) and 4OH-TAM cKO organoid growth over time. *Scale bars* = 200 μm. **(C)** Organoid survival curve. **(D)** In situ cell death (TUNEL) stain on day 11 control and 4OH-TAM cKO organoids. *Scale bars* = 200 μm (control), 100 μm (cKO). Values were compared by unpaired t test. n = 12. ****$p \leq 0.0001$.

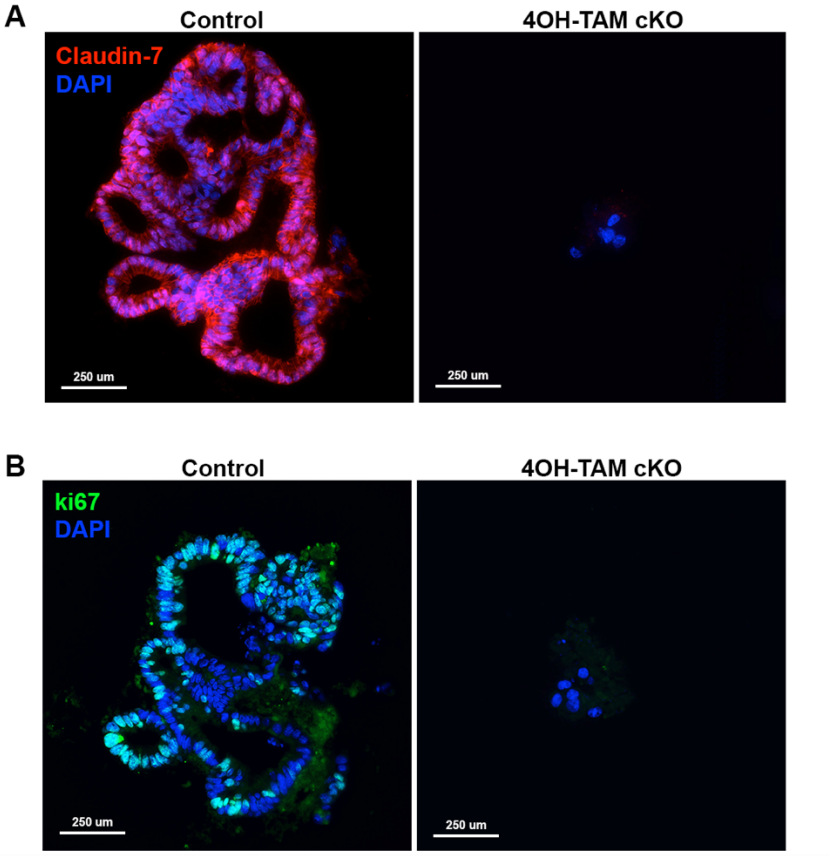

A
Control
4OH-TAM cKO
Claudin-7
DAPI
250 um
250 um
B
Control
4OH-TAM cKO
ki67
DAPI
250 um
250 um

Figure 3.10 Claudin-7 is successfully knocked out in culture. **(A)** Claudin-7 staining in control and 4OH-TAM cKO organoids. **(B)** Ki67 staining to mark proliferating cells in control and 4OH-TAM cKO organoids. *Scale bars* = 250 μm. n = 9.

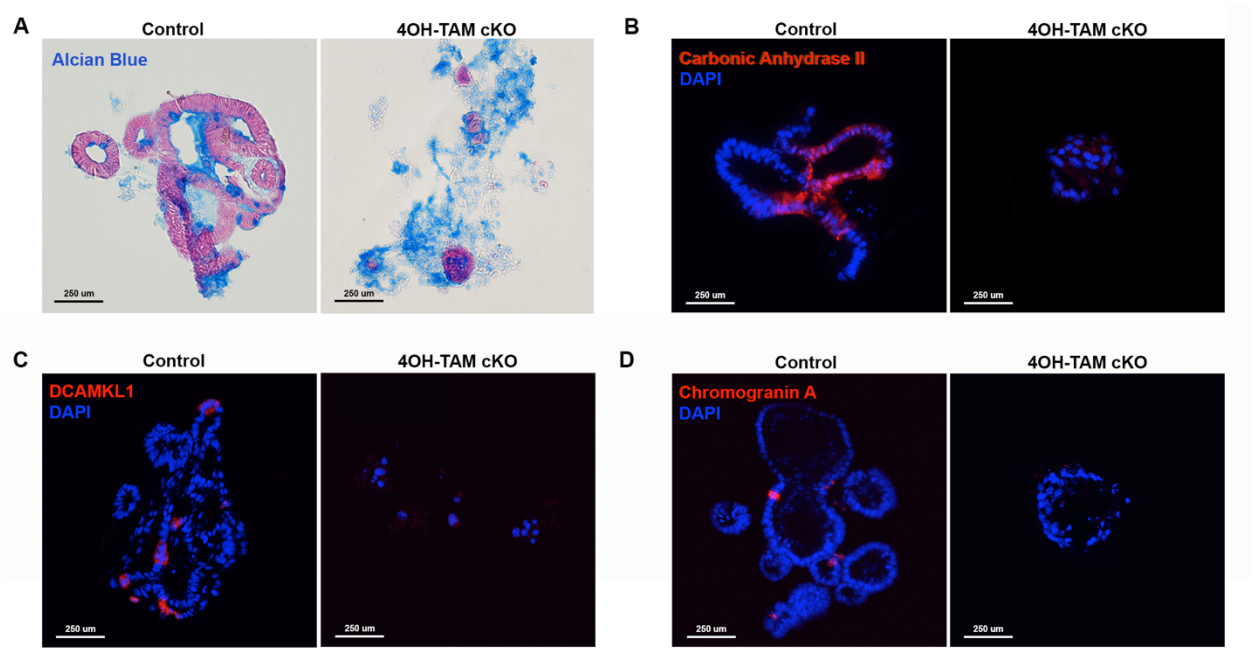

A
Control
4OH-TAM cKO
Alcian Blue
250 um
250 um
B
Control
4OH-TAM cKO
Carbonic Anhydrase II
DAPI
250 um
250 um
C
Control
4OH-TAM cKO
DCAMKL1
DAPI
250 um
250 um
D
Control
4OH-TAM cKO
Chromogranin A
DAPI
250 um
250 um

Figure 3.11 Control organoids differentiate into all epithelial cell types. (A) Alcian blue staining to mark mucus in goblet cells in control and 4OH-TAM cKO organoids. Blue staining outside of organoids may be due to dumping of mucus prior to cell death. **(B)** IF staining for carbonic anhydrase II to mark colonocytes in control and 4OH-TAM cKO organoids. **(C)** DCAMKL1 staining to mark Tuft cells in control and 4OH-TAM cKO organoids. **(D)** Staining for Chromogranin A to mark enteroendocrine cells in control and 4OH-TAM cKO organoids. *Scale bars* = 250 μm. n = 9.

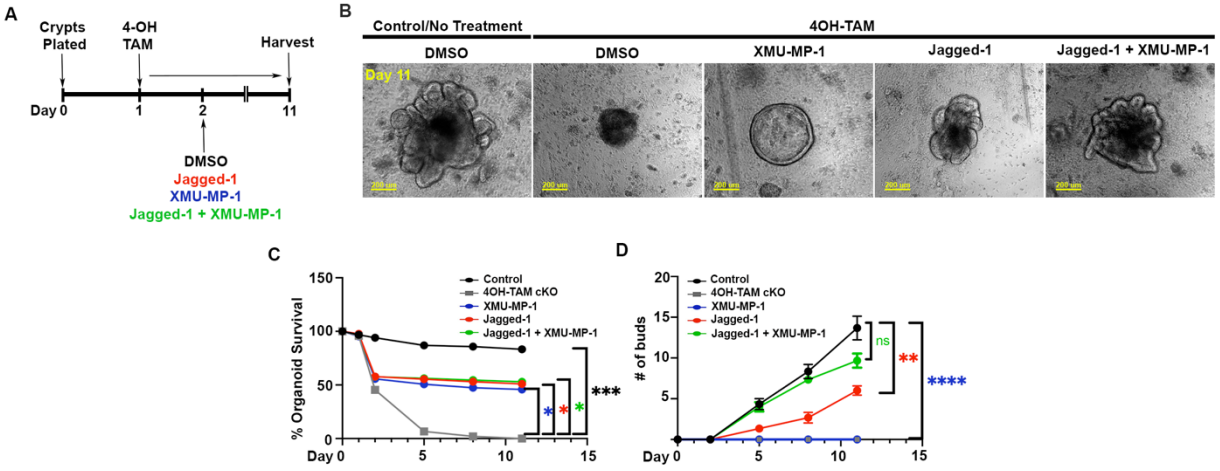

A
Crypts Plated
4-OH TAM
Harvest
Day 0
1
2
11
DMSO
Jagged-1
XMU-MP-1
Jagged-1 + XMU-MP-1
B
Control/No Treatment
4OH-TAM
DMSO
DMSO
XMU-MP-1
Jagged-1
Jagged-1 + XMU-MP-1
Day 11
C
% Organoid Survival
Control
4OH-TAM cKO
XMU-MP-1
Jagged-1
Jagged-1 + XMU-MP-1
***
*
*
*
D
# of buds
ns
**
****

Figure 3.12 Organoid survival can be rescued through modulation of Notch and Hippo signaling pathways. **(A)** Timeline of culture and treatments. **(B)** Representative images of day 11 control, KO and rescued organoids. **(C)** Percent of organoid survival through 11 days of culture. **(D)** Number of budding structures through 11 days of culture. *Scale bars* = 200 μm. n = 5. ns, not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

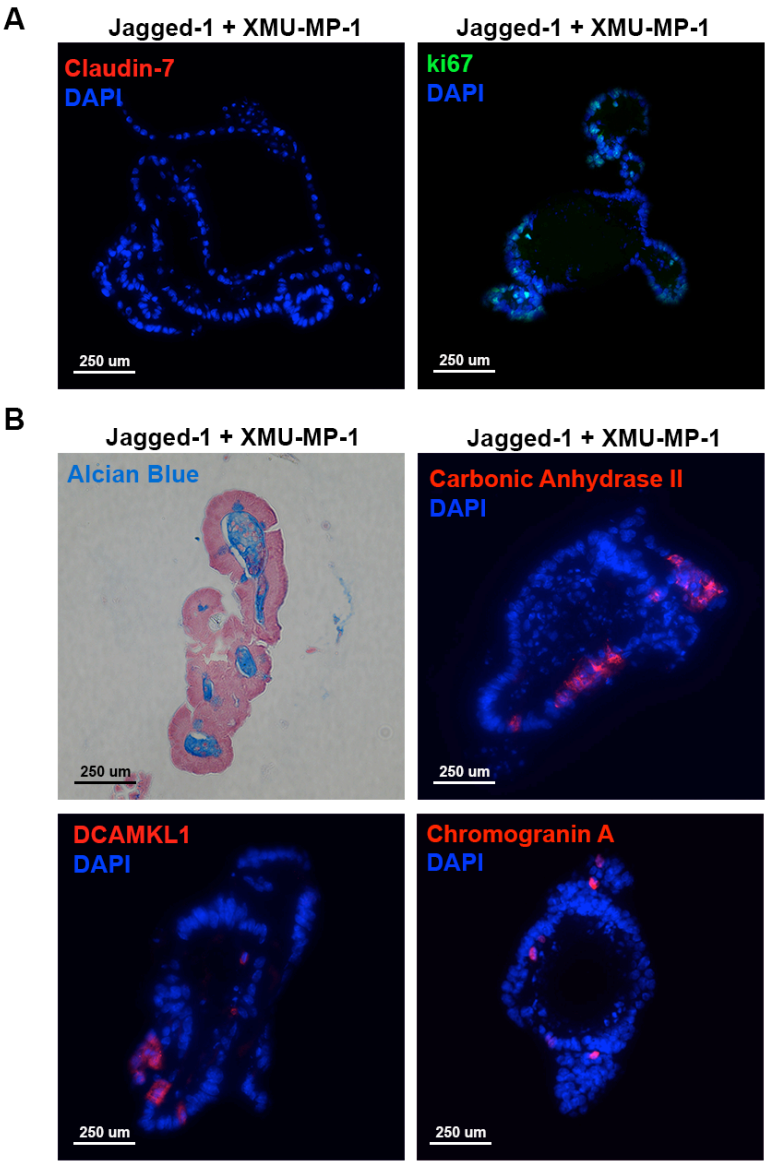
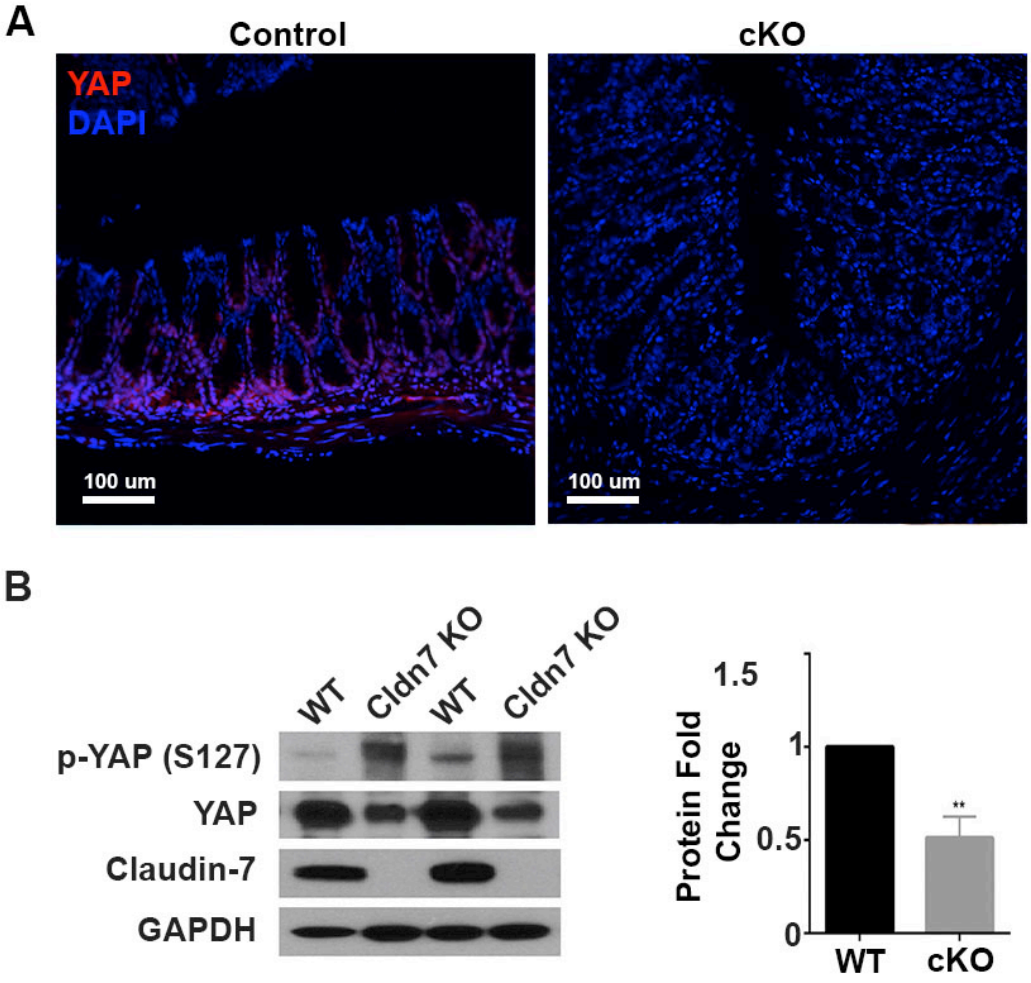

A
Jagged-1 + XMU-MP-1
Claudin-7
DAPI
250 um
Jagged-1 + XMU-MP-1
ki67
DAPI
250 um
B
Jagged-1 + XMU-MP-1
Alcian Blue
250 um
Jagged-1 + XMU-MP-1
Carbonic Anhydrase II
DAPI
250 um
DCAMKL1
DAPI
250 um
Chromogranin A
DAPI
250 um

Figure 3.13 Rescued organoids proliferate and can differentiate into all epithelial cell types.

(A) Claudin-7 and Ki67 staining in day 11 claudin-7 KO organoids rescued with Jagged-1 + XMU-MP-1. **(B)** Alcian blue, carbonic anhydrase II, DCAMKL-1 and chromogranin A staining to mark goblet cells, colonocytes, tuft cells, and enteroendocrine cells respectively, in day 11 claudin-7 KO organoids rescued with Jagged-1 + XMU-MP-1. *Scale bars* = 250 μm. n = 5.


A
Control
cKO
YAP
DAPI
100 um
100 um
B
WT
Cldn7 KO
WT
Cldn7 KO
p-YAP (S127)
YAP
Claudin-7
GAPDH
Protein Fold Change
1.5
1
0.5
0
**
WT
cKO

<u>Figure 3.14 Hippo signaling is altered in claudin-7 KO large intestine tissue.</u> (A) Immunofluorescent staining for YAP in WT and cKO colon tissue. **(B)** WB probing for YAP, phosphorylated YAP at Serine 127, and claudin-7 in WT and claudin-7 cKO colon tissue. **(C)** YAP protein fold change in WT and claudin-7 cKO colon. *Scale bars* = 50 μm. n = 3. ** $p \leq 0.01$.

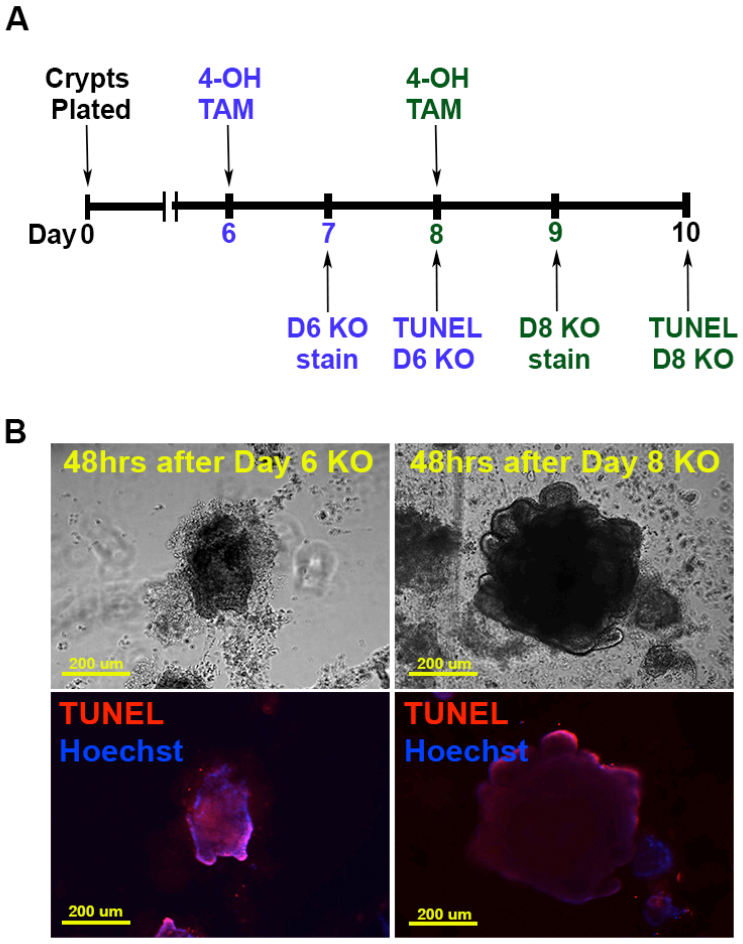

A
Crypts Plated
4-OH TAM
4-OH TAM
Day 0
6
7
8
9
10
D6 KO stain
TUNEL D6 KO
D8 KO stain
TUNEL D8 KO
B
48hrs after Day 6 KO
48hrs after Day 8 KO
200 um
200 um
TUNEL
Hoechst
TUNEL
Hoechst
200 um
200 um

Figure 3.15 Late KO of claudin-7 induces apoptosis within 48 hours. **(A)** Experimental timeline of 4OH-Tamoxifen treatments and harvesting times. TUNEL staining completed 48 hours after 4OH-TAM treatment. Organoids were harvested for immunofluorescent staining 24 hours after 4OH-TAM treatment. **(B)** TUNEL staining to examine apoptosis 48 hours after day 6 and day 8 4OH-TAM treatment. *Scale bars* = 200 μm. n = 5.

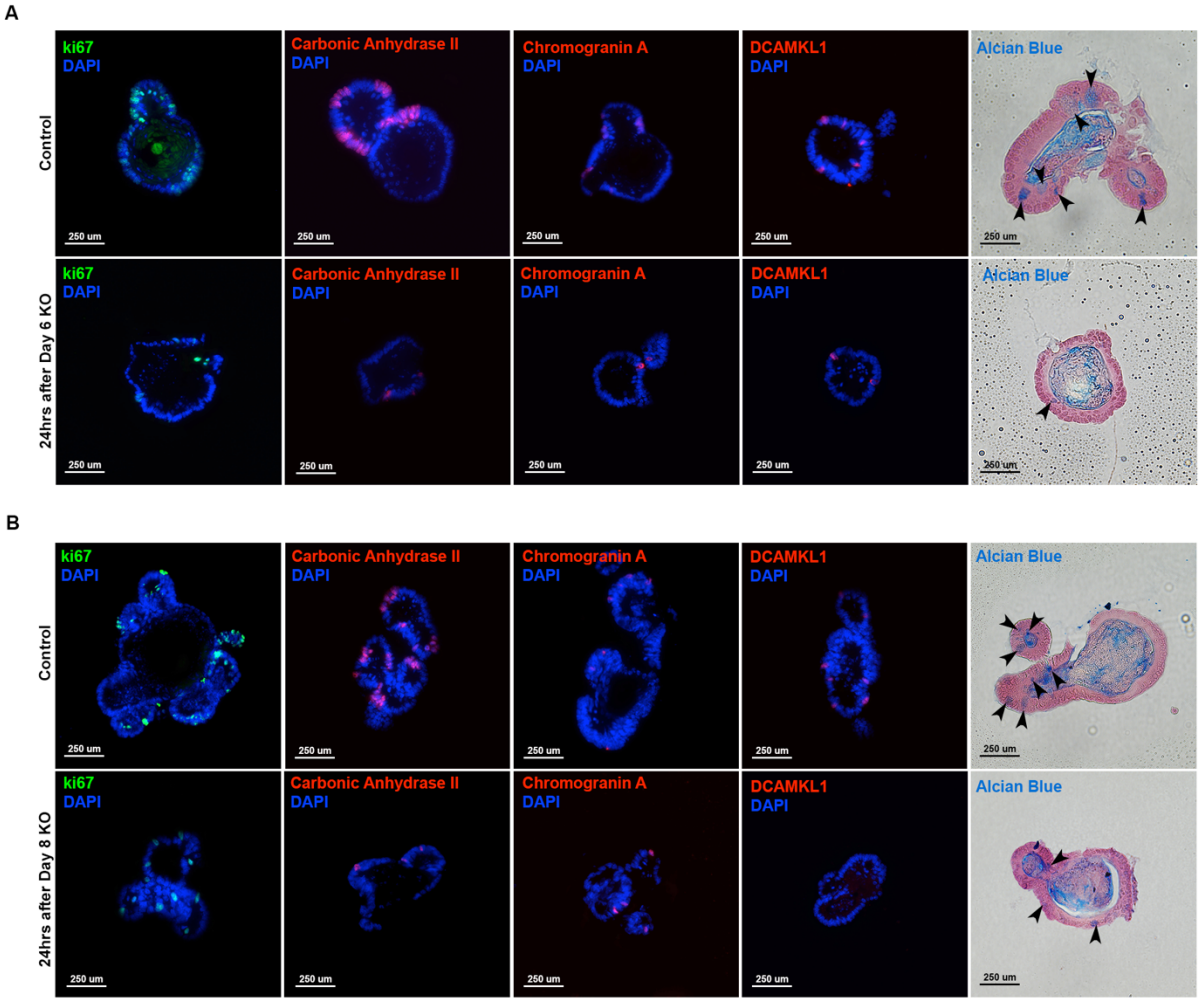

A
ki67
DAPI
Carbonic Anhydrase II
DAPI
Chromogranin A
DAPI
DCAMKL1
DAPI
Alcian Blue
Control
24hrs after Day 6 KO
250 um
B
24hrs after Day 8 KO
C
Control
24hrs after Day 6 KO
% of cells
****
***
Proliferating Cells
Colonocytes
Enteroendocrine Cells
Tuft Cells
D
24hrs after Day 8 KO

Figure 3.16 Claudin-7 is essential for organoid differentiation. **(A)** Immunofluorescent staining for epithelial cell types in control (day 7) and 24 hours after day 6 claudin-7 KO. Arrowheads point to mucus staining in goblet cells. **(B)** Immunofluorescent staining for epithelial cell types in control (day 9) and 24 hours after day 8 claudin-7 KO. *Scale bars* = 250 μm. Arrowheads point to mucus staining in goblet cells. **(C)** Quantification of percent of cells for each cell type in day 7 control and 24 hours after day 6 claudin-7 KO. **(D)** Quantification of percent of cells for each cell type in day 7 control and 24 hours after day 6 claudin-7 KO. n = 3.

