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The Role of Inflammation in Colitis-Associated Cancer

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Abstract

Colitis-associated cancer (CAC) is a major complication associated with Inflammatory bowel disease (IBD). Unfortunately, there are currently few, if any, effective chemopreventative strategies against CAC. Furthermore, the exact mechanism by which inflammation leads to CAC remains poorly understood. In this thesis, we focus on two inflammatory signaling pathways, the cyclooxygenase (COX) and NF-κB pathways, that have been shown to link inflammation and cancer. For instance, non-steroidal anti-inflammatory drugs (NSAIDs) that target COX-1 and/or -2 have previously been shown to be effective in chemoprevention of sporadic colorectal cancer. However, the ability of NSAIDs to prevent CAC has not fully been explored. Using the previously described *Dclk1*^{CreERT2}*;APC*$^{f/f}$ transgenic mouse model of CAC, we demonstrate that low-dose Aspirin prevents colitis-associated tumorigenesis by inhibiting epithelial-derived COX-1. Moreover, we identify that PGE_2, a major product of COX activity, and phospho-Akt are key inflammatory mediators that promote cellular plasticity of the intestinal epithelium. Specifically, using the *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mouse model, we have shown that PGE_2 and phospho-Akt are able to stimulate normally quiescent Dclk1+ tuft cells to repopulate the entire colonic crypt. Furthermore, we demonstrate that COX-1-derived PGE_2 and phosho-Akt are upregulated in colitis and cooperate to contribute to inflammation-associated dysplasia through the activation of Wnt signaling. In separate studies, we examined the role of canonical NF-κB signaling in the *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mouse model of CAC, which has been shown to link inflammation and cancer through the activity of IKKβ. We report the novel observation that IKKβ in Dclk1+ tuft cells serves a protective role in colitis and CAC. In summary, we have identified two novel mechanisms by which inflammation contributes to cancer and have shown that low-dose Aspirin serves as a safe and effective chemopreventative agent for the use against CAC.

Keywords

Akt (PKB)

Colitis

Colitis-associated cancer (CAC)

Colorectal cancer (CRC)

Cyclooxygenase (COX)

Doublecortin-like kinase 1 (Dclk1)

Inflammation

Inflammatory bowel disease (IBD)

Intestinal stem cells (ISCs)

Non-steroidal anti-inflammatory drugs (NSAIDs)

Tuft cells

Wnt signaling pathway

Summary for Lay Audience

Colorectal cancer is the second most common cause of cancer death in Canada. A major risk factor for this disease is prolonged inflammation of the gastrointestinal tract. This is primarily seen in patients who have Inflammatory bowel disease (IBD), which includes diseases such as Crohn's disease and Ulcerative colitis. Therefore, patients with IBD are at an increased risk for colitis-associated cancer (CAC). Despite the clear link between inflammation and cancer, exactly how colitis leads to CAC remains largely unknown. Similarly, there are currently few, if any, drugs that can aid in the prevention of CAC. Thus, the aim of this work is to identify how inflammation leads to cancer and determine how we can intervene to prevent this transformation. To do this, we focused on two known inflammatory pathways: the cyclooxygenase (COX) and NF-κB signaling pathways. Non-steroidal anti-inflammatory drugs, such as Aspirin, are some of the most commonly used drugs in the world and are known to inhibit the activity of COX enzymes. We report here the novel finding that low-dose Aspirin is safe and effective against CAC. We further identified that Aspirin works to prevent CAC by downregulating a known key mediator of inflammation, prostaglandin E2 (PGE_2). We show that in colitis, PGE_2 acts alongside Akt (Protein Kinase B) to stimulate normally non-dividing cells in the colon to give rise to cancer. We further show that PGE_2 and Akt promote inflammation-associated cancer by activating a pathway that is known to be abnormally activated in CRC, the Wnt signaling pathway. Furthermore, we identified that the NF-κB pathway serves a protective function against colitis and CAC. Overall, we have identified two novel ways that colitis leads to CAC and have identified that low-dose Aspirin can be safely and effectively used to prevent the transition from IBD to cancer.

Co-Authorship Statement

Hayley Good was the primary contributor to all experimental work presented in this thesis, unless indicated otherwise below. All chapters were written by Hayley Good and edited by Dr. Samuel Asfaha.

Chapter 3 is adapted from the following manuscript: Good HJ, Shin AE, Zhang L, Meriwether D, Worthley D, Reddy ST, Wang TC, and Asfaha S. PGE_2 and Akt promote tuft cell stemness to initiate inflammation-associated cancer. (In preparation). DM and the laboratory of STR performed the LC-MS inflammatory lipid panel in Figure 3.7 and Figure 3.8. LZ performed the Western Blots in Figure 3.9 and provided technical support. AES assisted with genotyping, mouse maintenance, and the multiplex cytokine array in Figure 3.5. DW and TCW assisted with manuscript revision.

Additional experiments included in **Chapter 3** were performed with assistance: Melody Ong and Sarah Chun, an undergraduate student and PEL student respectively, helped perform the tumor organoid experiments in Figure 3.23. Amanda Liddy additionally provided technical support for genotyping, mouse dissections, tissue processing, H&E staining, and imaging. Amber Harnett contributed to mouse genotyping and AOM/DSS experiments.

Chapter 4 is adapted from the following manuscript: Good HJ*, Shin AE*, Zhang L, and Asfaha S. NF-κB signaling in Dclk1+ cells is protective against colitis and colitis-associated cancer. (In preparation). *HJG and AES are co-first authors on this manuscript. AES performed all experiments in Figure 4.1, Figure 4.2, and Appendix 5 and assisted with genotyping. LZ performed the PCR DNA Assay in Appendix 6 and provided technical support.

Dedication

To my parents and family for their unconditional love and support.

To my late grandfather, Wilbur Wallace Good, who inspired my work in this field.

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List of Abbreviations

4-OHT	4-hydroxytamoxifen
15-PGDH	15-hydroxyprostaglandin dehydrogenase
AA	Arachidonic acid
AC	Adenylyl cyclase
ACF	Aberrant crypt foci
AKT	Protein kinase B
ANOVA	Analysis of variance
AOM	Azoxymethane
APC	Adenomatous polyposis coli
BAC	Bacterial artificial chromosome
BMP	Bone morphogenetic protein
bp	Base pairs
CAC	Colitis-associated colorectal cancer
cAMP	Cyclic adenosine monophosphate
CCL5	Chemokine ligand 5 (also RANTES)
CCND1	Cyclin D1
CD	Crohn’s disease
cDNA	Complementary DNA
CK1	Casein kinase 1
COX	Cyclooxygenase
COX-1	Cyclooxygenase 1 (also Ptgs1)
COX-2	Cyclooxygenase 2 (also Ptgs2)
CRC	Colorectal cancer
Cre	Cre recombinase
CreERT2	Cre recombinase estrogen-receptor fusion transgene
CTNNB1	Beta catenin
DAPI	Nuclear stain
DCLK1	Doublecortin-like kinase 1
DMH	Dimethylhydrazine
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DSS	Dextran sodium sulfate
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
Dvl	Disheveled
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EP	PGE_2 receptor
FAP	Familial adenomatous polyposis
FFPE	Formalin fixed paraffin embedded
FOSL1	Fos-related antigen 1
Fzd	Frizzled
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte-macrophage colony-stimulating factor
GEO	Gene expression omnibus
GFP	Green fluorescent protein
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GSK-3β	Glycogen synthase kinase 3 beta
H&E	Hematoxylin and eosin
HCl	Hydrochloric acid
HNPCC	Hereditary non-polyposis colorectal cancer
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IF	Immunofluorescence
IFN-γ	Interferon gamma
IHC	Immunohistochemistry
IKK2ca	Constitutively active IKKβ (IKK2)
IKKα	Inhibitor of nuclear factor kappa-B kinase subunit alpha (also IKK1)
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta (also IKK2)

IKKγ	Inhibitor of nuclear factor kappa-B kinase subunit gamma (also NEMO)
IL	Interleukin
ISC	Intestinal stem cell
K19	Keratin 19 (also Krt19)
KC	Keratinocyte chemoattractant
KRAS	Kirsten rat sarcoma viral oncogene homolog
LC-MS	Liquid chromatography mass spectrometry
LEF	Lymphoid enhancer-binding factor
LGR5	Leucine rich repeat containing G-protein coupled receptor 5
LiCl	Lithium chloride
LIF	Leukemia inhibitory factor
LRP	Low-density lipoprotein receptor-related protein
MCP-1	Monocyte chemoattractant protein 1
M-CSF	Macrophage colony stimulating factor
MIG	Monokine induced by interferon gamma
Min	Multiple intestinal neoplasia
MIP	Macrophage inflammatory protein
Miso	Misoprostol, PGE analogue
MPO	Myeloperoxidase
mRNA	Messenger RNA
mTmG	Membrane-targeted tandem dimer tomato membrane-targeted GFP
MYC	C-myc proto-oncogene, bHLH transcription factor
n	n-value, sample size
NaCl	Sodium chloride
NEMO	NF-kappa-B essential modulator (also IKKγ)
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-kappa-B-inducing kinase
NSAID	Non-steroidal anti-inflammatory drug
OCT	Optimal cutting temperature medium
p	p-value, probability
pAKT	Phosphorylated Akt
PBS	Phosphate-buffered saline

PFA	Paraformaldehyde
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin F_2 alpha
PGI_2	Prostacyclin
PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PPAR-δ	Peroxisome proliferator-activated receptor delta
PTEN	Phosphatase and tensin homolog
PTGES	Prostaglandin E synthase (also PGES)
PUFA	Polyunsaturated fatty acid
qRT-PCR	Quantitative real-time PCR (also qPCR)
RANTES	Regulated upon activation normal T cell expressed and secreted (also CCL5)
RFP	Red fluorescent protein
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROSA	Reverse orientation splice acceptor
RSPO	R-spondin
SC-560	COX-1 inhibitor
SC79	Akt activator
SEM	Standard error of mean
Ser	Serine
SMAD4	Mothers against decapentaplegic homolog 4
TAM	Tamoxifen
TCF	T-cell factor
tdTom	Tandem dimer tomato fluorescent protein

TGF-β	Transforming growth factor beta
Thr	Threonine
TNF-α	Tumor necrosis factor alpha
TXA_2	Thromboxane A2
TXB_2	Thromboxane B2
UC	Ulcerative colitis
UCN	Ulcerative colitis with associated neoplasia
VEGF	Vascular endothelial growth factor
Wnt	Wingless/integrated
WT	Wild-type

List of Appendices

Chapter 1

1 Introduction

1.1 Overview of Chapter 1

This thesis is focused on determining the mechanism by which colitis leads to colitis-associated cancer (CAC) by examining the role of related inflammatory signaling pathways in this transformation. In **Section 1.2**, the normal intestinal tract is introduced, with particular focus on homeostatic signaling pathways and cell types of interest. **Section 1.3** introduces colorectal cancer (CRC), the known pathogenesis of this cancer, and how normal signaling pathways can become dysregulated to contribute to CRC. **Section 1.4** expands on the role of inflammation as a risk factor for CRC, by introducing colitis and colitis-associated cancer (CAC). **Sections 1.5**, **1.6**, and **1.7** introduce the signaling pathways that are explored further in this thesis: cyclooxygenase (COX), PI3K/Akt, and NF-κB, and what is known about these pathways in relation to intestinal homeostasis, colitis, and colorectal cancer. Lastly, **Section 1.8** reviews the overall rationale, hypotheses, and project aims for this thesis.

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1.2 The Gastrointestinal (GI) Tract

1.2.1 *Anatomy of the GI Tract*

The alimentary gastrointestinal tract is composed of the mouth, pharynx, esophagus, stomach, small intestine, large intestine (colon), and the rectum. The small and large intestine form the two functionally and structurally distinct segments of the intestine. The adult small intestine is approximately six meters long and is composed of three regions known as the duodenum, jejunum, and ileum. The functions of the small intestine are to digest food, absorb nutrients, and maintain a barrier against potentially harmful luminal contents (Tortora and Derrickson, 2014). The adult colon, also known as the large bowel or the large intestine, is on average 1.5 meters long and has several distinct sections from the proximal to distal end: cecum, ascending colon, transverse colon, descending colon, and sigmoid colon. The colon is primarily responsible for the absorption of water from solid waste in addition to some nutrients and electrolytes (Azzouz and Sharma, 2021). Other functions include the propagation of solid feces to the rectum for excretion, and the production and absorption of vitamins (Azzouz and Sharma, 2021). The wall of the intestinal tract is composed of four separate tissue layers: the mucosa, submucosa, muscularis propria, and serosa/adventitia (Aldred et al., 2009).

The intestinal mucosa is composed of three compartments: the innermost epithelial layer, which has absorptive and secretory functions (further described in **Section 1.2.2**); the lamina propria, which is composed of connective tissue, small blood and lymphatic vessels, and immune cells; and the muscularis mucosae, a thin smooth muscle layer (Rao and Wang, 2010). The submucosa is a dense connective tissue layer that contains larger blood vessels, lymphatics, and nerves (Aldred et al., 2009). In particular, the submucosa contains part of the enteric nervous system known as Meissner's plexus (Shahrestani and M Das, 2021). The muscularis propria layer consists of two layers of muscle: a circular inner layer and a longitudinal outer layer. The contraction of these muscles is what orchestrates peristalsis to propagate solid waste for excretion. Between these muscle layers is Auerbach's (myenteric) plexus (Shahrestani and M Das, 2021). The serosa or adventitia is the outermost layer which is comprised of connective tissue. The enteric nervous system,

including Meissner's and Auerbach's plexus, controls the movement and secretion functions of the GI tract (Steadman et al., 2013).

1.2.2 *Intestinal Epithelium*

The intestinal epithelium is the innermost portion of the colonic mucosa which consists of a single layer of columnar intestinal epithelial cells (IECs). In the small intestine, the epithelium consists of two major compartments: crypts and villi. Intestinal crypts are invaginations of the epithelium into the lamina propria, whereas villi are finger-like protrusions extending into the lumen. Each villus is surrounded by at least six intestinal crypts and functions to increase the surface area available for nutrient absorption. In contrast to the small intestine, the mucosa of the colon is smooth due to its lack of villi. Colonic crypts are organized like a "row of test tubes" which is evident upon sections cut perpendicularly to the surface (**Figure 1.1**), where each crypt represents a single epithelium-lined "test tube" with a central lumen down the middle. Intestinal epithelial cells display a remarkable self-renewal rate and completely turn over every 3-5 days in the mouse, and every 7 days in the human (Barker, 2014; Leblond and Stevens, 1948). In fact, over 300 million new epithelial cells are generated in the small intestine every day (Barker, 2014). This rapid turnover is likely a result of the constant direct exposure of these epithelial cells to the potentially harmful chemicals and bacteria within the intestinal lumen.

The rapid self-renewal of the epithelium is driven by the activity of intestinal stem cells (ISCs), which reside in the stem cell zone located at the crypt base (further discussed in **Section 1.2.4**). As these cells differentiate, they move upwards along the crypt-villus axis into the transit-amplifying (TA) cell zone where they become a progenitor of either the absorptive or secretory lineage (**Figure 1.3a**). Absorptive progenitors will give rise to enterocytes, while secretory progenitors will give rise to mature secretory cell types. In the small intestine, secretory cells include Paneth cells, goblet cells, enteroendocrine cells, and tuft cells. Whereas in the colon, secretory cells include goblet, enteroendocrine, and tuft cells. Paneth cells are not present in the colon, however, cells which reside at the colonic

crypt base, known as deep secretory cells (DSCs), have been identified as potential Paneth-like cells (Sasaki et al., 2016). Mature epithelial cells will eventually be sloughed off into the intestinal lumen as they are replaced. Enterocytes, or colonocytes, are the most abundant differentiated epithelial cell type and are critical for nutrient absorption and the secretion of immunoglobulins (Kong et al., 2018). Goblet cells are the mucin-secreting cells of the intestine, which serve a protective function in the epithelium, aid in lubrication, and play a role in the presentation of luminal antigens to dendritic cells (Parikh et al., 2019). Enteroendocrine cells secrete hormones that regulate many digestive functions of the GI tract and serve as a link to the central neuroendocrine system (Worthington et al., 2018). Paneth cells are the only differentiated cell type that do not migrate up the crypt-villus axis but are localized to the crypt base. In contrast to the rapid turnover of other differentiated epithelial cell types, Paneth cells turn over every 3-6 weeks. Their role is to secrete anti-microbial peptides (e.g. defensins and lysozyme) and factors that maintain the intestinal stem cell niche (Sato et al., 2011). Tuft cells are rare long-lived cells in the epithelium and are described in more detail in **Section 1.2.2.1** below.

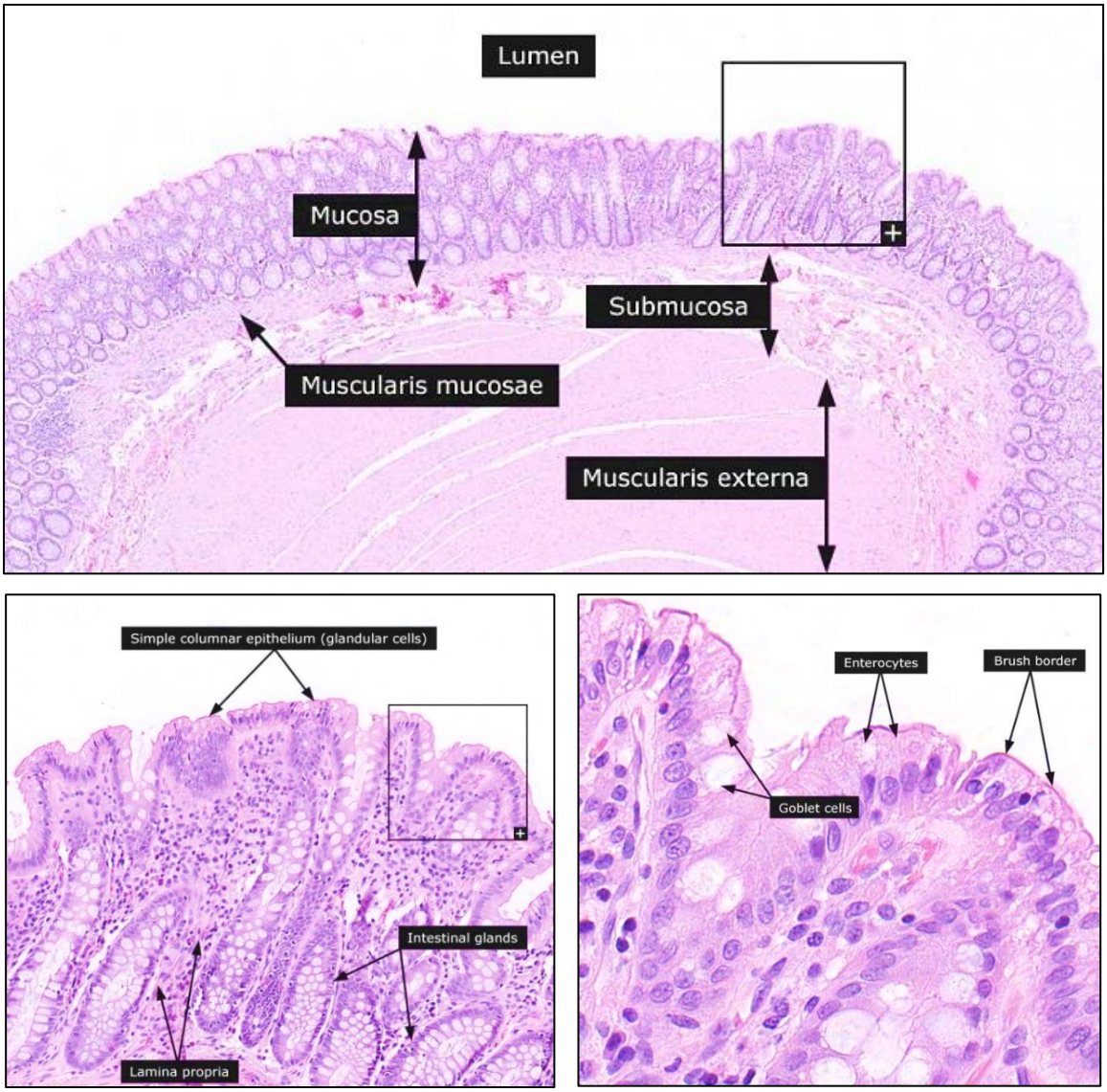

Figure 1.1 – Histology of the healthy human colon by H&E staining.

Images obtained from normal colon tissue slides at the Human Protein Atlas (https://v15.proteinatlas.org/learn/dictionary/normal/colon). Pontén, F., Jirström, K., and Uhlen, M. (2008). The Human Protein Atlas—a tool for pathology. J Pathol *216*, 387–393. DOI: 10.1002/path.2440; Uhlén, M., Fagerberg, L., Hallström, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., et al. (2015). Proteomics. Tissue-based map of the human proteome. Science *347*, 1260419. DOI: 10.1126/science.1260419.

1.2.2.1 *Tuft Cells*

Tuft cells are a rare, mature, secretory cell type of the intestinal epithelium which uniquely express the protein doublecortin-like kinase 1 (DCLK1) (Gerbe et al., 2011). The characterizing features of tuft cells are their long and blunt microvilli and their bulbous-like shape which is narrow at both the basal and apical ends. Although tuft cells have been extensively characterized by their morphology, their functional role in the epithelium has been less clear. Bezencon et al., 2008 published the first gene expression signature for tuft cells which included *Trpm5* and *α-gustducin*, members of the taste chemosensory pathways, as well as *Ptgs-1* and *Ptgs-2* (*Cox-1* and *Cox-2*), the enzymes responsible for prostaglandin synthesis (Bezençon et al., 2008). Interestingly, *Cox-1* and *-2* expression is specific to tuft cells in the intestinal and colonic epithelium during homeostasis (Gerbe et al., 2011; McKinley et al., 2017). More recently, tuft cells have been implicated in modulating immunity, particularly by serving as a primary source of IL-25 in the small intestine and by initiating type 2 immune responses to pathogens (Gerbe et al., 2016; von Moltke et al., 2016). Tuft cell-derived IL-25 stimulates group 2 innate lymphoid cells (ILC2s) to release IL-13, leading to tuft cell expansion and further IL-25 production. The resulting feedforward circuit and role of tuft cells in IL-25 production have been shown to be critical for the clearance of helminth infections (von Moltke et al., 2016). These findings were confirmed in the setting of *Tritrichomonas muris* infection, which is a protozoan that mounts a type 2 immune response (Howitt et al., 2016). Interestingly, mice lacking the taste chemosensory channel *Trpm5* were unable to induce tuft cell expansion in the setting of infection, indicating that there may be a link between the chemosensory and type 2 immunity functions of tuft cells (Howitt et al., 2016). In the setting of inflammatory or irradiation injury, ablation of either DCLK1 protein or *Dclk1*-expressing cells results in exacerbated disease severity, suggesting an important role for tuft cells in response to intestinal damage (May et al., 2014; Qu et al., 2015; Westphalen et al., 2015; Yi et al., 2019). Tuft cells have also been shown to expand in response to inflammation and carcinogenesis in the gastrointestinal tract, indicating that there may be a role for these cells in inflammation-associated tumorigenesis (Hayakawa et al., 2017; May et al., 2008; Tu et al., 2011; Saqui-Salces et al., 2011). Work by Westphalen et al., 2015 identified that a subset of tuft cells is extremely long-lived, remaining in the epithelium for up to 18

months (Westphalen et al., 2014). Importantly, these tuft cells can give rise to colorectal cancer in the setting of inflammation (described further in **Section 1.4.4.2**).

1.2.3 *Wnt Signaling in Intestinal Homeostasis*

The highly conserved Wnt signaling pathway plays a key role in embryogenesis and adult tissue homeostasis by regulating cellular proliferation, differentiation, and migration (Komiya and Habas, 2008). In particular, this pathway plays a critical role in maintenance of the intestinal epithelium. In mammals, there are 19 Wnt ligands and 10 seven-span transmembrane GPCRs known as Frizzled (FZD) receptors (MacDonald et al., 2009). Wnt signaling is mediated through the binding of Wnt ligands to Fzd receptors and a member of the LRP family of coreceptors, inducing a downstream signaling cascade (**Figure 1.2**). In canonical Wnt signaling, the main signal transducer is β-catenin, which is encoded by the *Ctnnb1* gene. In the absence of Wnt ligands, β-catenin primarily resides at the cell membrane, where it plays a role in cell-cell adhesion. Any remaining cytoplasmic β-catenin is tightly regulated by the destruction complex, which phosphorylates and tags β-catenin for ubiquitin-dependent proteasomal degradation. Members of the destruction complex include Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 beta (GSK-3β), and casein kinase 1 (CK1) (MacDonald et al., 2009). GSK-3β and CK1 are directly responsible for the phosphorylation and degradation of β-catenin, while Axin and APC serve as the scaffolds that bring β-catenin in proximity to these kinases (MacDonald et al., 2009). In the presence of Wnt ligand-receptor binding, the cytoplasmic Disheveled (DVL) protein blocks the activity of the destruction complex and allows for the stabilization of β-catenin (Komiya and Habas, 2008). Subsequently, β-catenin is able to accumulate in the cytoplasm and translocate into the nucleus. Here, β-catenin interacts with the TCF/LEF family of transcription factors that bind to DNA and other co-factors to induce Wnt target gene expression (MacDonald et al., 2009).