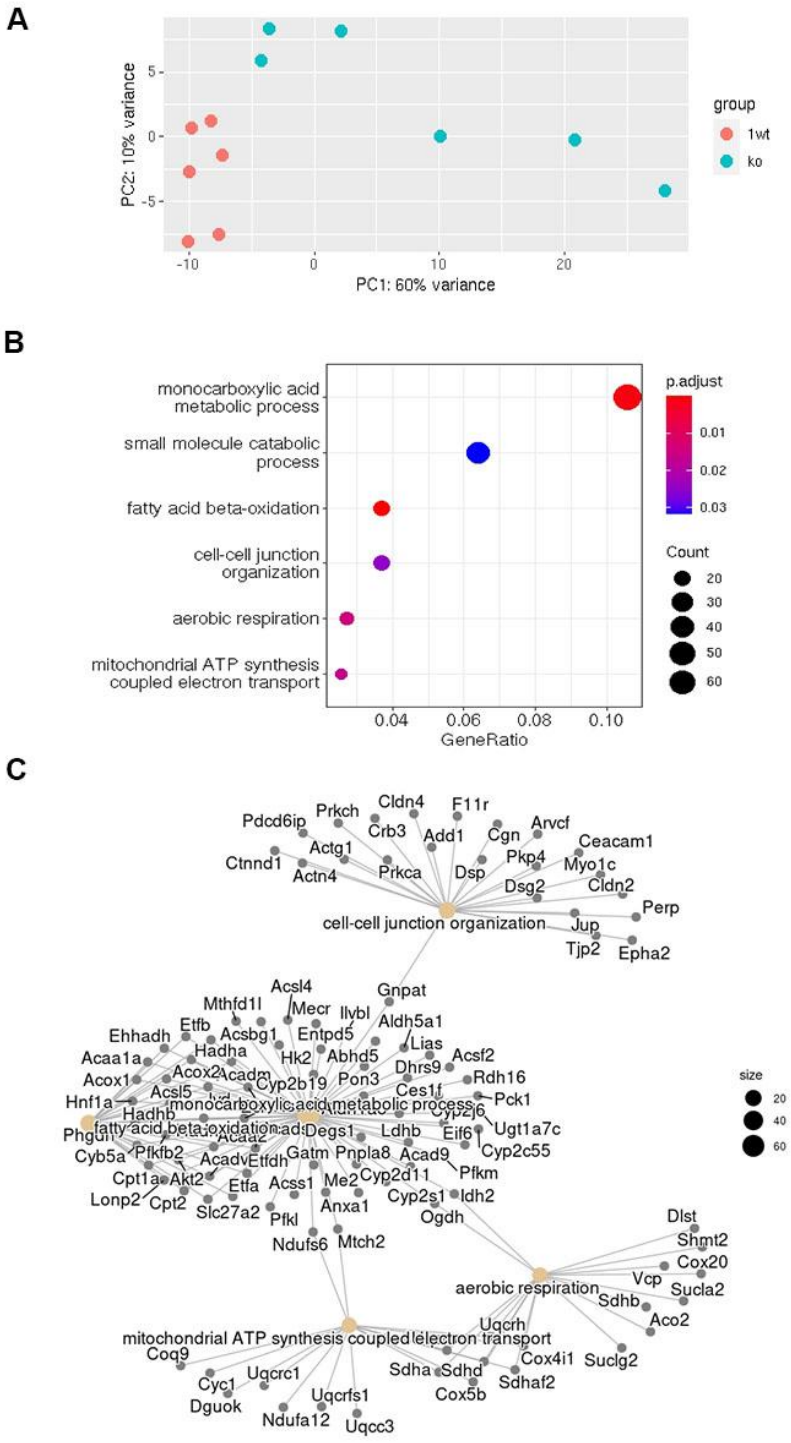

A
PC2: 10% variance
PC1: 60% variance
group
1wt
ko
B
monocarboxylic acid metabolic process
small molecule catabolic process
fatty acid beta-oxidation
cell-cell junction organization
aerobic respiration
mitochondrial ATP synthesis coupled electron transport
GeneRatio
p.adjust
Count
C
cell-cell junction organization
monocarboxylic acid metabolic process
fatty acid beta-oxidation
aerobic respiration
mitochondrial ATP synthesis coupled electron transport
size

Figure 3.17 Proteomics data reveals new avenues to study. **(A)** KEGG pathway analysis to create a PCA plot of protein expression between WT and claudin-7 KO samples. **(B)** GO-BP analysis. **(C)** Cnet plot depicting interconnected network of GO-BP analysis outcome and associated proteins.

Table 3.1 List of antibodies

Antibody	Species	Company (Catalog #)	Dilution
Primary			
Activated Notch	Rabbit	Abcam (ab8925)	1:1000 (WB)
Carbonic Anhydrase II	Rat	Novus Biologicals (MAB-2184)	1:100 (IF) 1:1000 (WB)
Chromogranin A	Mouse	Abcam (ab199014)	1:100 (IF)
Claudin-7	Mouse	Invitrogen (37-4800)	1:100 (IF)
Claudin-7	Rabbit	Immuno-Biological Laboratories (18875)	1:100 (IF) 1:750 (WB)
DCAMKL1	Rabbit	Abcam (ab37994)	1:100 (IF)
GAPDH	Rabbit	Cell Signaling (2118S)	1:2000 (WB)
Ki67	Rabbit	Cell Signaling (9129S)	1:100 (IF)
OLFM4	Rabbit	Novus Biologicals (NBP2-24535)	1:200 (IHC) 1:1000 (WB)
p-YAP (Ser127)	Rabbit	Cell Signaling (4911S)	1:1000 (WB)
Total Notch	Rabbit	Cell Signaling (3608S)	1:1000 (WB)
YAP	Rabbit	Cell Signaling (14074S)	1:200 (IHC) 1:750 (WB)
Secondary			
HRP Conjugate	Anti-Mouse	Promega (W402B)	1:2500 (WB)
HRP Conjugate	Anti-Rabbit	Promega (W401B)	1:2500 (WB)
Cy3	Anti-Mouse	Jackson Immunoresearch (715-165-150)	1:400 (IF)
Cy3	Anti-Rabbit	Jackson Immunoresearch (111-165-003)	1:400 (IF)
Cy3	Anti-Rat	Jackson Immunoresearch (112-165-003)	1:400 (IF)
FITC	Anti-Mouse	Jackson Immunoresearch (115-095-003)	1:400 (IF)
FITC	Anti-Rabbit	Jackson Immunoresearch (711-095-152)	1:400 (IF)

Table 3.2 List of primer sequences

Primer	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Atoh	GCCTTCCCGGACTG	TCTGTGCCATCATCGCTGTA
Chromogranin A	CAAGCTAGGAGCCCCATCTA	CACTCTGGCATTTCCATTACT
Claudin-7	TTCATTGTGGCAGGTCTTG	CCGATAAAGATGGCAGGT
DII4	TATTGGGCACCAACTCGTTC	ACCAAGAGAACCTTGGATGA
FABP-1	ATGAACTTCTCCGGCAAGTACC	CTGACACCCCCTTGATGTCC
GAPDH	CTTTGTCAAGCTCATTTCCTG	TCTTGCTCAGTGTCCTTGC
Hes1	GCTCACTTCGGACTCCATATG	GCTACGCATTTTACGGGTAGC
Hes2	CAGGGTCACTCCAAG	GAATCACTCCAATTTCCT
Hopx	GAGGACCAGGTGGAGATCCT	TCCGTAACAGATCTGCATTCC
Lgr5	ACCCGCCAGTCTCCTACATC	GCATCTAGGCGCAGGGATTG
Prom1	CACTCCTGACTGAAACACCAAAGC	TGCCATCCAGGTCTGAGAATGC

CHAPTER IV: NANOARCHITECTURE AND MOLECULAR INTERACTIONS OF INTESTINAL EPITHELIAL CELL JUNCTION PROTEINS REVEALED BY SUPER-RESOLUTION MICROSCOPY

This chapter is modified from Naser et al., Nanoarchitecture and Molecular Interactions of Epithelial Cell Junction Proteins Revealed by Super-Resolution Microscopy. Annals of the New York Academy of Sciences, 2022 July 12; 1-13. doi:10.1111/nyas.14855.

A. Summary

Epithelial cells are polarized with defined apical tight junctions (TJs), lateral adherens junctions (AJs), and basal integrin-matrix interactions. However, it is increasingly recognized that their characteristic resident proteins can be found in each other's territories with previously unrecognized functions. This study presents the nanoarchitecture and nano-colocalization of cell junction proteins in culture and in tissue by Stochastic Optical Reconstruction Microscopy (STORM). The Z-axial view of non-cancerous MDCK-II and PZ-HPV-7 cell-cell junctions resolved β-catenin and $p120^{ctn}$ localizations into milieu of TJ and AJ with $p120^{ctn}$ being apical to β-catenin and co-localizing with TJ protein claudin-7. Towards the basal direction, $p120^{ctn}$ and β-catenin become colocalized. This topography is lost in isogenic Ras-transformed MDCK cells and cancerous PC3 cells, in that $p120^{ctn}$ localization becomes basal to β-catenin. Interestingly, claudin-7 knockout in mice also altered the polarity of $p120^{ctn}$ to β-catenin localization, like that seen with normal to cancer cell phenotypic transformation. Claudin-7 deletion additionally redistributes and re-localizes other cell junction proteins including claudin-1, ZO-1, integrin α2, EpCAM, and FAK. Integrin α2 and FAK were surprisingly observed at apical-lateral compartment. Therefore,

STORM revealed the regional nanoarchitecture of cellular junctions that were previously unavailable, providing new insights in their potential trans-compartmental modulation of protein functions.

B. Introduction

Though it is one of the most common microscopic techniques, conventional light microscopy has a limited temporal resolution of 200–300 nm constrained by its diffraction limit (Xu et al. 2017). Owing to this limitation, two molecules can appear to be colocalized even if they are separated by hundreds of nanometers. These distances are not negligible, as they could have important implications when considering whether proteins are in sufficient proximity for molecular interactions. Super-resolution microscopy (SRM) techniques such as stochastic optical reconstruction microscopy (STORM) offer an experimental strategy to study molecules beyond the light microscopy diffraction limit, to visualize their localization and positional relationships. In STORM, individual fluorophores are repeatedly photo-activated, imaged, and photobleached utilizing photo-switchable fluorophores (Lin et al. 2018). A super-resolution image is then reconstructed by merging all the single molecule positions (Quang et. Al. 2015). This technique allows for a lateral (x,y) resolution of 20 nm and an axial (z) resolution of 50 nm (Xu et al. 2017). STORM is capable of multi-color imaging, enabling multiple proteins to be visualized within a single image Tam et al. 2015). Additionally, STORM offers superior resolution power with reduced phototoxicity, when compared to other SRM technologies (Hell and Wichmann, 1994; Gustaffson, 2000; Hein et al. 2008; Demmerle et al. 2017).

Cell–cell junctions link adjacent epithelial cells to each other and are vital for many physiological processes, such as tissue homeostasis, barrier functions, cytoskeleton regulation, cell

adhesion, and signaling (Friedl and Mayor, 2017; Slysmans et al. 2017; Garcia et al., 2018). Cell–cell junctions include tight junctions (TJs), adherens junctions (AJs), gap junctions (GJs), and desmosomes (Garcia et al. 2018). TJs, also called zonula occludens, are the most apical component of cell–cell contact regions; they help maintain tissue homeostasis and cell polarity through barrier and fence functions (Lu et al. 2013; Wang et al. 2018; Wang et al. 2019). The catenin family of proteins plays an important role within cell–cell junctions through interactions with cadherin molecules, especially by contributing to AJs and the underlying actin cytoskeleton (Nagafuchi, 2001).

Use of astigmatism-based three-dimensional SRM has demonstrated close proximity of cadherins to β-catenin and the adherens protein $p120^{ctn}$ (also called catenin delta-1) in normal cells (Bertocchi et al. 2017). Through cytoskeletal interactions, catenins function to regulate and coordinate cell–cell adhesion, as well as to modulate small Rho GTPases that induce numerous downstream signaling events (Reynolds, 2007; Meng and Takeichi, 2009). β-catenin is a key player in the canonical WNT signaling pathway, ultimately leading to transcriptional activation of target genes; $p120^{ctn}$ largely functions in stabilizing cadherins in cellular junctions and regulating membrane trafficking (Anastasidadis and Reynolds, 2000; Tortelote et al. 2017). It is well-established that both β-catenin and $p120^{ctn}$ bind to classical cadherins, exhibiting colocalization with AJs and participating in adhesion and motility during development and tissue homeostasis (McCrea and Gu, 2010). Although studies have shown $p120^{ctn}$ colocalization with claudins to form a novel hybrid TJs with AJ organization, the influence of TJ integrity on AJ proteins has not been visualized with light microscopy methods (Nunes et al. 2006).

Claudins, major components of TJs, function in controlling paracellular flux of ions and small molecules by acting as channels or tightening the paracellular pathway (Ding et al. 2012;

Ding et al. 2013; Xing et al. 2017). Deletion of TJ protein claudin-7 in mice causes cell–matrix loosening, chronic dehydration, induction of epithelial to mesenchymal transition (EMT) and severe intestinal defects including ulcerations, inflammation, hyperplasia, and adenomas, highlighting the importance of claudin-7 in maintaining epithelial integrity (Ding et al. 2012; Ding et al. 2013; Lu et al. 2015; Xing et al. 2017; Li et al. 2018; Wang et al. 2018; Xu et al. 2019). Integrin α2, involved in regulating cell proliferation and adhesion, interacts and colocalizes with claudin-7, as seen by conventional light microscopy (Ding et al. 2012). Deletion of claudin-7 results in altered expression and localization of integrin α2 and disruption of the integrin α2/claudin-1 complex (Ding et al. 2012). Focal adhesion kinase (FAK), typically localized with integrins, is predominantly involved in cellular adhesion and cell motility (Mitra et al. 2005; Schaller, 2010). Previous studies utilizing SRM investigated the spatial architecture of focal adhesions and identified protein-specific strata that comprise these integrin-based cell adhesions; however, changes in nanoarchitecture due to loss of cell adhesion proteins has not been examined (Kanchanawong et al. 2010).

In our study here, we used Nikon- or N-STORM technology to study the nanoarchitecture and molecular interactions of selective cell junction proteins, including β-catenin and $p120^{ctn}$, in MDCK-II cells, isogenic Ras-transformed MDCK cells (MDCKf3), human prostate epithelial cells (PZ-HPV-7), and human metastatic prostate carcinoma cells (PC3). We found that STORM can reveal previously unspecified changes of topographic relationships of junctional proteins and the potential involvement of TJ protein claudin-7 in controlling the AJ protein nanoarchitectures. We also evaluate junctional proteins in a claudin-7 gene conditional knockout (cKO) mouse model.

C. Materials and Methods

C.1 Cell culture

MDCK-II, PZ-HPV-7, and PC3 cells, were purchased from ATCC (Manassas, Virginia). Ras-transformed MDCKf3 cells were generously provided by J. Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands) (Chen et al. 2000). Cells were cultured according to ATCC instructions and as described[24] and maintained at 37°C with 5% CO_2 until 80–90% confluent. Cells were split with 0.25% Trypsin-EDTA (Gibco, Gaithersburg, Maryland) and grown on #1.5 coverslips (Electron Microscopy Sciences, Hatfield, PA) prior to staining.

C.2 Mouse tissue isolation

All animal experiments and procedures were approved by the East Carolina University (ECU) Animal Care and Use Committee (IACUC) and conducted in compliance with guidelines from the National Institutes of Health and ECU on laboratory animal care and use. The generation of inducible intestinal-specific claudin-7 gene cKO mice was described previously (Xing et al. 2020).

Small intestine was isolated from 3-month-old wild type (control) and claudin-7 cKO mice. Isolated tissues were rinsed in 1× phosphate buffered saline (PBS), transferred to a labeled block containing fresh optimal cutting temperature compound (OCT), frozen with 2-methylbutane and dry ice, and stored at -80°C until sectioned.

C.3 STORM immunofluorescence

Cells on coverslips were fixed in 4% paraformaldehyde (PFA) at room temperature, then treated with fresh 0.1% sodium borohydride for seven minutes. Cells were permeabilized in 0.2% Triton X-100, incubated with 100 mM Glycine, and blocked with 10% bovine serum albumin (BSA) at 37°C. Cells were incubated with the first primary antibody for one hour, rinsed with PBS and incubated with the appropriate secondary antibody for 30 minutes (Table 4.1). This process was repeated for the second primary antibody and second secondary antibody for the double staining. Cells were post-fixed with 4% PFA. Coverslips were imaged in OXEA buffer (50 mM β-mercaptoethylamine hydrochloride, 77% PBS, 20% Sodium DL-Lactate Solution, and 3% OxyFluor) at 100X using the Nikon Ti2-E inverted microscope with an L-APPS H-TIRF attachment and 4-line LUN-F laser module. Secondary antibody colors were changed for protein pairs throughout experiments to ensure that fluorophore specificity did not have an impact on the data collected. For example, Alexa 488 goat anti-rabbit and Alexa 647 goat anti-mouse were used as secondary antibodies in one experiment, then flipped to Alexa 488 goat anti-mouse and Alexa 647 goat anti-rabbit in another experiment.

Fresh frozen intestinal wild-type (control) and claudin-7 cKO tissues were sectioned, fixed with acetone at -20°C, rinsed with PBS, and assembled in a Sequenza rack with 0.2% Triton X-100. Sections were blocked with 5% BSA, then incubated overnight with respective primary antibodies at 4°C (Table 4.1). Tissue sections were washed with PBS before one hour incubation with appropriate secondary antibodies at room temperature. Sections were washed with PBS, post-fixed with 4% PFA, washed again with PBS and mounted with Vectashield (Vector Laboratories, H-1000). Stained tissue slides were then imaged at 100× on N-STORM. Secondary antibodies were changed for protein pairs throughout experiments to ensure that fluorophore specificity did

not have an impact on the data collected. Immunostaining for claudin-7 in claudin-7 cKO tissues served as a negative control to ensure antibody and fluorophore specificity.

C.4 STORM super-resolution imaging and analysis

X, *y*, and *z* calibrations and chromatic alignments were performed as stated in the NIS-Elements Advanced Research User's Guide menu; briefly, *x*, *y*, and *z* calibrations were completed via analyzing a Z-Series data set to determine the orientation and width of identified molecules. A calibration of Gaussian width in *x* and *y*, as a function of *z* position, was generated using a piezo stage insert to translate diffraction limited spots in the *z* dimension. TetraSpeck beads (100 nm) were used to calibrate the three reporter wavelengths both individually in *x*, *y*, and *z* dimensions, as well as used to correct for chromatic and warp aberrations in the *x*, *y*, and *z* dimensions. The alignment of multicolor asymmetric point spread functions allows for z calibration and color correction in the *x*, *y*, and *z* directions.

Three sets of images were taken for each double staining combination in cells and intestinal tissue. Each image consisted of 20,000 frames (10,000 frames per channel). Raw images were collected with the 3D STORM lens to a back-thinned Princeton Instruments Pro-EM-HS EMCCD 512×512 camera and analyzed using Nikon's NIS-Elements software. Images were first analyzed for "blinks" which indicate epifluorescent switching; blink thresholds were standardized to ensure that each image was analyzed equally. Thresholding based on peak intensity height allowed the elimination of potential background staining, as well as reduction of false positive signals. Processing of blinks generated a reconstructed STORM image from which further analysis was completed.

Within NIS-Elements, the distance between protein pairs and percent of protein overlap within layers was analyzed. The distance between protein signals, referred to as the colocalization, was determined using the Intensity Profile tool. Protein signals located along the cell-cell contact were selectively chosen for distance measurement. One hundred distance measurements were taken for each image then represented in figures as the mean ± SEM. Using the Cell Count (Molecular Count) function, the exact quantification of each molecule among the Z-stack layers was determined. Complete nano-colocalization was defined as signals less than 15 nm apart, at which point the yellow signals are no longer separated into red and green signals. This was used to determine the number of colocalizations present within each cell type.

Two-dimensional snapshots at various magnifications as well as three-dimensional reconstructions of specific regions of interest, such as the cell-cell junction and the cytoplasm, were taken as representative images for each protein staining combination. Twenty Z-stacks were taken for each protein staining combination (total of one hundred twenty Z-stacks) and exported as .nd2 files for analysis of colocalization distance using ImageJ (National Institutes of Health, Bethesda, MD). In ImageJ, a 3D projection to the brightest point was created for each Z-stack and a Gaussian Blur filter was placed over the image. A representative line of best fit through the dots was created to generate a Plot Profile of peak intensities for each channel. The absolute value of the difference between the *x*-value for the peak intensity of each channel was measured as the colocalization or the distance between protein signals. Ten calculated differences for each condition were used to determine average colocalization (distance measurement) of protein pairs. Values in figures were represented as mean ± SEM.

C.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8.4.1 for Mac (GraphPad Software, La Jolla, CA). Differences between and among groups were analyzed using the unpaired Student *t* test and two-way ANOVA. Data was presented as mean ± SEM. A *p* value less than 0.05 was considered statistically significant.

D. Results

D.1 STORM technology allows for visualization of nano-localization and protein interactions

STORM technology utilizes photo-switchable fluorophores to precisely localize particles (Quang and Lenne, 2015; Tam and Merino, 2015; Xu et al. 2017). In conventional microscopy, all fluorophores are activated at once, resulting in a low-resolution image; however, the photo-switchable nature of STORM fluorophores results in high resolution images (Figure 4.1A). Figure 2.1B depicts the series of analytical events that lead to the final reconstructed STORM image. Following data acquisition, a raw STORM image is obtained from which blinks—indicating epifluorescent switching—can be discerned (Figure 4.1B). The peak intensity of these blinks is used to set a minimum threshold for what NIS-Elements will count as a signal (Figure 4.1B). The program then analyzes each frame for blinks with a peak intensity higher than the set threshold to create a reconstructed image with the precise localization of the probed proteins (Figure 4.1B). Blinks represent the certainty of a protein being present in that location. A strong blink corresponds to high probability of a protein being present; therefore, the dot representing this protein in the reconstructed STORM image will be small and pinpointed. Weaker signals will result in larger and more unresolved dots. Since STORM is a three-dimensional super-resolution imaging technique, the obtained image can be viewed in *x*, *y*, and *z* dimensions to ascertain topographical

relationships between proteins that otherwise could not be illustrated utilizing conventional microscopy.

SRM technology was utilized to determine the regional architecture of cell–cell junction protein organization in both cells and tissues. For analysis of protein nano-localization in tissue samples, Z-stacks were generated for each image in NIS-Elements. Files were imported into ImageJ and separated into 3D projections of individual channels (Figure 4.1C). A Gaussian blur filter was placed over the image and a line of best fit was used to generate a plot profile based on peak intensity (Figure 4.1C). Colocalization was calculated by taking the absolute value of the peak intensity for the red channel subtracted by the peak intensity for the green channel (Figure 4.1C).

D.2 Claudin-7 distribution and colocalization with $p120^{ctn}$ altered in cancerous cells

We sought to investigate the topographic relationship among AJ and TJ proteins. TJ protein claudin-7 was found to be co-immunostained with β-catenin in MDCK-II and PZ-HPV-7 cells but not in MDCKf3 and PC3 cells. Two-dimensional STORM revealed an altered nanoarchitecture of β-catenin and claudin-7 in MDCKf3 and PC3 cells, as the normal β-catenin and claudin-7 localization in MDCK-II and PZ-HPV-7 cells to the cell–cell junction was largely disrupted (Figure 4.2A and Figure 4.3A, left panel, bottom image shows the high magnification). High resolution depiction of cell–cell junctions demonstrated decreased protein localization at the cell junction and increased distance between protein signals in MDCKf3 and PC3 cells (Figure 4.2A and Figure 4.3A; arrows indicate the region depicted in the high-resolution insert). Quantitatively, there were significantly fewer colocalizations, as well as increased distance between signals in

MDCKf3 cells compared with MDCK-II cells (Figure 4.2, B and C); this decreased colocalization and increased distance between signals were also observed in PC3 cells (Figure 4.4, B and C).

Previous studies highlighted p120^{ctn} interaction with claudins to form a novel hybrid TJ structure (Nunes et al. 2006). We evaluated this by co-immunostaining for claudin-7 and p120^{ctn} and visualizing at the super-resolution level. As previously reported, p120^{ctn} and claudin-7 exhibited strong distribution at the cell–cell junction as well as high colocalization in MDCK-II and PZ-HPV-7 cells (Figure 4.4A and Figure 4.5A, left panel; bottom panel, high magnification; high resolution inserts demonstrate nano-colocalization of these proteins. In contrast, MDCKf3 and PC3 cells exhibited disorganized cell–cell junctions with diminished colocalization of p120^{ctn} and claudin-7 (Figure 4.4A and Figure 4.5A, right panel; bottom panel, high magnification). There were both significantly fewer colocalizations of p120^{ctn} and claudin-7 and increased average distance in MDCKf3 and PC3 cells, compared with MDCK-II and PZ-HPV7 cells (Figure 4.4, B and C, and Figure 4.5, B and C). This suggests that p120^{ctn} interaction with TJ proteins is lost in cancerous cells.

D.3 TJ and AJ protein colocalization is disrupted in claudin-7 cKO intestinal tissue

To investigate the effect of TJ protein deficiency on AJ protein organization, we next explored the nanoarchitecture of TJ and AJ proteins at the tissue level, utilizing an inducible intestine-specific claudin-7 cKO model. β-catenin and p120^{ctn} were double labeled in control and cKO intestinal tissues and examined by N-STORM. Two-dimensional imaging of β-catenin and p120^{ctn} in control and cKO intestinal tissues revealed significant differences in organization and nano-colocalization (Figure 4.6). In control tissue, β-catenin and p120^{ctn} were organized in nanoclusters along the cell–cell junction (Figure 4.6A, left panel; arrows indicate cell–cell

junctions). β-catenin and p120^{ctn} were more disorganized in claudin-7 cKO tissues, exhibiting decreased localization to the cell–cell junction (Figure 4.6A, right panel; arrowheads indicate random non-junctional distribution).

Three-dimensional imaging highlighted the localization of β-catenin and p120^{ctn} both at the cell–cell junction and in the cytoplasm (Figure 4.6B), supporting previous hypotheses that β-catenin and p120^{ctn} may participate in cellular functions in non-junctional locations (Valenta et al. 2012; McCrea and Gottardi 2016). Junctional 3D images highlighted discernible nanoclusters of β-catenin and p120^{ctn}, as well as p120^{ctn} localization to the apical side in reference to β-catenin and more basal localization in cKO tissues (Figure 4.6B, top row; arrows indicate apical localization in control whereas arrowheads indicate basolateral localization in cKO). This observation substantiated the altered nanoarchitecture of β-catenin and p120^{ctn} from non-cancerous to cancerous cells in culture, in that TJ protein disruption is sufficient to promote p120^{ctn} redistribution from apical to basal of β-catenin. Quantitative analysis revealed a significant difference in the average colocalization (distance between protein signals) between β-catenin and p120^{ctn} in control versus cKO (Figure 4.6C). These results indicate that deletion of claudin-7 in intestines disrupts the nano-colocalization of β-catenin and p120^{ctn} at the cell–cell junction and shifts p120^{ctn} position from apical to basal of β-catenin.

Previous studies demonstrated that the TJ structure remains intact in claudin-7 KO intestinal epithelial cells as revealed by transepithelial resistance (TER) measurement (Ding et al. 2012). To determine whether the TJ protein organization also remains unaffected in claudin-7 cKO intestinal epithelia, the effect of claudin-7 deletion on other TJ proteins was examined by double labeling claudin-1 and ZO-1 in intestinal tissue using the STORM technology. Claudin-1 is vital in maintaining TJ integrity as its deletion in mice leads to severe water loss and dehydration

resulting in death within one day (Furuse et al. 2002; Bergmann et al. 2020). ZO-1 acts as a scaffolding protein to cross-link and anchor TJ proteins to the actin cytoskeleton (Van Itallie et al. 2009). As anticipated, control tissues exhibited colocalization of ZO-1 and claudin-1 along the cell membrane (Figure 4.7A, left panel; arrows yellow signals indicate colocalization). In contrast, significantly less colocalization of ZO-1 and claudin-1 and disrupted cell–cell junctions were observed in claudin-7 cKO tissues (Figure 4.7A, right panel; arrowheads, red signal clusters are separated from the green signals).

Three-dimensional examination of cell junctions versus the cytoplasm revealed similar findings. ZO-1 and claudin-1 were highly nano-colocalized at the cell–cell junction of control tissues, as indicated by concentrated clustering and yellow color due to signal overlap; in contrast, in the cKO intestinal tissues, ZO-1 and claudin-1 were found frequently separated from each other (Figure 4.7B, top row). Visually, there was also increased distance between ZO-1 and claudin-1 signals in cKO tissues (Figure 4.7A), which was confirmed by quantification of distance measurements (Figure 4.7C). In addition, expression of ZO-1 was increased in the cytoplasm of claudin-7 cKO tissues (Figure 4.7C, bottom row), suggesting that claudin-7 cKO may cause altered localization of ZO-1, leading to increased concentration in the cytoplasm.

D.4 Claudin-7 cKO leads to disruption of integrin α2, FAK, and EpCAM

The distance hierarchical relationship among β-catenin, $p120^{ctn}$, claudin-7, claudin-1, and ZO-1 raised questions about effects on other junctional protein topographical organization. Our previous study demonstrated that claudin-7 is not only localized at apical TJs but also distributed along the basolateral surface where it interacts with integrin $\alpha2$[25]. Deletion of claudin-7 in mouse intestines disrupts cell–matrix interactions (Ding et al. 2012).

Therefore, we stained control and claudin-7 cKO tissues for integrin α2, claudin-7, FAK, and epithelial cellular adhesion molecule (EpCAM). Claudin-7 was highly colocalized with integrin α2 in the control intestinal tissue, measured to be on average 40.17 ± 19.37 nm apart (Figure 4.8A, left panel; arrows indicate colocalization at cell-cell junctions). Similarly, claudin-7 colocalized with FAK, with peak intensity measurements of 49.42 ± 35.50 nm apart (Figure 4.8B, left panel; arrows indicate colocalization at cell–cell junctions). In contrast, colocalization of integrin α2 and FAK was disrupted in claudin-7 cKO (Figure 4.8, A and B, right panel). Three-dimensional analysis confirmed discontinuous integrin α2 distribution at the cell junction in cKO intestinal tissues (Figure 4.8C, top panel). Control tissues exhibited relatively uniform integrin α2 along the junction; however, there was more clustering of integrin α2 signal in the junction of cKO tissues (Figure 4.8C, top panel). Integrin α2 was present in the cytoplasm in both control and cKO (Figure 4.8C, bottom panel). FAK was localized to the cell–cell junction in relation to claudin-7 (Figure 4.8D, top panel); cKO tissues exhibited significantly increased cytoplasmic FAK and random distribution within the cells (Figure 4.8, B and D, right panel).

Investigation into the interaction between FAK and integrin α2 yielded similar results. Control tissues exhibited highly organized protein localization along the cell–cell junction (Figure 4.9A, left panel; arrows indicate cell–cell junctions). However, colocalization of integrin α2 and FAK was disrupted in cKO tissues (Figure 4.9A, right panel). Three-dimensional STORM revealed decreased FAK expression along the cell junction in cKO intestines, as well as increased FAK expression in the cytoplasm compared with control tissues (Figure 4.9B). The distance between integrin α2 and FAK signals was found to be significantly higher in claudin-7 cKO tissue (Figure 4.9C).

Additionally, integrin α2 and EpCAM were highly nano-colocalized at the cell junction in control tissues (Figure 4.10A, left panel; arrows depict colocalization at the junction). In contrast, cKO tissues exhibit disorganized integrin α2 colocalization (Figure 4.10A, right panel) and EpCAM. Similar to two-dimensional examination, three-dimensional analysis of integrin α2 and EpCAM in control tissue showed integrin α2 localized closely with EpCAM at the cell–cell junction; however, the two proteins were separated (both above and below) in cKO tissues (Figure 4.10B, top panel). Additionally, there was increased cytoplasmic integrin α2 and EpCAM in cKO intestinal tissues, compared with control tissue (Figure 4.10B, bottom panel). Analysis of nano-colocalization also demonstrated a significantly increased distance between integrin α2 and EpCAM in claudin-7 cKO intestines (Figure 4.10C).

E. Discussion

N-STORM investigation into the protein localization in the cell–cell contact area revealed, for the first time, claudin-7 effects on the nanoarchitecture of other junctional proteins (Figure 4.11). In intestinal tissues, we found β-catenin and $p120^{ctn}$ to be highly nano-colocalized at the cell–cell junction; however, localization at the cell junction was disrupted in claudin-7 cKO tissue. Discernable nanoclusters of β-catenin and $p120^{ctn}$ signals in the cytoplasm further support the idea that these proteins play other important roles outside of the cell junction (Anastasidadis and Reynolds, 2000; Valenta et al. 2012; Daniel, 2007). β-catenin is involved in WNT signaling from the plasma membrane to the nucleus (McCrea and Gottardi, 2016); this function explains the ubiquitous expression of β-catenin throughout the cell, especially since this distribution was not associated with $p120^{ctn}$. Previous studies reported that $p120^{ctn}$ may regulate WNT signaling–mediated nuclear transcription through Kaiso (Daniel, 2007). Further studies are needed to

decipher the exact nature of p120^{ctn}'s role and how it performs functions with β-catenin interactions at the cell–cell junction, but without it in WNT signaling.

Interestingly, we observed a topographical relationship between β-catenin and p120^{ctn} in which p120^{ctn} is localized apically of β-catenin. For mouse intestinal epithelial tissues, where p120^{ctn} is not down regulated as it is in MDCKf3 and PC3 cells, p120^{ctn} redistribution to the basal side in reference to β-catenin when claudin-7 was deleted in the cKO mice showed that the removal of the TJ integral membrane protein claudin-7 is sufficient to alter the p120^{ctn}–β-catenin topographical relationship. Currently, it is not known whether this displacement of p120^{ctn} contributes to the invasive properties of cancerous cells. The disorganization of p120^{ctn} in claudin-7 cKO mouse intestinal epithelium suggests that claudin-7 is essential in maintaining topographical relationships between AJ proteins and that p120^{ctn} likely has different pools, with some being intimately involved in TJ organization and function. Smalley-Freed *et al*. reported that p120^{ctn} conditional deletion leads to abnormal barrier function, increased inflammation, and disrupted epithelial homeostasis and survival in the intestine (Smalley-Freed et al. 2010). It is possible that the reduction of p120^{ctn} expression or its altered distribution in cancerous cells may contribute to the weakening of both TJs and AJs during epithelial-to-mesenchymal transition (EMT). We have previously reported that TJs form before AJs in Ras-transformed epithelial cells when the cancer-like cells reestablish cell–cell junctions after PD98095 treatment (Chen et al. 2000). Additionally, downregulation of claudin-7 induces colorectal cancer metastasis and invasion through promotion of EMT (Wang et al. 2019). Taken together, our results are consistent with the literature and highlight the potential roles of TJ proteins in influencing the nanoarchitecture and localization of AJ proteins in cancerous cells.

Previous studies have suggested an important interaction between claudin-7 and integrin α2 to form a functional complex (Ding et al. 2012). There is increasing evidence suggesting that integrins accumulate in the apical compartment in which they interact with TJ proteins and connexins (Peterson and Koval, 2021). Our use of N-STORM confirmed the colocalization of claudin-7 and integrin α2 and examined the nanoscale molecular architecture of integrin α2. Among claudin-7 cKO tissues, integrin α2 exhibited altered expression and nonhomogeneous distribution, as integrin α2 was largely found outside of the junction. Similarly, EpCAM showed clear dissociation at the cell–cell junction in claudin-7 cKO intestines. Focal adhesion kinase (FAK) organization was also disrupted in claudin-7 cKO intestinal tissues. Investigation into the interaction of claudin-7 with focal adhesion structures suggested localization of claudin-7 between integrin α2 and FAK, with preference towards the side of integrin α2 (Figure 4.8, 40.17 ± 19.37 nm versus 49.42 ± 35.50 nm). It is worth indicating that not only integrin α2 and EpCAM, but some FAK, also localized at apical side of cell junctions and interacted with claudin-7, a result owing to the fact that N-STORM imaging can only be focused on the apical region. In control intestinal tissues, claudin-1 and ZO-1 are highly colocalized at the cell-cell junction; however, deletion of claudin-7 resulted in significantly increased distance between STORM signals. These results suggest that claudin-7 may play a regulatory role in cell–cell junction composition, as claudin-7 cKO leads to displacement of many cell junction proteins.

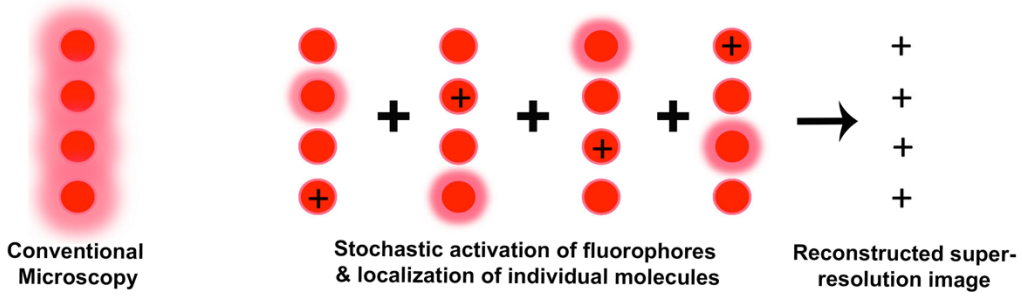
Use of STORM technology to reveal the regional architecture of cell–cell junction proteins in tissues, as well as comparison of non-cancerous and cancerous cells, is an example of how new technology can improve understanding of protein interactions and functions. In addition, previous data can be re-examined using SRM technology. However, although STORM technology is very powerful, it will not be able to fully eliminate other methods such as the protein complex

immunoprecipitation assays. STORM can evaluate proteins *in situ* and the close localization of two proteins at the nanoscale level. On the other hand, an immunoprecipitation assay requires disruption of the cell membrane and relies on the physical interaction between the molecules, which does not necessarily mean that only two proteins are interacting with each other. We envisage that STORM technology will be a complementary method to other biochemical assays.

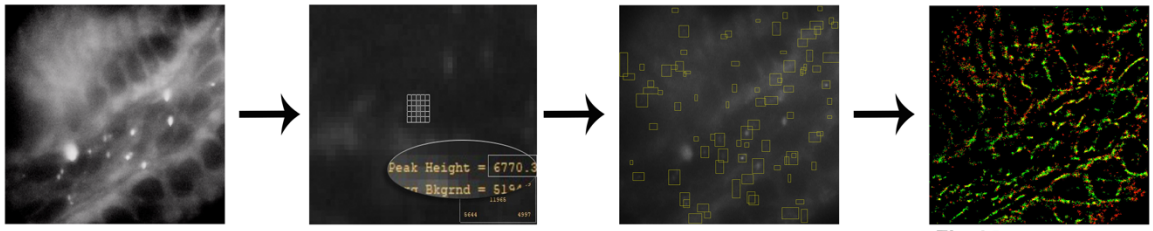
F. Acknowledgements

The authors thank Joani Zary Oswald and Christi Boykin for their technical assistance. This study was funded by NIH DK103166 and NIH GM146257.

A



B



C *Tissue Analysis:*

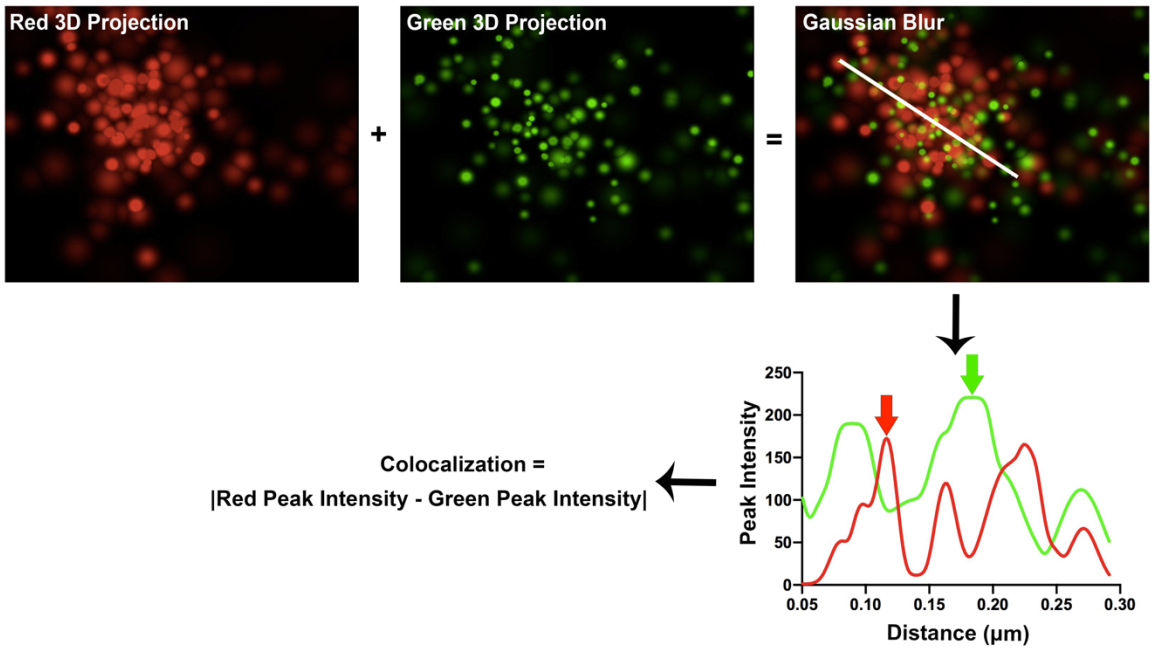


Figure 4.1 Graphic illustrating STORM technology and analyses. (A) Graphic comparison between conventional microscopy and STORM technology. **(B)** Process through which the final reconstructed STORM image is obtained. The representative image is from healthy intestinal tissue in which red represents integrin α2 signal and green marks focal adhesion kinase signal. **(C)** Workflow for analysis completed in tissue-based experiments.

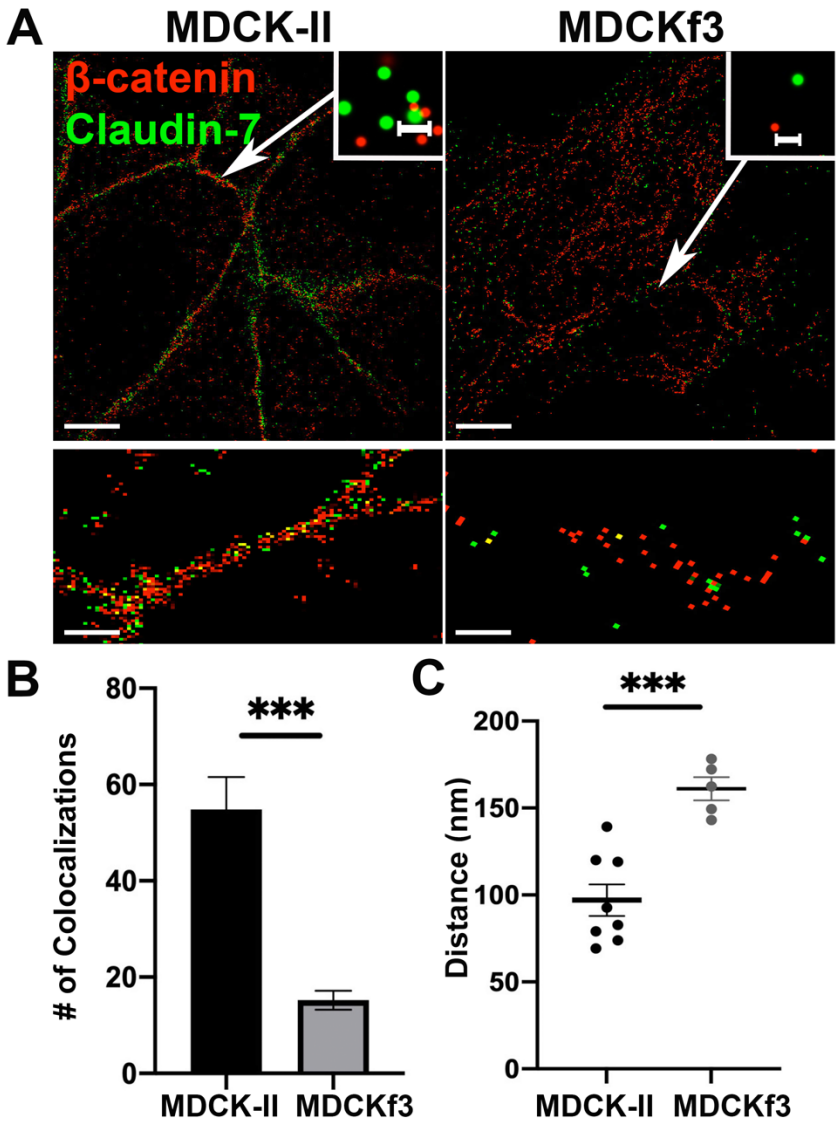

A
MDCK-II
MDCKf3
β-catenin
Claudin-7
B
# of Colocalizations
80
60
40
20
0
***
MDCK-II
MDCKf3
C
Distance (nm)
200
150
100
50
0
***
MDCK-II
MDCKf3

Figure 4.2 MDCKf3 cells exhibit altered distribution and reduced nano-colocalization of β-catenin and claudin-7. **(A)** β-catenin and claudin-7 in MDCK-II and MDCKf3. **(B)** Quantification of colocalizations between β-catenin and claudin-7. **(C)** Quantification of average distance between β-catenin and claudin-7. The distance between signals and number of colocalizations were compared by unpaired t test and plotted as mean ± SEM. Inserts of the region indicated by the arrow demonstrate separation of protein signals at high resolution. Scale bars: Top = 7 μm; Bottom = 500 nm; Inserts = 20 nm. n = 9. ***$p \leq 0.001$.