Studies performed in a variety of tissue types have displayed a critical role for Wnt signaling in stem cell activity. For example, ablation of *Ctnnb1* in the skin resulted in impaired differentiation of stem cells into follicular lineages (Huelsken et al., 2001), while

activation of Wnt signaling in hematopoietic stem cells resulted in an increased self-renewal capacity (Reya et al., 2003). The role of Wnt signaling in intestinal homeostasis has also been extensively studied. Disruption of Wnt signaling in the intestine by blockade of Wnt ligand-mediated signals, ablation of TCF transcription factors, or deletion of β-catenin results in complete loss of intestinal homeostasis, crypt structure, and viability as a result of terminally differentiated intestinal stem cells (van Es et al., 2012b; Fevr et al., 2007; Flanagan et al., 2015; Ireland et al., 2004; Korinek et al., 1998; Kuhnert et al., 2004). This indicates a critical and essential role for Wnt/β-catenin signaling in normal intestinal function.

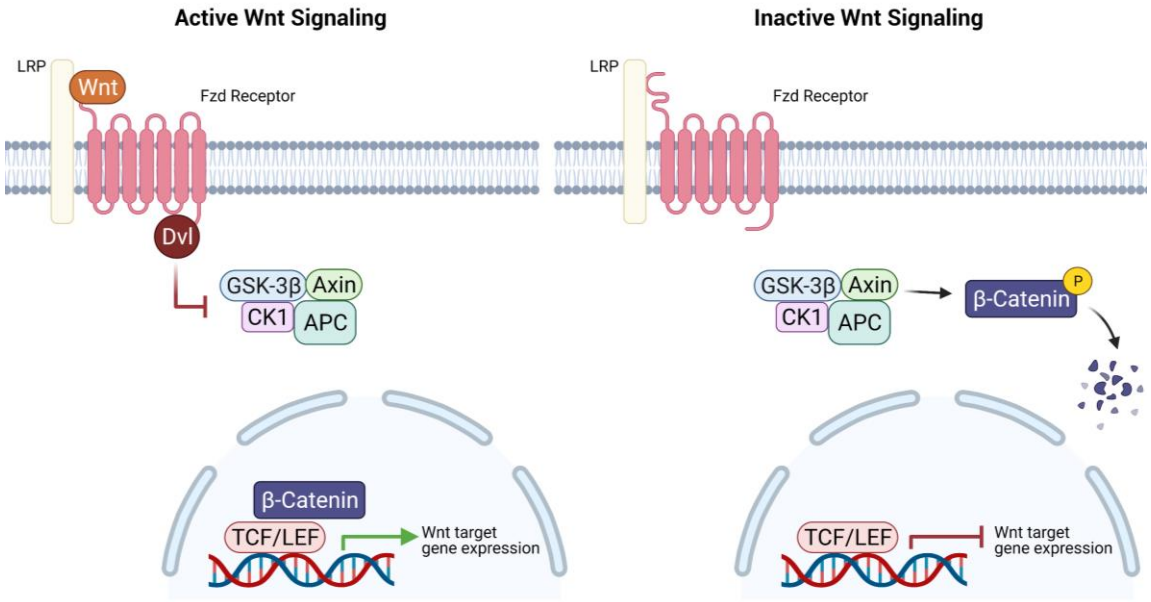

Figure 1.2 – The Wnt signaling pathway.

(**a**) Active Wnt signaling: Wnt signaling is activated by a Wnt ligand-Fzd receptor binding, which inhibits destruction complex-mediated degradation of β-catenin, allowing β-catenin to localize to the nucleus and upregulate the expression of Wnt target genes. (**b**) Inactive Wnt signaling in the absence of a Wnt ligand, β-catenin is tagged for proteasomal degradation by the destruction complex, and Wnt target gene expression is inhibited. Abbreviations: APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase 3 beta; CK1, casein kinase 1; Fzd, frizzled; LRP, low-density lipoprotein receptor-related protein; Dvl, dishevelled; TCF/LEF, T cell factor/lymphoid enhancer factor family. Adapted from "Wnt Signaling Pathway Activation and Inhibition", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

1.2.4 *Intestinal Stem Cells*

1.2.4.1 ISCs in intestinal homeostasis

Intestinal stem cells are defined by their ability to display the essential stem cell features: 1) self-renewal and 2) generation all differentiated epithelial cell types. Early studies attempting to characterize ISCs led to the development of two contrasting theories of stem cell identity. First, slow-cycling DNA label-retaining stem cells were identified and determined to be located at the +4 cell position relative to the crypt base, above Paneth cells (Potten et al., 1974). The observation that cells at the +4 position could retain the DNA label tritiated thymidine led to the conclusion that these cells were quiescent and slow-cycling, as non-dividing cells would not display this label-retaining ability. Alternatively, a population of actively dividing stem cells were described to be interspersed with Paneth cells in a "stem cell zone" at the crypt base. Due to their position within the crypt, these cells were termed crypt base columnar cells (CBCs) (Cheng and Leblond, 1974). However, the lack of specific markers for these cell populations and the inability to directly link their activity to epithelial regeneration served as a limitation for the proof of stemness in these models.

1.2.4.2 Methods to study ISCs

1.2.4.2.1 Cre-Lox Lineage Tracing

The development of the Cre-Lox system has allowed for the generation of *in vivo* models which can map the fate of different intestinal cell types, and therefore, aid in the identification of markers of intestinal cell populations. Cre recombinase is a site-specific DNA recombinase which recognizes target sequences known as loxP sites. Cre acts to recombine DNA between any two loxP sites, excising the sequence between them (Sauer, 1998). Cre activity can be induced in a cell- or tissue-specific manner by inducing Cre expression under control of a promoter of interest. Cre transgenes can either be constitutively expressed (i.e. Cre) or induced upon tamoxifen administration (i.e. Cre^{ERT2}). Cre^{ERT2} mice express a mutant estrogen receptor ligand binding region (ERT) Cre recombinase fusion protein which is inactive in the absence of tamoxifen (Kim et al., 2018).

Upon tamoxifen induction, Cre is able to translocate into the nucleus, recognize loxP sequences, and excise the target region of DNA. The mutated form of the estrogen receptor binding region allows for specific recognition of synthetic estrogens (i.e. tamoxifen or 4-hydroxytamoxifen) (Jaisser, 2000). The development of corresponding reporter transgenes that can be activated to express a fluorescent or visualizable protein upon Cre-recombinase-mediated excision of a STOP cassette (e.g. LacZ, RFP, or GFP) allows for the labeling of a cell population of interest. As this is a genetic labeling event, all cells expressing Cre and any of their progeny will be labelled, allowing for the fate of these cells to be mapped within the epithelium over time. Lineage tracing of the entire crypt villus axis, encompassing all intestinal cell types, would indicate that the targeted Cre-expressing cell of interest marks a stem cell population.

1.2.4.2.2 Organoid Culture Systems

Intestinal organoids are an *ex vivo* 3D model system that recapitulates the morphology and function of IECs *in vivo* (methodology described in greater detail within **Chapter 2**). The ability of certain cell populations to form long-lived and self-renewing organoids confirms their identity as stem cell populations in the intestine. Further identification of growth factors that are required for the long-term perpetuation of intestinal organoid cultures has revealed factors that are essential to the intestinal stem cell niche and cellular differentiation. These include Wnt, EGF, BMP, and Notch signaling pathways (Santos et al., 2018).

1.2.4.3 Markers of ISCs

A landmark study by Barker et al., 2007 identified that the Wnt target gene leucine-rich G-protein coupled receptor 5 (Lgr5) marks actively cycling CBC stem cells. Upon generation of the *Lgr5-eGFP-IRES-CreERT2* mouse model, they proved that Lgr5+ cells display the ability to self-renew and regenerate the gut as shown by sustained GFP+ lineage tracing of the entire intestinal epithelium (Barker et al., 2007). Sato et al., 2009 further described that Lgr5+ stem cells are capable of establishing and maintaining long-term intestinal organoid cultures, consistent with stem cell self-renewal capacity (Sato et al., 2009). Since that time,

additional markers for CBC stem cells have also been identified including, but not limited to, *Ascl2*, *Olfm4*, *Sox9*^{lo}, and *Smoc2* (van der Flier et al., 2009a, 2009b; Formeister et al., 2009; Carulli et al., 2014; Muñoz et al., 2012). Several proposed markers of stem cells at the +4 position, as described in **Section 1.2.4.1**, have also been identified such as *Bmi1*, *Hopx*, *mTert*, *Krt19*, *Clu*, *Mex3a*, *Dclk1*, *Sox9*^{hi} and *Lrig1* (Asfaha et al., 2015; Ayyaz et al., 2019; Barriga et al., 2017; Formeister et al., 2009; May et al., 2008; Montgomery et al., 2011; Powell et al., 2012; Roche et al., 2015; Sangiorgi and Capecchi, 2008; Takeda et al., 2011; Tian et al., 2011a; Wong et al., 2012). Using Cre-Lox technology, these populations have been shown to be relatively quiescent, occasionally contributing to epithelial turnover, yet display a high proliferative and stem cell capacity upon perturbations to actively cycling CBCs. These findings led to the conclusion that +4 stem cells represent a reserve intestinal stem cell population. Some +4 stem cell markers have also been detected in Lgr5+ cells, raising the question whether these truly represent distinct cell populations (Simons and Clevers, 2011; Wong et al., 2012). The inability to concurrently utilize more than one Cre-driven reporter in a single mouse line has limited the ability to distinguish between these different stem cell populations. However, some studies have confirmed a functional and spatial distinction between Lgr5+ cells and +4 markers (Asfaha et al., 2015; Yan et al., 2012). This has been achieved through the generation of the *Lgr5-eGFP-DTR* mouse model in which Lgr5+ cells express the diphtheria toxin receptor (DTR) and GFP, allowing for specific and targeted ablation of Lgr5-expressing cells using diphtheria toxin (DT) (Tian et al., 2011a). This mouse model allows for the simultaneous Cre-dependent labeling of Lgr5-negative cells in order to assess the function of other stem cell populations in the setting of CBC-loss.

1.2.4.4 ISCs in intestinal regeneration

The role of ISCs in regeneration or injury has recently been examined by a number of groups (Ayyaz et al., 2019; Metcalfe et al., 2014; Murata et al., 2020; de Sousa E Melo and de Sauvage, 2019; Tian et al., 2011). Interestingly, Lgr5+ cells appear to be highly sensitive to intestinal damage and are dispensable for epithelial regeneration. This has been shown through the functional and unimpaired ability of the intestinal epithelium to regenerate

upon Lgr5+ cells loss in the setting of injury or DT-mediated ablation (Asfaha et al., 2015; Ishibashi et al., 2018; Tian et al., 2011a; Yan et al., 2012). These findings suggest that non-Lgr5+ cells must have the capacity to regenerate the Lgr5+ stem cell pool during intestinal regeneration. Certain cell populations, such as those expressing Bmi1 or K19, have been shown to repopulate Lgr5+ cells and regenerate the entire small intestinal or colonic epithelium upon Lgr5+ cell ablation (Asfaha et al., 2015; Tian et al., 2011a). Other, more differentiated cell types of the absorptive or secretory lineages such as Dll1, Prox1, Alp1, and Neurog3 -expressing cells have also been shown to regenerate the intestinal epithelium upon Lgr5+ cell loss during intestinal injury (Buczacki et al., 2013; van Es et al., 2012a; Schonhoff et al., 2004; Tetteh et al., 2016; Yan et al., 2017). Fully differentiated cells, such as Paneth cells, have also been shown to reacquire stem cell capacity and repopulate the epithelium in the setting of intestinal injury (Schmitt et al., 2018; Yu et al., 2018). These studies suggest that there must be a certain degree of cellular plasticity within the gut, that particularly becomes relevant upon states of intestinal injury when Lgr5+ stem cells are lost.

Thus, the current working theory for ISCs today is that actively cycling Lgr5+ CBCs are responsible for the continual renewal of the gut epithelium, whereas +4 reserve stem cells divide more slowly, occasionally contributing to homeostasis, but become more important during regeneration of the epithelium after injury and/or CBC loss (**Figure 1.3**).

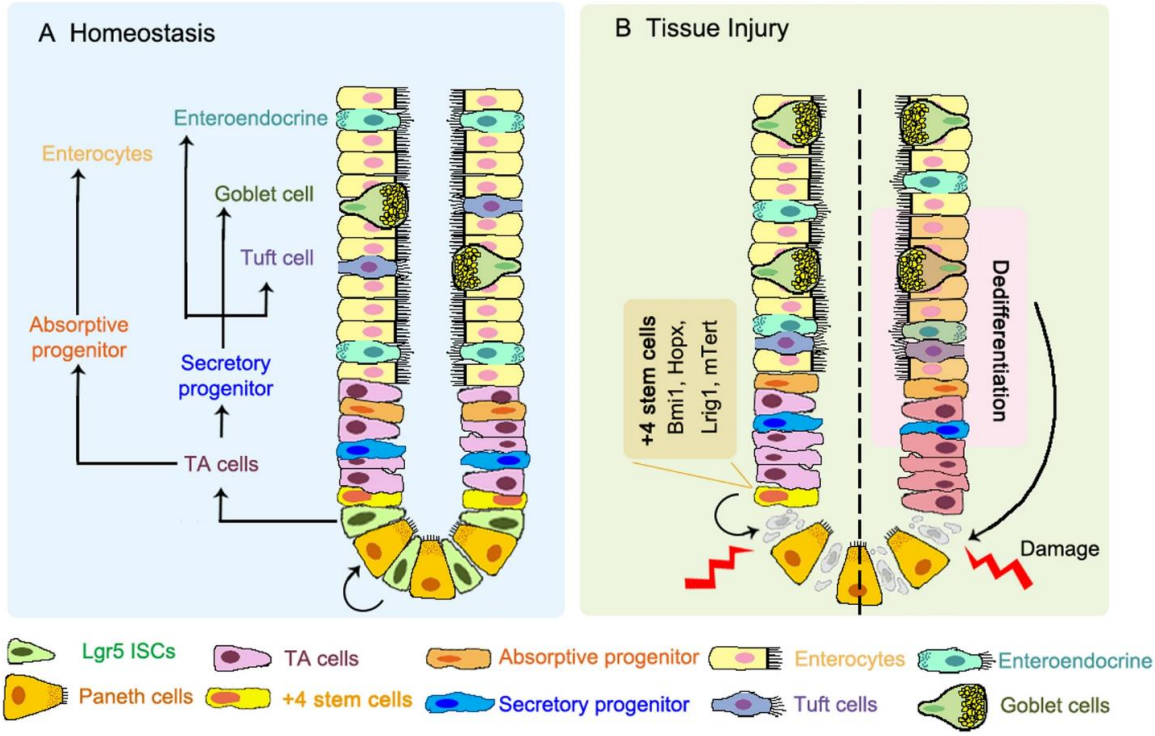

Figure 1.3 – The intestinal epithelium in homeostasis and injury.

(**a**) During homeostasis, Lgr5+ ISCs regenerate the intestinal epithelium, giving rise to progenitors and fully differentiated cell types. (**b**) During intestinal injury, Lgr5+ cells are lost, leading to either the dedifferentiation of progenitors or mature cell types, or the activation of +4 reserve stem cells to regenerate the Lgr5+ stem cell pool. Original figure obtained from: Liu, Y., and Chen, Y.-G. (2020). Intestinal epithelial plasticity and regeneration via cell dedifferentiation. Cell Regen *9*, 14. DOI: 10.1186/s13619-020-00053-5.

1.3 Colorectal cancer (CRC)

Colorectal cancer is a malignancy that arises from the cells in the wall of the colon or rectum. The 2018 GLOBOCAN estimates of global cancer statistics reported that colorectal cancer is the 3rd most commonly diagnosed cancer (10.2% of all cancers), after lung (11.6%) and breast (11.6%), and is the second most common cause of cancer death (9.2%) after lung cancer (18.4%) (Bray et al., 2018). The Canadian Cancer Society also reported that colorectal cancer was the 3rd most diagnosed cancer (11.9%) and the second most common cause of cancer death (11.6%) in Canada in 2020 (Brenner et al., 2020), accounting for 26,900 cancer diagnoses and 9,700 cancer deaths (Canadian Cancer Society, 2020). However, the incidence and mortality rates of colorectal cancer are declining, likely due to the increased implementation of screening programs which detect pre-cancerous polyps and reduce disease incidence (Brenner et al., 2020).

1.3.1 *Types of CRC*

Adenocarcinomas, malignancies arising from epithelial cells lining the colon or rectum, account for 95% of all colorectal cancer cases (Recio-Boiles and Cagir, 2021). Gastrointestinal carcinoid tumors that comprise ~1% of CRC cases are neuroendocrine tumors that arise from specialized enteroendocrine cells of the GI tract (Pinchot et al., 2008), while the remaining CRC cases are accounted by even less common sub-types which include: primary colorectal lymphoma, a type of Non-Hodgkin lymphoma arising from colonic lymphocytes; gastrointestinal stromal tumor (GIST), a sarcoma arising from the interstitial cells of Cajal; leiomyosarcoma, a sarcoma arising from the smooth muscle layer of the colon; and melanoma of the colon (Cuffy et al., 2006; Reddy and Fleshman, 2006) . Amongst adenocarcinomas, however, there are also less common cancer subtypes such as mucinous adenocarcinoma and Signet ring cell adenocarcinomas that account for 10-15% and <1% of all colorectal adenocarcinomas, respectively (Fleming et al., 2012; Hugen et al., 2014).

There are four major classifications of CRC: sporadic, inherited, colitis-associated, and familial. Sporadic colorectal cancer, accounting for approximately 70% of CRC cases, arises from point mutations and is not associated with inherited syndromes (Recio-Boiles and Cagir, 2021). Most sporadic CRC cases develop through the adenoma to carcinoma sequence (further described in **Section 1.3.3.1**). In contrast, about 5% of colorectal cancer cases are a result of inherited germline mutations that follow a Mendelian autosomal dominant inheritance pattern (Recio-Boiles and Cagir, 2021). These CRC cases can be classified as either polyposis or non-polyposis. Polyposis conditions primarily include familial adenomatous polyposis (FAP) in which 100-2500 adenomas form and proliferate within the colon. As adenomas are the most common precursor of CRC, these typically progress to malignancy by the age of 40-50 in patients with FAP (Groden et al., 1991; Lynch and de la Chapelle, 2003). Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, is the most common form of hereditary CRC. Lynch syndrome predisposes individuals to primary cancers of the intestine, endometrium, stomach, ovary, and urinary tract. These cancers arise from mutations in DNA repair machinery, such as the mismatch-repair genes *MSH2*, *MLH1*, *MSH6*, *PMS1*, and *PMS2*. However, 90% of these cases result from loss of *MLH1* or *MSH2* (Thibodeau et al., 1993). Hamartomatous polyposis syndromes, including Juvenile polyposis syndrome (JPS), Cowden syndrome (CS), and Peutz-Jeghers syndrome (PJS), are inherited syndromes that are characterized by multiple gastrointestinal polyps. These polyps are hamartomatous, indicating that they form from the overgrowth of normal cellular components. JPS is caused by germline mutations in *SMAD4*, whereas CD results from germline mutations in *PTEN*, and PJS is caused by germline mutations in *STK11* (Zbuk and Eng, 2007). These syndromes predispose individuals to intestinal cancer, however they account for fewer than 1% of all CRC cases (Nagy et al., 2004). Colitis-associated cancer (CAC) is a type of CRC that arises from areas of chronic intestinal inflammation, as seen in patients with Inflammatory Bowel Disease (IBD). CAC develops through a distinct mechanism of pathogenesis known as the dysplasia to carcinoma sequence (described further in **Section 1.4.3**), and accounts for approximately 1-2% of all CRCs (Munkholm, 2003). The remaining 25% of CRC cases are classified as familial CRC, in which inherited mutations are involved in disease incidence, but are not associated with known inherited syndromes.

The molecular mechanism of familial CRC is less clear but has been attributed to a combination of environmental and low-penetrance genetic factors. As a result, colonoscopy screening of individuals with first degree relatives affected by CRC should begin prior to the general population (Armelao and de Pretis, 2014).

1.3.2 *Risk Factors for CRC*

The lifetime probability of developing colorectal cancer is 4-5%, however there are various risk factors that can increase an individual's risk for and susceptibility to CRC (Howlader et al., 2019; Johnson et al., 2013). These include increased age, family history (as discussed above in **Section 1.3.1**), and lifestyle factors such as diet, smoking, alcohol consumption, and physical activity. Another major risk factor is chronic inflammation, which is most relevant to patients with Inflammatory Bowel Disease (IBD). Indeed, patients with IBD are at an increased risk for developing colitis-associated cancer (CAC), which is the type of CRC that develops in patients with IBD (discussed further in **Section 1.4**).

1.3.3 *Pathogenesis of CRC*

The current understanding of the development of sporadic CRC is that tumors arise due to accumulating genetic and epigenetic alterations that drive the progression from normal colonic epithelium to adenoma to adenocarcinoma (Kuipers et al., 2015). This is reflected in a series of histological changes depicted in **Figure 1.4** (Grady and Carethers, 2008). One of the key characteristics of the pathogenesis of CRC and an important contributor to disease progression is genomic instability. There are two predominant forms of genomic instability that contribute to CRC: chromosome instability (CIN) and microsatellite instability (MSI). CIN accounts for 80-85% of sporadic CRCs, whereas MSI-positive CRC accounts for the remaining 15% of cases (Grady and Carethers, 2008). CIN tumors are characterized by imbalances in chromosome number (aneuploidy) due to aberrant chromosomal segregation. When mutations occur in oncogenes and tumor suppressor genes, mitotic checkpoint and cell-cycle control mechanisms are lost, and chromosomal

segregation is dysregulated leading to aneuploidy and genomic instability (Kanneganti et al., 2011). CIN is a characteristic feature of FAP and sporadic CRC. In contrast, tumors that develop through the MSI pathway lose function of genes that are responsible for repairing the DNA base-pair mismatches which normally occur during DNA replication (e.g. HNPCC) (Kuipers et al., 2015).

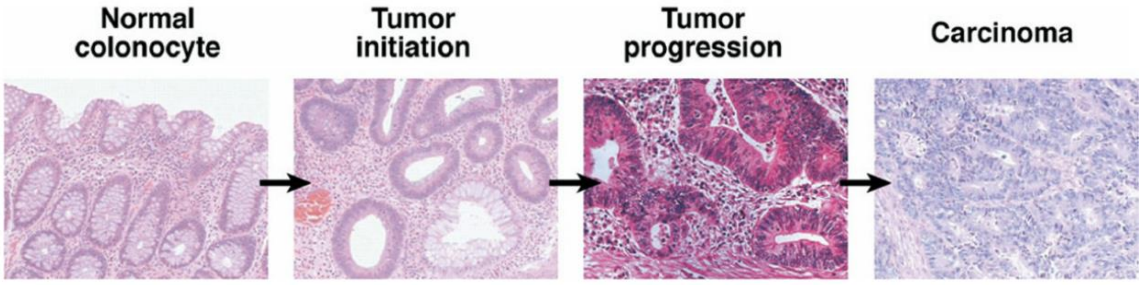

Figure 1.4 – Histological progression of sporadic CRC.

Adapted from: Grady, W.M., and Carethers, J.M. (2008). Genomic and epigenetic instability in colorectal cancer pathogenesis. Gastroenterology *135*, 1079–1099. DOI: 10.1053/j.gastro.2008.07.076.