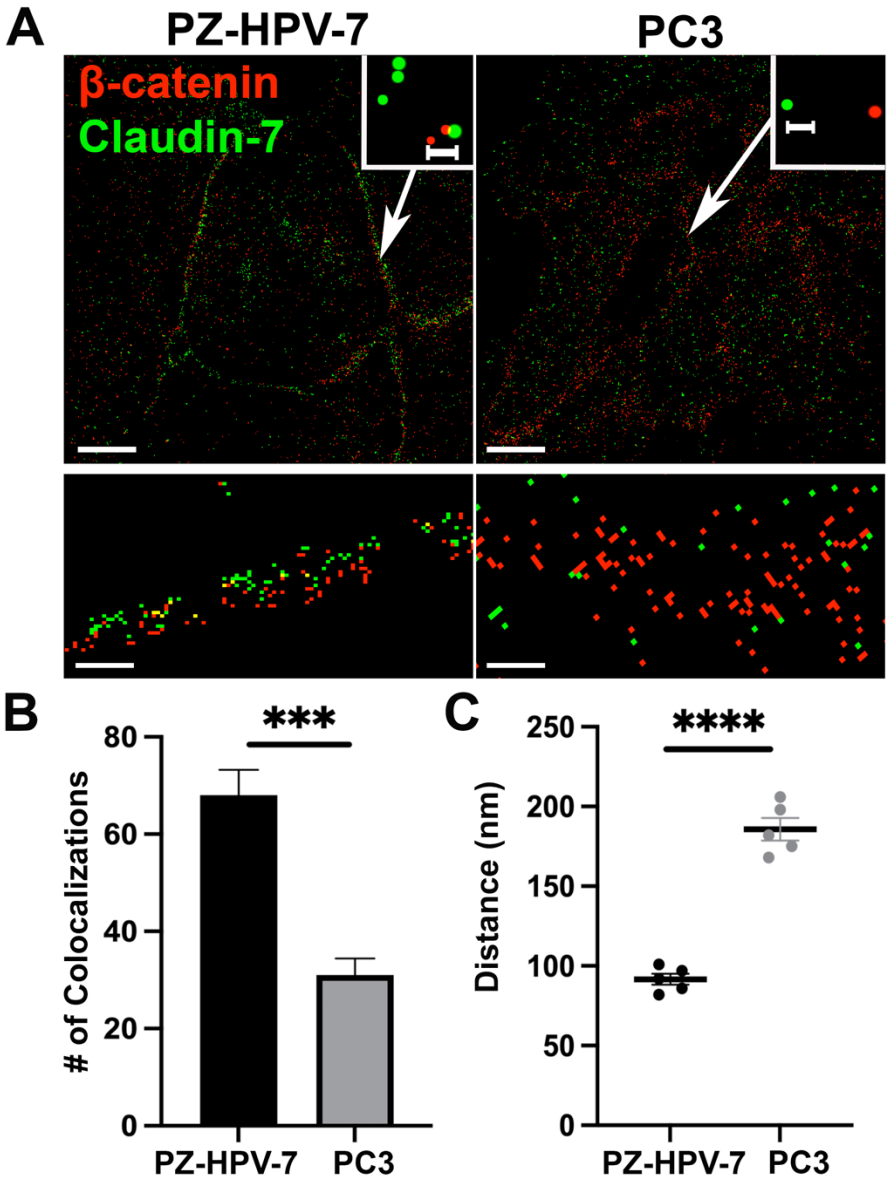

A
PZ-HPV-7
PC3
β-catenin
Claudin-7
B
***
# of Colocalizations
80
60
40
20
0
PZ-HPV-7
PC3
C
****
Distance (nm)
250
200
150
100
50
0
PZ-HPV-7
PC3

Figure 4.3 Altered distribution and reduced nano-colocalization of β-catenin and claudin-7 in cancerous PC3 cells. **(A)** β-catenin and claudin-7 in PZ-HPV-7 and PC3 cells. **(B)** Quantification of colocalizations between β-catenin and claudin-7. **(C)** Quantification of average distance between β-catenin and claudin-7. The distance between signals and number of colocalizations were compared by unpaired t test and plotted as mean ± SEM. Inserts of the region indicated by the arrow demonstrate separation of protein signals at high resolution. Scale bars: Top = 7 μm; Bottom = 500 nm; Inserts = 20 nm. n = 9. **** $p \leq 0.0001$, ***$p \leq 0.001$.

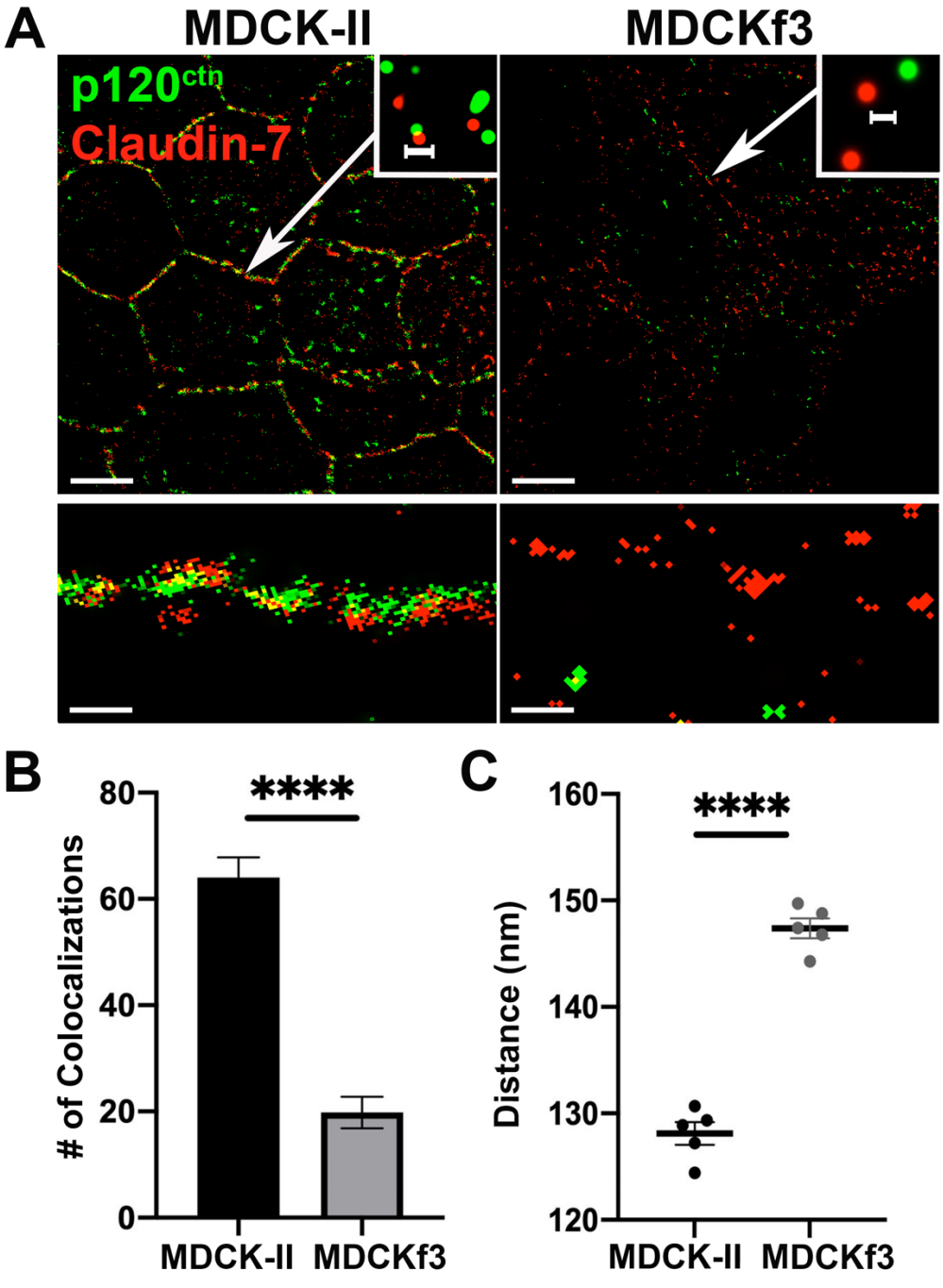

A
MDCK-II
MDCKf3
p120ctn
Claudin-7
B
# of Colocalizations
80
60
40
20
0
****
MDCK-II
MDCKf3
C
Distance (nm)
160
150
140
130
120
****
MDCK-II
MDCKf3

Figure 4.4 Disrupted distribution and reduced nano-colocalization of p120^{ctn} and claudin-7 in MDCKf3 cells. **(A)** p120^{ctn} and claudin-7 in MDCK-II and MDCKf3 cells. **(B)** Quantification of colocalizations. **(C)** Quantification of average distance. The distance between signals and number of colocalizations were compared by unpaired t test and plotted as mean ± SEM. Inserts of the region indicated by the arrow demonstrate separation of protein signals at high resolution. Scale bars: Top = 7 μm; Bottom = 500 nm; Inserts = 20 nm. n = 9. **** $p \leq 0.0001$.

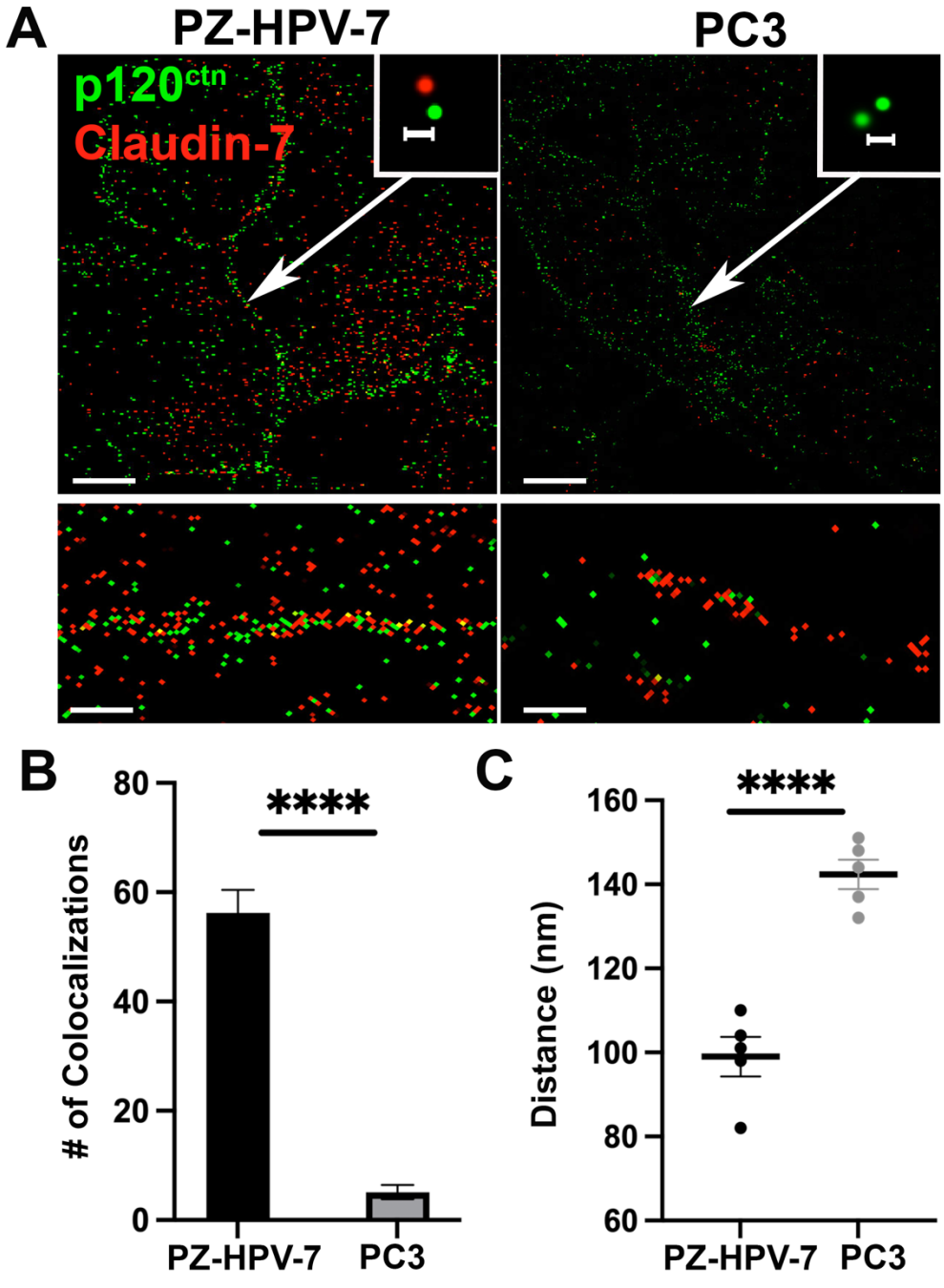

A
PZ-HPV-7
PC3
p120ctn
Claudin-7
B
# of Colocalizations
80
60
40
20
0
****
PZ-HPV-7
PC3
C
Distance (nm)
160
140
120
100
80
60
****
PZ-HPV-7
PC3

Figure 4.5 PC3 cells exhibit altered distribution and reduced nano-colocalization of $p120^{ctn}$ and claudin-7. (**A**) $p120^{ctn}$ and claudin-7 in PZ-HPV-7 and PC3 cells. **(B)** Quantification of colocalizations. **(C)** Quantification of average distance. The distance between signals and number of colocalizations were compared by unpaired t test and plotted as mean ± SEM. Inserts of the region indicated by the arrow demonstrate separation of protein signals at high resolution. Scale bars: Top = 7 μm; Bottom = 500 nm; Inserts = 20 nm. n = 9. **** $p \leq 0.0001$.

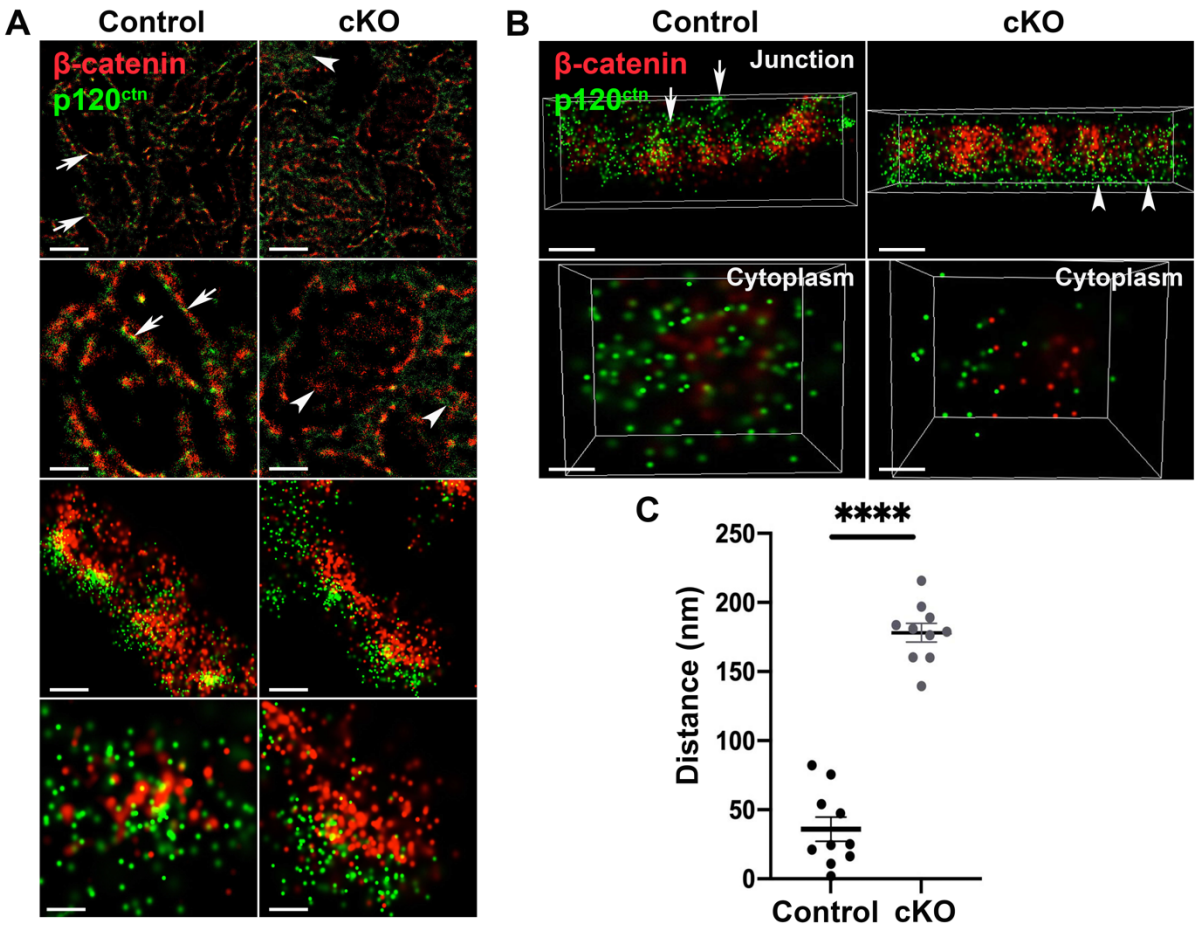

A
Control
cKO
β-catenin
p120ctn
B
Control
cKO
β-catenin
p120ctn
Junction
Cytoplasm
Cytoplasm
C
****
Distance (nm)
250
200
150
100
50
0
Control
cKO

Figure 4.6 Significantly reduced colocalization of β-catenin and p120^{ctn} in claudin-7 cKO intestinal tissues. (A) Two-dimensional snapshots of β-catenin and p120^{ctn}. Arrows: cell–cell junction; Arrowheads: random non-junctional distribution. **(B)** Three-dimensional snapshots of β-catenin and p120^{ctn} at the cell junction and cytoplasm. Arrows: apical localization of p120^{ctn}; Arrowheads: basolateral localization of p120^{ctn} in claudin-7 knockout intestines. **(C)** Quantification of average distance. The distance between signals was compared by unpaired *t* test and plotted as mean ±. SEM. 2D scale bars (top to bottom) = 5 μm, 2.5 μm, 500 nm, 250 nm; 3D scale bars = 100 px. n = 10. ****p ≤ 0.0001.

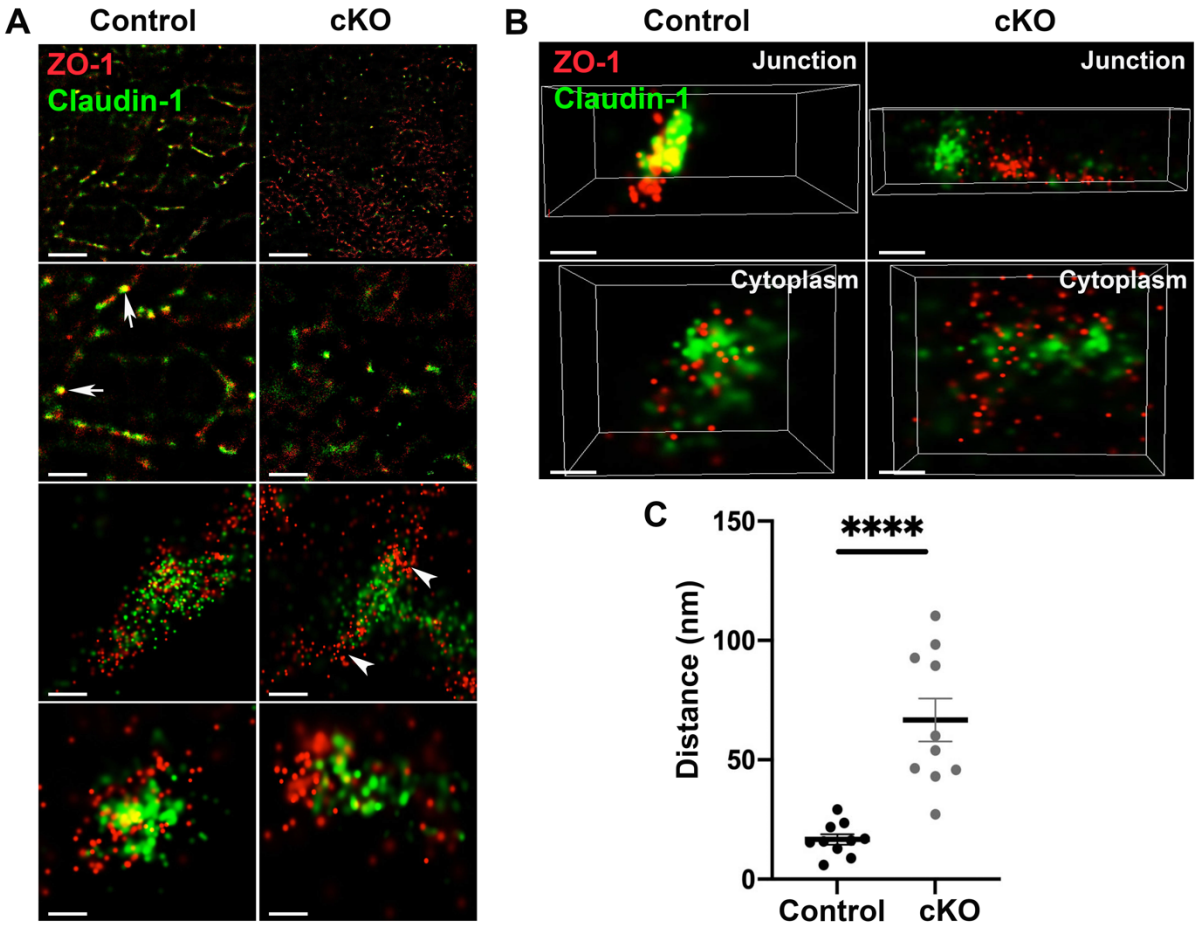

A
Control
cKO
ZO-1
Claudin-1
B
Control
cKO
ZO-1
Claudin-1
Junction
Junction
Cytoplasm
Cytoplasm
C
****
Distance (nm)
150
100
50
0
Control
cKO

Figure 4.7 Claudin-7 cKO intestinal tissues exhibit disrupted claudin-1 and ZO-1 localization. (A) Two-dimensional snapshots of ZO-1 and claudin-1; arrows: colocalization of ZO-1 and claudin-1; arrowheads: displacement of ZO-1 from claudin-1. **(B)** Three-dimensional snapshots of ZO-1 and claudin-1 at the junction and cytoplasm. **(C)** Quantification of average distance. The distance between signals was compared by unpaired *t* test and plotted as mean ±. SEM. 2D scale bars (top to bottom) = 5 μm, 2.5 μm, 500 nm, 250 nm; 3D scale bars = 100 px. n = 10. ****$p \leq 0.0001$.

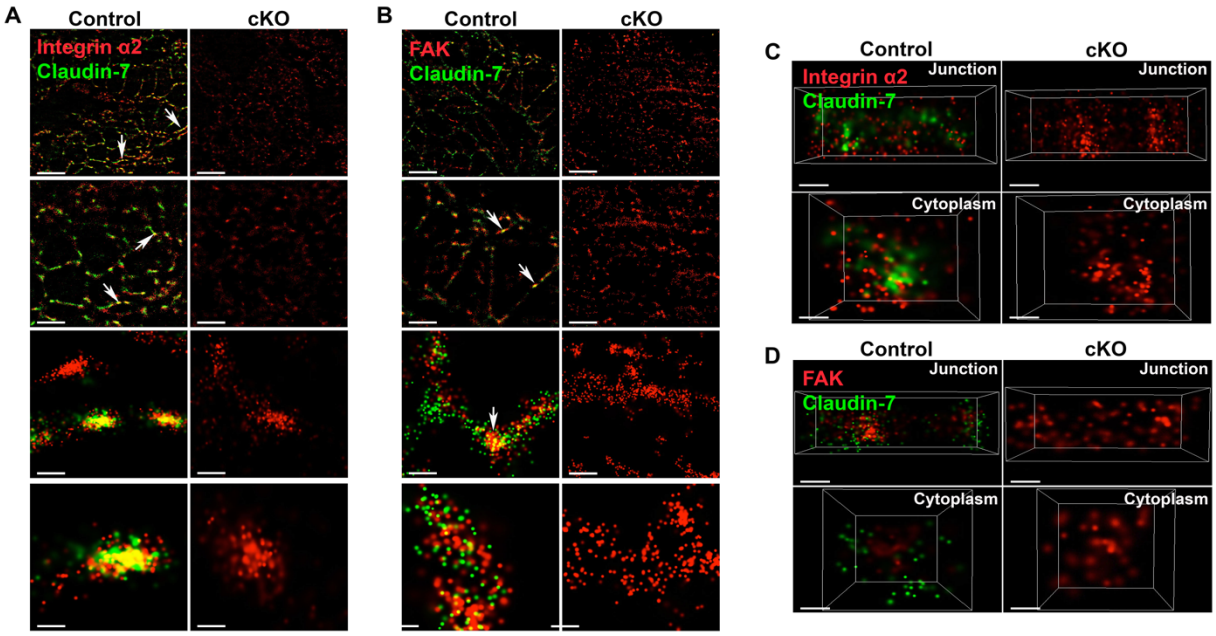

A
Control
cKO
Integrin α2
Claudin-7
B
Control
cKO
FAK
Claudin-7
C
Control
cKO
Integrin α2
Claudin-7
Junction
Junction
Cytoplasm
Cytoplasm
D
Control
cKO
FAK
Claudin-7
Junction
Junction
Cytoplasm
Cytoplasm

Figure 4.8 Disruption of integrin α2 and FAK organization due to loss of claudin-7 in intestine. (**A**) Two-dimensional snapshots of integrin α2 and claudin-7. (**B**) Two-dimensional snapshots of FAK and claudin-7. (**C**) Three-dimensional snapshots of integrin α2 and claudin-7. (**D**) Three-dimensional snapshots of FAK and claudin-7. Arrows: cell–cell junctions. 2D scale bars (top to bottom) = 5 μm, 2.5 μm, 500 nm, 250 nm; 3D scale bars = 100 px. n = 10.

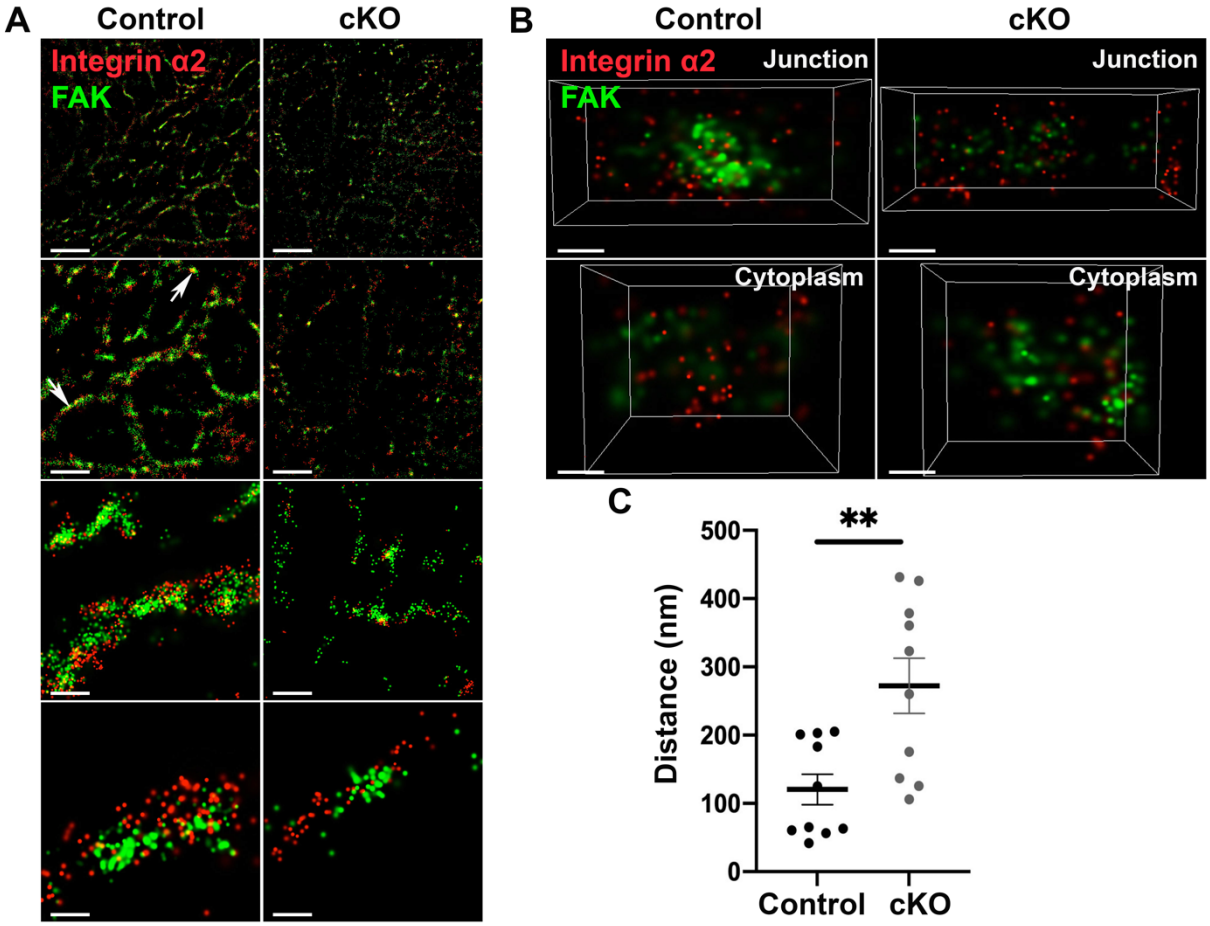

A
Control
cKO
Integrin α2
FAK
B
Control
cKO
Integrin α2
FAK
Junction
Junction
Cytoplasm
Cytoplasm
C
**
Distance (nm)
500
400
300
200
100
0
Control
cKO

Figure 4.9 Altered integrin α2 and FAK localization in claudin-7 cKO intestinal tissues. (A) Two-dimensional snapshots of integrin α2 and FAK. **(B)** Three-dimensional snapshots of integrin α2 and FAK. **(C)** Quantification of average distance between integrin α2 and FAK. The distance between signals was compared by unpaired *t* test and plotted as mean ± SEM. Arrows: cell–cell junctions; Arrowheads: separation of signals at cell junction. 2D scale bars (top to bottom) = 5 μm, 2.5 μm, 500 nm, 250 nm; 3D scale bars = 100 px. n = 10. **p ≤ 0.01.

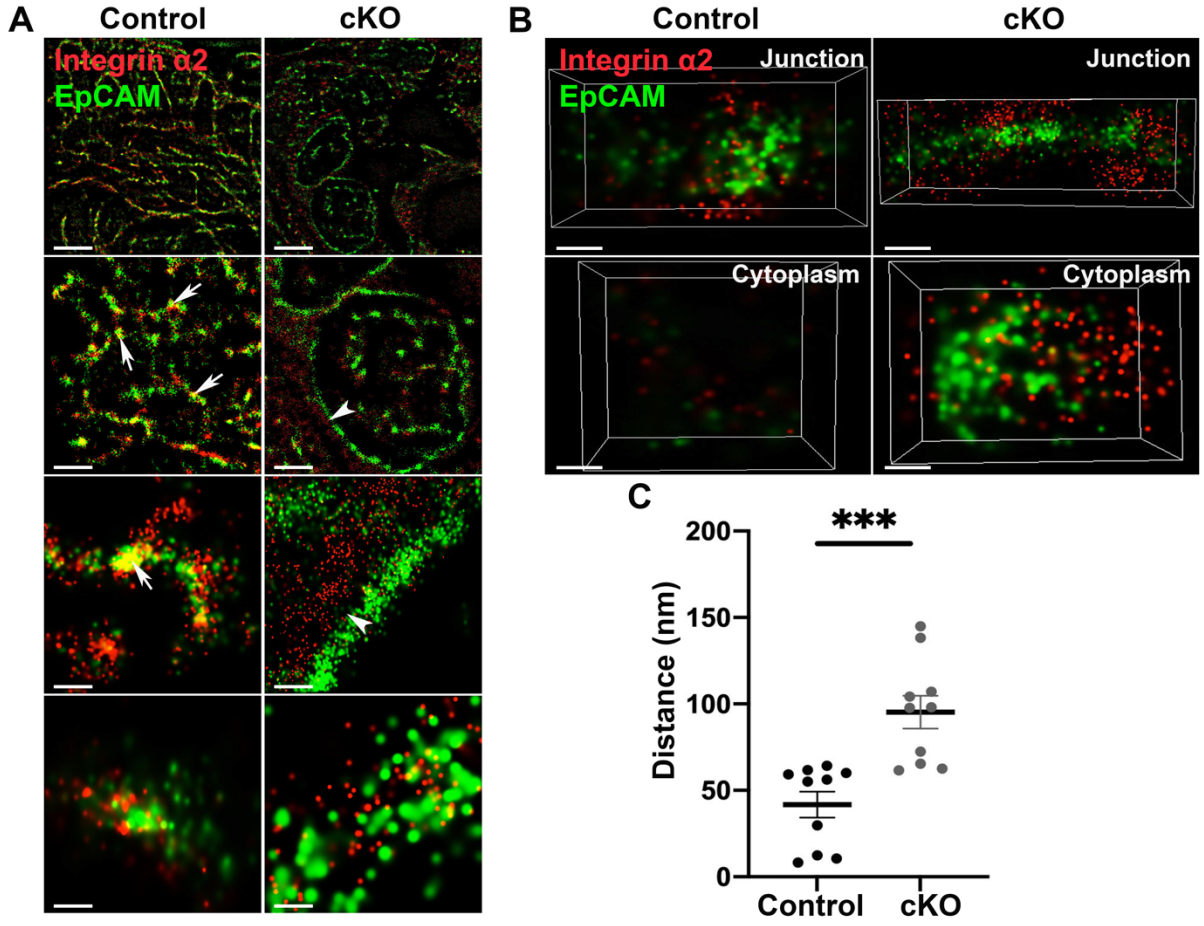

A
Control
cKO
Integrin α2
EpCAM
B
Control
cKO
Integrin α2
EpCAM
Junction
Junction
Cytoplasm
Cytoplasm
C
***
Distance (nm)
200
150
100
50
0
Control
cKO

Figure 4.10 Claudin-7 cKO intestinal tissues exhibit disrupted cell-cell junctions and altered integrin α2 and EpCAM distribution. **(A)** Two-dimensional snapshots of integrin α2 and EpCAM. **(B)** Three-dimensional snapshots of integrin α2 and EpCAM. **(C)** Quantification of average distance between integrin α2 and EpCAM. The distance between signals was compared by unpaired *t* test and plotted as mean ± SEM. Arrows: cell–cell junctions; Arrowheads: separation of signals at cell junction. 2D scale bars (top to bottom) = 5 μm, 2.5 μm, 500 nm, 250 nm; 3D scale bars = 100 px. n = 10. ***$p \leq 0.001$.

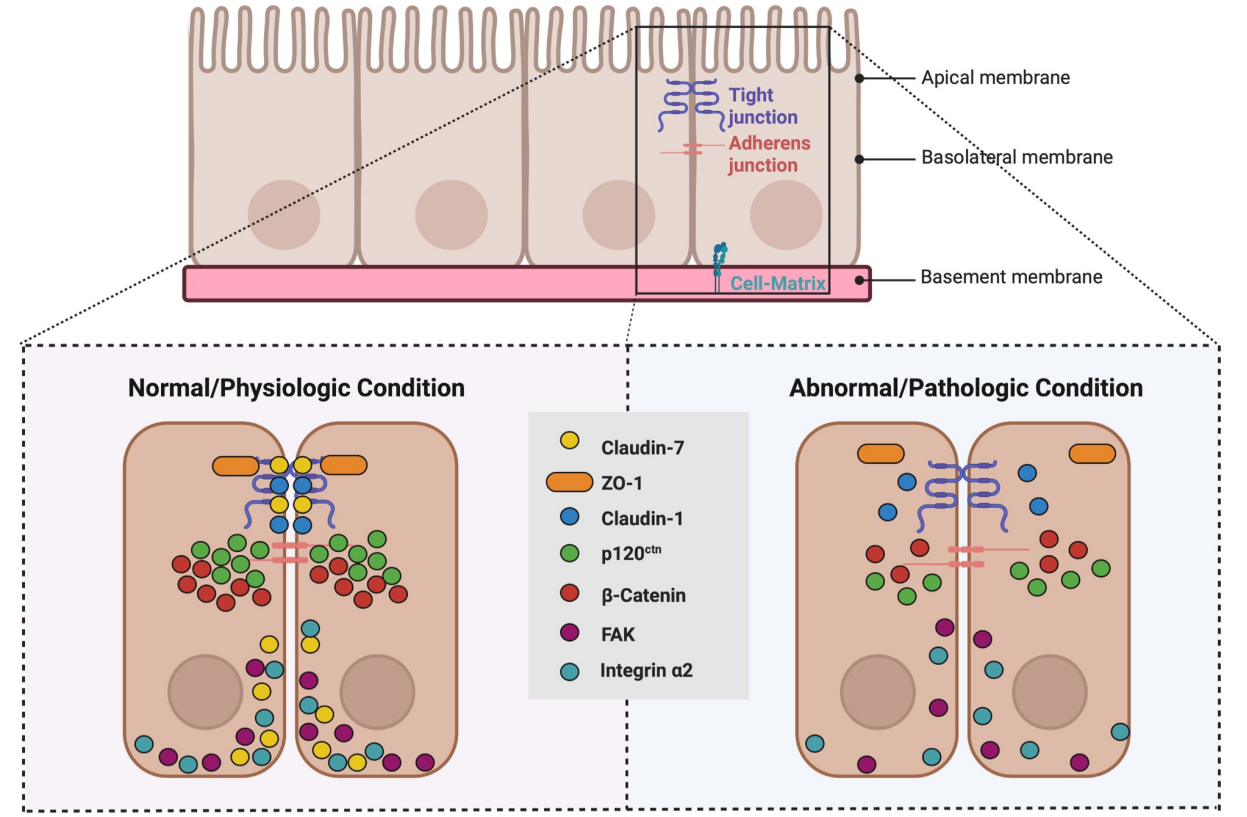

Apical membrane
Tight junction
Adherens junction
Basolateral membrane
Cell-Matrix
Basement membrane
Normal/Physiologic Condition
Abnormal/Pathologic Condition
Claudin-7
ZO-1
Claudin-1
p120ctn
β-Catenin
FAK
Integrin α2

Figure 4.11 Graphical summary. Graphic depicting altered protein organization and hierarchical relationship between normal/physiologic conditions (MDCKII, PZ-HPV-7 and control small intestine) and abnormal/pathologic conditions (MDCKf3, PC3 and claudin-7 conditional knockout intestine).

Table 4.1 List of Antibodies

Antibody	Species	Source/Catalog #	Dilution
		Primary	
β-catenin	Rabbit	Sigma-Aldrich(C2206)	1:100 (IF)
β-catenin	Mouse	BD Transduction Labs (610153)	1:100 (IF)
Claudin-1	Rabbit	Invitrogen (71-7800)	1:50 (IF)
Claudin-7	Rabbit	Immuno-Biological Laboratories (18875)	1:100 (IF)
Claudin-7	Rabbit	Invitrogen (34-9100)	1:100 (IF) 1:1000 (WB)
Claudin-7	Mouse	Invitrogen (37-4800)	1:100 (IF)
E-Cadherin	Rat	Invitrogen (13-1900)	1:100 (IF)
E-Cadherin	Mouse	BD Transduction Labs (610181)	1:500 (WB)
EpCAM	Rabbit	Cell Signaling (93790)	1:100 (IF)
Focal adhesion kinase	Rabbit	Cell Signaling (3285S)	1:100 (IF)
GAPDH	Mouse	Calbiochem (CB1001)	1:2000 (WB)
Integrin α2	Hamster	Chemicon (CBL1345)	1:100 (IF)
P120^{ctn}	Mouse	BD Transduction Labs (610134)	1:100 (IF) 1:1000 (WB)
ZO-1	Rat	Invitrogen (41-9776-82)	1:100 (IF)
		Secondary	
Atto 488	Goat Anti-Rabbit	Rockland (611-152-122)	1:500 (IF)
Alexa Fluor 488	Goat Anti-Rabbit	Invitrogen (A-11008)	1:500 (IF)
Alexa Fluor 488	Goat Anti-Hamster	Invitrogen (A-21110)	1:500 (IF)
Alexa Fluor 488	Goat Anti-Mouse	Invitrogen (A-11001)	1:500 (IF)
Alexa Fluor 568	Goat Anti-Rat	Invitrogen (A-11077)	1:500 (IF)
Alexa Fluor 647	Goat Anti-Hamster	Invitrogen (A-21451)	1:500 (IF)
Alexa Fluor 647	Goat Anti-Mouse	Invitrogen (A-21236)	1:500 (IF)
Alexa Fluor 647	Goat Anti-Rat	Invitrogen (A-21247)	1:500 (IF)
Alexa Fluor 647	Goat Anti-Rabbit	Invitrogen (A-21244)	1:500 (IF)
HRP Conjugate	Anti-Mouse	Promega (w402b)	1:2500 (WB)
HRP Conjugate	Anti-Rabbit	Promega (w301b)	1:2500 (WB)

CHAPTER V: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Inflammatory bowel disease (IBD)—encompassing Crohn's disease (CD) and Ulcerative colitis (UC)—and colorectal cancer (CRC) are major pathologies of the colon which have all been linked to dysregulation of claudin-7 as loss of claudin-7 disrupts the epithelial barrier, leading to severe inflammation, epithelial to mesenchymal transition, and formation of adenomas (Ding et al. 2012; Bhat et al. 2015; Li et al. 2018; Kim et al. 2019). Claudin-7 knockout mice exhibit hyperproliferative epithelium, which may potentially lead to the formation of adenomas and progression to CRC. Interestingly, our microarray data highlighted an increase in the CRC biomarker OLFM4 in claudin-7 knockout mice, as well as enrichment of inflammatory response, gastrointestinal disease, and CRC. In humans, expression level of claudins can be used to predict cancer prognosis–lower claudin-7 expression correlates with increased disease grade and poor prognosis (Karabulut et al. 2015; Quan et al. 2020; Gowrikumar et al. 2021). Given these results, our claudin-7 conditional knockout mice with increased OLFM4 and hyperproliferation can represent a precancerous model.

For the first time, we uncovered a novel role for claudin-7 in the large intestine regulating stem cell survival, self-renewal, proliferation, and differentiation utilizing *in vivo* claudin-7 global (gKO) and inducible, intestinal-specific (cKO) knockout mouse models, and *ex vivo* colon organoid culture. Gene set enrichment analysis (GSEA) of microarray data, qRT-PCR, and fluorescent in situ hybridization highlighted a shift in the crypt stem cell population from Lgr5 positive active stem cells to the quiescent and progenitor cell types. Colon cancer stem cells (CSCs) are believed to arise from progenitor cell types, suggesting that this shift in crypt cell population may lead to production of a CSC population which will hyperproliferate, ultimately leading to tumorigenesis. This shift from active to quiescent stem cells also contributes to loss of regenerative

ability which may further contribute to injury and pathogenesis. Specifically, loss of the active stem cell pool was associated with impaired differentiation ability; therefore claudin-7 knockout mice lacked vital epithelial cell types such as colonocytes for absorption. Lack of absorptive ability manifested phenotypically as rapid weight loss and lack of abdominal adipose tissue in claudin-7 knockout mice, ultimately leading to death. Interestingly, our proteomics study underlined metabolic changes with loss of claudin-7, suggesting that there was a shift in the metabolic pathways utilized for nutrient absorption in claudin-7 knockout mice, potentially towards a pathway using fat as an energy source, such as the ketogenesis. Future directions include further investigation into the metabolic role of claudin-7.

In addition to changes in metabolic pathways, claudin-7 knockout mice exhibit changes in Notch and Hippo signaling cascades. GSEA demonstrated poor enrichment in Notch target genes with loss of claudin-7 which was further corroborated through western blot and qRT-PCR examining Notch ligands and target genes. Activation of the Notch signaling pathway in culture rescued organoid survival, proliferation, and differentiation highlighting the vital role of the Notch pathway in stem cell function, as well as the regulation of Notch signaling by claudin-7. Interestingly, claudin-1 and claudin-11 play roles in intestinal epithelial homeostasis and osteoblast regulation, respectively, through modulation of Notch signaling (Pope et al. 2014; Lindsey et al. 2019). These studies further emphasize the interactions between the claudin family of proteins and Notch signaling. Additionally, Hippo signaling was altered with knockout of claudin-7 as shown through immunofluorescence, western blot, and organoid culture. YAP expression was significantly decreased in claudin-7 knockout mice, suggesting that the Hippo signaling pathway is turned on, leading to phosphorylation and degradation of YAP, therefore resulting in no expansion of the stem cell compartment. Inhibiting the phosphorylation of YAP in

our organoid model system through XMU-MP-1, rescued stem cell survival and proliferation highlighting the important role of the Hippo cascade in expansion of the stem cell compartment. Taken together, claudin-7 is vital for stem cell functions including survival, proliferation, and differentiation through both the Notch and Hippo pathways.

In addition to stem cell function, claudin-7 has been previously shown to interact with other junctional proteins to modulate barrier function and cell-matrix interactions. Here we demonstrated the change in nanoarchitecture of intestinal epithelial junctional proteins with deletion of claudin-7 utilizing super-resolution Stochastic Optical Reconstruction Microscopy (STORM). Loss of claudin-7 led to disorganized cell-cell junctions, as well as altered localization of TJ proteins claudin-1 and ZO-1, adherens junction proteins β-catenin and p120^{ctn}, and focal adhesion proteins integrin $\alpha 2$ and focal adhesion kinase. These changes in the nanoarchitecture of junctional proteins due to loss of claudin-7 may have serious implications on cellular functions. For example, adherens junction protein β-catenin is involved in the canonical Wnt/β-catenin signaling cascade; aberrant Wnt signaling has been implicated in cancers, metabolic diseases, and stem cell function, among many other processes (Clevers et al. 2012; Xing et al. 2020). In addition to changes in protein localization and distribution, knockout of claudin-7 led to increased distance between associated proteins, suggesting that their functional interaction may be diminished. Examining these proteins at the nanoscale level provides new insight into their true interactions and may challenge previous data suggesting that two proteins closely interact, when in fact they may not. This study demonstrates an example in which new technology can be used to better understand protein interactions and functional changes. Further evaluation of these protein interactions and complexes is necessary to fully understand what cellular processes may be altered due to these changes in nanoarchitecture with deletion of claudin-7. Additionally, given the

functional interaction between claudin-7 and focal adhesion protein integrin $\alpha 2$, studying the reciprocal role—through knockout of integrin—would provide new information about their interaction. Future studies may include investigation into the effect of integrin $\alpha 2$ on claudin-7 localization, expression, and function.

Understanding the many roles of claudin-7 in addition to its traditional TJ function provides new information to be considered when examining the development of intestinal disorders such as CD and UC, as well as etiology of CRC. Here we demonstrated the vital role of claudin-7 in large intestine IESC function, regulating stem cell survival, self-renewal, proliferation, and differentiation through modulation of the Notch and Hippo signaling pathways. As balance of the IESC pool and niche is key for regeneration, especially in the case of injury such as inflammation, these findings provide new therapeutic targets for IBD treatment, regenerative medicine, and CRC treatment.