1.3.3.1 Adenoma to Carcinoma Sequence

In 1990, a model of CRC pathogenesis was proposed by Fearon and Vogelstein termed the adenoma to carcinoma sequence (Fearon and Vogelstein, 1990). This sequence describes a multi-step sequence of accumulating mutations in oncogenes and tumor suppressor genes that correlates with and drives CRC progression. In this model, initiating events in *APC* stimulate the formation of an adenoma, followed by activating mutations in the oncogene *KRAS*, and inactivating mutations in *SMAD4* and *TP53*, allowing for the eventual progression to an invasive carcinoma (**Figure 1.5**). Loss of function mutations in the tumor

suppressor *APC* occur early in disease progression and are thought to be the initiating event for polyp formation (Powell et al., 1992). Through its role in β-catenin degradation and negative regulation of the Wnt signaling pathway, *APC* acts as a tumor suppressor gene. As a result, mutations triggering the truncation and inactivation of APC lead to aberrant Wnt signaling and uncontrolled cellular proliferation. The earliest identifiable preneoplastic lesion at the histological level is termed an aberrant crypt focus (ACF) which is a small lesion with morphological abnormalities such as a hyperplastic or dysplastic appearance (Clapper et al., 2020). As loss of APC is observed in ACF, this is supportive of the finding that *APC* mutations are an early event in colonic tumorigenesis (Souglakos, 2007). The formation of polyps that occur upon APC-loss "sets the stage" for the accumulation of subsequent mutations, as most carcinomas are known to develop from pre-existing adenomas or polyps. This was highlighted in the National Polyp Study in 1993 which showed that the removal of polyps decreased the incidence of colorectal malignancy by 76-90% and prevented death from colorectal cancer (Winawer et al., 1993). The next mutational change described in the adenoma-carcinoma sequence is activation of the oncogene *KRAS* (Kirsten rat sarcoma viral oncogene homolog). Of the three-rat sarcoma virus (*RAS)* oncogenes (*KRAS*, *HRAS*, and *NRAS*), *KRAS* is the most commonly altered in CRC, with activating mutations observed in approximately 40% of CRC cases (Fleming et al., 2012). *KRAS* encodes a GTPase protein, which acts to convert GTP into GDP and propagate signaling as part of the RAS/MAPK signaling pathway (Hymowitz and Malek, 2018). Single nucleotide substitutions, as seen in CRC, lead to constitutive activation of *KRAS* and uncontrolled activation of downstream effectors that contribute to carcinogenesis. These mutations are observed in adenocarcinomas and larger adenomas but not smaller polyps, suggesting that *KRAS* alterations occurs after polyp initiation (Souglakos, 2007). Another common mutational change observed in CRC is loss of heterozygosity in the chromosome region of 18q21. One of the genes located at chromosome 18q is the tumor suppressor *SMAD4* (Mothers against decapentaplegic homolog 4), which plays an important role in regulating the TGF-β signaling pathway (Woodford-Richens et al., 2001). Loss of function mutations in SMAD4 are seen in about 30% of CRC cases and results in impaired transcriptional regulation of genes required for cell cycle control, leading to the emergence of cells resistant to apoptosis (Ma et al., 2014;

Woodford-Richens et al., 2001). Lastly, mutations in the tumor suppressor *TP53* have been described as one of the most important events in CRC. The role of p53 is to induce cell cycle arrest under conditions of ineffective DNA repair (Lakin and Jackson, 1999). Therefore, inactivation of p53 promotes the progression from adenoma to carcinoma. This is highlighted by evidence showing that mutations in *TP53* are infrequent in adenomas and ACF, yet are present in as many as 75% of CRC tumors (Souglakos, 2007).

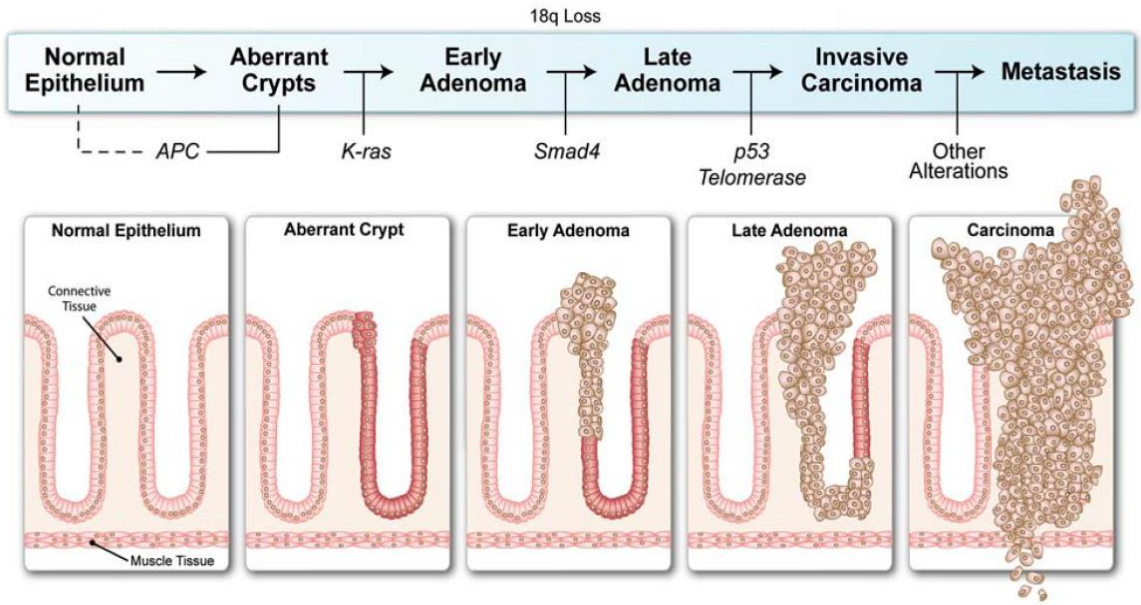

Figure 1.5 – Adenoma to carcinoma sequence of sporadic CRC development.

The normal colonic epithelium transforms to an invasive carcinoma through a stepwise accumulation of mutations in oncogenes and tumor suppressor genes that drives disease progression. Sequentially, these alterations include inactivation of APC, activation of K-ras, inactivation of Smad4, and inactivation of p53. Adapted from: Roig, A.I., Wright, W.E., and Shay, J.W. (2009). Is telomerase a novel target for metastatic colon cancer? Curr Colorectal Cancer Rep *5*, 203–208. DOI: 10.1007/s11888-009-0028-3.

1.3.4 *Wnt Signaling in CRC*

Wnt signaling is one of the major signaling pathways that is dysregulated in colorectal cancer (Bienz and Clevers, 2000; Kolligs et al., 2002). In 2012, The Cancer Genome Atlas (TCGA) consortium estimated that 93% of colonic tumors displayed aberrantly activated Wnt signaling, and this was a result of inactivating mutations in *APC* or activating mutations in *CTNNB1* (gene encoding β-catenin) in the majority of cases (Cancer Genome Atlas Network, 2012). Further studies identified mutations in *RNF43* and translocations of RSPO, both regulators of Wnt signaling, as key drivers in colorectal cancer. Interestingly, these mutations were mutually exclusive with *APC* mutations (Seshagiri et al., 2012; Giannakis, 2014), highlighting the importance of Wnt signaling in the pathogenesis of CRC. The importance in Wnt signaling in CRC was initially recognized by the detection of frequent inactivating mutations in *APC* (Ashton-Rickardt et al., 1989; Groden et al., 1991). Indeed, loss of function mutations leading to the truncation and inactivation of APC are common initiating events in sporadic colorectal cancer, as described in **Section 1.3.3.1** and are responsible for FAP (Groden et al., 1991; Kinzler et al., 1991).

1.3.5 *Mouse Models of CRC*

1.3.5.1 Chemically induced models

Certain chemical agents are known carcinogens and can be administered to mice to induce tumorigenesis. These include azoxymethane (AOM) (Maltzman et al., 1997), 1,2-dimethylhydrazine (DMH) (Wargovich et al., 1983) and methyl-azoxymethane (MAM). AOM, through its ability to alkylate DNA, is the most commonly used carcinogen for the induction of colonic tumorigenesis in mice (Kanneganti et al., 2011). When 6-8-week-old mice are administered six weekly intraperitoneal injections of AOM, spontaneous colonic tumors will develop within 30 weeks (Neufert et al., 2007). AOM-induced colonic tumors resemble human CRC due to their distal localization in the colon and their aberrant Wnt pathway activity as shown by truncating mutations in APC and the nuclear localization of β-catenin (Maltzman et al., 1997; Takahashi et al., 2000).

1.3.5.2 Genetically engineered models

The earliest genetic mouse model of colorectal cancer was the *Apc*$^{Min+/-}$ (multiple intestinal neoplasia) mouse model. The generation of this model was a result of screening for germline mutations after administering *N*-ethyl-*N*-nitrosourea to mice (Moser et al., 1990). This resulted in the identification of a nonsense mutation in codon 850 of the *Apc* gene. In this model, a heterozygous mutation in *Apc* induces Wnt activation and spontaneous tumor development upon loss of heterozygosity (Moser et al., 1990; Su et al., 1992). Most *Apc*$^{Min+/-}$ mice will only live up to 120 days due to the development of over 50 adenomas in the intestinal tract, however most of these occur in the small intestine (Fodde and Smits, 2001). Through the development of gene knockout technology, various other models containing Apc mutations have been developed that lead to polyp formation. These include the *Apc*$^{\Delta 716}$ model which contains a truncating mutation at codon 716 (Oshima et al., 1995; Oshima et al., 1997) and the *Apc*^{1638N} model which introduces a mutation at codon 1638 (Fodde et al., 1994; Smits et al., 1998).

The development of the Cre-Lox recombination system (as described in **Section 1.2.4.2.1**) has allowed for the generation of mouse models in which loss of function mutations in tumor suppressor genes or activating mutations in oncogenes could be induced in a conditional and tissue-specific manner. For example, the first conditional knockout mice were generated by the introduction of loxP sites into introns 13 and 14 of the *Apc* gene, in which Cre-mediated recombination leads to truncation and inactivation of APC (Shibata et al., 1997). Since then, multiple other conditional *Apc*-floxed mice have been generated, including the *Apc*^{CKO} mice generated by Dr. Raju Kucherlapati's group (Kuraguchi et al., 2006). These *Apc*^{CKO} mice harbour loxP sites flanking exon 14 and are utilized as part of the studies described in this thesis (see **Section 2.2** for further information). The crossing of mice harboring floxed alleles to mice with Cre-recombinase allows for conditional and tissue-specific gene recombination. As a result, genetic crosses of mice with floxed oncogenes or tumor suppressor genes, such as *Apc*, to mice with Cre expression under control of cell-specific promoters has allowed for the elucidation of the cell-of-origin of intestinal cancer (described further in **Section 1.3.6**).

1.3.6 *Cellular Origin of Intestinal Cancer*

Due to the rapid turnover of intestinal epithelial cells, the identity of the cellular origin of intestinal cancer has been long thought to be an intestinal stem cell. As stem cells are much longer-lived than differentiated cell types and display persistent clonogenic potential required to sustain intestinal homeostasis, the idea has emerged that stem cells may have the capacity to accumulate mutations and initiate cancer. The Cre-Lox system and the identification of stem cell markers (as described in **Section 1.2.4.3**) has allowed for the generation of mouse models in which mutations can be introduced into specific stem cell populations to examine their ability to initiate cancer. Indeed, *Apc*-deletion in Lgr5+ or Lrig1+ cell populations (Barker et al., 2009; Powell et al., 2012), and activation of β-catenin in Bmi1+ or Prom1+ cell populations resulted in the formation of intestinal tumors (Sangiorgi and Capecchi, 2008; Zhu et al., 2009). In contrast, *Apc* mutations in differentiated non-stem cells resulted in either non-progressing microadenomas or did not lead to intestinal tumors (Barker et al., 2009; Schwitalla et al., 2013). These studies have supported the idea that stem cells are the cell-of-origin of intestinal cancer and highlights the importance for Wnt activation in adenoma formation.

However, other studies have further examined the role for non-stem cells in the initiation of intestinal cancer. Schwittala et al., 2013 proved that the combined activation of NF-κB and Wnt signaling can result in the dedifferentiation of post-mitotic intestinal epithelial cells to a stem cell-like state to initiate tumorigenesis (Schwitalla et al., 2013). Westphalen and Asfaha et al., 2014 identified that normally quiescent post-mitotic Dclk1+ tuft cells can act as facultative stem cells and initiate tumor formation upon APC-loss, but this only occurs in the setting of inflammation (Westphalen et al., 2014) (further described in **Section 1.4.4.2**). Furthermore, aberrant epithelial expression of the BMP antagonist GREM1 resulted in adenoma formation arising from differentiated cells of the intestine (Davis et al., 2015). These studies suggest that if mutations in non-stem cells are able to persist over time, a “second hit” through inflammation or additional mutations may result in the initiation of cancer.

1.4 Inflammation and CRC

The link between inflammation and cancer was initially proposed by Rudolf Virchow in 1863, who suggested that cancer originated from sites of chronic inflammation (Balkwill and Mantovani, 2001). Since then, inflammation has been classified as key hallmark of cancer (Hanahan and Weinberg, 2011). Although the mechanism by which inflammation contributes to cancer is not entirely known, evidence suggests that inflammatory mediators can promote cancer by inducing mutations, promoting proliferation, preventing apoptosis, or stimulating angiogenesis (Shacter and Weitzman, 2002). Upon activation by a pathogen, inflammatory cells can generate reactive oxygen species which can contribute to tumorigenesis by inducing DNA damage. Inflammatory cells also release cytokines and chemokines at sites of inflammation that can promote tumorigenesis by inducing a proliferative response (Shacter and Weitzman, 2002). Indeed, many chronic inflammatory conditions are associated with neoplasia. These relationships include: asbestosis and mesothelioma, bronchitis and lung carcinoma, cystitis and bladder carcinoma, gingivitis and oral squamous cell carcinoma, pancreatitis and pancreatic carcinoma, Barrett's esophagus and esophageal carcinoma, hepatitis and hepatocellular carcinoma, and inflammatory bowel disease and colorectal cancer (Coussens and Werb, 2002).

1.4.1 *Inflammatory Bowel Disease*

Inflammatory Bowel Disease (IBD) is a term describing a group of conditions characterized by chronic and relapsing inflammation of the gastrointestinal tract (Khor et al., 2011). There are two primary diseases of IBD: Crohn's Disease (CD) and Ulcerative colitis (UC). The etiology of IBD is still largely unknown, but it is currently accepted to be a result of the interaction between and individual's genetics, environment, microbiome, and immune response. If the integrity of the intestinal epithelium is compromised, this can result in dysfunctional immunity, leading to aberrant activation and/or repression of cell signaling pathways, and ultimately to inflammation (Khor et al., 2011). In 2018, Canadian statistics reported that there were 270,000 people living with IBD in Canada, and this

number is expected to rise to 400,000 people, which accounts for 1% of the population, by 2030 (Kaplan et al., 2019).

1.4.1.1 DSS model of colitis

There are currently many experimental models of IBD, but the most widely used model of murine colitis is with the agent dextran sodium sulfate (DSS). The ability of DSS to induce colonic inflammation in mice was initially described by Okayasu et al., 1990 (Okayasu et al., 1990) after reports of its ability to induce colitis in hamsters (Ohkusa, 1985). DSS is a negatively charged polysaccharide that induces the clinical and histopathological features of human IBD in mice. DSS is typically administered to mice in the drinking water (*ad libitum*), with varying doses and duration of exposure. Doses of DSS are typically within the range of 1-5% (wt/vol) in mice and are administered for 5-7 days. However, multiple intermittent doses can be administered to model the chronic and relapsing characteristics of IBD in humans (Eichele and Kharbanda, 2017). The clinical features observed in mice after DSS treatment include weight loss, diarrhea, bloody stool, decreased appetite, reduced motility, and anemia (Perše and Cerar, 2012). If DSS treatment is not ceased, these disease manifestations can lead to death. Histological features of DSS-colitis include ulceration of the mucosa, edema, goblet cell loss, crypt distortion and/or destruction, and infiltration of inflammatory and immune cells into the colonic tissue (Perše and Cerar, 2012; Solomon et al., 2010). The ability of DSS to induce experimental colitis and the localization of this inflammation within the colon depends on the molecular weight of DSS. For example, it has been shown that low-weight DSS (5 kDa) induces inflammation in the cecal and proximal colon, whereas mid-weight DSS (40 kDa) induces colitis of the greatest severity in the mid and distal colon, and high-weight DSS (500 kDa) does not induce colitis in mice (Kitajima et al., 2000). Mid-weight DSS is the most commonly used for the induction of experimental colitis and all future mention of DSS will be referring to this molecular weight. The advantages of DSS as a model of IBD include its pathological similarities to IBD in humans – particularly UC – in addition to its low cost, ease of administration, and ability to induce both acute and chronic forms of the disease. Although DSS is widely and commonly used for induction of colitis in mice, the exact mechanism by which DSS

induces inflammation is still unknown. One theory is that DSS disrupts intestinal permeability, allowing for infiltration of luminal bacteria into the submucosa, leading to the induction of an inflammatory response (Venkatraman et al., 2000). DSS can also be administered to mice harbouring genetic mutations or alongside carcinogens in order to stimulate the initiation of colonic tumors and serve as model colitis-associated cancer (further described in **Section 1.4.4**).

1.4.2 *Colitis-Associated Cancer (CAC)*

Patients with IBD are at an increased risk for colitis-associated colorectal cancer (CAC) (Eaden et al., 2001; Ekbom et al., 1990; Kraus and Arber, 2009; Rutter et al., 2004; Shanahan, 2001), which is a malignancy that arises in the colorectum as a result of IBD. If colitis is uncontrolled, the risk of CAC is estimated to be as high as 5-20% of patients with IBD (Eaden et al., 2001; Jess et al., 2006; Lakatos and Lakatos, 2008; Lutgens et al., 2013), or 20 to 30-fold greater risk than the general population (Castaño-Milla et al., 2014). There is a strong association between the risk of cancer and the severity and duration of inflammation, highlighted by the incidence of CAC increasing with the number of years since IBD diagnosis (Lakatos and Lakatos, 2008; Ullman and Itzkowitz, 2011). Although CAC is not common prior to 7 years after initial IBD diagnosis, cancer risk increases approximately 0.5-1% every year thereafter (Coussens and Werb, 2002). Importantly, CAC is the most lethal complication for patients with IBD, with death resulting in over 50% of those diagnosed (Lakatos and Lakatos, 2008). Furthermore, CAC is typically characterized by an earlier age of onset, poorer prognosis, and advanced disease state when compared to sporadic CRC (Baars et al., 2012; Mutaguchi et al., 2019; Soh et al., 2019; Watanabe et al., 2011). Therefore, patients with IBD require frequent colonoscopies and tissue biopsies in order to screen for dysplastic changes in their colonic mucosa and detect early stage cancer.

1.4.3 *Histopathology of CAC*

Both sporadic and colitis-associated colorectal cancers initiate from the abnormal cells of pre-cancerous dysplastic lesions. However, in contrast to the adenomas or polyps that precede sporadic CRC, early lesions commonly occur in the form of flat, less apparent, dysplastic lesions in CAC (Itzkowitz and Yio, 2004). This led to the development of the dysplasia-carcinoma sequence of CAC pathogenesis, which contrasts the adenoma-carcinoma sequence for sporadic CRC. In this sequence, CAC carcinogenesis occurs through a sequence of events in the transformation from colitis to indefinite dysplasia, low-grade dysplasia, high-grade dysplasia, and lastly to carcinoma (Itzkowitz and Yio, 2004). The progression from normal epithelium to dysplasia in CAC is influenced by reactive oxygen and nitrogen species, chemokines, and cytokines that are produced as a result of chronic inflammation (Kraak et al., 2015). Inflammation is associated with both sporadic and colitis-associated CRCs, however inflammation typically follows tumor development in sporadic CRC, whereas it precedes and initiates tumorigenesis in CAC. The frequencies of colitis-associated cancers displaying chromosomal instability or microsatellite instability are similar to what is observed in sporadic CRC, accounting for approximately 85% and 15% of cases, respectively (Mattar et al., 2011). The key molecular changes that occur in the progression of sporadic CRC also play a role in the dysplasia-carcinoma sequence of CAC, such as alterations in Wnt/β-catenin signaling, KRAS, TP53, TGF-β, and MMR proteins (Terzić et al., 2010). However, these mutations differ in frequency and timing throughout disease progression (**Figure 1.6**). For example, an early and frequent mutation in sporadic CRC is APC, yet in CAC, this alteration occurs later in disease progression and is only observed in 15-30% of cases (Fujita et al., 2017; Umetani et al., 1999). One of the most common mutations in CAC is *TP53*, observed in approximately 60-80% of CAC cases and is an early genetic event in the neoplastic progression from colitis (Brentnall et al., 1994; Burmer et al., 1992). The mutational disparities between sporadic and colitis-associated cancer suggest that IBD drives a pathological mechanism of cancer that differs from the initiation and progression of sporadic CRC.

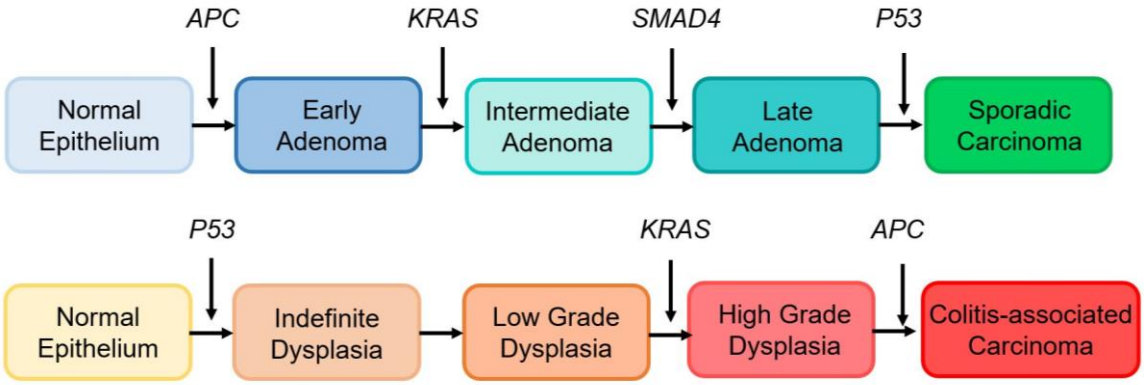

Figure 1.6 – Pathogenesis of sporadic and colitis-associated colorectal cancers.

Multistep progression in the pathogenesis of sporadic CRC (top) and colitis-associated CRC (bottom). Both diseases develop through a stepwise accumulation of mutations, however the sequence of these mutations differs between sporadic and colitis-associated CRC. Abbreviations: APC, Adenomatous polyposis coli; KRAS, Kirsten rat sarcoma viral oncogene homolog; SMAD4, Mothers against decapentaplegic homolog 4; P53 (TP53), tumor protein p53. Adapted from: Kameyama, H., Nagahashi, M., Shimada, Y., Tajima, Y., Ichikawa, H., Nakano, M., Sakata, J., Kobayashi, T., Narayanan, S., Takabe, K., et al. (2018). Genomic characterization of colitis-associated colorectal cancer. World J Surg Oncol *16*, 121. DOI: 0.1186/s12957-018-1428-0.

1.4.4 *Mouse Models of CAC*

1.4.4.1 AOM DSS model of CAC

As described in **Section 1.3.5.1**, chemical agents can be administered to animals to induce reproducible models of tumorigenesis. For example, six doses of the carcinogen AOM stimulates sporadic colonic tumorigenesis by week 30. However, work by Tanaka et al., 2003 showed that a single injection of AOM followed by one or more cycles of DSS resulted in the formation of macroscopic colonic tumors after just 20 weeks (Tanaka et al., 2003). The generation of this model highlighted the importance of inflammation in colonic tumorigenesis, and since then, has become one of the most commonly used murine models of CAC. AOM DSS-induced colonic tumors display nuclear β-catenin, indicative of active Wnt signaling, and no immunoreactivity for p53 (De Robertis et al., 2011).

1.4.4.2 *Dclk1^{CreERT2};APC$^{f/f}$* model of CAC

Westphalen, Asfaha et al., 2014 generated a novel transgenic mouse model of CAC and elucidated a cellular origin of colitis-associated cancer as the Dclk1+ tuft cell (Westphalen et al., 2014), a fully differentiated secretory cell type (as discussed in **Section 1.2.2.2**). Generation of *Dclk1^{CreERT2}* mice enabled Cre-Lox-mediated genetic labeling of Dclk1-expressing tuft cells within the intestinal epithelium. Interestingly, a subset of these Dclk1+ cells could be detected within the epithelium even 18 months after labelling. This indicated that, in contrast to the rapid turnover of most differentiated IECs, Dclk1+ cells can serve as long-lived cells, giving rise to the idea that this cell may be able to acquire a propagate mutations leading to cancer. However, even upon introduction of allelic loss of *Apc* in Dclk1+ cells with the generation of *Dclk1^{CreERT2};APC^{ff}* mice, Dclk1+ cells remained long-lived and resistant to proliferation. As inflammation is a risk factor for CRC, they next analyzed the effect of inducing colitis in mice with APC-deficient Dclk1+ cells. Upon colitis induction, 100% of *Dclk1^{CreERT2};APC^{ff}* mice developed colonic tumors by 14 weeks. Colonic tumors derived from *Dclk1^{CreERT2};APC^{ff}* were confirmed to be derived from Dclk1+ cells through genetic lineage tracing assessment of tumors (Westphalen et al., 2014).

1.5 Cyclooxygenase Pathway

The cyclooxygenase (COX) pathway includes the COX enzymes and their downstream prostanoids. This pathway is known to be a key player in intestinal homeostasis, inflammation, and colorectal cancer. Importantly, non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX activity, are chemopreventative agents against CRC. This section will explore these topics and the relevance of COX signaling to intestinal homeostasis, inflammation, and cancer in further detail.

1.5.1 *COX Pathway in Homeostasis*

The cyclooxygenase enzyme has two distinct isoforms, COX-1 and COX-2. The COX isoforms display approximately 60-65% sequence homology and are similar in size, consisting of 576 and 587 amino acids for the mature COX-1 and -2 proteins, respectively (Smith et al., 2000). The COX isoforms are products of different genes and therefore display differential gene and protein regulation (Kang et al., 2007; Smith et al., 2000; Tanabe and Tohnai, 2002). COX-1 is constitutively expressed in almost all tissue types, and therefore, has been characterized as a housekeeping gene that functions to maintain homeostasis by promoting intestinal mucosal barrier function, platelet aggregation, vasodilation, and renal homeostasis (Simmons et al., 2004). In contrast, COX-2 expression is restricted at baseline, but is upregulated in the setting of inflammation or injury to produce mediators that are involved in pain, inflammation, and fever (Simmons et al., 2004).

The COX enzymes display similar catalytic activity in the synthesis of physiologically active lipid mediators, known as prostanoids, from arachidonic acid (AA) or other 20 carbon fatty acids (Smith et al., 2011). AA is a polyunsaturated fatty acid (PUFA) which is released from the cell membrane by phospholipase A2 (PLA_2). Once released, AA contributes to the synthesis of eicosanoids: prostanoids, leukotrienes, and epoxyeicosatrienoic acids by the action of COX, lipoxygenase (LOX), and epoxygenase enzymes, respectively (Smyth et al., 2009). In the synthesis of prostanoids, the dual

cyclooxygenase and peroxidase activity of the COX enzymes catalyzes the conversion of AA to PGG_2, followed by the reduction of PGG_2 to the intermediate PGH_2. PGH_2 is acted upon by prostanoid-specific terminal synthases that metabolize PGH_2 to series-2 prostanoids: prostaglandin E2 (PGE_2), prostaglandin D2 (PGD_2), prostaglandin F2a ($PGF_{2\alpha}$), prostacyclin (PGI_2), or thromboxane A2 (TXA_2) (**Figure 1.7**). The COX enzymes can also act upon other PUFAs, such as dihomo-γ-linolenic acid (DGLA) and eicosapentanoic acid (EPA) to produce series-1 and series-3 prostaglandins, respectively (Schröder et al., 2012; Yang et al., 2014). Prostaglandins play a key role in various physiological functions including inflammation, blood clotting, labor, wound healing, and ovulation (Ricciotti and FitzGerald, 2011). Due to their short half-life, they are produced in various tissue types and act in an autocrine or paracrine manner to influence their surrounding environment. The array of prostaglandins that are produced are dependent on the availability of prostanoid-specific synthases within a particular cell or tissue type. For example, platelets primarily produce TXA_2 (Weksler, 2015), endothelial cells mainly produce PGI_2 (Vane and Botting, 1995), and mast cells primarily produce PGD_2 (Tilley et al., 2001). To exert their biological effects, prostaglandins will bind to prostanoid-specific G protein coupled receptors (GPCRs), which are coupled to heterotrimeric G proteins containing stimulatory or inhibitory subunits. These prostanoid-specific GPCRs include EP1-4 for PGE_2, DP1 and DP2 for PGD_2, FP for $PGF_{2\alpha}$, IP for PGI_2, and TP for TXA_2. Prostanoids binding to a member of their prostanoid-specific receptor family results in G-protein-mediated downstream signaling cascades, such as modulating the activity adenyl cyclase, cAMP, or PLC (**Table 1.1**). In the intestine, a recent study identified EP4 as the PGE_2 receptor with the highest expression in both the small intestine and colon of mice, and in the colon of humans (Crittenden et al., 2021).

The most abundant prostaglandin is PGE_2 (Legler et al., 2010). PGH_2 is rapidly converted into PGE_2 by 3 distinct synthases: microsomal PGES-1 (mPGES-1 or PTGES), mPGES-2, and cytosolic PGES (cPGES) (Hara et al., 2010). mPGES is usually induced alongside COX-2 to increase PGE_2 levels during inflammation (Jakobsson et al., 1999). Metabolism of PGE_2 is controlled by 15-PGDH (15-hydroxyprostaglandin dehydrogenase), a prostaglandin degrading enzyme, which acts to catalyzes the 15(S)-hydroxyl group of PGE_2 to produce inactive 15-keto PGE_2 (Tai, 2011). The role of 15-PGDH in PGE_2

metabolism was confirmed upon the finding of increased tissue levels of PGE_2 upon genetic deletion of 15-PGDH (Coggins et al., 2002). PGE_2 is involved in the redness, swelling, and pain that classically characterizes inflammation (Legler et al., 2010).

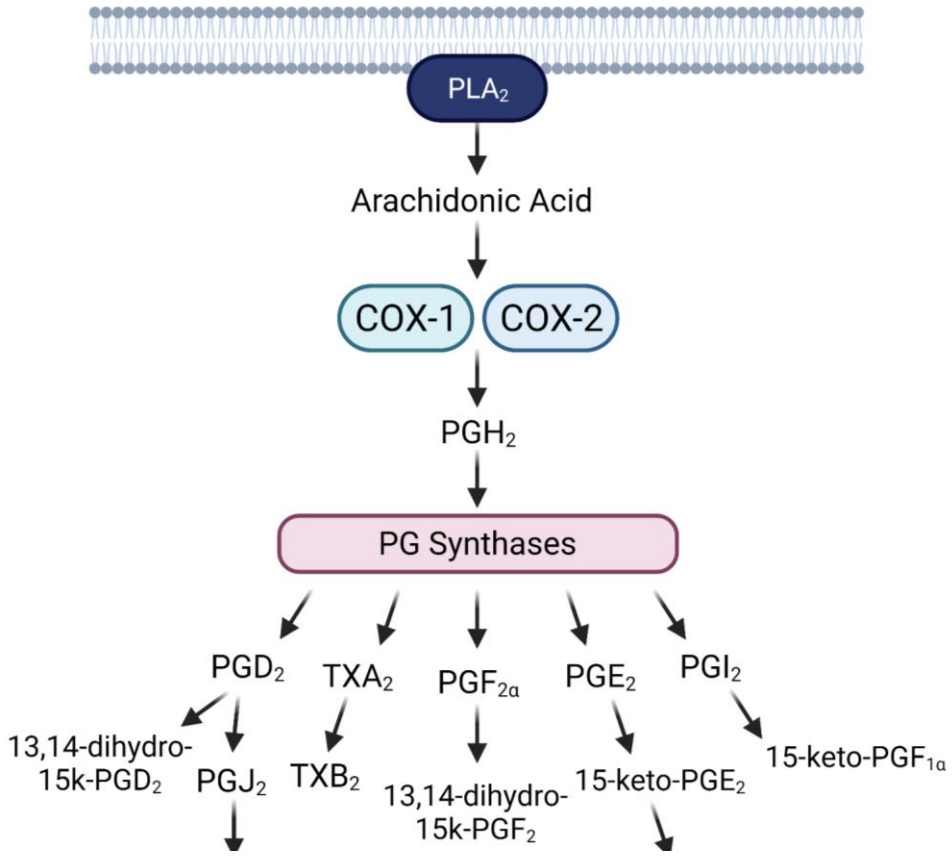

Figure 1.7 – Pathway of cyclooxygenase-derived prostanoid synthesis.

Cyclooxygenase enzymes act upon polyunsaturated fatty acids to produce downstream biologically active lipid mediators. COX acts predominantly on arachidonic acid derived from the cell membrane by PLA_2 to produce PGH_2. PGH_2 is then acted upon by tissue and prostaglandin-specific synthases to produce downstream series-2 prostanoids. Due to their short half life, prostanoids are rapidly degraded to their downstream metabolites. Abbreviations: PLA_2, phospholipase A2; COX, cyclooxygenase; PG, prostaglandin; PGH_2, prostaglandin H2; PGD_2, prostaglandin D2; PGJ2, prostaglandin J2; TXA_2, thromboxane A2; TXB_2, thromboxane B2; $PGF_{2\alpha}$, prostaglandin F2alpha; PGE_2, prostaglandin E2; PGI_2, prostacyclin. Created in Biorender.com.

Table 1.1 – Prostanoid synthases, GPCRs, and downstream signaling cascades.

Prostanoid	Synthase	GPCR	G protein	Downstream signaling[1]
PGE_2	mPGES-1 mPGES-2 cPGES	*EP1*	$G\alpha_q$	• PLC activation → ↑ intracellular Ca^{2+} → PKC activation → NFAT, NF-κB, and MAPK-mediated gene transcription
		EP2	$G\alpha_s$	• AC activation → ↑ cAMP → PKA activation • Inhibition of GSK-3β → β-catenin activation → Wnt target gene expression • PI3K activation → Akt activation
		EP3	$G\alpha_i$* $G\alpha_s$ $G_{12/13}$	• AC inhibition → ↓ cAMP → PKA inhibition; ↑ intracellular Ca^{2+} • AC activation → ↑ cAMP → PKA activation • Rho activation
		EP4	$G\alpha_s$	• AC activation → ↑ cAMP → PKA activation • PI3K activation → GSK-3β inhibition → β-catenin activation → Wnt target gene expression
PGD_2	PGDS	*DP1*	$G\alpha_s$	• AC activation → ↑ cAMP
		DP2	$G\alpha_i$	• AC inhibition → ↓ cAMP → PKA inhibition; ↑ intracellular Ca^{2+}
$PGF_{2\alpha}$	PGFS	*FP*	$G\alpha_q$	• PLC activation → ↑ intracellular Ca^{2+}
PGI_2	PGIS	*IP*	$G\alpha_s$	• AC activation → ↑ cAMP → PKA activation
TXB_2	TXAS	*TP*	$G\alpha_q$	• AC inhibition → ↓ cAMP → PKA inhibition

[1]Fujino et al., 2002; Hertzel et al., 2020; O'Callaghan and Houston, 2015; Sugimoto and Narumiya, 2007

*predominant isoform

Abbreviations: AC, adenylyl cyclase; Ca^{2+}, calcium; cAMP, cyclic AMP; PKC. protein kinase C; PKA. protein kinase A; PLC, phospholipase C; Akt, protein kinase B.

1.5.2 *COX Pathway in Colitis*

COX-2 has consistently been described as the dominant source of prostaglandins in inflammation (Futaki et al., 1993; Myers et al., 2000; Ochi et al., 2003; Vane et al., 1994). However, some studies suggest a role for both isoforms during inflammation and colitis (Chen et al., 2008; Langenbach et al., 1995; Langenbach et al., 1999; McAdam et al., 2000; Wallace et al., 1998). Indeed, COX-2 expression is upregulated in patients with active IBD and in mouse models of colitis (Shattuck-Brandt et al., 2000; Singer et al., 1998), while other studies also report the upregulation of COX-1 (Tessner et al., 1998). COX-derived prostaglandins can also modulate intestinal inflammation and have been shown to correlate with disease severity in patients with IBD and in mouse models of colitis (Carty, 2000; Rampton and Hawkey, 1984; Wiercińska-Drapało et al., 1999; Yamashita, 1993). In particular, PGE_2 has been described as a key mediator of colitis. The upregulation of PGE_2 in patients with UC was first described in 1978 (Sharon et al., 1978), and this finding has been confirmed in various further studies in both humans and mouse models of IBD (Gould et al., 1981; MacDermott, 1994; Melgar et al., 2006; Raab et al., 1995; Sheibanie et al., 2007; Tessner et al., 1998). Further analyses of patients with IBD also suggest that PGE_2 levels increase with disease severity (Sharon et al., 1978; Wiercinska-Drapalo et al., 1999), and that polymorphisms in the *Ptger4* gene (encoding EP4) are associated with upregulated EP4 expression and increased colitis severity (Barrett et al., 2008; Libioulle et al., 2007). As PGE_2 displays the ability to both perpetuate and resolve inflammation, likely in part due to the differences in EP receptor responses, it has been suggested to play a dual role in colitis (Nakanishi and Rosenberg, 2013). The first study demonstrating the direct link between PGE_2 and colonic inflammation was shown by Sheibanie et al., 2007. Here, they proved that exogenous administration of PGE_2 analogues during murine colitis exacerbates disease severity by promoting colonic infiltration of neutrophils and T-helper17 (Th17) cells (Sheibanie et al., 2007). More recently, PGE_2 was shown to enhance colonic inflammation by inhibiting regulatory T cells (Tregs) in a microbiota-dependent mechanism (Crittenden et al., 2021). However, other studies have shown that genetic deletion of COX-1, COX-2, or mPGES-1 exacerbates inflammation in mice with experimental colitis (Hara et al., 2010; Ishikawa et al., 2011; Montrose et al., 2015; Morteau et al., 2000). These findings have been supported by other studies demonstrating

that administration of PGE_2 or EP4 agonists can ameliorate DSS-colitis or improve epithelial repair after injury (Jiang et al., 2007; Miyoshi et al., 2017; Nitta et al., 2002). Furthermore, EP receptor knockout mice have been developed to investigate the role of PGE_2 signaling in colitis. A study by Kabashima et al., 2002 reported that of all EP receptors, only EP4-deficient mice developed severe DSS-induced colitis, suggesting that PGE_2-EP4 binding is the major protective response against inflammation (Kabashima et al., 2002). The role for EP4 in reducing colitis severity has been attributed to the ability of PGE_2 to promote proliferation and regeneration of the intestinal epithelium (Jiang et al., 2007; Peng et al., 2017; Tessner et al., 1998; Zhang et al., 2015). In fact, treatment with an EP4 agonist in a Phase II clinical trial resulted in histological improvements of UC in patients resistant to 5-aminosalicylic acid, a medication commonly used to treat IBD (Nakase et al., 2010). However, the ability of PGE_2 to promote tissue regeneration has led to the idea that it may also contribute to cancer in the setting of chronic inflammation.

1.5.3 *COX Pathway in CRC*

The cyclooxygenase pathway has been strongly implicated in the pathogenesis of CRC (Wang and Dubois, 2010a). Both COX enzymes are aberrantly over-expressed in the non-neoplastic colonic mucosa of patients with CRC (Jensen et al., 2018), while COX-2 has been consistently shown to be overexpressed in colorectal tumors (Eberhart et al., 1994; Kunzmann et al., 2013; Wang and Dubois, 2010b) and in UC-associated neoplasia (Agoff et al., 2000). COX-2 overexpression in CRC has also been inversely associated with prognosis and survival (Ogino et al., 2008). Further evidence of a role for COX in cancer has been elucidated with the use of animal models. Deletion of either *Cox-1* and/or *Cox-2* genes resulted in decreased tumor formation in the $Apc^{Min+/-}$ and $Apc^{\Delta 716}$ mouse models of CRC (Chulada et al., 2000; Oshima et al., 1996). In contrast, however, Ishikawa & Herschman, 2010 reported that neither COX-1 nor COX-2 expression was required for the initiation of AOM/DSS-derived tumors (Ishikawa and Herschman, 2010). COX-derived prostaglandins have also been strongly associated with CRC, particularly PGE_2, through its ability to promote cellular proliferation, stimulate angiogenesis, prevent apoptosis, promote invasion and motility, and suppress immune responses (Wang and Dubois, 2006).

In fact, PGE_2 is the most abundant prostaglandin found in human CRC (Rigas et al., 1993) and has been shown to be upregulated and associated with poorer prognoses in colon, breast, lung, and head and neck cancers (Hambek et al., 2007; Mal et al., 2011; McLemore et al., 1988; Wang and Dubois, 2004). Levels of PGE_2 are dependent on both the activity of COX enzymes and on the activity of the enzyme that metabolizes PGE_2, 15-PDGH. Incidentally, 15-PDGH is reduced in human CRC (Backlund et al., 2005) and 15-PDGH knockout mice showed a significant increase in AOM-induced colonic tumorigenesis (Myung et al., 2006). Further studies have determined that exogenous administration of PGE_2 leads to increased small and large intestinal adenomas in the *Apc*$^{Min+/-}$ model (Wang et al., 2004), increased AOM-induced colonic tumor number and size (Kawamori et al., 2003), and increased inflammation-associated colonic tumorigenesis (Hernandez et al., 2010; Kohno et al., 2005). Genetic ablation of microsomal PGES-1 (*mPGES-1*) resulted in decreased intestinal tumors in both *Apc*$^{Min+/-}$ and AOM models of CRC (Nakanishi et al., 2008, 2011; Sasaki et al., 2012). Furthermore, studies investigating the role of EP receptors in CRC have supported the importance of PGE_2 in colonic tumorigenesis. The use of an EP1 antagonist or genetic knockout of EP1 inhibited polyp number in APC-deficient mice and prevented aberrant crypt foci (ACF) in AOM-treated mice (Kawamori et al., 2005; Kitamura et al., 2003; Watanabe et al., 1999; Watanabe et al., 2000). Similarly, EP2 deletion led to a decrease in the number and size of intestinal polyps in the *Apc*$^{\Delta 716}$ mouse model (Sonoshita et al., 2001), and marked inhibition of tumorigenesis in the AOM/DSS model of CAC (Ma et al., 2015). The role for EP3 in colorectal tumorigenesis is still unclear, likely due to the opposing downstream effects of the different EP3 isoforms (**Table 1.1**). EP3 knockout mice showed no effect on tumor formation in the *Apc*$^{\Delta 716}$ mouse model (Sonoshita et al., 2001), whereas AOM-induced tumor incidence was increased in EP3 knockout mice (Shoji et al., 2004). Lastly, EP4 antagonists and EP4 knockout mice resulted in reduced polyp development in the *Apc*$^{Min+/-}$ model and decreased AOM-induced ACF (Kitamura et al., 2003; Mutoh et al., 2002).

Finally, some of the strongest evidence suggesting a critical role for the COX pathway in CRC is the ability of non-steroidal anti-inflammatory drugs (NSAIDs), which act to inhibit COX activity, to greatly reduce the risk of CRC. In the *Apc*$^{Min+/-}$ model of colonic tumorigenesis, this NSAID-induced regression of adenomas was prevented upon PGE_2

administration, suggesting that NSAIDs prevent CRC through COX inhibition (Hansen-Petrik et al., 2002). The role of NSAIDs in the prevention of CRC is further described in **Section 1.5.4.1**.

1.5.4 *Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)*

Non-steroidal anti-inflammatory drugs (NSAIDs) are used for the management of pain and inflammation due to their anti-inflammatory, analgesic, and antipyretic effects. NSAIDs are some of the most commonly used medications accounting for 5-10% of prescribed medications every year (Onder et al., 2004). The mechanism of action of NSAIDs is through inhibition of the enzymatic activity of COX-1 and/or COX-2, preventing the downstream synthesis of prostaglandins (Vane, 1971). Some of the best known traditional NSAIDs include acetylsalicylic acid (Aspirin), ibuprofen (Advil), and naproxen (Aleve), which are non-selective and act to inhibit both COX-1 and COX-2. Aspirin displays the unique ability to irreversibly inhibit COX activity, through acetylation of Ser-530 on COX-1 and Ser-516 on COX-2. Additionally, Aspirin can be administered at a low dose (81-100mg) to prevent cardiovascular events such as myocardial infarction or stroke by inhibiting COX-1 in platelets and preventing the downstream synthesis of the prothrombotic TXA_2 (Antithrombotic Trialists' (ATT) Collaboration et al., 2009; Patrignani et al., 1982).

The prolonged use of traditional NSAIDs at standard doses can result in adverse effects due to the wide range of effects of prostaglandins in physiological processes. These include nausea, dyspepsia, gastritis, abdominal pain, peptic ulcers, and GI bleeds (Wolfe et al., 1999). These side effects were attributed to inhibition of COX-1 due to the role of COX-1 in gastrointestinal homeostasis and mucosal protection, and therefore led to the development of COX-2 selective inhibitors. These COX-2 inhibitors were termed COXIBs which include drugs such as celecoxib (Celebrex), rofecoxib (Vioxx), and valdecoxib (Bextra). The aim of develping COX-2-selective inhibitors was to prevent the GI-associated adverse effects of traditional NSAIDs while maintaining their therapeutic properties. Initial studies showed that COXIBs were able to exert comparable analgesic

and anti-inflammatory responses in patients with arthritis as compared to traditional NSAIDs, but with a reduced incidence of gastrointestinal toxicity (Bombardier et al., 2000; Silverstein et al., 2000; Simon et al., 1998). However, concerns were raised regarding the increased risk of cardiovascular events that appeared to be associated with the use of these drugs (Curfman et al., 2005). Further investigation confirmed that COXIBs led to an increased risk of heart attack or stroke, likely as a result of the dysregulated balance between the prothrombotic COX-1-derived TXA_2 and the antithrombotic PGI_2 upon COX-2-selective inhibition (Bombardier et al., 2000; Bresalier et al., 2005; Grosser et al., 2006; Nussmeier et al., 2005; Solomon et al., 2005). As a result, rofecoxib and valdecoxib were withdrawn from the market in 2004 and 2005, respectively. Celecoxib remains on the market today with advisory warnings for the potential increased risk of cardiovascular events.

1.5.4.1 NSAIDs in CRC Chemoprevention

In addition to the anti-inflammatory properties of NSAIDs, their use has also been associated with reduced incidence and recurrence of various cancers including colon, breast, lung, liver, and prostate (Wong, 2019). Of all NSAIDs, Aspirin displays the strongest and most consistent chemopreventative effect against CRC. This idea initiated from case control studies that associated regular Aspirin use with decreased risk of CRC (Kune et al., 1988; Rosenberg et al., 1991; Thun et al., 1991). Several further observational, case control, and cohort studies supported the correlation between Aspirin use and reduced incidence of colonic tumors and CRC-related death (Chan et al., 2005; Chan et al., 2008; Flossmann and Rothwell, 2007; Giovannucci et al., 1994; Giovannucci et al., 1995; Peleg et al., 1996; Ruder et al., 2011; Schreinemachers and Everson, 1994; Thun et al., 1993). Since then, numerous randomized controlled trials (RCTs) have provided further evidence for Aspirin use in the prevention of both adenomatous polyps and CRC (Baron et al., 2003; Benamouzig et al., 2003; Flossmann and Rothwell, 2007; Ishikawa et al., 2014; Logan et al., 2008; Sandler et al., 2003). Results of these studies and meta-analyses have suggested that Aspirin can reduce the risk of CRC by 20-50% if consistently used for at least 10 years (Bosetti et al., 2020; Chan et al., 2005; Chan et al., 2008; Cole et al., 2009; Cuzick et al.,

2015; Dubé et al., 2007; Giovannucci et al., 1995; Nan et al., 2015). The long-term benefit of Aspirin use has been highlighted in clinical trials that did not initially show any benefit at earlier pre-determined time points, but displayed a significant risk reduction when patient outcomes were assessed at extended follow-up time points (Burn et al., 2011; Cook et al., 2013). This further suggests that studies that did not detect a significant preventative effect against CRC with Aspirin use may be due to premature analysis (Gann et al., 1993; Sturmer et al., 1998). In contrast, some studies have suggested a benefit after just 5 years of consistent Aspirin use (Cook et al., 2005; Cook et al., 2013; Rothwell et al., 2010; Rothwell et al., 2011). Notably, low-dose Aspirin has been shown to be as effective as higher standard (i.e. anti-inflammatory) doses for the prevention of cancer (Baron et al., 2003; Cole et al., 2009; Flossmann and Rothwell, 2007; Rohwer et al., 2020; Rothwell et al., 2011). This was incidentally shown through the analysis of long-term follow-up data of RCTs that were initially designed to analyze the effects of low-dose Aspirin on the prevention of cardiovascular disease (Burn et al., 2008; Cook et al., 2005; Rothwell et al., 2010). Indeed, additional studies have shown that low-dose Aspirin use over a period of 5 years can reduce the risk of CRC by about 30% (Friis et al., 2015). Interestingly, some studies have demonstrated that low-dose Aspirin, but not traditional anti-inflammatory doses, reduces the risk of colonic adenomas (Baron et al., 2003; Cole et al., 2009). These findings have led the U.S. Preventative Services Take Force to recommend low-dose Aspirin for the primary prevention colorectal cancer and cardiovascular disease (CVD) in individuals aged 50-59 years with 10% or greater risk of CVD and no increased risk of bleeding (Bibbins-Domingo, 2016; Chubak et al., 2016). The UK National Institute for Health and Care Excellence (NICE) has also recommended daily Aspirin as a preventative measure against CRC in individuals with Lynch Syndrome (NICE (Natl. Inst. Health Care Excellence)., 2020). Recent studies have emerged suggesting that an individualized approach should be considered in the recommendation low-dose Aspirin for chemoprevention. For example, and individual's body mass index (BMI) may influence the ability of low-dose Aspirin to exert chemopreventative effects due to changes in bioavailability (Petrucci et al., 2019; Rothwell et al., 2018), whereas smoking status has been linked to inhibiting Aspirin's chemopreventative effects (Drew et al., 2016). Additionally, the risks of Aspirin use in older individuals (over 70-years-old) may

outweigh the perceived benefit as shown by the surprising findings from the ASPREE trial that reported that low-dose Aspirin trended towards increased all-cause mortality after just 5 years of use (McNeil et al., 2018), and the recent findings that concluded that Aspirin does not reduce the risk of CRC when initiated in an older age group (Zhang et al., 2021). Therefore, higher doses of Aspirin may be required for chemoprevention in individuals with a higher BMI and low-dose Aspirin may only be advisable in those who are non-smokers and under 70-years-old without risk of cardiovascular events.