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APPENDIX A: ANIMAL USE PROTOCOLS

ECU

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27354 - 4354
252-744-2436 office | 252-744-2355 fax

October 14, 2020

Yan-Hua Chen, Ph.D.
Department of Anatomy and Cell Biology, ECU

Dear Dr. Chen:

Your Animal Use Protocol entitled, "Investigating the role of claudin-7 in cellular functions and inflammation." (AUP #A172e) was reviewed by this institution's Animal Care and Use Committee on 10/09/2020. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION APRIL, 2017

Project Title:

Investigating the role of claudin-7 in cellular functions and inflammation

	Principal Investigator	Secondary Contact
Name	Yan-Hua Chen	Rodney Tatum
Dept.	Anatomy and Cell Biology	Anatomy and Cell Biology
Office Ph #	252-744-1341	252-744-2866
Cell Ph #	252-327-2828	252-412-8515
Pager #	Click here to enter text.	Click here to enter text.
Home Ph #	Click here to enter text.	Click here to enter text.
Email	cheny@ecu.edu	tatumr@ecu.edu

For IACUC Use Only

AUP #	A172e		
New/Renewal	Renewal		
Full Review/Date 9/21/2020	DR/Date		
Approval Date	10/9/2020		
Study Type	Kidney		
Pain/Distress Category	D		
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC	EHS
OHP Enrollment			
Mandatory Training			
Amendments Approved			

I. Personnel

A. Principal Investigator(s):

Yan-Hua Chen

B. Department(s):

Anatomy and Cell Biology

C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom: Please add degrees to all personnel and roles and responsibilities for Amna – please provide a response

Name/Degree/Certification	Position/Role(s)/ Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
Yan-Hua Chen	PI (PhD), All procedures	Yes	14 years plus received animal (mice) training class from ECU
Rodney Tatum	Tech (BS), All procedures	Yes	14 years plus received animal (mice) training class from ECU
Amna Naser	Graduate Student (BS), All procedures	Yes	3 years plus received animal (mice) training class from ECU
Click here to enter text.	Click here to enter text.	Choose an item.	Click here to enter text.
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II. Regulatory Compliance

A. Non-Technical Summary

Using language a non-scientist would understand, please provide a clear, concise, and sequential description of animal use. Additionally, explain the overall study objectives and benefits of proposed research or teaching activity to the advancement of knowledge, human or animal health, or good of society. (More detailed procedures are requested later in the AUP.)
Do not cut and paste the grant abstract.

Disruption of epithelial tight junctions can result in many human diseases including hypertension and inflammatory bowel disease (IBD). To understand the proteins that regulate the epithelial barrier function is of particular importance for the development of different therapeutic strategies. We now have evidence that one such factor is the tight junction protein claudin-7. Claudin-7 is localized in the kidney and intestines. Recent studies from this laboratory established that claudin-7 is involved in cell-cell and cell-matrix interactions. In this project, we will use a claudin-7 knockout mouse model generated in our laboratory to investigate the role of claudin-7 in kidney disease and intestinal inflammation.

In order to investigate the role and function of tight junction proteins, the mouse tissues including kidneys, intestines, and lung from WT and KO will be collected for analysis using different methods such as RT-PCR, western blotting and immunostaining. We will study and compare the changes at the histological, proteins, and mRNAs levels in both WT and KO animals.

We are also interested in determining if the life expectancy of the KO pups can be extended by supplementing nursing pups with dextrose and saline.

In the past 3 years, we identified that claudin-7 is essential for epithelial cell differentiation. However, we do not know which signaling pathways that claudin-7 interact with and how claudin-7 regulates the cellular functions. In the next 3 years, we aim to investigate the molecular mechanism underlying claudin-7 deletion mediated tissue defects.

B. Ethics and Animal Use

B.1. Duplication

Does this study duplicate existing research? No

If yes, why is it necessary? (note: teaching by definition is duplicative)

Click here to enter text.

B.2. Alternatives to the Use of Live Animals

Are there less invasive procedures, other less sentient species, isolated organ preparation, cell or tissue culture, or computer simulation that can be used in place of the live vertebrate species proposed here? No

If yes, please explain why you cannot use these alternatives.

Click here to enter text.

B.3. Consideration of Alternatives to Painful/Distressful Procedures

a. Include a literature search to ensure that alternatives to all procedures that may cause more than momentary or slight pain or distress to the animals have been considered.

1. Please list all of the potentially painful or distressful procedures in the protocol:

Dextrose/Saline Injection: Inject subcutaneously 0.45% NaCl with 2.5% Dextrose into 3-9 days old of Claudin-7 mouse pups. Claudin-7 null mouse pups suffer from dehydration and loss of electrolytes and die typically before day 7. The injection of 0.45% NaCl with 2.5% Dextrose may increase vitality and enable the pups to survive beyond 7-days of age. Dextrose supplement may be required since claudin-7 KO mice have disrupted villi and may not be able to absorb nutrition from their mother's milk beyond 3 days after birth. Keeping the pups alive longer will allow us to study the impact of claudin-7 deletion on intestinal epithelial cell differentiation. In addition, this is a rescue experiment. We would like to see whether it is the dehydration that causes the early death of the animals. Although this was listed in our previous submission we have not performed these experiments so far.

2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):

Date Search was performed:	8/30/2020
Database(s) searched:	Pubmed
Time period covered by the search (i.e. 1975-2013):	1986-Present
Search strategy (including scientifically relevant terminology):	knockout mouse model, tight junctions, claudins, Dextrose/saline injection, Fasting mouse pups
Other sources consulted:	Click here to enter text.

3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress.

Our study makes use of a unique claudin-7 KO mouse model that enables us to investigate intestinal and kidney functions in the absence of this protein to elucidate its function in the cell differentiation. There is no alternative to studying these systems in the intact animal that differs from wild type only in this single genetic trait. We will use the minimum number of mice that are needed for the experiments (reduction). The animals will be euthanized according to the current protocol. The tissues will be collected for western blot, RT-PCR and immunostaining. Click here to enter text. Refinement: When mouse pups are removed from dam, pups will be euthanized.

C. Hazardous Agents

1. Protocol related hazards (chemical, biological, or radiological):

Please indicate if any of the following are used in animals and the status of review/approval by the referenced committees:

HAZARDS	Oversight Committee	Status (Approved, Pending, Submitted)/Date	AUP Appendix I Completed?
Radioisotopes	**Radiation**	Click here to enter text.	No
Ionizing radiation	**Radiation**	Click here to enter text.	No
Infectious agents (bacteria, viruses, rickettsia, prions, etc.)	**IBC**	Click here to enter text.	No
Toxins of biological origins (venoms, plant toxins, etc.)	**IBC**	Click here to enter text.	No
Transgenic, Knock In, Knock Out Animals---breeding, cross breeding or any use of live animals or tissues	**IBC**	Approved – 03/06/2019	No
Human tissues, cells, body fluids, cell lines	**IBC**	Click here to enter text.	Choose an item.

Viral/Plasmid Vectors/Recombinant DNA or recombinant techniques	**IBC**	Click here to enter text.	Choose an item.
Oncogenic/toxic/mutagenic chemical agents	**EH&S**	Click here to enter text.	Choose an item.
Nanoparticles	**EH&S**	Click here to enter text.	Choose an item.
Cell lines, tissues or other biological products injected or implanted in animals	**DCM**	Click here to enter text.	Choose an item.
Other agents		Approved 9/18/2020	No

2. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

N/A

III. Animals and Housing

A. Species and strains:

Claudin-7-deficient mice (129/Ola)

B. Weight, sex and/or age:

Birth to adult, female and male

C. Animal numbers:

1. Please complete the following table:

Total number of animals in treatment and control groups	**Additional animals (Breeders, substitute animals)**	**Total number of animals used for this project**
300	+500	=800

2. Justify the species and number (use statistical justification when possible) of animals requested:

Claudin-7 knockout mice are needed in order to study claudin-7 functions in vivo. 300 experimental mice are needed for studying claudin-7 functions in kidneys, lungs and intestines.

These numbers of animal are necessary in order to obtain the statistically significant data using student-t test. Our experiments need WT and KO pups. We estimate that 25% of pups will be KO and 25% will be WT. The remaining hets will be used for breeding or sacrificed. The breeders use adult mice and the experiments use pups. Although 25% should be born, neonatal deaths are expected therefore ~ 10 % survive to day 3.

The 800 mice include all animals - breeding mice and pups for experiments and pups that are not used. Each litter will have 1-10 pups. Therefore, we need to enough animal numbers to cover these pups that may or may not be used in the experiments.

3. Justify the number and use of any additional animals needed for this study:
Claudin-7 breeders (-/-, +/-, +/+): 500

500 mice are needed for breeders over three years since these mice are generated in ECU and are not commercially available.

Breeding colony for claudin-7. This KO mouse model is not available commercially so a breeding colony will be maintained in DCM's barrier facility and Brody facility. Since this KO has small litters and are difficult to breed, therefore, 500 animals are necessary over 3 years to maintain the colony and produce the necessary experimental animals. These 500 animals also include the animals that don't express the WT or KO and are subsequently culled from the study. All animals will be tail snipped as per DCM protocol to determine genotype.

The breeders use 500 +/- mice and the experiments use 300 +/+ and -/- pups. Of which around 250 pups will be used for tissue collection and around 50 pups will be used to extend the life expectancy.

a. For unforeseen outcomes/complications:
Click here to enter text.

b. For refining techniques:
Click here to enter text.

c. For breeding situations, briefly justify breeding configurations and offspring expected:

Wildtype, Heterozygous, knockout pups are expected in 1:2:1 ratio.

d. Indicate if following IACUC tail snip guidelines: Yes
(if no, describe and justify)
Click here to enter text.

1. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health, behavioral, physical abnormalities, or cause debilitating effects in experimental manipulations? Yes **(if yes, describe)**
Claudin-7 knockout mice typically die by day 7 due to kidney and intestinal defects.

2. Are there any deviations from standard husbandry practices?
Yes **If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, prolonged weaning times, wire-bottom cages, etc.):**
Cluadin-7 mice will be provided with cages and accessories that have been autoclaved along with irradiated feed and bedding. We are trying to keep the breeding colony free of diseases.

3. The default housing method for social species is pair or group housing (including mice, rats, guinea pigs, rabbits, dogs, pigs, monkeys). Is it necessary for animals to be singly housed at any time during the study?
Yes **(If yes, describe housing and justify the need to singly house social species):**
Male mice will also have to be separated from the female mice before birth to alleviate stress during birth and reduce the possibility of the female getting pregnant while nursing her pups. Male adult mice may be singly housed to prevent fighting and injury.

4. Are there experimental or scientific reasons why routine environmental enrichment should not be provided? No
(If yes, describe and justify the need to withhold enrichment)
Click here to enter text.

5. If wild animals will be captured or used, provide permissions (collection permit # or other required information):
Click here to enter text.

6. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.
Brody 7N-80

IV. Animal Procedures

A. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design. Briefly state surgical plans in this section. Surgical procedures can be described in detail in IV.S.

The study's objective is to compare WT and KO. This will be accomplished by sacrificing WT and KO pups between 0-9 days of age.

Genotyping will be performed on neonatal pups by tail snips according to the IACUC guidance.

1) When mouse pups are removed from dam, if no milk spot is present, pups will be euthanized. We intend to use around 250 pups for this experiment.

All pups will be euthanized according to the current protocol. The tissues will be collected for western blot, RT-PCR and immunostaining. Click here to enter text.

2) In addition, we would like to investigate whether the KO pups can be rescued. We intend to use around 50 pups for this experiment. The pups will be remained with the dam and be provided with the following.

Saline Injection: Inject subcutaneously 0.45% NaCl with 2.5% Dextrose into 3-9 days old of Claudin-7 mouse pups. The site of the injection will be alternated daily to avoid repeated injections in the same location. Claudin-7 null mouse pups suffer from dehydration and loss of electrolytes and die typically before day 7. The injection of 0.45% NaCl with 2.5% Dextrose may increase vitality and enable the pups to survive beyond 7-days of age. Dextrose supplement may be required since claudin-7 KO mice have disrupted villi and may not be able to absorb nutrition from their mother's milk beyond 7 days after birth. If any pups are observed to be in distress or rejected by dam, they will be sacrificed. Although this was listed in our previous submission, we have not performed these experiments yet.

In sections IV.B-IV.S below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.

Please refer to DCM and IACUC websites for relevant guidelines and SOPs.

B. Anesthesia/Analgesia/Tranquilization/Pain/Distress Management For Procedures Other than Surgery:

For all procedures, provision of pre-emptive (pre-procedural) analgesia is required, unless specifically exempted by DCM veterinarians. For major survival

surgical procedures and extensive non-surgical procedures requiring anesthesia, post-procedural analgesia must be provided for a minimum of 3 full days following anesthetic recovery, unless specifically exempted by DCM veterinarians. Analgesic administration should be continued for at least 1 full day following recovery from minor surgical and non-surgical procedures. Please contact DCM veterinary staff for recommendations and guidance when formulating anesthetic regimens.

Adequate records describing anesthetic monitoring and recovery must be maintained for all species. Please see Guidelines for Intra-operative and Intra-procedural Monitoring on the IACUC website.

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:
Click here to enter text.

1. **Describe the pre-procedural preparation of the animals:**
   a. **Food restricted for** 0 **hours**
   b. **Food restriction is not recommended for rodents and rabbits and must be justified:**

   The mouse pups will not need to be fasted since we now study the large intestine.

   c. Water restricted for 0**hours**
   d. Water restriction is not recommended in any species for routine pre-op prep and must be justified:
   No water restriction.

2. Anesthesia/Analgesia *for Procedures Other than Surgery*

	Agent	**Concentration**	**Dose (mg/kg)**	**Max Volume**	**Route**	**Frequency**	**Number of days administered**
Pre-procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to	Click here to	Click here to enter text.	Click here to enter text.

				enter text.	enter text.		
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Reason for administering agent(s):
Click here to enter text.
4. For which procedure(s):
Click here to enter text.
5. Methods for monitoring anesthetic depth:
Click here to enter text.
6. Methods of physiologic support during anesthesia and recovery:
Click here to enter text.
7. Duration of recovery:
Click here to enter text.
8. Frequency of recovering monitoring:
Click here to enter text.
9. Specifically what will be monitored?
Click here to enter text.
10. When will animals be returned to their home environment?
Click here to enter text.
11. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:
Click here to enter text.

C. Use of Paralytics

1. Will paralyzing drugs be used? No

2. For what purpose:
Click here to enter text.

3. Please provide scientific justification for paralytic use:
Click here to enter text.

4. Paralytic drug:
Click here to enter text.

5. Dose:
Click here to enter text.

6. Method of ensuring appropriate analgesia during paralysis:
Click here to enter text.

D. Blood or Body Fluid Collection

1. Please fill out appropriate sections of the chart below:

	Location on animal	Needle/catheter size	Volume collected	Frequency of procedure	Time interval between collections
Blood Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Body Fluid Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

E. Injections, Gavage, & Other Substance Administration

1. Please fill out appropriate sections of the chart below:

	Compound	Location & Route of admin	Needle/catheter/gavage size	Max volume admin	Freq of admin (ie two times per day)	Number of days admin (ie for 5 days)	Max dosages (mg/kg)
Injection/ Infusion	Physiological Saline 0.45% NaCl + 2.5% Dextrose	s.c. injection	26G	Up to 100ul	Once/day Starting from day 3 after birth	6 days; Pups will be sacrificed at day 9	500mg /kg

Gavage	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. **Pharmaceutical grade drugs, biologics, reagents, and compounds are defined as agents approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been written/established by any recognized pharmacopeia such as USP, NF, BP, etc. These standards are used by manufacturers to help ensure that the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy. For all injections and infusions for CLINICAL USE, PHARMACEUTICAL GRADE compounds must be used whenever possible. Pharmaceutical grade injections and infusions for research test articles are preferred when available. If pharmaceutical grade compounds are not available and non-pharmaceutical grade agents must be used, then the following information is necessary:** Pharmaceutical grade saline and dextrose will be used.

a. **Please provide a scientific justification for the use of ALL non-pharmaceutical grade compounds. This may include pharmaceutical-grade compound(s) that are not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.**
b. **Indicate the method of preparation, addressing items such as purity, sterility, pH, osmolality, pyrogenicity, adverse reactions, etc. (please refer to ECU IACUC guidelines for non-pharmaceutical grade compound use), labeling (i.e. preparation and use-by dates), administration and storage of each formulation that maintains stability and quality/sterility of the compound(s).**

Click here to enter text.

F. <u>Prolonged restraint with mechanical devices</u>

Prolonged restraint in this context means *beyond routine care and use procedures* for rodent and rabbit restrainers, and large animal stocks. Prolonged restraint also includes *any* use of slings, tethers, metabolic crates, inhalation chambers, primate chairs and radiation exposure restraint devices.

1. **For what procedure(s):**

Click here to enter text.

2. **Explain why non-restraint alternatives cannot be utilized:**

Click here to enter text.

3. **Restraint device(s):**

Click here to enter text.

4. **Duration of restraint:**

Click here to enter text.

5. **Frequency of observations during restraint/person responsible:**

Click here to enter text.

6. **Frequency and total number of restraints:**

Click here to enter text.

7. **Conditioning procedures:**

Click here to enter text.

8. **Steps to assure comfort and well-being:**

Click here to enter text.

9. **Describe potential adverse effects of prolonged restraint and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

G. Tumor Studies, Disease Models, Toxicity Testing, Vaccine Studies, Trauma Studies, Pain Studies, Organ or System Failure Studies, Shock Models, etc.

1. **Describe methodology:**

Click here to enter text.

2. **Expected model and/or clinical/pathological manifestations:**

Click here to enter text.

3. **Signs of pain/discomfort:**

Click here to enter text.

4. **Frequency of observations:**

Click here to enter text.

5. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

H. Treadmills/Swimming/Forced Exercise

1. Describe aversive stimulus (if used):
Click here to enter text.
2. Conditioning:
Click here to enter text.
3. Safeguards to protect animal:
Click here to enter text.
4. Duration:
Click here to enter text.
5. Frequency:
Click here to enter text.
6. Total number of sessions:
Click here to enter text.
7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

I. Projects Involving Food and Water Regulation or Dietary Manipulation

(*Routine pre-surgical fasting not relevant for this section)*

1. Food Regulation

a. Amount regulated and rationale:
Click here to enter text.
b. Frequency and duration of regulation (hours for short term/weeks or months for long term):
Click here to enter text.
c. Frequency of observation/parameters documented (i.e. recording body weight, body condition, etc.):
Click here to enter text.
d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

2. Fluid Regulation

a. Amount regulated and rationale:
No fluid regulation.
b. Frequency and duration of regulation (hours for short term/weeks or months for long term):
Click here to enter text.
c. Frequency of observation/parameters documented (body weight, hydration status, etc.):
Click here to enter text.
d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

3. Dietary Manipulations

a. Compound supplemented/deleted and amount:
Click here to enter text.
b. Frequency and duration (hours for short term/week or month for long term):
Click here to enter text.
c. Frequency of observation/parameters documented:
Click here to enter text.
d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

J. Endoscopy, Fluoroscopy, X-Ray, Ultrasound, MRI, CT, PET, Other Imaging

1. Describe animal methodology:
Click here to enter text.

2. Duration of procedure:
Click here to enter text.
3. Frequency of observations during procedure:
Click here to enter text.
4. Frequency/total number of procedures:
Click here to enter text.
5. Method of transport to/from procedure area:
Click here to enter text.
6. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.
7. Please provide or attach appropriate permissions/procedures for animal use on human equipment:
Click here to enter text.

K. Polyclonal Antibody Production

1. **Antigen/adjuvant used and justification for adjuvant choice:**
   Click here to enter text.
2. **Needle size:**
   Click here to enter text.
3. **Route of injection:**
   Click here to enter text.
4. **Site of injection:**
   Click here to enter text.
5. **Volume of injection:**
   Click here to enter text.
6. **Total number of injection sites:**
   Click here to enter text.
7. **Frequency and total number of boosts:**
   Click here to enter text.
8. **What will be done to minimize pain/distress:**
   Click here to enter text.
9. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
   Click here to enter text.

L. Monoclonal Antibody Production

1. Describe methodology:

Click here to enter text.

2. Is pristane used: Choose an item.

Volume of pristane:

Click here to enter text.

3. Will ascites be generated: Choose an item.

i. Criteria/signs that will dictate ascites harvest:

Click here to enter text.

ii. Size of needle for taps:

Click here to enter text.

iii. Total number of taps:

Click here to enter text.

iv. How will animals be monitored/cared for following taps:

Click here to enter text.

4. What will be done to minimize pain/distress:

Click here to enter text.

5. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

M. Temperature/Light/Environmental Manipulations

1. Describe manipulation(s):

Click here to enter text.

2. Duration:

Click here to enter text.

3. Intensity:

Click here to enter text.

4. Frequency:

Click here to enter text.

5. Frequency of observations/parameters documented:

Click here to enter text.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

N. Behavioral Studies

1. Describe methodology/test(s) used:
Click here to enter text.
2. Will conditioning occur? If so, describe:
Click here to enter text.
3. If aversive stimulus used, frequency, intensity and duration:
Click here to enter text.
4. Length of time in test apparatus/test situation: *(i.e., each test is ~10 mins)*
Click here to enter text.
5. Frequency of testing and duration of study: *(i.e., 5 tests/week for 6 months)*
Click here to enter text.
6. Frequency of observation/monitoring during test:
Click here to enter text.
7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

O. Capture with Mechanical Devices/Traps/Nets

1. Description of capture device/method:
Click here to enter text.
2. Maximum time animal will be in capture device:
Click here to enter text.
3. Frequency of checking capture device:
Click here to enter text.
4. Methods to ensure well-being of animals in capture device:
Click here to enter text.
5. Methods to avoid non-target species capture:
Click here to enter text.
6. Method of transport to laboratory/field station/processing site and duration of transport:
Click here to enter text.
7. Methods to ensure animal well-being during transport:
Click here to enter text.
8. Expected mortality rates:
Click here to enter text.
9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

P. Manipulation of Wild-Caught Animals in the Field or Laboratory

1. Parameters to be measured/collected:
Click here to enter text.
2. Approximate time required for data collection per animal:
Click here to enter text.
3. Method of restraint for data collection:
Click here to enter text.
4. Methods to ensure animal well-being during processing:
Click here to enter text.
5. Disposition of animals post-processing:
Click here to enter text.
6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

Q. Wildlife Telemetry/Other Marking Methods

1. Describe methodology (including description of device):
Click here to enter text.
2. Will telemetry device/tags/etc. be removed? Choose an item. **If so, describe:**
Click here to enter text.
3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

R. Other Animal Manipulations

1. Describe methodology:
2. Describe methods to ensure animal comfort and well-being:
3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

S. Surgical Procedures

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species. Please see Guidelines for Intra-operative and Intra-procedural Monitoring on the IACUC website.

1. Location of Surgery (Building & Room #):
Click here to enter text.

2. Type of Surgery (check all that are appropriate):
Click here to enter text.

☐ **Non-survival surgery (animals euthanized without regaining consciousness)**
☐ **Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function)**
☐ **Minor survival surgery**
☐ **Multiple survival surgery**
If yes, provide scientific justification for multiple survival surgical procedures:
Click here to enter text.

3. Describe the pre-op preparation of the animals:

a. **Food restricted for** Click here to enter text. **hours**
b. **Food restricted is not recommended for rodents and rabbits and must be justified:**
Click here to enter text.
c. Water restricted for Click here to enter text. **hours**
d. Water restriction is not recommended in any species for routine pre-op prep and be justified:
Click here to enter text.

4. Minimal sterile techniques will include (check all that apply):

Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species and type of surgery (survival vs. non-survival).

☐ **Sterile instruments**

How will instruments be sterilized?

Click here to enter text.

If serial surgeries are done, how will instruments be sterilized between surgeries:

Click here to enter text.

☐ **Sterile gloves**
☐ **Mask**
☐ **Cap**
☐ **Sterile gown**
☐ **Sanitized operating area**
☐ **Clipping or plucking of hair or feathers**
☐ **Skin preparation with a sterilant such as betadine**
☐ **Practices to maintain sterility of instruments during surgery**
☐ **Non-survival (clean gloves, clean instruments, etc.)**

5. Describe all surgical procedures:

a. Skin incision size and site on the animal:

Click here to enter text.

b. Describe surgery in detail (include size of implant if applicable):

Click here to enter text.

c. Method of wound closure:

Click here to enter text.

i. Number of layers

Click here to enter text.

ii. Type of wound closure and suture pattern:

Click here to enter text.

iii. Suture type/size/wound clips/tissue glue:

Click here to enter text.

iv. Plan for removing of skin sutures/wound clip/etc:

Click here to enter text.