Non-Aspirin NSAIDs have also been shown to reduce the risk of CRC. Sulindac reduces the number and size of polyps in patients with FAP or previous colonic polyps (Cruz-Correa et al., 2002; Giardiello et al., 1993; Takayama et al., 2011), yet does not display a chemopreventative effect in patients without prior adenoma development (Giardiello et al., 2002). Ibuprofen can also reduce the risk of CRC in patients with Lynch syndrome (Ait Ouakrim et al., 2015). Several clinical research studies have concluded that COX-2 inhibitors can reduce the risk of CRC (Arber et al., 2006; Bertagnolli et al., 2006; Phillips et al., 2002; Steinbach et al., 2000), suggesting that Aspirin may prevent sporadic CRC in a COX-2-dependent manner. However, many of these studies raised concerns regarding the safety of COX-2 inhibitors for chemoprevention due to their association with increased risk of cardiovascular and gastrointestinal adverse events (Arber et al., 2006; Baron et al., 2006; Bertagnolli et al., 2006). As a result, risk-benefit analysis suggests that COX-2 inhibitors are not an ideal agent for the prevention of CRC in individuals, unless they are part of a high-risk group (e.g. patients with FAP) (Katona and Weiss, 2020). These clinical findings have been supported by multiple additional studies demonstrating that both Aspirin and non-Aspirin-NSAIDs can prevent sporadic CRC in various animal models (Barnes and Lee, 1998; Berkel et al., 1996; Evans, 2003; Jacoby et al., 2000; Kitamura et al., 2002; Lew et al., 2002; Oshima et al., 1996; Reddy et al., 1993; Rohwer et al., 2020; Williamson et al., 1999; Yan et al., 2004; Zhao et al., 2020).

The mechanism of NSAID chemoprevention is still not entirely known. However, the ability for NSAIDs to inhibit COX activity and downregulate prostaglandin synthesis has been linked to their mechanism of cancer prevention. Studies have shown that Aspirin use is associated with reduced risk of colonic tumors expressing higher levels of COX-2 (Chan

et al., 2007), and Aspirin use has been shown to be most efficacious against preventing CRC in patients with high mucosal 15-PGDH expression (Fink et al., 2014) or elevated urinary levels of the PGE_2 metabolite, PGE-M (Bezawada et al., 2014). Low-dose Aspirin has also been suggested to prevent CRC through inhibition of COX-1 in platelets (Thun et al., 2012). The role of COX signaling in CRC is previously discussed in **Section 1.5.3**.

1.5.4.2 NSAIDs in Colitis and CAC

In the context of IBD, patients are often advised to use caution with NSAIDs as administration of COX-inhibitors at anti-inflammatory doses can exacerbate the symptoms of IBD in patients (Allison et al., 1992; Bonner, 2001; Cipolla et al., 2002; Evans et al., 1997; Gornet et al., 2002; Kefalakes et al., 2009; Kurahara et al., 2001; Meyer et al., 2006; Thiéfin and Beaugerie, 2005) and worsen colitis severity in mouse models (Berg et al., 2002; Okayama et al., 2007; Singh et al., 2004). This is particularly true of COX-2 inhibitors which are associated with GI toxicity and exacerbation of colitis (Allison et al., 1992; Evans et al., 1997; Meyer et al., 2006). The restricted use of NSAIDs in patients with IBD has limited the ability to determine their chemopreventative capacity against CAC, and therefore the role of COX inhibition in the prevention of CAC has not been extensively studied. However, the harmful effects of NSAIDs in patients with IBD have been attributed to standard anti-inflammatory doses, and the effect of low-dose NSAIDs on IBD exacerbation are still unclear. Recent reports have indicated that daily low-dose Aspirin use does not impact clinical outcomes of patients with IBD (Patel et al., 2021), indicating that NSAIDs at low doses may be safe for use in these patient populations.

Some studies have analyzed the chemopreventative effects of NSAIDs in mouse models of CAC with varying results. Low-dose Aspirin appears to be effective against colonic tumorigenesis in mouse models of CAC (Guo et al., 2016, 2017; Rohwer et al., 2020; Tian et al., 2011b), whereas COX-2 selective inhibitors have resulted in both prevention and exacerbation of CAC (Hegazi et al., 2003; Lee et al., 2020; Setia et al., 2014a). This may be a result of increased colitis severity that is a well-documented effect of COX-2 inhibition during colonic inflammation in both humans and mice (Biancone et al., 2003; Bonner,

2001; Hegazi et al., 2003; Ishikawa et al., 2011; Matuk et al., 2004; Meyer et al., 2006; Okayama et al., 2007; Shafiq et al., 2005; Tsubouchi et al., 2006, 2006; Zhang et al., 2008). More research is needed to determine whether low-dose NSAIDs are safe and effective in the chemoprevention of CAC, and the mechanism by which this occurs.

1.5.4.3 COX and the Wnt/β-Catenin Pathway

As the majority of CRC cases display both aberrant COX expression and active Wnt signaling (as discussed in **Section 1.5.3** and **Section 1.3.4**, respectively), exploration into a potential connection between COX and Wnt signaling pathways has been of great importance. Castellone et al., 2005 was one of the first groups to identify a direct link between COX and Wnt pathways in colorectal cancer. Here, these authors showed that PGE_2 can increase the activation of Tcf/Lef transcription factors to active canonical Wnt signaling in colorectal carcinoma cells. They identified a mechanism by which PGE_2 can lead to the phosphorylation and inactivation of GSK-3β, resulting in reduced degradation of β-catenin, and allowing for its nuclear accumulation and activation of Wnt signaling (Castellone et al., 2005). The link between PGE_2 and Wnt signaling was further supported by Shao et al., 2005, who also showed that PGE_2 can activate β-catenin/Tcf-dependent transcription in a colorectal cancer cell line (Shao et al., 2005). This was mediated through activation of cAMP/PKA and inactivation of GSK-3β, resulting in the stabilization of β-catenin. Interestingly, they also noted that PGE_2 can act in a synergistic manner with mutated β-catenin to upregulate Wnt target genes, indicating that PGE_2 may be able to promote tumorigenesis through stimulation of Wnt/β-catenin-mediated transcription. However, a limitation of these studies is their use of immortalized CRC cell lines, leading to the question whether this interaction may be a result of aberrant pathway regulation specific to cancer. This limitation was addressed by Goessling et al., 2009 who showed the first evidence for *in vivo* regulation of PGE_2 on Wnt signaling in vertebrate development and organ regeneration (Goessling et al., 2009). Here, they showed that PGE_2 can regulate Wnt signaling through cAMP/PKA-mediated GSK-3β phosphorylation and stabilization of β-catenin (**Figure 1.8**). Various other studies have demonstrated COX and Wnt signaling crosstalk: stimulation of EP2 and EP4 can lead to the PKA-dependent activation of Tcf/Lef

signaling through phosphorylation of GSK-3β in HEK-293 cells (Fujino et al., 2002), inhibition of GSK-3β stimulates COX-2 expression in gastric cancer (Thiel et al., 2006), and PPAR-δ has been identified as a key link between prostaglandin and Wnt signaling in the promotion of colonic tumor growth (Wang et al., 2004).

The ability of PGE_2 to promote Wnt signalling via nuclear translocation of β-catenin correlates with additional findings demonstrating the ability of PGE_2 to promote stemness and proliferation. PGE_2 can promote hematopoietic stem cell number in mice and zebrafish (Frisch et al., 2009; Goessling et al., 2009; North et al., 2007), the expansion of human colon stem cells *in vitro* (Jung et al., 2011), and the stemness of human lung and skin cancer cell lines (Terzuoli et al., 2017). In colonic organoids from mice, the addition of PGE_2 promoted organoid growth, proliferation, the number of Lgr5+ intestinal stem cells, and the expression of stem cell markers *Sox9*, *Axin2*, and *Cd44* (Fan et al., 2014).

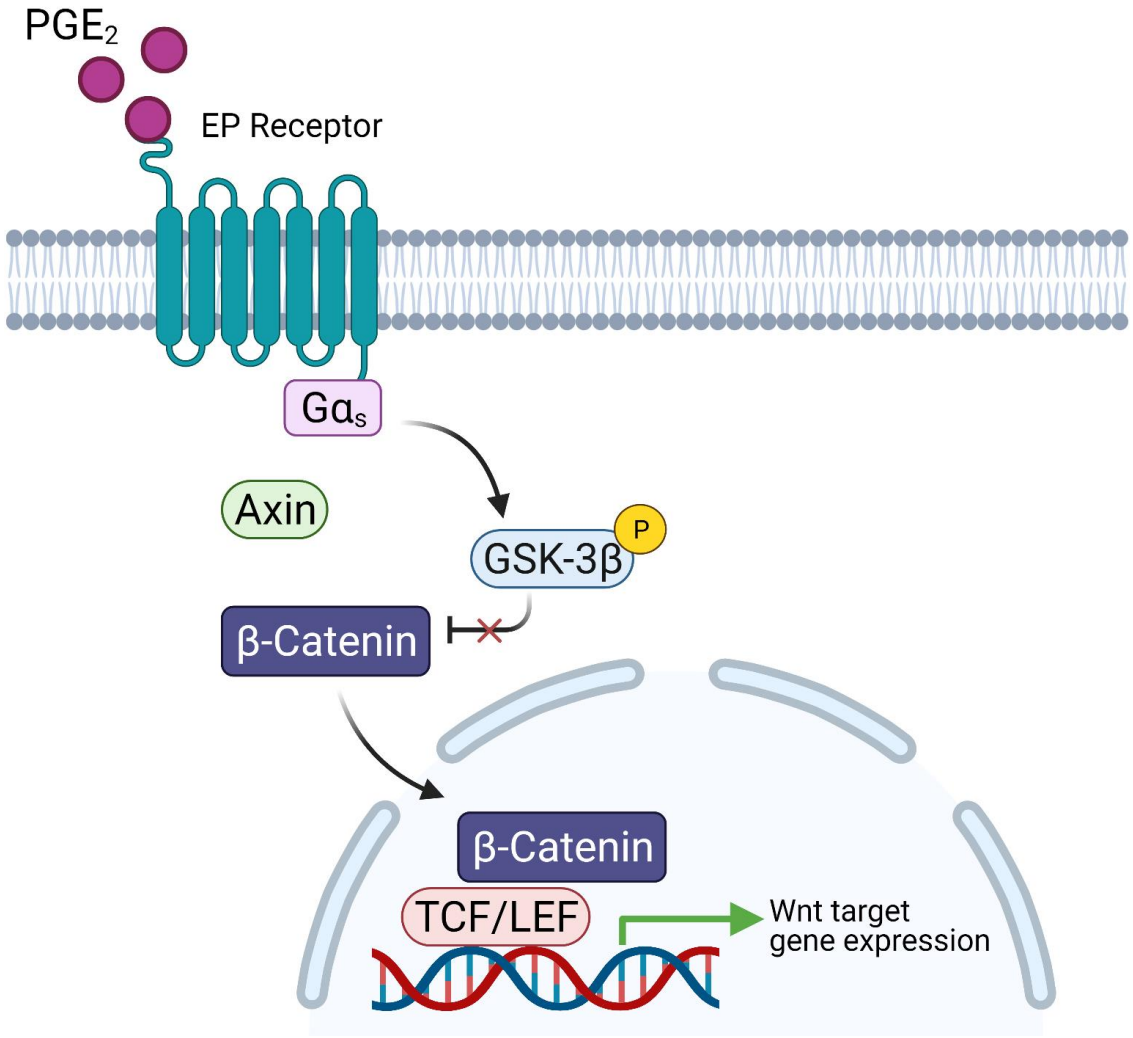

Figure 1.8 – Mechanism of PGE_2-mediated activation of Wnt signaling.

PGE_2 acts in a paracrine or autocrine manner to bind to PGE_2-specific GPCRs (EP receptors) and stimulate downstream phosphorylation and inactivation of GSK-3β. This results in the stabilization and nuclear localization of β-catenin, leading to the upregulation of Wnt target gene expression. Abbreviations: PGE_2, prostaglandin E2; GSK-3β, glycogen synthase kinase 3 beta; P, phosphorylated; TCF/LEF, T cell factor/lymphoid enhancer factor; GPCR, G-protein coupled receptor. Created in Biorender.com.

1.6 PI3K/Akt Signaling Pathway

The serine-threonine Akt kinase (also known as protein kinase B) is the major downstream mediator of the phosphoinositide 3-kinase (PI3K) signaling cascade. Upon activation by growth factor receptors or adaptors, Class I PI3K phosphorylates the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2), converting it to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) (Cantley, 2002). PIP_3 then acts to recruit proteins containing pleckstrin-homology (PH) domains, which bind phospholipids, to the plasma membrane. These include Akt and phosphoinositide-dependent kinases (PDK). Once in close proximity, Akt is then phosphorylated and activated by PDK1 and/or PDK2 (Vanhaesebroeck and Waterfield, 1999). Upon phosphorylation of both Thr308 and Ser473 residues, Akt is fully activated and released from the membrane to propagate downstream signaling activity. The kinase activity of Akt mediates many effectors that contribute to cell growth, survival, proliferation, and migration (Scheid and Woodgett, 2003). There are three separately encoded isoforms of Akt (Akt1, Akt2, Akt3) which all contain the threonine and serine residues required for activation. Akt proteins contain three domains: N-terminal PH domain, catalytic domain, and C-terminal hydrophobic motif domain (Vanhaesebroeck and Waterfield, 1999).

1.6.1 *PI3K/Akt Pathway in Intestinal Homeostasis*

Distinct regulatory signaling pathways exist as gradients along the crypt-villus axis in order to regulate stemness and differentiation of the intestinal epithelium. These include the opposing Wnt and BMP signaling gradients. Wnt signaling is highest at the crypt base and functions to promote proliferation and stemness, whereas BMP signaling restricts stemness, promotes differentiation, and increases upwards along the crypt-villus axis. BMP signaling is critical in the prevention of hyperproliferation of the stem cell zone by restricting the stemness of ISCs (Qi et al., 2017). A positive and functional relationship between BMP and PTEN, the negative regulator of the PI3K/Akt signaling pathway, has been previously described in which BMP is able to prevent the degradation of PTEN

(Waite and Eng, 2003). He et al., 2004 further identified that the ability of BMP signaling to balance epithelial self-renewal occurs through activation of PTEN and subsequent inhibition of PI3K/Akt signaling and β-catenin transcriptional activity (He et al., 2004). In this mechanism, PTEN mediates the interaction of BMP and Wnt signaling pathways to sustain intestinal homeostasis by modulating Akt activity and preventing the nuclear localization of β-catenin. These authors further describe that cells in the CBC stem cell position were positive for p-Akt, indicating that Akt signaling may be important for ISC function (He et al., 2004). Further roles for Akt in maintaining intestinal homeostasis have been well-described through the promotion of epithelial proliferation (Engelman et al., 2006; Kim et al., 2002; Sheng et al., 2003; Stiles et al., 2004), modulation of differentiation (Laprise et al., 2002), and regulation of cell-cycle progression (He et al., 2007).

1.6.2 *PI3K/Akt Signaling and the Wnt/β-catenin Pathway*

A molecular link between the PI3K/Akt and Wnt/β-catenin signaling pathways has been well-described. Previous work has attributed this link to one of the targets of Akt kinase activity, GSK-3β (Desbois-Mouthon et al., 2001; Frame and Cohen, 2001; Sutherland et al., 1993). Upon Akt-mediated phosphorylation, GSK-3β is inhibited, allowing for the activation of pathways that are normally downregulated by GSK-3β activity. As described previously (**Section 1.2.3**), GSK-3β is a member of the destruction complex that acts to repress β-catenin levels and inhibit Wnt signaling. Therefore, the ability of Akt to phosphorylate GSK-3β suggests that Akt activity can upregulate the Wnt signaling pathway by indirect activation of β-catenin. However, Frame and Cohen, 2001 described that GSK-3β exists in two distinct pools and that Akt and Wnt-mediated phosphorylation of GSK-3β result in exclusive and independent biological downstream effects (Frame and Cohen, 2001). In contrast to this theory, however, Akt has also been shown to modulate Wnt signaling through direct activation of β-catenin. Studies have identified that the Ser552 residue on β-catenin is an Akt-specific phosphorylation site that stimulates the cytoplasmic and nuclear localization of β-catenin, resulting in increased transcriptional activity (Fang et al., 2007; He et al., 2007). This finding is supported by multiple additional studies that have shown that Akt can directly induce the nuclear accumulation of β-catenin (Fang et al.,

2007; He et al., 2007; Kaler et al., 2009) and Wnt target gene expression (Muise-Helmericks et al., 1998). Phopsho-Akt levels can also be induced upon activation of Wnt signaling to enhance or sustain GSK-3β phosphorylation through association with Dishevelled (Fukumoto et al., 2001). In this study, Akt was identified as an enhancer as opposed to an initiator of Wnt signaling. Furthermore, expression of PTEN, a PI3K/Akt negative regulator, results in decreased β-catenin transcriptional activity, whereas loss of PTEN induces β-catenin levels, supporting the link between Akt activation and Wnt target gene expression (Persad et al., 2001; Sharma et al., 2002).

1.6.3 *Akt in Colorectal Cancer*

There exists a clear oncogenic role for Akt in driving the progression of many types of human malignancies (Bellacosa et al., 2005; Vivanco and Sawyers, 2002). This is particularly true of colorectal cancer as 30-40% of colonic tumors display aberrant activation of the PI3K/Akt signaling pathway (Carpten et al., 2007; Parsons et al., 2005). Aberrant PI3K/Akt activity in CRC frequently occurs due to activating mutations in *PIK3A* or inactivating mutations in *PTEN* (Cancer Genome Atlas Network, 2012), resulting in uncontrolled cellular proliferation, survival, and cell cycle progression. Indeed, studies have shown that all three Akt isoforms are upregulated in CRC (Buikhuisen et al., 2021; Rychahou et al., 2008). The PI3K/Akt pathway has also been implicated in the initiation of intestinal cancer in mice with the use of PTEN-deficient models. Although homozygous knockout of *Pten* is embryonically lethal in mice, heterozygous *Pten* loss results in the initiation of dysplastic lesions in the colonic epithelium (Di Cristofano et al., 1998). Further studies that assessed the effects of PTEN-loss in IECs observed that multiple small intestinal polyps formed as a result of Akt-mediated phosphorylation and nuclear localization of β-catenin (Byun et al., 2011; He et al., 2007). As the tumors arising from mice with epithelial loss of PTEN display constitutive activation of both Wnt and Akt signaling, this suggests that the previously described link between Wnt and Akt (**Section 1.6.2**) may also function to promote tumorigenesis. This idea was supported by Marsh et al., 2008 who reported that IECs deficient for both PTEN and APC resulted in the rapid

formation of colonic adenocarcinomas that displayed strong pAkt expression (Marsh et al., 2008).

1.6.4 *Akt in Colitis & Colitis-Associated Cancer*

The PI3K/Akt signaling pathway has been identified as an important mediator of colitis in both patients with IBD and in experimental mouse models. Phospho-Akt levels are increased in the colonic epithelium of patients with UC (Dahan et al., 2008; Huang et al., 2011) and in the DSS model of colitis (Fu et al., 2017; Gutiérrez-Martínez et al., 2019; Lee et al., 2010). Inhibition of the PI3K/Akt signaling pathway using the compound wortmannin resulted in reduced levels of inflammation and improved severity of DSS-colitis in mice (Huang et al., 2011). The upregulation of pro-inflammatory cytokines in colitis can also stimulate Akt activation. Particularly, TNF-α and IFN-γ, which exacerbate inflammation and are known to be elevated in human IBD and DSS-colitis (Akazawa et al., 2002; Andreou et al., 2020; Ishiguro, 1999; Obermeier et al., 1999; Scaldaferri et al., 2010), can contribute to intestinal inflammation in an Akt and Wnt/β-catenin dependent manner (Nava et al., 2010). Interestingly, the association between Akt activity and active inflammation has been linked to the ability of Akt to promote β-catenin transcriptional activity. A study by Lee *et al.*, 2010 analyzed the effect of deletion of *Pik3r1*, the gene encoding class IA PI3K, in all intestinal epithelial cells. This resulted in effective inhibition of PI3K/Akt signaling activity and reduced nuclear β-catenin localization in a mouse model of colitis (Lee et al., 2010). Their findings indicated that the upregulation of PI3K-mediated Akt activity in inflammation is required to stimulate canonical Wnt signaling through stabilization of β-catenin by phosphorylation at Ser552. Interestingly, levels of pAkt and p-β-catenin^{522} were increased during the progression from colitis to dysplasia, which was prevented upon deletion of *Pik3r1*, highlighting a role for the PI3K/Akt signaling pathway in the initiation of colitis-associated cancer (Lee et al., 2010). This indicates that the previously discussed link between PI3K/Akt and Wnt signaling (**Section 1.6.2**) is relevant in both the setting of inflammation and in CAC. The role of Akt in CAC was supported by further studies showing that pAkt levels increased in CD68+ macrophages during the progression from colitis to cancer, which was prevented upon PI3K inhibition (Khan et al.,

2013). Setia et al., 2014 demonstrated that several members of the PI3K/Akt pathway are upregulated in the DMH+DSS mouse model of colitis-associated cancer, including PI3K and Akt, which correlated with increased β-catenin and Wnt pathway activity (Setia et al., 2014b).

1.6.5 *PI3K/Akt Signaling & the COX Pathway*

Previous studies have established a link between the PI3K/Akt and COX signaling pathways in the intestine. This association was initially described as an anti-apoptotic mechanism in colorectal cancer where PI3K activity resulted in upregulated COX-2 expression, while PI3K inhibition reduced COX-2 expression and PGE_2 synthesis (Di Popolo et al., 2000; Kaur and Sanyal, 2010). The converse relationship between these two pathways has also been described in CRC, where exogenous PGE_2 administration is able to upregulate pAkt levels to promote proliferation and inhibit apoptosis (Buchanan et al., 2003; Cen et al., 2020; Leone et al., 2007; Tessner et al., 1998). Conflicting evidence suggests that the mechanism by which PGE_2 activates Akt may be in a cAMP/PKA-dependent manner (Leone et al., 2007) or through Src-mediated transactivation of the EGFR signaling pathway (Buchanan et al., 2003). This link was further established in studies showing that in CRC, PGE_2 can activate pAkt to stimulate the nuclear localization of β-catenin and augment Wnt signaling through GSK-3β inhibition (Castellone et al., 2005; Hsu et al., 2017). This, importantly, supports a connection between COX, PI3K/Akt, and Wnt signaling pathways in the pathogenesis of CRC (**Figure 1.9**). The ability for COX signaling to activate Akt has also been shown in various other tissue and cancer types (Chang et al., 2019; Chen et al., 2017; Fujino et al., 2002; George et al., 2007; Glynn et al., 2010; Uddin et al., 2010; Vo et al., 2013; Yang et al., 2020). Interestingly, Aspirin use in patients with PIK3CA-mutated CRC results in improved survival as compared to patients with WT PIK3CA CRC (Elwood et al., 2016; Liao et al., 2012; Paleari et al., 2016), supporting a mechanistic cross-talk between PI3K/Akt and COX signaling pathways in CRC. A link between Akt and COX has also been described in the setting of intestinal inflammation, particularly mediated through the activity of PGE_2. Peng et al., 2017 showed that in the setting of colitis, the COX-1/PGE_2/EP4 signaling axis upregulates pAkt to

prevent mucosal injury (Peng et al., 2017). Whereas similarly, Yao et al., 2013 showed that PGE_2 can promote Akt phosphorylation in T cells to modulate immune responses by upregulating regulators of Th1-cell differentiation such as IL-12 and IFN-γ in a model of IBD (Yao et al., 2013). Overall, strong evidence suggests that the PI3K/Akt signaling pathway can be upregulated as a result of PGE_2 activity.

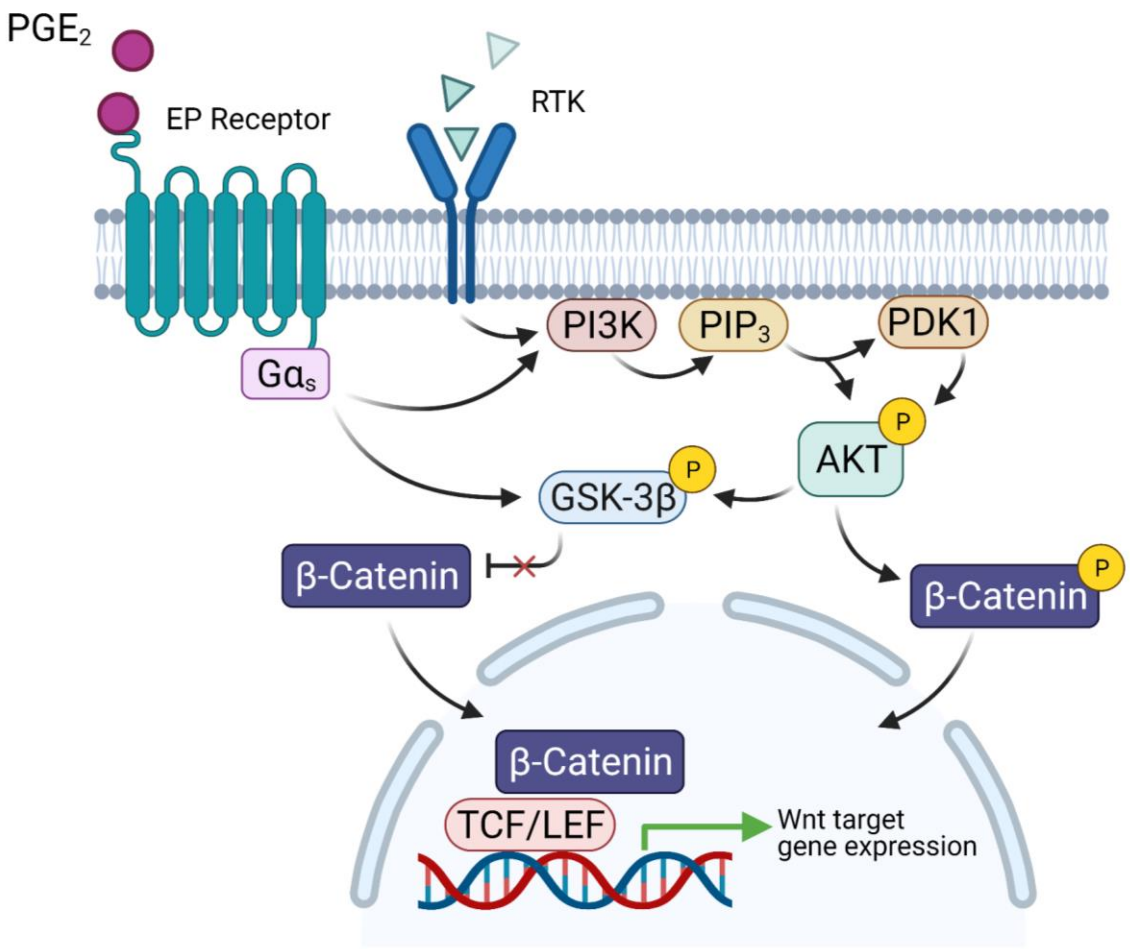

Figure 1.9 – Schematic of the compiled mechanisms of crosstalk between PGE_2, PI3K/Akt, and Wnt signaling pathways.

PGE_2 can lead to phosphorylation and inactivation of GSK-3β, leading to the stabilization and nuclear localization of β-catenin (as described in **Figure 1.8**). PGE_2 can also lead to activation of the PI3K pathway resulting in upregulated phospho-Akt levels, which can also be induced by activation by growth factors through RTKs. Phospho-Akt can also lead to the phosphorylation and inactivation of GSK-3β, or it can phosphorylate β-catenin on the Akt-specific Ser522 residue, resulting in its stabilization and nuclear localization. Abbreviations: PGE_2, prostaglandin E2; RTK, receptor tyrosine kinase; AKT, protein kinase B; GSK-3β, glycogen synthase kinase 3 beta; PI3K, phosphoinositide 3-kinase; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; PDK1, phosphatidylinositide-dependent protein kinase 1; P, phosphorylated. Created in Biorender.com.

1.7 NF-κB Signaling Pathway

The NF-κB signaling pathway is an evolutionarily conserved pathway that triggers and coordinates inflammatory, immune, and anti-apoptotic responses (Karin and Lin, 2002). NF-κB signaling is driven by a series of five transcription factors that all contain a Rel-homology domain, enabling them to dimerize and bind DNA for regulation of target gene expression (Oeckinghaus and Ghosh, 2009). In mammals, these transcription factors include p65 (RelA), p50 (p105), p52 (p100), c-Rel, and RelB, which form 15 different NF-κB homo- and heterodimers (Herrington et al., 2016). The effect of NF-κB signaling is determined by the subunit composition. For example, positive regulation of gene expression can be induced by p65, c-Rel, and RelB as they contain transcription activation domains. Whereas p50 and p52, which are synthesized from their precursor proteins p105 and p100, respectively, downregulate transcription unless they are dimerized with another protein (Ghosh and Hayden, 2008). NF-κB dimers are normally inactivated and retained in the cytoplasm by IκB inhibitory proteins, including IκBα, IκBβ, IκBϵ, IκBγ, p100 and p105 precursor proteins, or Bcl-3, IκBζ and IκBδ (IκBNS) atypical proteins. These proteins all contain an ankyrin repeat domain that enables them to bind and sequester NF-κB dimers, preventing them from localizing to the nucleus (Ghosh et al., 1998; Hinz et al., 2012; Pasparakis, 2009). NF-κB signaling activity is initiated by the IKK-complex that phosphorylates IκB inhibitory proteins on serine residues and tags them for proteasomal degradation. This causes NF-κB dimers to freely move into the nucleus and induce target gene transcription (**Figure 1.10**). The IKK-complex consists of the two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), and the regulatory protein NEMO/IKKγ. Although both IKKα and IKKβ are catalytically active, they have been shown to induce different downstream physiological effects and activation of the two distinct arms of the NF-κB signaling pathway. Canonical NF-κB signaling is thought to be predominantly mediated by IKKβ, which is activated by various extracellular pro-inflammatory signals such as luminal bacteria-producing LPS or inflammatory cytokines (e.g. TNF-α, IL-1β). IKKβ activation results in IκBα degradation and the nuclear accumulation of p50, p65, and c-Rel dimers (Dejardin et al., 2002; Pasparakis, 2009; Senftleben et al., 2001). However, in the setting of IKKβ-loss, IKKα has also been shown to initiate canonical NF-κB signaling

through a compensatory mechanism (Lam et al., 2008; Luedde et al., 2005). Canonical NF-κB signaling leads to the upregulation of a wide range of genes involved in inflammation, immunity, cell adhesion, antioxidant processes, cell survival, and cell proliferation (Ghosh and Karin, 2002). Another target gene of canonical NF-κB signaling is IκBα, which generates a negative feedback loop to regulate signaling activity (Oeckinghaus and Ghosh, 2009). Non-canonical, or alternative, NF-κB signaling is activated by IKKα, in a NEMO- and IKKβ-independent mechanism. This leads to the activation of NF-κB-inducing kinase (NIK), which triggers IKKα-dependent phosphorylation and processing of p100 to p52 and the nuclear translocation of p52/RelB dimers (Saccani et al., 2003; Xiao et al., 2001). Non-canonical NF-κB signaling is activated by members of the tumor necrosis factor receptor (TNFR) superfamily and upregulates the expression of genes involved in lymphoid organogenesis, B cell maturation, and bone metabolism (Senftleben et al., 2001).

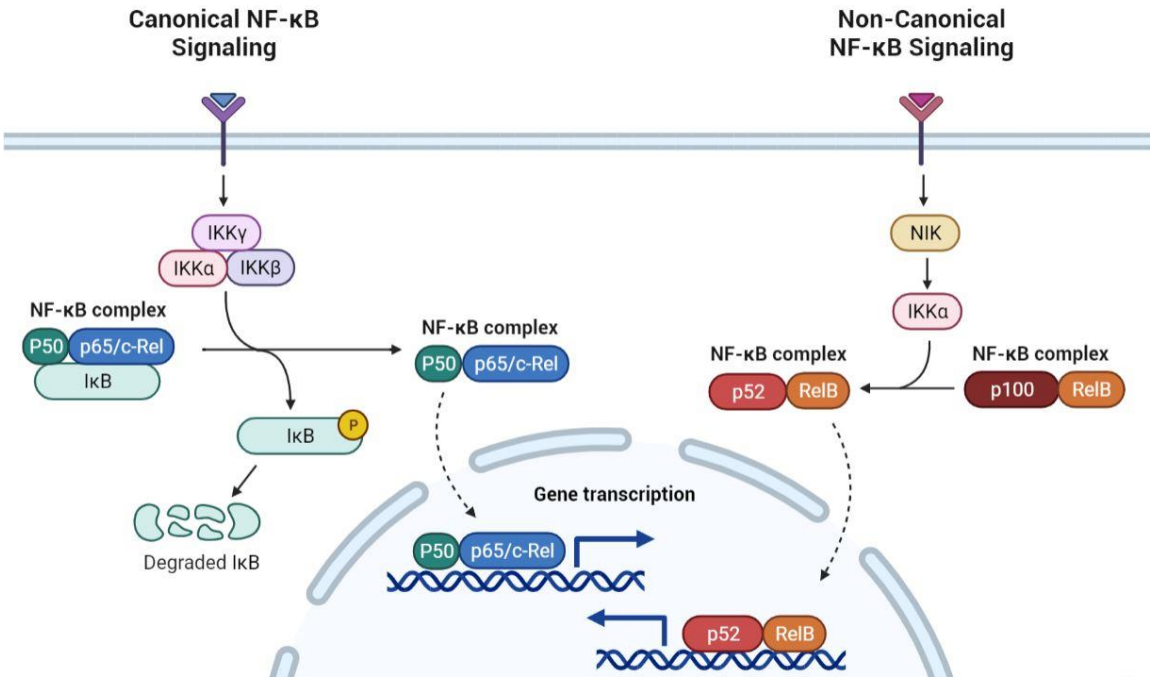

Figure 1.10 – Canonical and non-canonical NF-κB signaling pathways.

Canonical NF-κB signaling (left) is mediated through the IKKβ-dependent inactivation of IκBa, allowing for p50/p65 or p50/c-Rel NF-κB dimers to localize to the nucleus and upregulate genes involved in immune regulation. Non-canonical NF-κB signaling (right) is mediated through IKKα, in a IKKβ and IKKγ-independent mechanism, leading to the processing of p100 to p52, and subsequent nuclear localization of p52/RelB NF-κB dimers. Abbreviations: IKK, inhibitor of nuclear factor kappa-B kinase; IκB, inhibitor of nuclear factor kappa B; NIK, NF-κB inducing kinase. Adapted from "NF-κB Signaling Pathway", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

1.7.1 *NF-κB Signaling in Intestinal Homeostasis*

Increased NF-κB signaling activity is often observed in the setting of chronic inflammation, where it is believed to contribute to disease pathogenesis by upregulating and sustaining the inflammatory response (Liu et al., 2017). However, NF-κB signaling is also important for anti-apoptotic responses, and evidence suggests that inhibition of NF-κB signaling can cause perturbations to intestinal homeostasis and induce basal inflammation. This suggests that NF-κB also plays a key role in regulating tissue homeostasis. Nenci et al., 2007 showed that IEC-specific deletion of IKKγ/NEMO resulted in spontaneous intestinal inflammation and increased expression of various inflammatory cytokines. The authors also observed extensive TNF-mediated apoptosis of NEMO-deficient IECs, which they attributed to the loss of anti-apoptotic regulation by NF-κB signaling (Nenci et al., 2007). Interestingly, mice deficient for either IKKα or IKKβ in IECs did not result in spontaneous colitis, whereas mice deficient for both epithelial IKK subunits displayed intestinal inflammation comparable to IKKγ/NEMO-deficient mice, suggesting a potential compensatory mechanism between IKKα and IKKβ subunits (Nenci et al., 2007). Intestinal mucosal homeostasis is dependent on the barrier established between the luminal bacteria and underlying epithelial and immune cells. If this barrier is compromised, bacteria infiltrate into the submucosa and lead to inflammation and tissue damage. Interestingly, IKKγ/NEMO-loss in IECs also led to decreased expression of antimicrobial peptides, suggesting that NF-κB signaling in IECs is also important for maintaining epithelial barrier integrity (Nenci et al., 2007). Overall, this study showed that loss of NF-κB signaling in IECs results in epithelial apoptosis, which leads to reduced expression of antimicrobial peptides and compromised barrier function. Loss of barrier function resulted in infiltration of luminal bacteria into the mucosa, ultimately triggering an inflammatory response (Nenci et al., 2007). Further studies have confirmed that canonical NF-κB inhibition results in spontaneous intestinal inflammation as a result of increased IEC apoptosis (Lee et al., 2000; Vlantis et al., 2016). Interestingly, a recent study showed that mice deficient for IκBα, a key inhibitor of NF-κB, also resulted in spontaneous intestinal inflammation (Mikuda et al., 2020), highlighting the dual pro- and anti-inflammatory role for NF-κB signaling in the intestine. Other studies have identified that canonical NF-κB signaling may contribute to intestinal homeostasis by regulating intestinal proliferation. P65 has been shown to be

localized to the bottom third of the crypt base near intestinal stem cells (Inan et al., 2000; Steinbrecher et al., 2008), and mice deficient for *Nfkb1*, which encodes p105/p50, displayed reduced NF-κB activity and a hyperproliferative epithelium. These mice also had upregulated TNF-α expression, which may have contributed to the enhanced proliferative response (Inan et al., 2000). These studies suggest that NF-κB signaling may play a role in intestinal homeostasis by suppressing epithelial proliferation.

1.7.2 *NF-κB Signaling in Intestinal Injury and Inflammation*

As the NF-κB signaling pathway is an inflammation-related pathway, it has been associated with many inflammatory disease states, particularly IBD. Aberrant canonical NF-κB activation has been detected in colonic tissue samples from patients with active CD or UC (Ellis et al., 1998; Rogler et al., 1998; Schreiber et al., 1998) and the degree of NF-κB activation has been shown to correlate with disease severity (Han et al., 2017). Known targets of NF-κB signaling activity, such as the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6, are also upregulated in IBD (Sanchez-Munoz et al., 2008). Moreover, certain pharmacological agents used in the treatment of IBD have been shown to inhibit NF-κB signaling activity (Barnes and Karin, 1997; Wahl et al., 1998) (Weber, 2000; Egan, 1999). The first study that demonstrated that NF-κB signaling plays a pathogenic role in IBD was work done by Neurath, et al., 1996, which showed that pharmacological inhibition of NF-κB signaling using a p65 antisense oligonucleotide was protective against experimental colitis in rodents (Neurath et al., 1996). This finding was confirmed in further studies demonstrating that pharmacological inhibition of NF-κB signaling using a NEMO-binding domain (NBD) peptide, a p65 antisense oligonucleotide, or a selective IKK inhibitor were preventative against murine models of colitis (Davé et al., 2007; MacMaster et al., 2003; Murano et al., 2000; Shibata et al., 2007). However, these studies did not address whether pathogenic NF-κB signaling in colitis was derived from the epithelial and/or non-epithelial (e.g. immune, stromal) cell populations. The generation of intestinal cell-specific perturbations in NF-κB signaling has revealed a more complicated role for this pathway during intestinal injury, with varying effects based on the targeted NF-κB mediator. NF-κB signaling is considered a pro-inflammatory pathway as it upregulates the expression of

many cytokines and chemokines. However, it also plays an important role in regulating anti-apoptotic mechanisms. As a result, inhibition of NF-κB signaling can lead to increased IEC apoptosis, impaired intestinal barrier function, and ultimately promote inflammation due to tissue damage, as discussed in **Section 1.7.1**. Therefore, NF-κB signaling can play a dual role in contributing to both pro-inflammatory and protective functions during intestinal injury. This has been highlighted by multiple studies analyzing the effects of IEC-specific canonical NF-κB inhibition or activation on intestinal damage responses. IEC-specific loss of IKKβ resulted in increased susceptibility to tissue damage and apoptosis during gut ischemia-reperfusion injury (Chen et al., 2003), increased inflammatory response to *C. difficile* or *T. muris* infection (Chae et al., 2006; Zaph et al., 2007), exacerbated mucosal damage in a thermal skin injury model (Chen et al., 2007), and increased sensitivity to irradiation injury (Egan et al., 2004). These studies provide strong support for a protective role of canonical NF-κB signaling in IECs.

The role of NF-κB in IECs during colitis, however, is less clear. Greten et al., 2004 showed that IEC-specific loss of IKKβ resulted in exacerbated colitis severity as a result of decreased epithelial survival and impaired barrier function (Greten et al., 2004). This finding was confirmed in additional studies using mice with loss of p65 (RelA) in IECs, which resulted in increased colitis severity and aberrant epithelial apoptosis and proliferation. The authors suggest that these findings may be a result of reduced levels of the protective cytokines IL-18 and IL-11, leading to impaired mucosal healing (Steinbrecher et al., 2008). Eckmann et al., 2007 reported that IKKβ-loss in the intestinal epithelium resulted in increased DSS-colitis severity during the acute phase of injury, yet led to attenuated disease severity during chronic inflammatory responses (Eckmann et al., 2008). Whereas further studies analyzing the effects of IEC-specific activation of canonical NF-κB signaling in DSS-colitis also led to exacerbated disease states, highlighting the dual role for canonical NF-κB in the intestinal epithelium (Guma et al., 2011; Vereecke et al., 2010). Burkitt et al., 2015 analyzed the role of both canonical and non-canonical NF-κB signaling pathways in DSS-colitis using mice that were deficient in p50, p52, or c-Rel. Interestingly, their results showed that loss of p52 was protective against DSS-colitis, whereas c-Rel and p50-deficient mice showed a moderate increase in colitis severity (Burkitt et al., 2015), indicating that non-canonical and canonical NF-κB signaling may

play opposing roles in DSS injury. In parallel to this, studies that activated non-canonical NF-κB signaling in IECs resulted in increased DSS-colitis severity, however this was reported to be a consequence of a compensatory upregulation of canonical NF-κB signaling (Allen et al., 2012; Zaki et al., 2011). Recent reports by Chawla et al., 2021 indicated that inhibition of non-canonical NF-κB signaling using mice with p52-deficient IECs resulted in improved DSS-colitis severity, but this was due to a corresponding decrease in canonical p65-mediated activity (Chawla et al., 2021). In contrast, Giacomin et al., 2015 showed that mice with IKKα-deficient IECs worsened intestinal inflammation in DSS and *C. rodentium* models of injury, while IKKβ-deficiency in IECs had no effect (Giacomin et al., 2015). Macho-Fernandez et al., 2015 reported that IEC-specific inhibition of non-canonical NF-κB signaling resulted in worsened DSS-colitis severity (Macho-Fernandez et al., 2015). The stark contrast in findings from studies analyzing the effects of activation or inhibition of NF-κB signaling in IECs during DSS-colitis is exemplified in work done by Ramakrishan et al, 2019. Here, they showed that both activation and inhibition of the non-canonical mediator NIK in IECs worsened colitis severity (Ramakrishnan et al., 2019). Overall, the results from these various studies suggest that the NF-κB signaling pathway in IECs plays various roles in the setting of intestinal injury or inflammation. Further work is required to elucidate the cellular and pathophysiological mechanisms that regulate the opposing roles of this pathway in intestinal injury.

1.7.3 *NF-κB Signaling in CRC and CAC*

NF-κB signaling activity regulates several of the essential hallmarks of cancer as described by Hanahan and Weinberg (Hanahan and Weinberg, 2011). These functions include regulating immune responses, preventing of apoptosis, stimulating proliferation, angiogenesis, and differentiation, and inducing resistance to chemo- and radiotherapy (Soleimani et al., 2020). Indeed, constitutive activation of canonical NF-κB signaling has been detected in human colorectal cancer tissue biopsies and colorectal cancer cell lines (Hardwick et al., 2001; Lind et al., 2001; Voboril and Weberova-Voborilova, 2006). Aberrant NF-κB signaling activity also correlates with tumor progression and poor prognoses in patients with CRC (Kojima et al., 2004; Lin et al., 2012). A functional role

for NF-κB signaling in colonic tumorigenesis was proven when older mice developed spontaneous colonic tumors in the setting of constitutive activation of IKKβ in IECs (Vlantis et al., 2011). Tumorigenesis is enhanced and accelerated when IEC-specific loss of *Apc* is introduced in combination with constitutive NF-κB activity (Shaked et al., 2012). Given the association between NF-κB signaling and colonic tumorigenesis, and the role of this pathway in inflammation, a strong link between NF-κB and colitis-associated cancer has been established. The first study proving this connection was work done by Greten et al., 2004, where inhibition of canonical NF-κB signaling mediated through genetic IKKβ-loss resulted in fewer colonic tumors in the AOM/DSS model of CAC (Greten et al., 2004). Their utilization of mouse models harbouring cell-specific loss of IKKβ further showed that NF-κB signaling in both epithelial and myeloid cell populations contributes to CAC, although through different mechanisms. Epithelial cell-specific loss of IKKβ prevented tumorigenesis by inhibiting NF-κB-mediated anti-apoptotic gene expression during tumor initiation, whereas loss of IKKβ in myeloid cells reduced tumor number and size by downregulating the expression of pro-inflammatory cytokines (Greten et al., 2004). These NF-κB derived cytokines, such as IL-6 and TNF-α, have been shown to fuel proliferation and directly promote CAC initiation and growth (Grivennikov et al., 2009; Popivanova et al., 2008). Non-canonical NF-κB signaling has also been associated with the pathogenesis of CRC. Constitutive p105 pathway activation has been detected in colorectal carcinoma tissue samples (Lauscher et al., 2010). Interestingly, this has been linked to upregulation Wnt/β-catenin signaling activity, which is well-described in the pathogenesis of CRC (See **Section 1.3.4**). Genetic loss of *Nlrp12*, a negative regulator of non-canonical NF-κB activity, results in increased tumor number and size in the AOM/DSS model of CAC, which correlated with upregulated non-canonical NF-κB signaling activity (Allen et al., 2012). However, in alignment with the dual function of NF-κB in intestinal homeostasis and injury (as described in **Section 1.7.1** and **1.7.2**), conflicting results in studies analyzing the role of NF-κB in colonic tumorigenesis have also been described. Burkitt et al., 2015 revealed that *p52*-null mice display reduced polyp number in the AOM/DSS model of CAC, whereas *c-Rel*-deficient mice display increased polyp number, and *p50*-null mice displayed no change in tumor number relative to controls (Burkitt et al., 2015). Overall, there is evidence for a strong link between NF-κB signaling and colonic tumorigenesis that

is attributable to the pro-inflammatory, anti-apoptotic, and pro-proliferative functions of this signaling pathway.

1.8 Summary of Objectives

1.8.1 *General Overview & Hypothesis*

The overall scope of this thesis is to assess the role of two major pro-inflammatory signaling pathways (cyclooxygenase and NF-κB) in the pathogenesis of colitis-associated cancer. We have capitalized on our mouse model of CAC in which the cell-of-origin is mature Dclk1+ tuft cells. This has allowed us to further investigate the role of these signaling pathways in the dedifferentiation of tuft cells to initiate cancer in the setting of inflammation. I hypothesize that cyclooxygenase and NF-κB signaling pathways contribute to the pathogenesis of colitis-associated cancer by promoting the stemness of mature tuft cells during inflammation.

1.8.2 *Effect of COX-Inhibition on Colitis-Associated Cancer*

NSAIDs are known chemopreventative drugs against sporadic CRC. This is particularly true of Aspirin when given at a low (sub-anti-inflammatory) dose. In the context of IBD, however, patients are often advised to use caution with NSAIDs, as standard anti-inflammatory doses of these drugs are associated with GI toxicity and exacerbation of colitis (Allison et al., 1992; Evans et al., 1997; Meyer et al., 2006). Thus, the role of COX inhibition for the prevention of CAC has not been well studied. Westphalen and Asfaha et al., 2014 previously showed that Dclk1+ tuft cells can serve as a cell-of-origin of CAC, and this cell are known to express very high levels of both COX-1 and COX-2 enzymes (Westphalen et al., 2014). Thus, I hypothesized that low-dose NSAIDs are effective in the prevention of colitis-associated cancer through inhibition of COX-1 and/or COX-2 in tuft cells. In **Chapter 3**, two mouse models of CAC are utilized to test the effects of pharmacological and genetic inhibition of COX on the pathogenesis of CAC. Although

studies have demonstrated a chemopreventative effect of low-dose Aspirin against sporadic CRC, the exact mechanism by which this occurs is still unclear. Hence, in **Chapter 3** we further aim to determine the mechanism by which COX inhibition influences colitis-associated tumorigenesis. We have focused on PGE_2, which is the most upregulated prostaglandin in CRC (Rigas, 1993), and has been shown to promote stemness (Fan et al., 2014; Goessling et al., 2009; Jung et al., 2011; Terzuoli et al., 2017), as well as activate Wnt signaling (Castellone et al., 2005; Shao et al., 2005). Therefore, I hypothesized that PGE_2 promotes colitis-associated cancer by promoting the stemness of non-stem cells (such as the Dclk1+ tuft cell) to initiate tumorigenesis during intestinal injury.