6. Anesthetic Protocol:

For all procedures, provision of pre-emptive (pre-procedural) analgesia is required, unless specifically exempted by DCM veterinarians. For major survival surgical procedures and extensive non-surgical procedures requiring anesthesia, post-procedural analgesia must be provided for a minimum of 3 full days following anesthetic recovery, unless specifically exempted by DCM veterinarians. Analgesic administration should be continued for at least 1 full day following recovery from minor surgical and non-surgical procedures. Please contact DCM veterinary staff for recommendations and guidance when formulating anesthetic regimens.

a. If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:

Click here to enter text.

b. Anesthesia/Analgesia For Surgical Procedures

	Agent	Dose (mg/kg or %)	Volume	Route	Frequency	Number of days administered
Pre-operative analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post-operative Analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

c. Methods that will be used to monitor anesthetic depth (include extra measures employed when paralyzing agents are used):

Click here to enter text.

d. Methods of physiologic support during anesthesia and immediate post-op period (fluids, warming, etc.):

Click here to enter text.

e. List what parameters are monitored during immediate post-op period. Provide the frequency and duration:

Click here to enter text.

f. Describe any other manipulations that will be used to alleviate pain, distress, and/or discomfort during the immediate post-op period (soft bedding, long sipper tubes, food on floor, dough diet, etc.):

Click here to enter text.

g. List criteria used to determine when animals are adequately recovered from anesthesia and when the animals can be returned to their home environment:

Click here to enter text.

7. Recovery from Surgical Manipulations (after animal regains consciousness and is returned to its home environment)

Click here to enter text.

a. What parameters (behavior, appetite, mobility, wound healing, etc.) will be monitored:

Click here to enter text.

b. How frequently (times per day) will animals be monitored:

Click here to enter text.

c. How long post-operatively (days) will animals be monitored:

Click here to enter text.

8. Surgical Manipulations Affecting Animals

a. Describe any signs of pain/discomfort/functional deficits resulting from the surgical procedure:

Click here to enter text.

b. What will be done to manage any signs of pain or discomfort (include pharmacologic and non-pharmacologic interventions):

Click here to enter text.

c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

V. Euthanasia

Please refer to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition and DCM Guidelines to determine appropriate euthanasia methods.

A. Euthanasia Procedure*. All investigators, even those conducting non-terminal studies, must complete this section in case euthanasia is required for humane reasons.*

1. Physical Method- If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a scientific justification must be provided:
CO2 or isoflurane followed by cervical dislocation or decapitation

2. Inhalant Method- Choose an item.
(if other, describe the agent and delivery method)
CO2 euthanasia for adults or isoflurane using the open drop method in a fumehood followed by cervical dislocation or decapitation.

3. Non-Inhalant Pharmaceutical Method (injectables, MS-222, etc.)- Please provide the following:

a. Agent:
Ketamine /Xylazine

b. Dose or concentration:
Ketamine 18mg/ml Xylazine 2mg/ml Administer .05ml/10g b.w. I.P. Over dose 50ul for 2g pup and 75ul for 3g pup.

c. Route:
Subcutaneous

B. Method of ensuring death (can be physical method, such as pneumothorax or decapitation for small species and assessment method such as auscultation for large animals):
If there is no reflex, pups will be decapitated and adults will either be decapitated or have cervical dislocation

C. Describe disposition of carcass following euthanasia:

Carcasses are bagged and the bags are placed in the DCM cooler for disposal.

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature: Yan-Hua Chen **Date:** 10/5/2020

Veterinarian: DocuSigned by: Cecile Baccanale 4833444F3627C423... **Date:** 10/14/2020

IACUC Chair: **Date:** 10/12/2020

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION APRIL, 2017

AUP#196 Function of junctional complex on kidney and intestinal epithelial cells

	Principal Investigator	Secondary Contact
Name	Yan-Hua Chen	Rodney Tatum
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For IACUC Use Only

AUP #	A196b		
New/Renewal	Renewal		
Full Review/Date 01/24/2022	DR/Date		
Approval Date	2/2/2022		
Study Type	Gastrointestinal system		
Pain/Distress Category	D		
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
~~Hazard Approval/Dates~~	~~Rad~~	~~IBC 03/16/2019~~	EHS 2/2/2022
OHP Enrollment			
Mandatory Training			
Amendments Approved	#1 03/25/2022		

I. Personnel

A. Principal Investigator(s):

Yan-Hua Chen

B. Department(s):

Anatomy and Cell Biology

C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name/Degree/Certification	Position/Role(s)/ Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
Yan-Hua Chen, PhD	Principal Investigator: Lead investigator supervising the project	Yes	18 years plus received animal handling class from ECU
Rodney Tatum, BS	Research Tech: Tail snips, injections, harvesting tissues	Yes	16 years, East Carolina University Training 16 years animal experience: mouse handling, mouse tag, tail snip
Amna Naser , BS	Graduate Student: Tail snips, injections, harvesting tissues	Yes	3 years, Received ECU animal surgery training on 1/9/2019
Click here to enter text.	Click here to enter text.	Choose an item.	Click here to enter text.
Click here to enter text.	Click here to enter text.	Choose an item.	Click here to enter text.

II. Regulatory Compliance

A. Non-Technical Summary

Using language a non-scientist would understand, please provide a clear, concise, and sequential description of animal use. Additionally, explain the overall study objectives and benefits of proposed research or teaching activity to the advancement of knowledge, human or animal health, or good of society. (More detailed procedures are requested later in the AUP.)

Do not cut and paste the grant abstract.

The integrity of intestinal mucosa is required for nutrition absorption and defense against pathogens. Disruption of intestinal epithelial homeostasis leads to a variety of intestinal disorders, such as inflammatory bowel disease (IBD) and cancer. This AUP is intended to investigate the roles of claudin-7 in gastrointestinal (GI) system and other related organ system such as kidneys. Claudin-7 is highly expressed in both small and large intestines. Deletion of claudin-7 in intestines leads to severe mucosal ulcerations, epithelial cell sloughing, and inflammation. Deletion of claudin-7 affects intestinal stem cells and disrupts epithelial differentiation and evokes the inflammatory response. In this study, we will use our global claudin-7 knockout mice and inducible, intestinal-specific claudin-7 knockout mice to determine the roles of claudin-7 in intestinal stem cell functions and inflammatory signaling.

The proposed experiments involved with animals will be:

1. Isolate intestinal crypt regions (organ culture) from adult claudin-7 WT and KO intestines;
2. Inject biotin tracer into claudin-7 WT and KO intestinal lumens;
3. Inject Tamoxifen to induce claudin-7 deletion in adult mice for organoid culture;
4. Isolate intestines and kidneys from claudin-7 WT and KO for qRT-PCR, Western blotting, and immunostaining.

4-hydroxytamoxifen (4-OH TAM) treatments have been performed on organoid cultures to induce claudin-7 deletion in vitro. Lipopolysaccharide (LPS) have been used to treat intestinal epithelial cells in culture to induce inflammation with or without claudin-7 to study the signaling pathway alterations. We will continue to perform these experiments to understand the role of claudin-7 in intestines and other organs such as kidneys.

Amendment#1: Recently, we have determined that it is not necessary to inject Sulfo-NHS-biotin tracer into the intestinal lumen of a live animal. Therefore, we wish to switch from a non-survival surgery to a post-euthanasia administration. Sulfo-NHS-biotin and PBS will be injected into the intestinal lumen after euthanasia

B. Ethics and Animal Use

B.1. Duplication

Does this study duplicate existing research? No

If yes, why is it necessary? (note: teaching by definition is duplicative)

Click here to enter text.

B.2. Alternatives to the Use of Live Animals

Are there less invasive procedures, other less sentient species, isolated organ preparation, cell or tissue culture, or computer simulation that can be used in place of the live vertebrate species proposed here? No

If yes, please explain why you cannot use these alternatives.

Click here to enter text.

B.3. Consideration of Alternatives to Painful/Distressful Procedures

a. Include a literature search to ensure that alternatives to all procedures that may cause more than momentary or slight pain or distress to the animals have been considered.

1. Please list all of the potentially painful or distressful procedures in the protocol:

Click here to enter text.

2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):

Date Search was performed:	12/1/2021
Database(s) searched:	Pubmed
Time period covered by the search (i.e. 1975-2013):	1969-2021
Search strategy (including scientifically relevant terminology):	Tight junctions, Claudin-7 knock out model, alternative, intestinal epithelial cells, inflammation, tamoxifen
Other sources consulted:	Google Scholar

3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress. While a search is not necessary, please address the 3Rs for the other procedures including tamoxifen administration.

Replacement – no alternatives to using a live animal model. Reduction – the minimum number of animals will be used based on our past studies. Refinement – following tamoxifen injection, mice will be monitored daily, any showing signs of difficulty breathing, anorexia, or not drinking, or difficulty ambulating will be euthanized.

C. Hazardous Agents

1. Protocol related hazards (chemical, biological, or radiological):

Please indicate if any of the following are used in animals and the status of review/approval by the referenced committees:

HAZARDS	Oversight Committee	Status (Approved, Pending, Submitted)/Date	AUP Appendix I Completed?
Radioisotopes	**Radiation**	Click here to enter text.	Choose an item.
Ionizing radiation	**Radiation**	Click here to enter text.	Choose an item.
Infectious agents (bacteria, viruses, rickettsia, prions, etc.)	**IBC**	Click here to enter text.	Choose an item.
Toxins of biological origins (venoms, plant toxins, etc.)	**IBC**	Click here to enter text.	Choose an item.
Transgenic, Knock In, Knock Out Animals---breeding, cross breeding or any use of live animals or tissues	**IBC**	Approved 3/16/2019	No
Human tissues, cells, body fluids, cell lines	**IBC**	Click here to enter text.	Choose an item.
Viral/Plasmid Vectors/Recombinant DNA or recombinant techniques	**IBC**	Click here to enter text.	Choose an item.
Oncogenic/toxic/mutagenic chemical agents	**EH&S**	Approved 2/2/2022	Yes
Nanoparticles	**EH&S**	Click here to enter text.	Choose an item.
Cell lines, tissues or other biological products injected or implanted in animals	**DCM**	Click here to enter text.	Choose an item.
Other agents		Click here to enter text.	Choose an item.

2. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

Click here to enter text.

III. Animals and Housing

A. Species and strains:

Mus Musculus , C57BL6/ Cldn-7+/+ (Wild-type, WT), Cldn-7+/- (Hetero) and Cldn-7-/- (Knockout, KO)

cldn7 fl/fl (homozygous) – mice with claudin-7 sequence flanked by LoxP sites
cldn7 fl/wt (heterozygous)
cldn7 fl/fl creT – Inducible tissue specific claudin-7 KO mice
cldn7 fl/wt creT

The strain of the mice is C57BL/6.

B. Weight, sex and/or age:

Postnatal day 1-21, males and females; 1g-20g
Adult mice, 1 month – 1.5 years old, males and females; 20g-30g

C. Animal numbers:

1. Please complete the following table:

Total number of animals in treatment and control groups	Additional animals (Breeders, substitute animals)	Total number of animals used for this project

Task 1: Isolate adult intestinal epithelial cells: Inducible KO mice: 25 Wild-type controls: 25 Pup KO: 25 Pup WT: 25 Task 2: Epithelial barrier function of Knockout intestines Biotin Injections: Knockout Biotin : 20 Knockout PBS : 6 Wild-type Biotin : 20 Wild-type PBS : 6 Task 3: Tamoxifen injection: Adult Knockout: Tamoxifen 100 Control: Sunflower oil 100 Task 4: Pup tissue collections Knockout: 100 Wild-type: 100	+Additional mice for breeding Cldn-7+/- (Hetero): 60 cldn7 fl/fl creT: 60	Task 1: 100 mice Task 2: 52 mice Task 3: 200 mice Task 4: 200 mice Additional mice for breeding: 120 Total mice: 672

2. Justify the species and number (use statistical justification when possible) of animals requested:

Claudin-7 knockout mice are needed in order to study claudin-7 functions in vivo. 552 experimental mice are needed for studying claudin-7 functions in intestines and kidneys. These numbers of animal are necessary in order to obtain the statistically significant data using student-t test and One-way ANOVA analysis to reach a *p*-value of < 0.05. Our experiments need WT and KO mice. We estimate that 12.5-25% of pups will be KO and 12.5-25% will be WT. The remaining hets will be used for breeding or sacrificed.

We request to use the claudin-7 KO colonies to study the roles of claudin-7 in stem cell functions and inflammation.

Task 1, 2, and 3: We request the use of 276 adult mice total for inducible KO and WT.
Task 1, 2, and 4: We request the use of 276 total for KO and WT pups.

The number of the animals requested are required so that the results we receive will be statistically significant.

3. Justify the number and use of any additional animals needed for this study:
We request additional 120 mice for setting up breeders.

120 mice are needed for breeders over three years since these mice are generated in ECU and are not commercially available.

Breeding colony for claudin-7. We have two claudin-7 colonies:

1. global claudin-7 KO colony: Cldn-7+/- (Hetero) x Cldn-7+/- (Hetero)
2. inducible, intestinal-specific claudin-7 KO colony: cldn7 fl/fl creT x cldn7 fl/fl creT

These claudin-7 KO mouse models are not available commercially, so breeding colonies will be maintained in DCM's barrier facility and Brody facility. The 120 animals are necessary over 3 years to maintain the colonies and produce the necessary experimental animals. All animals will be tail snipped as per IACUC protocol to determine genotypes.

a. For unforeseen outcomes/complications:
Click here to enter text.

b. For refining techniques:
Click here to enter text.

c. For breeding situations, briefly justify breeding configurations and offspring expected:

1. global claudin-7 KO colony: Cldn-7+/- (Hetero) x Cldn-7+/- (Hetero).
   Offspring: WT KO Hetero
2. inducible, intestinal-specific claudin-7 KO colony: cldn7 fl/fl creT x cldn7 fl/fl creT
   Offspring: WT KO Hetero

d. Indicate if following IACUC tail snip guidelines: Choose an item.
(if no, describe and justify)
Click here to enter text.

1. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health, behavioral, physical abnormalities, or cause debilitating effects in experimental manipulations? Yes **(if yes, describe)**

All C57BL/6 Cldn7 Knockout mice do not live beyond 12 days after birth. All experiments for this study will be performed on new-born and mice younger than 12 days.

2. Are there any deviations from standard husbandry practices?
No **If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, prolonged weaning times, wire-bottom cages, etc.):**
Click here to enter text.

3. The default housing method for social species is pair or group housing (including mice, rats, guinea pigs, rabbits, dogs, pigs, monkeys). Is it necessary for animals to be singly housed at any time during the study?

No **(If yes, describe housing and justify the need to singly house social species):**
Click here to enter text.

4. Are there experimental or scientific reasons why routine environmental enrichment should not be provided? No
(If yes, describe and justify the need to withhold enrichment)
Click here to enter text.

5. If wild animals will be captured or used, provide permissions (collection permit # or other required information):
N/A

6. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.
Animal facility, 7N-80

IV. Animal Procedures

A. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design. Briefly state surgical plans in this section. Surgical procedures can be described in detail in IV.S.

Task 1: For the first task, we will need to isolate intestinal and/or kidney epithelial cells from the wild-type (WT, Cldn7+/+) and knockout (KO, Cldn7-/-) mice to be grown and studied in vitro. Cldn7 WT and KO pups at 3-4 days old or 2-5-month-old inducible control and KO mice will be euthanized, and intestines and kidneys will be removed from abdominal cavity. The cells will then be isolated from intestines and kidneys and cultured in vitro.

Task 2: To determine whether epithelial barrier function of KO intestines is intact, intestinal lumen will be injected with biotin tracer. Biotin is a compound that labels proteins on the cell surface. Biotins are incapable of diffusing through the intact tight junctions. The protocol for setting up the biotin assay can be found in Methods Mol Biol. 2011; 762: 91-100. Mouse pups ages 0, 3, and 6 days old from WT and KO pups or the inducible control and KO mice at 2-5-month-old will be injected with Biotin in the intestinal lumen. Mice will be euthanized using Isoflurane overdose and secondary method of euthanasia. After ensuring the mouse is dead, the abdominal cavity is exposed to inject Sulfo-NHS-Biotin diluted in phosphate buffered saline (PBS) using a low-pressure syringe pump with a 25G needle connected to polyethylene tubing running at 50 µl/min up to 10 mins. A second group of mice will be injected with PBS as a control. Then the intestines will be dissected to

be embedded on O.C.T. compound to be studied under the microscope with immunohistochemistry staining.

This procedure will also be our lab general method to detect in vivo tight junction function.

Task 3: Tamoxifen injection is used to induce claudin-7 deletion. Tamoxifen (Sigma) will be dissolved in 10 ml Sunflower oil to make a 0.2g/10ml stock solution and then filtered to sterile it. Up to 8-month old of mice will be injected with up to 7 doses of 2mg Tamoxifen /mouse by I.P. over the period of 12- 14 days to induce claudin-7 tissue-specific KO mice. The injected mice will be monitored daily. The mice will be euthanized if they show the sign of breath problem, not eating or drinking, or not moving.

Sunflower oil will be given to the control mice separately to serve as vehicle control since Tamoxifen is dissolved in Sunflower oil. Up to 8-month old of control mice will be injected with up to 7 doses of up to 200 ul /mouse by I.P. over the period of 12- 14 days. The injected mice will be monitored daily. The mice will be euthanized if they show the sign of breath problem, not eating or drinking, or not moving.

Task 4: The wild-type (WT, Cldn7+/+) and knockout (KO, Cldn7-/-) pups from 0-10 days after birth will be euthanized to collect tissues including intestines and kidneys.

We identified that the milk proteins in pup's stomach and intestines affect our experimental results. Therefore, the mouse pups need to be fasted for up to 6 hours in order to get rid of the milk proteins in the stomach and proximal region of the intestines. This needs to be done so that the intestinal samples and our experimental results will not be affected by the milk proteins.
The neonates will be separate from the mother and fasted for the duration of up to 6 hours. During this time, they will be kept warm on a heating pad and stimulated with a Q-tip hourly to defecate and urinate. Animals will be euthanized according to the current protocol.

There is no alternative method. To minimize the discomfort, they will be kept warm on a heating pad and a cotton Q-tip will be used to rub gently the genital area of the pups hourly to facilitate the urination and defecation. We will use the minimum number of mice to do the experiments. We do not expect to result in adverse effects since these mice will be euthanized right after fasting.

Up to 8-month-old inducible mice will be euthanized to collect tissues including intestines and kidneys.

In sections IV.B-IV.S below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.

Please refer to DCM and IACUC websites for relevant guidelines and SOPs.

B. Anesthesia/Analgesia/Tranquilization/Pain/Distress Management For Procedures Other than Surgery:

For all procedures, provision of pre-emptive (pre-procedural) analgesia is required, unless specifically exempted by DCM veterinarians. For major survival surgical procedures and extensive non-surgical procedures requiring anesthesia, post-procedural analgesia must be provided for a minimum of 3 full days following anesthetic recovery, unless specifically exempted by DCM veterinarians. Analgesic administration should be continued for at least 1 full day following recovery from minor surgical and non-surgical procedures. Please contact DCM veterinary staff for recommendations and guidance when formulating anesthetic regimens.

Adequate records describing anesthetic monitoring and recovery must be maintained for all species. Please see Guidelines for Intra-operative and Intra-procedural Monitoring on the IACUC website.

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:
Click here to enter text.

1. **Describe the pre-procedural preparation of the animals:**
   a. **Food restricted for** Click here to enter text. **hours**
   b. **Food restriction is not recommended for rodents and rabbits and must be justified:**
   Click here to enter text.
   c. Water restricted for Click here to enter text. **hours**
   d. Water restriction is not recommended in any species for routine pre-op prep and must be justified:
   Click here to enter text.

2. Anesthesia/Analgesia *for Procedures Other than Surgery*

	Agent	Concentration	Dose (mg/kg)	Max Volume	Route	Frequency	Number of days administered
Pre- procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre- anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Reason for administering agent(s):
Click here to enter text.

4. For which procedure(s):
Click here to enter text.

5. Methods for monitoring anesthetic depth:
Click here to enter text.

6. Methods of physiologic support during anesthesia and recovery:
Click here to enter text.

7. Duration of recovery:
Click here to enter text.

8. Frequency of recovering monitoring:
Click here to enter text.

9. Specifically what will be monitored?
Click here to enter text.

10. When will animals be returned to their home environment?
Click here to enter text.

11. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:
Click here to enter text.

C. Use of Paralytics

1. Will paralyzing drugs be used? No

2. For what purpose:

Click here to enter text.

3. Please provide scientific justification for paralytic use:

Click here to enter text.

4. Paralytic drug:

Click here to enter text.

5. Dose:

Click here to enter text.

6. Method of ensuring appropriate analgesia during paralysis:

Click here to enter text.

D. Blood or Body Fluid Collection

1. Please fill out appropriate sections of the chart below:

	Location on animal	Needle/catheter size	Volume collected	Frequency of procedure	Time interval between collections
Blood Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Body Fluid Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

E. Injections, Gavage, & Other Substance Administration

1. Please fill out appropriate sections of the chart below:

	Compound	Location & Route of admin	Needle/catheter/gavage size	Max volume admin	Freq of admin (ie two times per day)	Number of days admin (ie for 5 days)	Max dosages (mg/kg)
Injection/ Infusion	Task 1: Sulfo-NHS-LC-Biotin	Lumen intestine	25G needle	Up to 200 ul	Once	1 day	2000mg /kg
	PBS	Lumen intestine	Click here to enter text. 25 G needle	Up to 200 ul	Once	1 day	

	Task 2: Tamoxifen Sunflower oil	I.P. I.P.	25 G needle 25 G needle	2mg/ 200ul/ Mouse Up to 200 ul	Once per day, Up to 7 doses Once per day, Up to 7 doses	Up to 14 days Up to 14 days	80mg/kg
Gavage	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. **Pharmaceutical grade drugs, biologics, reagents, and compounds are defined as agents approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been written/established by any recognized pharmacopeia such as USP, NF, BP, etc. These standards are used by manufacturers to help ensure that the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy. For all injections and infusions for CLINICAL USE, PHARMACEUTICAL GRADE compounds must be used whenever possible. Pharmaceutical grade injections and infusions for research test articles are preferred when available. If pharmaceutical grade compounds are not available and non-pharmaceutical grade agents must be used, then the following information is necessary:**

a. **Please provide a scientific justification for the use of ALL non-pharmaceutical grade compounds. This may include pharmaceutical-grade compound(s) that are not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.**

We purchase Tamoxifen from Sigma, T5648, and its purity ≥99%. However, this product is not a pharmaceutical grade compound. There is no pharmaceutical grade Tamoxifen available at this purity.
There is no pharmaceutical grade of Sulfo-NHS-Biotin available.

b. Indicate the method of preparation, addressing items such as purity, sterility, pH, osmolality, pyrogenicity, adverse reactions, etc. (please refer to ECU IACUC guidelines for non-pharmaceutical grade compound use), labeling (i.e. preparation and use-by dates), administration and storage of each formulation that maintains stability and quality/sterility of the compound(s).

We will purchase Tamoxifen from Sigma, T5648, and its purity ≥99%. This product is not a pharmaceutical grade compound.

Tamoxifen (Sigma) will be dissolved in 10 ml Sunflower oil to make a 0.2g/10ml stock solution and then passed through a 0.2 μm filter to sterilize. Up to 8-month old of mice will be injected with 2mg Tamoxifen /mouse by I.P. once per day for up to 7 doses over the period of up to 14 days to induce claudin-7 tissue-specific mice. The injected mice will be monitored daily after tamoxifen injections. The mice will be euthanized if they show the sign of breath problem, not eating or drinking, or not moving.

There are no adverse reactions at the concentration that we will use. The compound will be made as needed, store at -20℃, protect from the light. The pH will not be assessed since it is dissolved in Sunflower oil.