1.8.3 *The Role of NF-κB in Colitis-Associated Cancer*

A key link between inflammation and cancer is the NF-κB signaling pathway. However, NF-κB signaling has been shown to play a dual role in intestinal homeostasis and inflammation due to its pro-inflammatory and anti-apoptotic functions (**Section 1.7**). Previous work has identified that constitutive activation of NF-κB signaling in intestinal epithelial cells leads to spontaneous inflammation and tumorigenesis (Vlantis et al., 2011), whereas inhibition of NF-κB in IECs prevents tumorigenesis in a mouse model of CAC (Greten et al., 2004). Therefore, I hypothesized that NF-κB signaling in Dclk1+ cells plays a key role in the pathogenesis of CAC. In **Chapter 4**, we aimed to test the effects of Dclk1+ cell-specific activation and inhibition of NF-κB signaling on CAC.

1.9 References

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Chapter 2

2 General Materials and Methods for Chapters 3 & 4

2.1 General Maintenance of Mice

All animal procedures were approved by the Animal Care and Use Committee at The University of Western Ontario in accordance with the Canadian Council of Animal Care. Experimental mice were housed in in 12hr-light/12hr-dark cycles with controlled temperature (18-24°C) and humidity (40-60%). Mice were housed with littermates of the same sex, up to a maximum of five mice per cage. All mice had access to standard laboratory chow and drinking water *ad libitum*. Health checks were performed daily by animal care staff and changes in animal health were reported using Sick Animal Response (SAR) protocols. Mice were euthanized if they reached a humane endpoint as indicated by algorithm or veterinarian assessment. Designated breeder pairs were generated by mating one male with 1-2 females per cage. Breeders were refreshed with their resulting offspring after approximately 5 months of breeding. Pups were weaned at 3 weeks of age, sexed, and ear-tagged for identification purposes. Tail samples were collected upon weaning for genotyping. Experimental animals were randomly assigned to control or treatment groups, with equivalent males and females in each group.

2.2 Transgenic Mouse Lines

All transgenic mouse models were generated on a C57Bl/6 background. *Dclk1*^{CreERT2} BAC transgenic mice were previously generated (Westphalen et al., 2014). Briefly, a BAC clone containing 50kb 5' sequence of the *Dclk1* gene-coding region and a *CreTM-FrtNeoFrt* cassette was inserted upstream of exon 2 in the *Dclk1* gene using BAC recombineering and homologous recombination, as previously reported (Westphalen et al., 2014).

As previously described, *Dclk1*^{CreERT2} mice were crossed with B6.129(Cg)-*Gt(ROSA)26Sor*$^{tm4(ACTBtdTomato,-EGFP)Luo}$/J (*Rosa26*^{mTmG}) (Jackson Laboratories, 007576) or B6;129S6-*Gt(ROSA)26Sor*$^{tm9(CAGtdTomato)Hze}$/J (*Rosa26*^{tdTomato}) (Jackson Laboratories, 007905) reporter strains in order to label Dclk1+ cells and their progeny upon tamoxifen administration (Westphalen et al., 2014). ROSA26 is a locus that allows for ubiquitous and constitutive expression of a particular transgene in mice (Soriano, 1999). *Dclk1*^{CreERT2}*;Rosa26*^{mTmG} *and Dclk1*^{CreERT2}*;Rosa26*^{tdTomato} mice were further crossed to B6:*Apctm2Rak* (*APC*$^{f/f}$) mice (Kuraguchi et al., 2006) from the National Cancer Institute (NCI) (Bethesda, MD) as previously reported (Westphalen et al., 2014). Resulting pups allow for a Dclk1+ cell-specific Cre recombinase-mediated loss of APC and simultaneous genetic labeling of Dclk1+ cells and any progeny with GFP or RFP expression, respectively upon tamoxifen treatment (*Dclk1*^{CreERT2}*;Rosa26*^{mTmG}*;APC*$^{f/f}$ and *Dclk1*^{CreERT2}*;Rosa26*^{tdTomato}*;APC*$^{f/f}$). See **Table 2.1**.

In **Chapter 3**, *Dclk1*^{CreERT2}*;Rosa26*^{tdTomato}*;APC*$^{f/f}$ mice were crossed to B6;129S4-*Ptgs1*$^{tm1.1Hahe}$/J (*COX-1*$^{f/f}$) mice (Jackson Laboratories, 030884) to generate *Dclk1*^{CreERT2}*;R26*^{tdTomato}*;APC*$^{f/f}$*;COX-1*$^{f/f}$ mice. Tamoxifen treatment allows for a Dclk1+ cell-specific loss of both APC and COX-1, while labeling Dclk1+ cells and their progeny with tdTomato (RFP) expression. Previously generated K19$^{Cre(BAC)}$ mice (Asfaha et al., 2015) were also crossed to B6;129S4-*Ptgs1*$^{tm1.1Hahe}$/J (*COX-1*$^{f/f}$) (Jackson Laboratories, 030884) to generate pups with constitutive loss of COX-1 in K19-expressing cells. In the intestine and colon, K19 labels all intestinal epithelial cells (Asfaha et al., 2015). See **Table 2.2**.

In **Chapter 4**, *Dclk1^{CreERT2};Rosa26^{tdTomato};APC$^{f/f}$* mice were crossed to IKKβ$^{f/f}$ mice (Park et al., 2002) to generate the following groups: control (*Dclk1^{CreERT2};R26^{tdTomato};APC$^{f/f}$; or IKKβ$^{+/+}$*), heterozygous for IKKβ$^{f/f}$ (*Dclk1^{CreERT2};R26^{tdTomato};APC$^{f/f}$;IKKβ$^{+/f}$; or IKKβ$^{+/f}$*), and homozygous for IKKβ$^{f/f}$ (*Dclk1^{CreERT2};R26^{tdTomato};APC$^{f/f}$;IKKβ$^{f/f}$; or IKKβ$^{f/f}$*). Tamoxifen treatment allows for a Dclk1+ cell-specific loss of both APC and IKKβ, while labeling Dclk1+ cells and their progeny with tdTomato (RFP) expression. *Dclk1^{CreERT2};APC$^{f/f}$* mice were also crossed to B6.Cg-Gt(ROSA)26Sor$^{tm4(Ikbkb)Rsky}$/J (*R26$^{IKK2ca\text{-}eGFP}$*) mice (Jackson Laboratories, 008242) to generate the following groups: control (*Dclk1^{CreERT2};APC$^{f/f}$; or IKK2$^{+/+}$*), heterozygous for IKK2ca (*Dclk1^{CreERT2};APC$^{f/f}$;R26IKK2ca-eGFP$^{+/f}$; or IKK2ca$^{+/mut}$*), and homozygous for IKK2ca (*Dclk1^{CreERT2};APC$^{f/f}$;R26IKK2ca-eGFP$^{f/f}$* or *IKK2ca$^{mut/mut}$*). *R26-IKK2ca-eGFP* mice express a FLAG-tagged constitutively active form of IKKβ (IKK2ca) and eGFP under control of the ROSA26 locus. However, this bicistronic sequence is preceded by a loxP-flanked STOP cassette that prevents downstream expression of IKK2ca and eGFP in the absence of active Cre-recombinase. Upon tamoxifen treatment, activation of Cre recombinase in Dclk1+ cells will remove this STOP cassette and induce expression of the constitutively active form of IKKβ and eGFP expression specifically in Dclk-expressing cells. See **Table 2.2**.

For mouse lines with tamoxifen-inducible Cre-recombinase, tamoxifen (MP Biomedicals, 02156738) was dissolved in corn oil and administered by oral gavage (3 doses of 6mg q.a.d.). Mouse genotypes were confirmed using PCR genotyping (further described in **Section 2.3**).

Table 2.1 – Mouse lines.

Mouse Line	Source	Description
C57Bl/6	Jackson Labs (000664)	Wild-type mice.
Dclk1^{CreERT2}	Previously generated (Westphalen et al., 2014)	Tamoxifen-inducible Cre recombinase specific to Dclk1+ cells.
APC$^{fl/fl}$	NCI (Kuraguchi et al., 2006)	Conditional allele of APC with LoxP sites flanking exon 14. Cre-mediated deletion of exon 14 results in a truncated and inactive APC protein.
Rosa26^{tdTomato}	Jackson Labs (007905)	Cre reporter with LoxP sites flanking a STOP cassette preventing transcription of red fluorescent protein (tdTomato) under control of the ubiquitous ROSA26 locus. Cre-mediated deletion of cassette results in tdTomato expression.
Rosa26^{mTmG}	Jackson Labs (007576)	Ubiquitous membrane tdTomato expression under control of the ROSA26 locus at baseline. Cre-mediated recombination results in replacement of tdTomato with membrane eGFP expression.
K19$^{Cre(BAC)}$	Previously generated (Asfaha et al., 2015)	Constitutive Cre-recombinase activity in K19+ cells (all intestinal epithelial cells).
IKKβ$^{fl/fl}$	Previously generated (Park et al., 2002)	Conditional allele of IKKβ with LoxP sites flanking exon 3 (encodes ATP binding site of the kinase domain).
COX-1$^{fl/fl}$	Jackson Labs (030884)	Conditional allele of COX-1 (*Ptgs1*) with LoxP sites flanking exons 3-5.
R26$^{IKK2ca\text{-}eGFP}$	Jackson Labs (008242) (originally generated by Sasaki et al., 2006)	Conditional allele containing a LoxP-flanked STOP cassette preventing transcription of constitutively active IKKβ (IKK2ca) and eGFP expression under control of the ubiquitous ROSA26 locus. Cre-mediated deletion of cassette results in IKK2ca and eGFP expression.

Table 2.2 – Genetic crosses of transgenic mouse lines.

Mouse Line Cross	Phenotype
Dclk1^{CreERT2};Rosa26^{mTmG}	Tamoxifen treatment results in replacement of RFP expression with GFP expression specifically in Dclk1+ cells and their progeny.
Dclk1^{CreERT2};APC$^{fl/fl}$	Tamoxifen treatment results in truncation (inactivation) of APC specifically in Dclk1+ cells and their progeny.
Dclk1^{CreERT2};APC$^{fl/fl}$; Rosa26^{tdTomato}	Tamoxifen treatment results in truncation (inactivation) of APC and tdTomato expression specifically in Dclk1+ cells and their progeny.
Dclk1^{CreERT2};APC$^{fl/fl}$; Rosa26^{mTmG}	Tamoxifen treatment results in truncation (inactivation) of APC and replacement of RFP expression with GFP expression specifically in Dclk1+ cells and their progeny.
K19$^{Cre(BAC)}$;COX-1$^{fl/fl}$	Constitutive COX-1 loss in K19-expressing cells (all intestinal epithelial cells).
Dclk1^{CreERT2};APC$^{fl/fl}$; Rosa26^{tdTomato};IKKβ$^{fl/fl}$	Tamoxifen treatment results in truncation (inactivation) of APC, tdTomato expression, and loss of IKKβ specifically in Dclk1+ cells and their progeny.
Dclk1^{CreERT2};APC$^{fl/fl}$; Rosa26^{tdTomato};COX-1$^{fl/fl}$	Tamoxifen treatment results in truncation (inactivation) of APC, tdTomato expression, and loss of COX-1 specifically in Dclk1+ cells and their progeny.
Dclk1^{CreERT2};APC$^{fl/f}$; R26$^{IKK2ca-eGFP}$	Tamoxifen treatment results in truncation (inactivation) of APC constitutive activation of IKKβ, and eGFP expression specifically in Dclk1+ cells.

2.3 Genotyping of Mice

Tail samples obtained at time of weaning (post-natal day 21) were used to determine mouse genotypes by polymerase chain reaction (PCR). Approximately 2-5mm of tail from each mouse was incubated in 75µL of Solution 1 (25mM NaOH/0.2 mM EDTA) for 1 hour at 95°C. Tails were cooled to 15°C before adding 75µL of Solution 2 (40mM Tris HCl, pH 5.5). Isolated tail DNA (1µL) was combined with the appropriate gene-specific primers, 2x Taq FroggaMix (FroggaBio, FBTAQM), and nuclease-free water to a total volume of 20µL before amplification using a thermocycler (Thermo Fisher). PCR conditions were dependent on primers used (**Table 2.3**). Amplified PCR products were run on a 2% agarose gel containing ethidium bromide (Sigma, E1510). Gels were visualized using a GelDoc UV Trans Illuminator (Bio-Rad).

Table 2.3 – Primer sequences for PCR genotyping of transgenic mouse lines.

Target	Primer Sequences (5' to 3')	Band Size (bp)	Annealing Temp. (°C)
Dclk1^{CreERT2}	F: TGACACCTTGAGAGGATGTGACTG R: GGATAGTTTTTACTGCCAGACCGC	550	57
APC$^{fl/fl}$	F: GAGAAACCCTGTCTCGAAAAAA R: AGTGCTGTTTCTATGAGTCAAC	WT 320 Mut 430	60
mTomato	F: CCCGGCTACTACTACGTGGA R: GCGGATGAACTCTTTGATGA	200	58
Rosa26^{mTmG}	F: CTCTGCTGCCTCCTGGCTTCT WT R: CGAGGCGGATCACAAGCAATA Mut R: TCAATGGGCGGGGGTCGTT	WT 330 Mut 250	61
Rosa26^{tdTomato}	WT F: AAGGGAGCTGCAGTGGAGTA WT R: CCGAAAATCTGTGGGAAGTC Mut F: GGCATTAAAGCAGCGTATCC Mut R: CTGTTCCTGTACGGCATGG	WT 297 Mut 196	61
K19$^{Cre(BAC)}$	F: TCTCCCTCCTCATCATGTCC R: CATGTTTAGCTGGCCCAAAT	320	55
IKKβ$^{fl/fl}$	F: GTCATTTCCACAGCCCTGTGA R: CCTTGTCCTATAGAAGCACAAC	WT 220 Mut 310	55
COX-1$^{fl/fl}$	F: GTTCACGTACCGCTGTCTCA WT R: AGGTCACCAGGAAATGGTCA Mut R: GCGCAACGCAATTAATGA	WT 288 Mut 168	55
R26$^{IKK2ca\text{-}eGFP}$	WT F: GAGCTGCAGTGGAGTAGGCG WT R: CCAGATGACTACCTATCCTC Mut F: AGGGCGAGCTCTGCACGGAA Mut R: ACGATGTCCACTTCGCTCTT	WT 350 Mut 375	55

Abbreviations: WT, wild-type; Mut, mutant; F, forward; R, reverse.

2.4 Disease Models

2.4.1 *DSS Model of Colitis*

To induce experimental colitis, 8-week-old mice were treated with 2.5% (wt/vol) DSS (molecular weight 36 000 – 50 000 kDa, Gojira Fine Chemicals, 9011181) in the drinking water for 5 days. Animals were monitored closely and scored based on clinical features of colitis and body weight loss (**Appendix 8**). Mice were analyzed acutely at 1 or 3 days post-DSS (day 6 or 8) or sub-acutely at 2 weeks post-DSS (day 19).

2.4.2 *AOM/DSS Model of CAC*

As previously described (Tanaka et al., 2003) (see **Section 1.4.4.1**), 6-week-old C57Bl/6J mice were treated with the carcinogen azoxymethane (10mg/kg, i.p.; Sigma, A5486) on day 0, followed by 5 days of 2.5% DSS in the drinking water starting on day 7 in order to induce tumorigenesis. Colonic and tumor tissues were harvested at week 20 for analysis (**Figure 2.1a**)

2.4.3 *Dclk1^{CreERT2};APC$^{f/f}$ Model of CAC*

As previously described (Westphalen et al., 2014), 6-week-old *Dclk1^{CreERT2};APC$^{f/f}$* mice (see **Section 1.4.4.2, Section 2.2**) were treated with tamoxifen (6mg; MP Biomedicals, 0215673894) on days 0, 2, and 4 to induce Cre recombinase activity and APC-loss in *Dclk1*-expressing cells. Mice were treated with 2.5% DSS in the drinking water for 5 days starting on day 14 to induce colonic tumorigenesis specifically from Dclk1-expressing cells. Colonic and tumor tissues were harvested at week 16 for analysis (**Figure 2.1b**).

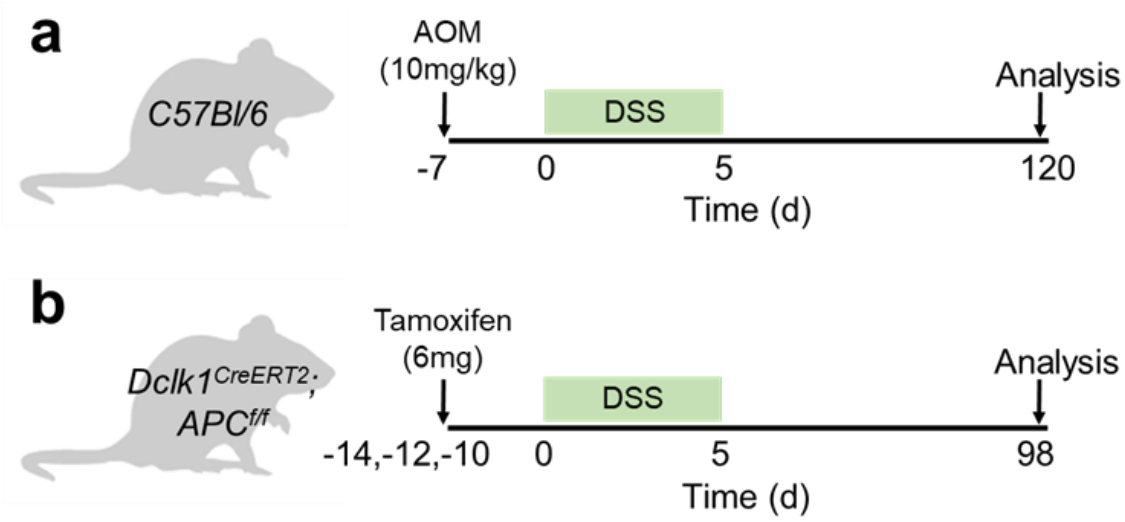

Figure 2.1 – Schematic of experimental timelines for the previously established mouse models of CAC.

(**a**) AOM/DSS mouse model: wild-type mice are administered a single dose of AOM (10mg/kg; i.p.), followed by DSS (dissolved in drinking water, *ad libitum*) for 5 days starting one week post-AOM. (**b**) *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mouse model: *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice are administered 3 doses of tamoxifen (6mg; p.o.) every other day, followed by DSS (dissolved in drinking water, *ad libitum*) for 5 days starting two weeks after the first dose of tamoxifen. Abbreviations: AOM, azoxymethane; DSS, dextran sodium sulfate.

2.5 Tissue Harvesting, Fixation, and Processing

2.5.1 *Tissue Harvesting*

Experimental animals were euthanized by CO_2 asphyxiation and cervical dislocation. Small intestinal and colonic tissues were flushed with cold PBS and opened longitudinally. Using the 'Swiss roll' technique (Moolenbeek and Ruitenberg, 1981), small intestinal and colonic tissues were rolled from the distal to proximal end onto the plastic portion of a cotton swab. Tissue rolls were secured in labeled histology cassettes using biopsy sponge pads. For tumor experiments, tumors were counted, measured, and collected in histology cassettes between filter paper and biopsy sponge pads. Tissue cassettes were immediately submersed in the appropriate fixative.

2.5.2 *Tissue Fixation & Processing*

2.5.2.1 Generation of frozen tissue blocks

Tissues were fixed in 4% paraformaldehyde (Carson's PFA, Electron Microscopy Sciences, 1573310) for 6-8 hours at 4°C in the dark and cryopreserved in 30% sucrose for at least 24 hours. Tissues were embedded in OCT (Sakura, 4583) on dry ice and stored at -80°C until further use. Frozen tissues were sectioned at 5µm using the Leica CM3050 cryostat (Leica Biosystems) onto glass slides and stored at -20°C.

2.5.2.2 Generation of formalin-fixed paraffin-embedded (FFPE) tissue blocks

Tissues were fixed in 10% formalin solution (Fisher, SF984) overnight at 4°C before being transferred to 70% ethanol. Tissues were dehydrated using increasing concentrations of ethanol, cleared using xylene, and infiltrated with paraffin either manually or using an automatic tissue processor. Tissues were then embedded in paraffin. FFPE blocks were cooled on ice before being sectioned at 5µm onto glass slides and stored at room temperature.

2.6 Histological Staining & Analysis

2.6.1 *Endogenous Fluorescence*

To analyze endogenous fluorescence from $R26^{tdTomato}$ or $R26^{mTmG}$ reporters, frozen tissue sections were rehydrated with PBS for 5 minutes at room temperature in the dark and mounted using Vectashield Mounting Medium containing DAPI (Vector Laboratories, H-2000). Slides were imaged using the EVOS FL Auto Imaging System (Thermo Fisher).

2.6.2 *Hematoxylin & Eosin (H&E) Staining*

For FFPE sections, tissues were deparaffinized in xylene (Fisher, X3P1GAL) and rehydrated in decreasing concentrations of ethanol. Sections were stained using CAT hematoxylin (Biocare Medical, BCCATHEM), Tacha's Bluing Solution (Biocare Medical, HTBLUMX), and Eosin Y (Sigma, 230251). For frozen sections, tissues were rehydrated in PBS for 5 minutes at room temperature, and stained using Mayer's Hematoxylin (Sigma, MHS32), Tacha's Bluing Solution (Biocare Medical, HTBLUMX), and Eosin Y (Sigma, 230251). Slides were subsequently dehydrated in increasing concentrations of ethanol, cleared in xylene, and mounted using Permount (Thermo Fisher Scientific, SP15). Slides were analyzed using the EVOS FL Auto Imaging System (Thermo Fisher). Histological damage scores were determined based on the percent damaged area which was calculated using the sum of manually determined areas with epithelial damage over the total epithelial area for each section.

2.6.3 *Immunofluorescence (IF) Staining*

For FFPE sections, tissues were deparaffinized in xylene (Fisher, X3P1GAL) and rehydrated in decreasing concentrations of ethanol. Heat-induced antigen retrieval was performed by microwaving slides in boiling Tris-EDTA buffer (pH 9.0) for 12 minutes on power 1. For frozen sections, tissues were rehydrated in PBS for 5 minutes at room temperature and antigen retrieval was performed by microwaving slides in boiling sodium

citrate buffer (pH 6.0) for 5 minutes. Tissues were permeabilized with 0.2% Triton-X (BioShop, TRX777) in PBS for 10 minutes and blocked using 10% normal goat serum (Vector Laboratories, S1000) for 30 minutes at room temperature. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. Secondary antibodies were diluted in blocking solution and incubated for 1 hour at room temperature in the dark. Slides were counterstained and mounted using Vectashield Mounting Medium containing DAPI (Vector Laboratories, H-2000). Tissue sections were imaged using the EVOS FL Auto Imaging System (Thermo Fisher). See **Table 2.4** for complete list of antibodies used.

2.6.4 *Immunohistochemical (IHC) Staining*

For FFPE sections, tissues were deparaffinized in xylene (Fisher, X3P1GAL) and rehydrated in decreasing concentrations of ethanol. Heat-induced antigen retrieval was performed by microwaving slides in boiling Tris-EDTA buffer (pH 9.0) for 12 minutes on power 1. Slides were rinsed in PBS and incubated in 3% hydrogen peroxide (BioShop, HYP001) in methanol to quench endogenous peroxidase activity. Slides were rinsed with PBS and blocked with 5% normal horse serum (Vector Laboratories, S2000). Primary antibodies were diluted in blocking solution and slides were incubated overnight at 4°C. Staining was performed using the R.T.U. Vectastain Universal Elite ABC Kit (Vector Laboratories, PK7200): biotinylated secondary antibody was applied for 1hr at room temperature followed by ABC reagent for 30 minutes. ImmPACT DAB Substrate (Vector Laboratories, SK4105) was used as the peroxidase substrate solution. Slides were counter-stained with CAT hematoxylin (Biocare Medical, BCCATHEM), rehydrated, cleared, and mounted using Permount (Thermo Fisher Scientific, SP15). Slides were imaged using the EVOS FL Auto Imaging System (Thermo Fisher). See **Table 2.4** for complete list of antibodies used.

Table 2.4 – Antibodies used in this study.