To prepare 1 mg/ml Sulfo-NHS-Biotin (Pierce Chemical Co., Molecular weight: 443.43; Catalog # 21217), we will take Sulfo-NHS-Biotin out from −20°C freezer and let it sit at room temperature for 15 min before opening the cap. Dissolve Biotin in PBS to make 1 mg/ml solution and then passed through a 0.2 μm filter to sterilize.

The Biotin solution will be made fresh on the day of injection. PBS pH will be 7.4 and adding Biotin will not change the PBS pH.

F. Prolonged restraint with mechanical devices

Prolonged restraint in this context means *beyond routine care and use procedures* for rodent and rabbit restrainers, and large animal stocks. Prolonged restraint also includes *any* use of slings, tethers, metabolic crates, inhalation chambers, primate chairs and radiation exposure restraint devices.

1. For what procedure(s):
Click here to enter text.

2. Explain why non-restraint alternatives cannot be utilized:
Click here to enter text.

3. Restraint device(s):
Click here to enter text.

4. Duration of restraint:
Click here to enter text.
5. Frequency of observations during restraint/person responsible:
Click here to enter text.
6. Frequency and total number of restraints:
Click here to enter text.
7. Conditioning procedures:
Click here to enter text.
8. Steps to assure comfort and well-being:
Click here to enter text.
9. Describe potential adverse effects of prolonged restraint and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

G. Tumor Studies, Disease Models, Toxicity Testing, Vaccine Studies, Trauma Studies, Pain Studies, Organ or System Failure Studies, Shock Models, etc.

1. Describe methodology:
Click here to enter text.
2. Expected model and/or clinical/pathological manifestations:
Click here to enter text.
3. Signs of pain/discomfort:
Click here to enter text.
4. Frequency of observations:
Click here to enter text.
5. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

H. Treadmills/Swimming/Forced Exercise

1. Describe aversive stimulus (if used):
Click here to enter text.
2. Conditioning:
Click here to enter text.
3. Safeguards to protect animal:
Click here to enter text.

4. Duration:
Click here to enter text.
5. Frequency:
Click here to enter text.
6. Total number of sessions:
Click here to enter text.
7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

I. Projects Involving Food and Water Regulation or Dietary Manipulation

(*Routine pre-surgical fasting not relevant for this section)*

1. Food Regulation

a. Amount regulated and rationale:

We identified that the milk proteins in pup's stomach and intestines affect our experimental results. Therefore, the mouse pups need to be fasted for up to 6 hours in order to get rid of the milk proteins in the stomach and proximal region of the intestines. This needs to be done so that the intestinal samples and our experimental results will not be affected by the milk proteins.
The neonates will be separate from the mother and fasted for the duration of up to 6 hours. During this time, they will be kept warm on a heating pad and stimulated with a Q-tip hourly to defecate and urinate. Animals will be euthanized according to the current protocol.

There is no alternative method. To minimize the discomfort, they will be kept warm on a heating pad and a cotton Q-tip will be used to rub gently the genital area of the pups hourly to facilitate the urination and defecation. We will use the minimum number of mice to do the experiments. We do not expect to result in adverse effects since these mice will be euthanized right after fasting.

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):
Up to 6 hours fasting

c. Frequency of observation/parameters documented (i.e. recording body weight, body condition, etc.):
Hourly using a Q-tip

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

The pups will be euthanized if they are not moving

2. Fluid Regulation

a. Amount regulated and rationale:

Click here to enter text.

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):

Click here to enter text.

c. Frequency of observation/parameters documented (body weight, hydration status, etc.):

Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

3. Dietary Manipulations

a. Compound supplemented/deleted and amount:

Click here to enter text.

b. Frequency and duration (hours for short term/week or month for long term):

Click here to enter text.

c. Frequency of observation/parameters documented:

Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

J. Endoscopy, Fluoroscopy, X-Ray, Ultrasound, MRI, CT, PET, Other Imaging

1. Describe animal methodology:

Click here to enter text.

2. Duration of procedure:
Click here to enter text.
3. Frequency of observations during procedure:
Click here to enter text.
4. Frequency/total number of procedures:
Click here to enter text.
5. Method of transport to/from procedure area:
Click here to enter text.
6. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.
7. Please provide or attach appropriate permissions/procedures for animal use on human equipment:
Click here to enter text.

K. Polyclonal Antibody Production

1. **Antigen/adjuvant used and justification for adjuvant choice:**
   Click here to enter text.
2. **Needle size:**
   Click here to enter text.
3. **Route of injection:**
   Click here to enter text.
4. **Site of injection:**
   Click here to enter text.
5. **Volume of injection:**
   Click here to enter text.
6. **Total number of injection sites:**
   Click here to enter text.
7. **Frequency and total number of boosts:**
   Click here to enter text.
8. **What will be done to minimize pain/distress:**
   Click here to enter text.
9. **Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
   Click here to enter text.

L. Monoclonal Antibody Production

1. Describe methodology:

Click here to enter text.

2. Is pristane used: Choose an item.

Volume of pristane:

Click here to enter text.

3. Will ascites be generated: Choose an item.

i. Criteria/signs that will dictate ascites harvest:

Click here to enter text.

ii. Size of needle for taps:

Click here to enter text.

iii. Total number of taps:

Click here to enter text.

iv. How will animals be monitored/cared for following taps:

Click here to enter text.

4. What will be done to minimize pain/distress:

Click here to enter text.

5. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

M. Temperature/Light/Environmental Manipulations

1. Describe manipulation(s):

Click here to enter text.

2. Duration:

Click here to enter text.

3. Intensity:

Click here to enter text.

4. Frequency:

Click here to enter text.

5. Frequency of observations/parameters documented:

Click here to enter text.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

N. Behavioral Studies

1. Describe methodology/test(s) used:

Click here to enter text.

2. Will conditioning occur? If so, describe:
Click here to enter text.
3. If aversive stimulus used, frequency, intensity and duration:
Click here to enter text.
4. Length of time in test apparatus/test situation: *(i.e., each test is ~10 mins)*
Click here to enter text.
5. Frequency of testing and duration of study: *(i.e., 5 tests/week for 6 months)*
Click here to enter text.
6. Frequency of observation/monitoring during test:
Click here to enter text.
7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

O. Capture with Mechanical Devices/Traps/Nets

1. Description of capture device/method:
Click here to enter text.
2. Maximum time animal will be in capture device:
Click here to enter text.
3. Frequency of checking capture device:
Click here to enter text.
4. Methods to ensure well-being of animals in capture device:
Click here to enter text.
5. Methods to avoid non-target species capture:
Click here to enter text.
6. Method of transport to laboratory/field station/processing site and duration of transport:
Click here to enter text.
7. Methods to ensure animal well-being during transport:
Click here to enter text.
8. Expected mortality rates:
Click here to enter text.
9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

P. Manipulation of Wild-Caught Animals in the Field or Laboratory

1. Parameters to be measured/collected:
Click here to enter text.

2. Approximate time required for data collection per animal:
Click here to enter text.

3. Method of restraint for data collection:
Click here to enter text.

4. Methods to ensure animal well-being during processing:
Click here to enter text.

5. Disposition of animals post-processing:
Click here to enter text.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

Q. Wildlife Telemetry/Other Marking Methods

1. Describe methodology (including description of device):
Click here to enter text.

2. Will telemetry device/tags/etc. be removed? Choose an item. **If so, describe:**
Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

R. Other Animal Manipulations

1. Describe methodology:
Click here to enter text.

2. Describe methods to ensure animal comfort and well-being:
Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

S. Surgical Procedures

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species. Please see Guidelines for Intra-operative and Intra-procedural Monitoring on the IACUC website.

1. Location of Surgery (Building & Room #):
Click here to enter text.

2. Type of Surgery (check all that are appropriate):
Click here to enter text.

☒ **Non-survival surgery (animals euthanized without regaining consciousness)**

☐ **Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function)**

☐ **Minor survival surgery**

☐ **Multiple survival surgery**

If yes, provide scientific justification for multiple survival surgical procedures:
Click here to enter text.

3. Describe the pre-op preparation of the animals:

a. **Food restricted for** Click here to enter text. **hours**
b. **Food restricted is not recommended for rodents and rabbits and must be justified:**
Click here to enter text.
c. **Water restricted for** 0 **hours**
d. **Water restriction is not recommended in any species for routine pre-op prep and be justified:**
Click here to enter text.

4. Minimal sterile techniques will include (check all that apply):

Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species and type of surgery (survival vs. non-survival).

☐ **Sterile instruments**

How will instruments be sterilized?

Click here to enter text.

If serial surgeries are done, how will instruments be sterilized between surgeries:

Click here to enter text.

- ☐ **Sterile gloves**
- ☐ **Mask**
- ☐ **Cap**
- ☐ **Sterile gown**
- ☐ **Sanitized operating area**
- ☐ **Clipping or plucking of hair or feathers**
- ☐ **Skin preparation with a sterilant such as betadine**
- ☐ **Practices to maintain sterility of instruments during surgery**

Non-survival (clean gloves, clean instruments, etc.)

5. **Describe all surgical procedures:**

a. Skin incision size and site on the animal:

~~about 1 cm on the abdominal~~

b. Describe surgery in detail (include size of implant if applicable):

c. Method of wound closure:

Click here to enter text.

i. Number of layers

Click here to enter text.

ii. Type of wound closure and suture pattern:

Click here to enter text.

iii. Suture type/size/wound clips/tissue glue:

Click here to enter text.

iv. Plan for removing of skin sutures/wound clip/etc:

Click here to enter text.

6. **Anesthetic Protocol:**

For all procedures, provision of pre-emptive (pre-procedural) analgesia is required, unless specifically exempted by DCM veterinarians. For major survival surgical procedures and extensive non-surgical procedures requiring anesthesia, post-procedural analgesia must be provided for a minimum of 3 full days following anesthetic recovery, unless specifically

exempted by DCM veterinarians. Analgesic administration should be continued for at least 1 full day following recovery from minor surgical and non-surgical procedures. Please contact DCM veterinary staff for recommendations and guidance when formulating anesthetic regimens.

a. If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:
Click here to enter text.

b. Anesthesia/Analgesia For Surgical Procedures

	Agent	Dose (mg/kg or %)	Volume	Route	Frequency	Number of days administered
Pre-operative analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre- anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	~~Isoflurane~~	~~4%~~	Click here to enter text.	~~inhalation~~	~~once~~	~~1~~
Post- operative Analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

c. Methods that will be used to monitor anesthetic depth (include extra measures employed when paralyzing agents are used):
Click here to enter text.

d. Methods of physiologic support during anesthesia and immediate post-op period (fluids, warming, etc.):
Click here to enter text.

e. List what parameters are monitored during immediate post-op period. Provide the frequency and duration:
~~N/A~~

f. Describe any other manipulations that will be used to alleviate pain, distress, and/or discomfort during the immediate post-op period (soft bedding, long sipper tubes, food on floor, dough diet, etc.):

~~N/A~~

g. List criteria used to determine when animals are adequately recovered from anesthesia and when the animals can be returned to their home environment:

~~N/A~~

7. Recovery from Surgical Manipulations (after animal regains consciousness and is returned to its home environment)

~~N/A~~

a. What parameters (behavior, appetite, mobility, wound healing, etc.) will be monitored:

Click here to enter text.

b. How frequently (times per day) will animals be monitored:

Click here to enter text.

c. How long post-operatively (days) will animals be monitored:

Click here to enter text.

8. Surgical Manipulations Affecting Animals

a. Describe any signs of pain/discomfort/functional deficits resulting from the surgical procedure:

Click here to enter text.

b. What will be done to manage any signs of pain or discomfort (include pharmacologic and non-pharmacologic interventions):

Click here to enter text.

c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

V. Euthanasia

Please refer to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition and DCM Guidelines to determine appropriate euthanasia methods.

A. Euthanasia Procedure. ***All investigators, even those conducting non-terminal studies, must complete this section in case euthanasia is required for humane reasons.***

1. Physical Method- If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a scientific justification must be provided:

2. Inhalant Method- Other
(if other, describe the agent and delivery method)

Mice will be euthanized with isoflurane overdose followed by cervical dislocation, pneumothorax, or decapitation.

3. Non-Inhalant Pharmaceutical Method (injectables, MS-222, etc.)- Please provide the following:

a. Agent:

Click here to enter text.

b. Dose or concentration:

Click here to enter text.

c. Route:

Click here to enter text.

B. Method of ensuring death (can be physical method, such as pneumothorax or decapitation for small species and assessment method such as auscultation for large animals):

Ensure death with cervical dislocation, pneumothorax, or decapitation.

B. Describe disposition of carcass following euthanasia:

All animal carcasses will be discarded in biohazard bags and incinerated through ECU Hazardous Waste Management.

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature: Yan-Hua Chen **Date:** 3/24/2022

Veterinarian: DocuSigned by: Cecile Baccanale 483344F5627C423... 3/28/2022 | 10:55 AM EDT

IACUC Chair: _____ **Date:** 03/28/2022

PI Name: Yan-Hua Chen/Signature: Yan-Hua Chen
Date: 09/18/2020

Laboratory Safety Plan for Isoflurane Administration for Anesthesia and Euthanasia

***Process**	Isoflurane administration for anesthesia or euthanasia in animals.
***Hazardous Chemical/ Chemical Class**	Isoflurane: anesthetic liquid/vapor Specific target organ toxicity - single exposure (central nervous system) Specific target organ toxicity – repeated exposure, inhalation (central nervous system, cardio-vascular system) **Target organs:** central nervous system, cardio-vascular system
***Hazardous Equipment**	Anesthesia jar (small glass container with a metal tea strainer to hold a cotton ball with the isoflurane on it).
***Potential Hazards**	Inhalation may cause shortness of breath, respiratory depression, drowsiness or dizziness, may cause damage to organs (cardio-vascular system, central nervous system) through prolonged or repeated exposure. May be harmful if ingested.
***Personal Protective Equipment**	Safety glasses/goggles, nitrile or other chemical compatible gloves (no latex), lab coat, long pants and closed-toed/heeled shoes.
***Engineering and Ventilation Controls**	Administration of isoflurane will be done through a nose cone from a precision vaporizer that is scavenged via an activated charcoal filter canister in a certified chemical fume hood or designated chamber.
Designated Use Area for Carcinogens, Reproductive Toxins or Acute Toxins	Brody 7N-80 where administration will occur.
Special Use Procedures	Avoid inhalation, use small amounts. When using Isoflurane is it suggested do so in an approved, certified chemical fume hood or designated chamber.
Special Handling and Storage Requirements	Protect from sunlight, store in well-ventilated place. Avoid prolonged or repeated exposure. Keep container tightly closed.
***Spill and Accident Procedures**	Clean spills only if proper materials are available and if researcher is properly trained to do so; all other spills should be reported to EH&S for clean-up. For minor spills: ventilate area; if spill occurs outside hood, cover liquid with absorbent material; slowly brush into dustpan and place in plastic bag; do not breath the dust from absorbent. Contact EH&S for removal of sealed waste clean-up material. Do not place clean up materials in regular waste. Broken glass fragments will be collected with a scoop/brush and placed in the sharps container. Contact EH&S for removal of any contaminated materials.
***Waste Minimization Plan**	Minimize the amount of chemical used whenever possible.
***Hazardous Waste Disposal**	Full filter canisters will be disposed in a biohazard bag. Disposal of hazardous waste shall be completed by EH&S Hazardous Waste Management. Keep waste in an approved container with secure lid. Attach a Hazardous waste tag and complete the information required. Remember the signature at the bottom. Contact EH&S for pick up by emailing safety@ecu.edu. Any biological waste should be placed in a red bio-hazard bag and request a pick-up through Prospective Health.
Decontamination Procedures	Work area will be decontaminated at the end of each work session by removing the paper liner and wiping the work surfaces from back to front. (PPE as listed above must be worn during decontamination.)
Animal Care Precautions	Animal care workers in this area should wear standard required PPE.
***Chemical Procurement**	Order only minimum amounts needed for the experiments. Isoflurane becomes inactivated over extended periods of time at room temperature.
***Revision Date**	09/2020

PI Name: Yan-Hua Chen/Signature: Yan-Hua Chen
Date approved: 09/18/2020

Laboratory Safety Plan for Carbon Dioxide Euthanasia Administration in Animals

***Process**	Carbon Dioxide Euthanasia Administration
***Hazardous Chemical/ Chemical Class**	Gas under pressure (compressed gas), liquefied gas, simple asphyxiant **Target Organs:** Lungs Class 9
***Hazardous Equipment**	N/A
***Potential Hazards**	Inhalation may cause rapid suffocation by displacing oxygen. Contains gas under high pressure; may explode if heated.
***Personal Protective Equipment**	Safety glasses/goggles, nitrile or other chemical compatible gloves (no latex), full-buttoned lab coat, long pants and closed-toed/heeled shoes.
***Engineering and Ventilation Controls**	When preparing, handling and administering Carbon dioxide, do so in a certified chemical fume hood or designated chamber (Brody 7N-80). The floor of the hood will be covered with plastic backed paper liner. All extraneous equipment will be removed from the hood before work begins. Do not administer outside of a certified fume hood or designated chamber.
Special Use Procedures	Cylinders should be inspected before each use. Include cylinder, piping, safety relief devices, valves, protection caps and stems. Use regulators that are rated for this cylinder. Close valve after each use and when empty use the valve protection cap. Protect from damage; examples include dragging, rolling or dropping. Ensure cylinders are secured with 2 chains.
Special Handling and Storage Requirements	Store in well ventilated areas. Keep upright, secured with 2 chains (1/3 top, 1/3 bottom) attached to a fixed surface. Keep capped and labeled when not in use. Wash hands immediately after handling or using the material. Avoid inhalation and exposure to skin and eyes. Keep away from flames and heat sources. If heated the increased pressure can cause the cylinder to explode.
***Spill and Accident Procedures**	If a leak occurs, shut the regulator valve if possible, contact the Medical Storeroom and/or vendor that provided cylinder and EH&S at 328-6166. Shut the door on your way out and evacuate the area. Do not return to area until issue has been resolved. If cylinder falls and is not damaged, upright and secure it immediately. If cylinder is damaged evacuate the area and contact the Medical Storeroom and/or vendor.
***Waste Minimization Plan**	Minimize the amount required whenever possible.
***Hazardous Waste Disposal**	Cylinders should be capped when not in use or empty and labeled as such. Contact the medical storeroom located in the BSOM to have the vendor pick up the empty cylinder and replace it with a full one. Any biological waste should be placed in a red bio-hazard bag or bio-hazard sharps container (syringes/needles etc.) and request a pick-up through Prospective Health.
Decontamination Procedures	Make sure to wash hands thoroughly after use. If necessary, remove equipment from fume hood and wipe from back to front.
Animal Care Precautions	DCM personnel/animal care workers should wear standard PPE required.
***Chemical Procurement**	Carbon dioxide will be procured in the minimum quantity necessary.
***Revision Date**	06/2020

PI Name/Signature: Yan-Hua Chen
Date approved: 2/21/2019

Laboratory Safety Plan for Ketamine/Xylazine Solutions Preparation and Administration

***Process**	Preparation and use of Ketamine/Xylazine solutions for anesthesia
***Hazardous Chemical/ Chemical Class**	Ketamine hydrochloride: **Controlled substance (schedule III)** acute toxicity, skin irritation, eye irritation, specific target organ toxicity (single exposure) Xylazine hydrochloride: acute toxicity, skin irritation, eye irritation, specific target organ toxicity (single exposure) **Target Organs**: respiratory system
***Hazardous Equipment**	Syringes
***Potential Hazards**	Ketamine hydrochloride: harmful if swallowed, causes skin irritation, causes serious eye irritation, may cause respiratory irritation. Xylazine hydrochloride: toxic if swallowed, causes skin irritation, causes serious eye irritation, may cause respiratory irritation.
***Personal Protective Equipment**	Safety glasses/goggles, nitrile or other chemical compatible gloves (no latex), fully buttoned lab coat, long pants and closed-toed/heeled shoes.
***Engineering and Ventilation Controls**	All handling and preparation of the Ketamine (18mg/ml)/Xylazine (2mg/ml) solution will be done in a certified chemical fume hood. The floor of the hood will be covered with a plastic backed paper liner. All extraneous equipment will be removed from the hood before work begins. Containers containing these materials can only be removed from the fume hood if tightly capped and the exterior is wet wiped. Injections will be performed under the fume hood. All equipment required for the injections will be placed in the hood prior to beginning work.
Designated Use Area for Carcinogens, Reproductive Toxins or Acute Toxins	7N-80
Special Use Procedures	Drug delivery will be accomplished in a closed loop system. Syringes used for delivery will be sized large enough so that they are not full when the entire drug dose is present. Never re-cap needles. Syringes will be disposed at the end of administration in a sharps disposal container. If moving the chemical, placed in secondary containment with absorbent paper and lid. Use freight elevator or stairs to transport.
Special Handling and Storage Requirements	The storage site in the lab **will be secured at all times (DEA regulation)**. Containers will be properly labelled and segregated from other chemicals by secondary containment. Avoid prolonged or repeated exposure. Keep tightly closed. Each use of the solution will be recorded in the drug use log book.
***Spill and Accident Procedures**	Small spill: Clean up with proper materials if trained and materials available or contact EH&S at 328-6166 for cleanup and disposal. Large spill: evacuate the area and contact EH&S for cleanup and disposal. If a spill occurs under the fume hood, the substance will be wiped up with absorbent gauze pads and the sill area cleaned 3 times with soap and water and wiped down with bleach 1:5. Any broken glass fragments will be collected with a scoop and brush and placed in the sharps container. Contaminated items and clean-up material will be double bagged and placed in a red bag for incineration. Red bags must be marked with the cancer hazard symbol. Contact EH&S for removal of any contaminated materials.
***Waste Minimization Plan**	Minimize the amount of chemical purchased and used whenever possible.
***Hazardous Waste Disposal**	Disposal of hazardous waste shall be completed by EH&S Hazardous Waste Management. Keep waste in an approved container with secure lid. Attach a Hazardous waste tag and complete the information required. Contact EH&S for pick up by emailing safety@ecu.edu. **Empty bottles should be returned to the person holding the controlled substances license in your department.** Any biological waste should be placed in a red bio-hazard bag and request a pick up through Prospective Health.
Decontamination Procedures	If necessary, hood will be decontaminated by removing the paper liner and wiping the hood interior surfaces with 1:5 bleach from the top to bottom and back to front. PPE as listed above must be worn during decontamination.
Animal Care Precautions	Animal care workers in this area should wear standard required PPE.
***Chemical Procurement**	Chemicals will be procured in the minimum quantity necessary for the procedures.
***Revision Date**	02-2019

APPENDIX B: PERMISSION TO PUBLISH

Dear William Guiler:

I am completing a doctoral dissertation at East Carolina University Brody School of Medicine titled "Investigating Non-Tight Junction Functions of Claudin-7: Regulating Large Intestine Stem Cell Functions and Niche Maintenance, and Junctional Protein Nanoarchitecture". I would like your permission to reprint in my dissertation excerpts from the following:

- Nanoarchitecture and Molecular Interactions of Epithelial Cell Junction Proteins Revealed by Super-Resolution Microscopy published in Annals of the New York Academy of Sciences in July 2022.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by UMI. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below. Thank you very much.

Sincerely,

Amna Naser

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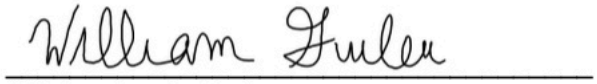

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10/10/2022

Date

Dear Dr. Yan-Hua Chen:

I am completing a doctoral dissertation at East Carolina University Brody School of Medicine titled "Investigating Non-Tight Junction Functions of Claudin-7: Regulating Large Intestine Stem Cell Functions and Niche Maintenance, and Junctional Protein Nanoarchitecture". I would like your permission to reprint in my dissertation excerpts from the following:

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Yan-Hua Chen

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Dear Tiaosi Xing:

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Dear Dr. Qun Lu:

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Sincerely,

Amna Naser

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Qun Lu

Click here to enter text.

November 4, 2022

Date