Antibody	Type	Concentration	Vendor	CAT No.
Anti-DCAMKL1	Rabbit polyclonal	1:200	Abcam	ab31704
Anti-Ki67	Rabbit monoclonal	1:200	Abcam	ab16667
Anti-RFP	Rabbit polyclonal	1:200	Rockland Inc	Ab124754
Anti-β-catenin	Mouse monoclonal	1:100	BD Biosciences	610154
Alexa Fluor 488 (Goat anti-rabbit)	Goat polyclonal	1:200	Thermo Fisher	A11008
Alexa Fluor 594 (Goat anti-rabbit)	Goal polyclonal	1:200	Thermo Fisher	A11012
Alexa Fluor 488 (Goat anti-mouse)	Goat polyclonal	1:200	Thermo Fisher	A11001
Alexa Fluor 594 (Goat anti-mouse)	Goat polyclonal	1:200	Thermo Fisher	A11005
Anti-phospho-Akt (Ser473)	Rabbit polyclonal	1:1000	Cell Signaling Technology	9271S
Anti-Akt	Rabbit polyclonal	1:1000	Cell Signaling Technology	9272S
Anti-β-Tubulin	Rabbit polyclonal	1:1000	Cell Signaling Technology	2146S

2.7 RNA Isolation & Real-time Quantitative PCR (qRT-PCR)

2.7.1 *RNA Isolation*

Colonic tissue was harvested and collected in Trizol (Invitrogen, 15596026). Tissues were snap frozen on dry ice if not to be used immediately. Tissue samples were homogenized in Trizol using the Bullet Blender 5 Storm (Next Advance) and 0.5mm RNase Free Stainless Steel Beads (Next Advance, SSB05-RNA). Tissue homogenate was subsequently used for RNA isolation according to the manufacturer's instructions for Trizol. Briefly, tissue homogenate was incubated with chloroform for phase separation, the RNA-containing upper aqueous phase was collected, and isopropanol was added for RNA precipitation. RNA was purified with lithium chloride as described previously, with some modifications (Viennois et al., 2013). Briefly, RNA was incubated with 8M LiCl (BioShop, LIT704) for 45 minutes at -20°C, centrifuged at 15,000 rpm for 15 minutes, washed with 75% ethanol, and resuspended in nuclease-free water. RNA concentration was determined using the NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific).

2.7.2 *Quantitative Real-Time PCR (qRT-PCR)*

RNA (1µg) isolated from colonic tissue was transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad, 1708890). RT-PCR was carried out in triplicate using PowerUp SYBR Green Master Mix (Thermo Fisher, A25741) and ViiA QuantStudio 5 (Thermo Fisher). Relative expression values were determined using the delta delta CT method and were normalized to the housekeeping gene *Gapdh*. See **Table 2.5** for complete list of primers used.

Table 2.5 – Primer sequences for gene expression analysis by qRT-PCR.

mRNA Target	Primer Sequences (Forward and Reverse, 5' to 3')
Gapdh	F: GACATCAAGAAGGTGGTGAAGCAG R: ATACCAGGAAATGAGCTTGACAAA
Cox-1	F: CACAACACTTCACCCACCAG R: AGAGCCGCAGGTGATACTGT
Cox-2	F: GCTGCCCGACACCTTCAACATT R: CACATTTCTTCCCCCAGCAACC
Lgr5	F: GACGCTGGGTTATTTCAAGTTCAA R: CAGCCAGCTACCAAATAGGTGCTC
Dclk1	F: AGCACTGCAGCAGGAGTTTCTG R: AGTCCTCCGATTCCGAGTTCAA
Il-1β	F: CAAGCAACGACAAAATACCTGTG R: AGACAAACCGTTTTTCCATCTTCT
Il-6	F: CCGGAGAGGAGACTTCACAGAG R: CTGCAAGTGCATCATCGTTGTT
Il-13	F: ATTGCATGGCCTCTGTAACC R: GTGGGCTACTTCGATTTTGG
Il-25	F: AGTGTCCGGCATGTACCAG R: CACGATCATTGCCAAGAATG
Tnf-α	F: TGGCCCAGACCCTCACACTCAG R: ACCCATCGGCTGGCACCACT
Ifn-γ	F: AGCAACAGCAAGGCGAAAAAG R: CGCTTCCTGAGGCTGGATTC
C-myc	F: CCCACCACCAGCAGCGACTC R: CAGTGGGCTGTGCGGAGGTT
Ccnd1	F: GCAGAAGGAGATTGTGCCATCC R: AGGAAGCGGTCCAGGTAGTTCA
Ppar-δ	F: ATGGAACAGCCACAGGAGGA R: ATCACAGCCCATCTGCAGCT

2.8 Myeloperoxidase (MPO) Activity Assay

Colonic MPO activity was measured as previously described (Kim et al., 2012; Krawisz et al., 1984). Briefly, fresh distal colonic tissue was harvested and weighed. Samples were homogenized in 0.5% hexadecyltrimethylammonium bromide (HTAB) (Sigma, H9151) in 50mM potassium phosphate buffer (pH 6.0). Homogenate was centrifuged at 13,400 rpm for 6 minutes at 4°C. Supernatant was collected and loaded in triplicate to a 96-well plate. 0.167mg/mL *o*-dianisidine (Sigma, D9143) was dissolved in 100mL of 50mM potassium phosphate buffer (pH 6.0). Hydrogen peroxide (0.0005%) was added to the *o*-dianisidine buffer and 200µL of this was loaded into each well containing sample. Immediately, absorbance was measured at 450nm using the Perkin Elmer Wallac 1420 Victor2 Microplate Reader (GMI). MPO activity was calculated as units of MPO activity/mg tissue, where one unit of MPO activity is equal to 1 µmol of peroxidase degraded per minute at 25°C. Remaining homogenate supernatant was stored at -80°C.

2.9 Intestinal Organoid Cultures

2.9.1 *Small Intestinal (Enteroid) Organoid Cultures*

Small intestinal crypt cultures were performed as previously described (Sato et al., 2009). Briefly, the small intestine was harvested, villi were scraped off, and tissue was washed 5 times with cold PBS. Tissues were incubated for one hour in 2.5mM EDTA in PBS at 4°C in a rotator. Tissue was transferred to cold 10% fetal bovine serum (FBS) in PBS and subjected to mechanical agitation to remove intestinal crypts from the mesenchyme. Intestinal crypts were subsequently passed through a 70µM filter (Fisher, 087712) and pelleted by centrifugation at 800 rpm for 5 minutes. Pelleted crypts were resuspended in conditioned media and centrifuged at 600 rpm for 5 minutes. Crypts were embedded in Matrigel (Corning, 356231) and plated on pre-warmed 48-well plates. After 15-30 minutes of polymerization in a 37°C incubator, 250µL of Dulbecco's Modified Eagle's Medium/F12 (DMEM) (Gibco, 21331020) containing N2 supplement (1x; Thermo Fisher

Scientific, 17502048), B27 supplement (1x; Thermo Fisher Scientific, 17502048), N-acetylcysteine (1µM; Sigma, A7250), Glutamax (1x; Thermo Fisher Scientific, 35050061), HEPES (10µM; Gibco, 15630106), and penicillin/streptomycin (500 µg/mL; Thermo Fisher Scientific, 15070063) was added to plated crypts and refreshed every 4 days. Media was supplemented with EGF (50 ng/ml; Thermo Fisher Scientific, PMG8041), mNoggin (100 ng/ml; Peprotech, 25038), and R-Spondin 1 µg/ml every 2 days. Organoids were kept in a 37°C incubator. Organoids were passaged after 10-14 days of culture. Briefly, media was removed and cold DMEM was used to collect the Matrigel and organoids into a 15mL Falcon tube on ice. A narrowed glass pipette was used to dissociate the organoids by pipetting up and down 10-15 times. The organoids were centrifuged at 600 rpm for 5 minutes and the resulting supernatant was discarded. Cold DMEM and Matrigel (1:1 ratio) was used to resuspend the organoids for re-seeding. Organoids were replated and incubated for 15-30 minutes to allow for Matrigel polymerization. Fresh conditioned media and growth factors were added. For experiments using organoids with tamoxifen-inducible Cre-recombinase, 4-hydroxytamoxifen (100nM; Sigma, H6278) was added to conditioned media for 48 hours. Organoids were imaged and analyzed using the EVOS FL Auto Imaging System (Thermo Fisher).

2.9.2 *Colonic Tumor Organoid Cultures*

Tumor organoid cultures were performed as previously described with some modifications (Xue and Shah, 2013). Briefly, colonic tumors were dissected and incubated with EDTA-chelation buffer for 60 minutes at 4°C to remove non-tumor intestinal epithelial cells. Tumor fragments were washed in cold PBS, and incubated with DMEM conditioned media containing 2.5% FBS, amphotericin B (2.5ng/mL; Sigma, PHR1662), type IV collagenase (200U/ml; Worthington, LS004186) and type II dispase (125µg/mL; Sigma, 42613332) for 90 minutes at 37°C to allow for tumor cells to separate from the mesenchyme. Supernatant containing tumor cells was collected and centrifuged at 200xg for 3 minutes, washed in cold PBS, and subsequently centrifuged at 200xg for 3 minutes. Tumor cells were embedded in Matrigel (Corning, 356231), and plated on pre-warmed 48-well plates. After polymerization, 250µL of Dulbecco's Modified Eagle's Medium/F12 (Gibco, 21331020)

containing N2 supplement (1x; Thermo Fisher Scientific, 17502048), B27 supplement (1x; Thermo Fisher Scientific, 17502048), N-acetylcysteine (1 µM; Sigma, A7250), Glutamax (1x; Thermo Fisher Scientific, 35050061), HEPES (10µM; Gibco, 15630106), penicillin/streptomycin (500 µg/mL; Thermo Fisher Scientific, 15070063), and amphotericin B (2.5ng/ml; Sigma, PHR1662) was added to plated cells and refreshed every 4 days. Media was supplemented with EGF (50 ng/ml; Thermo Fisher Scientific, PMG8041) and mNoggin (100 ng/ml; Peprotech, 25038) every 2 days. Organoids were imaged and analyzed using the EVOS FL Auto Imaging System (Thermo Fisher).

2.10 Statistics

All data are presented as mean ± SEM, unless indicated otherwise. Analysis was performed using GraphPad Prism, version 9.0 (GraphPad Software, Inc.). Unpaired Student's t-test was used when comparing 2 groups, one-way ANOVA was used when comparing 3 or more groups, and two-way ANOVA was used when comparing groups with 2 independent variables. Data was considered statistically significant when $p < 0.05$.

2.11 References

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Chapter 3

3 Low-dose Aspirin prevents colitis-associated cancer by inhibiting the stemness of tuft cells in a PGE_2 and Akt-dependent manner

Portions of text and figures from this chapter have been adapted from the following manuscript to conform to the format of this thesis:

Good HJ, Shin AE, Zhang L, Meriwether D, Worthley D, Reddy ST, Wang TC, Asfaha S. Prostaglandin E2 and Akt promote stemness of tuft cells to initiate inflammation-associated cancer. In preparation.

*Western Blots in Figure 3.9 were performed by LZ. LC-MS in Figure 3.7 and Figure 3.8 was performed by DM and the laboratory of STR.

3.1 Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer death in North America (Ferlay et al., 2019; Bray et al., 2018). A major risk factor for CRC is Inflammatory Bowel Disease (IBD), including Crohn's disease and ulcerative colitis, which is characterized by chronic colonic inflammation (Eaden et al., 2001; Ekbom et al., 1990; Kraus and Arber, 2009; Rutter et al., 2004; Shanahan, 2001). If colitis is uncontrolled, the risk of colitis-associated cancer (CAC) is estimated to be as high as 5-20% of patients with IBD (Eaden et al., 2001; Jess et al., 2006; Lakatos and Lakatos, 2008; Lutgens et al., 2013). Importantly, CAC is the most lethal complication for IBD patients and is typically characterized by an earlier age of onset, poorer prognosis, and advanced disease state when compared to sporadic CRC (Baars et al., 2012; Mutaguchi et al., 2019; Soh et al., 2019; Watanabe et al., 2011). Despite the clear link between inflammation and cancer, the mechanism by which colitis leads to cancer remains largely unknown. Thus, there remains a pressing need to elucidate the mechanism by which colitis leads to CAC and in doing so, identify chemopreventative strategies that reduce the risk of CAC.

In sporadic CRC, one cell-of-origin was previously shown to be Lgr5+ stem cells (Barker et al., 2007). Lgr5+ cells rapidly give rise to colonic tumors upon loss of *APC*, a tumor suppressor gene responsible for negative regulation of the Wnt signaling pathway (Barker et al., 2009). In the case of CAC, however, a cell-of-origin has not yet been defined. We previously examined the role of mature, long-lived, quiescent tuft cells that are found throughout the gastrointestinal epithelium and marked by expression of Dclk1. We reported that these tuft cells can give rise to colonic tumors upon APC-loss, but only following colitis (Westphalen et al., 2014). These data identified Dclk1+ tuft cells as a cell-of-origin for CAC. Interestingly, Dclk1+ cells are also known to express high levels of the enzymes cyclooxygenase (COX)-1 and -2 (Bezençon et al., 2008; Gerbe et al., 2011).

COX-1 and -2 enzymes catalyze the synthesis of prostaglandins from the precursor arachidonic acid, and in the colonic epithelium, COX-1 is constitutively expressed, whereas COX-2 expression is largely induced in inflammation or upon injury. COX has

also been implicated in the pathogenesis of CRC as it is aberrantly over-expressed in colorectal tumors and the non-neoplastic colonic mucosa of CRC patients (Eberhart et al., 1994; Jensen et al., 2018). Furthermore, non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit COX activity have been shown to be chemopreventative against sporadic CRC. In particular, Aspirin and some COX-2-selective inhibitors have been shown to prevent sporadic CRC in several epidemiological and animal studies (Bosetti et al., 2012; Chulada et al., 2000; Cole et al., 2009; Flossmann and Rothwell, 2007; Gupta and DuBois, 2001; Oshima et al., 1996; Peek, 2004; Phillips et al., 2002; Rothwell et al., 2010; Zhao et al., 2020).

However, the role of COX inhibition for the prevention of CAC has not been well-studied as, patients with IBD are often advised to use caution with NSAIDs (Allison et al., 1992; Evans et al., 1997; Meyer et al., 2006) This is particularly true of the COX-2 inhibitors that are associated with GI toxicity and exacerbation of colitis in humans and mice (Biancone et al., 2003; Bonner, 2001; Hegazi et al., 2003; Matuk et al., 2004; Meyer et al., 2006; Tsubouchi et al., 2006). Notably, in sporadic CRC, low-dose Aspirin has been shown to be as effective as high (i.e. anti-inflammatory) doses for the prevention of cancer (Baron et al., 2003; Cole et al., 2009; Flossmann and Rothwell, 2007; Rohwer et al., 2020; Rothwell et al., 2011). Thus, we hypothesized that low-dose NSAIDs may be safe to use in the setting of colitis and effective in inhibiting colitis-associated cancer through inhibition of COX-1 and/or COX-2 in tuft cells.

Here, we report the finding that low-dose Aspirin, but surprisingly, not COX-2 selective inhibitors, prevents colitis associated cancer. We further demonstrate that colitis is associated with increased PGE_2 and Akt signaling that act on quiescent Dclk1+ tuft cells to acquire stemness. We further describe that the inhibition of colitis-associated cancer by Aspirin is due to effects on COX-1, rather than COX-2- derived PGE_2, that subsequently acts in concert with Akt to promote tumorigenesis.

3.2 Materials & Methods

3.2.1 *Mouse Models*

Dclk1^{CreERT2} mice were previously generated and crossed to *APC*$^{flox/flox}$, *Rosa26-mTomato/mGFP* (*R26-mTmG*), and *Rosa26-tdTomato* strains as previously described (Westphalen et al., 2014). *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice were further crossed to COX-1$^{f/f}$ mice (JAX#030884). Tamoxifen was administered by oral gavage (3 doses of 6mg q.a.d.) to induce Cre recombinase activity in *Dclk1*-expressing cells. K19Cre (BAC) transgenic mice were previously generated (Asfaha et al., 2015) and crossed to COX-1$^{flox/flox}$ mice (Crescente et al., 2020) (JAX#030884). Mice were housed in 12hr-light/12hr-dark cycles with controlled temperature and humidity. All animal procedures were performed in accordance with the Animal Care and Use Committee at The University of Western Ontario. Further information on these mouse models is provided in **Section 2.2**.

3.2.2 *DSS Colitis Model & Drug Treatments*

To induce experimental colitis, 8-week-old mice were treated with 2.5% (wt/vol) DSS (molecular weight 36 000 – 50 000 kDa, Gojira) in the drinking water for 5 days. Mice were treated with NSAIDs 3 times during DSS and every day post-DSS until experimental endpoints. Mice were sacrificed 12-14 weeks post-DSS for tumor experiments, and 1 or 3 days post-DSS for acute experiments. NSAIDs used were: Aspirin (25mg/kg; Sigma), celecoxib (6mg/kg, 50mg/kg; Sigma), rofecoxib (5mg/kg, 15mg/kg; ApexBio), SC-560 (10mg/kg; Abcam), indomethacin (1mg/kg; Abcam), and respective vehicles. The drug doses were selected based on those that were previously reported to be equivalent to low and high-dose NSAIDs in patients based on plasma concentrations, calculated human equivalent doses, and/or equipotent COX inhibition (El-Medany et al., 2005; Laudanno et al., 2001; Nair and Jacob, 2016; Oshima et al., 2001; Rohwer et al., 2020; Smith et al., 1998). For lineage tracing studies, mice were treated with Misoprostol (15µg i.p., Cayman Chemicals) or SC79 (20mg/kg i.p., Cayman Chemicals) daily for 5 consecutive days.

3.2.3 *Myeloperoxidase (MPO) Assay*

Colonic MPO activity was measured as previously described (Kim et al., 2012; Krawisz et al., 1984). Briefly, fresh colonic tissue was harvested and weighed. Samples were homogenized in hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM potassium phosphate buffer, pH 6.0) and centrifuged at 13 400 rpm for 6 minutes at 4°C. Supernatant was collected and loaded in triplicate to a 96-well plate. *O*-dianisidine dihydrochloride solution (0.167mg/mL *o*-dianisidine in 50mM potassium phosphate buffer, pH 6.0) containing 0.0005% hydrogen peroxide was added to each well. Absorbance was measured at 450nm using the Perkin Elmer Wallac 1420 Victor2 Microplate Reader (GMI). MPO activity was calculated as units of MPO activity/mg tissue, where one unit of MPO activity is equal to 1 µmol of peroxidase degraded per minute at 25°C. See **Section 2.8** for further information.

3.2.4 *Quantitative RT-PCR (qRT-PCR)*

Total RNA was extracted from colonic tissue using TRIzol reagent (Invitrogen). RNA from DSS-treated mice was purified with lithium chloride as previously described, with some modifications (Viennois et al., 2013). Briefly, RNA was incubated with 8M LiCl for 45 minutes at -20°C, centrifuged at 15 000 rpm for 15 minutes, washed with 75% ethanol, and resuspended in nuclease-free water. RNA concentration was determined using the NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using 1µg RNA and was performed using iScript cDNA Synthesis Kit (Bio-Rad). RT-PCR was carried out in triplicate using PowerUp SYBR Green Master Mix (Thermo Fisher) and ViiA QuantStudio 5 (Thermo Fisher). See **Table 2.5** for complete list of primers used and **Section 2.7** for further information.

3.2.5 *Histology, Immunohistochemistry, & Immunofluorescence*

Tissues were collected, fixed in formalin, embedded in paraffin, and sectioned at 5µm thick onto glass slides. Tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. For histology, tissues were stained with CAT hematoxylin (Biocare Medical) and Eosin Y (Sigma) and were subsequently rehydrated and mounted using Permount (Thermo Fisher Scientific) for imaging. Percent damaged area was calculated based on the sum of areas with epithelial damage over total tissue area for each section. For immunohistochemistry and immunofluorescence, antigen retrieval was performed by boiling slides the microwave in Tris-EDTA buffer (pH 9.0) for FFPE sections and in sodium citrate buffer (pH 6.0) for frozen sections. For IHC, slides were incubated in 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Slides were rinsed with PBS and blocked with 5% normal horse serum. Primary antibodies were diluted in blocking solution and slides were incubated overnight at 4°C. Using R.T.U. Vectastain Universal Elite ABC Kit (Vector Laboratories), biotinylated secondary antibody was applied for 1hr at room temperature, followed by ABC reagent for 30min. ImmPACT DAB Substrate (Vector Laboratories) was used as the peroxidase substrate solution. Slides were counter-stained with CAT hematoxylin, rehydrated, and mounted using Permount. For immunofluorescence, slides were permeabilized in 0.2% Triton-X in PBS and blocked using 10% normal goat serum for 30 minutes at room temperature. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. Secondary antibodies were diluted in blocking solution and incubated for 1hr at room temperature. Slides were counterstained and mounted using Vectashield Mounting Medium containing DAPI (Vector Laboratories). See **Table 2.4** for complete list of antibodies used and refer to **Section 2.6** for further information.

3.2.6 *Organoid Culture Systems*

Small intestinal crypt organoids were cultured as previously described (Sato et al., 2009). Detailed protocol description can be found in **Section 2.9**. Organoid media was supplemented with EGF (50 ng/ml; Thermo Fisher Scientific), mNoggin (100 ng/ml; Peprotech), and R-Spondin 1 µg/ml every 2 days. For lineage tracing experiments, organoids were treated with 4-hydroxytamoxifen (100nM; Sigma) for 48 hours, washed, and treated with PGE_2 (10µM; Abcam) and/or SC79 (8µg/mL; Cayman Chemicals) in fresh media. For epithelial injury experiments, organoids were treated with doxorubicin (0.5 µg/mL; Sigma) dissolved in the media for 3 hours. Organoids were then washed 3 times with PBS before being treated with PGE_2 (10µM; Abcam) and/or SC79 (8µg/mL; Cayman Chemicals) dissolved in normal media. Colonic tumor organoids were cultured as previously described (Xue and Shah, 2013). Detailed protocol description can be found in **Section 2.9**. Tumor organoid media was supplemented with EGF (50 ng/ml; Thermo Fisher Scientific) and mNoggin (100 ng/ml; Peprotech) every 2 days. For experiments, tumor organoids were treated with NSAIDs (Aspirin: 0.5mM, 1mM, 5mM, or 10mM; SC-560: 5µM, 12.5µM, 25µM, 50µM, or 100µM; celecoxib: 12.5µM, 25µM; 50µM; rofecoxib: 12.5µM, 25µM, 50µM, 100µM) or PGE_2 (1µM) added to the media.

3.2.7 *AOM/DSS Model of Tumorigenesis*

As previously described (Tanaka et al., 2003), 6-week-old wild-type mice were treated with azoxymethane (10mg/kg, i.p.) on day 0, followed by 5 days of 2.5% DSS in the drinking water starting on day 7. Colons were harvested at week 20 for analysis. Further information is provided in **Section 2.4**.

3.2.8 *Multiplex Cytokine Array*

Colonic tissue was harvested and snap frozen on dry ice. Thawed tissue samples were homogenized in RIPA buffer with 0.5mM NaF and 0.1mM Na_3VO_4 using the Bullet Blender 5 Storm (Next Advance) and 0.5mm RNase Free Stainless Steel Beads (Next Advance, SSB05-RNA). Total protein concentration of homogenized tissue was determined using the DC Protein Assay (Bio-Rad). Levels of eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL-17E, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-28B, IL-31, IL-33, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, MIP-3a, RANTES, TNF-α, and VEGF were determined using a Multiplexing LASER Bead Assay (Eve Technologies Corporation).

3.2.9 *MRM LC-MS Inflammatory Lipid Signaling Panel*

Levels of eicosanoids were measured in colonic tissue using Multiple Reaction Monitoring (MRM) liquid chromatography-mass spectrometry (LC-MS) as previously described (Meriwether et al., 2019).

3.2.10 *Western Blot*

Western Blots were performed as previously described (Tamming et al., 2020) with some modifications. Briefly, 25-30mg of colonic tissue was collected and homogenized in 200µL standard RIPA buffer with 0.5mM NaF and 0.1mM Na_3VO_4 for protein isolation. Protein was quantified using a Bradford assay (Bio-Rad). 20µg protein lysates were loaded on 8% SDS-PAGE gel and transferred to nitrocellulose membrane (Bio-Rad) using a wet electroblotting system (Bio-Rad). The membrane was incubated using the manufacturer protocols for p-Akt (S473) (CAT# 9271S), Akt (CAT# 9272S), and beta-tubulin (CAT#2146S) antibodies (Cell Signaling Technologies). The membrane was rinsed briefly in enhanced chemiluminescence substrate (Bio-Rad) and imaged using the Bio-Rad ChemiDoc MP Image System.

3.2.11 *Imaging*

Imaging was performed using the EVOS FL Auto Imaging System (Thermo Fisher) or Nikon Confocal Microscope.

3.2.12 *Statistics*

Statistical analysis was performed using a 2-tailed Student's *t* test when comparing 2 groups, a one-way ANOVA when comparing 3 or more groups, or a two-way ANOVA when comparing groups with 2 independent variables. A P-value of less than 0.05 was considered statistically significant.

3.3 Results

3.3.1 *Low-dose Aspirin, but not COX-2 inhibitors, prevents colitis-associated cancer initiation.*

To examine the effects of low-dose Aspirin on colitis-associated cancer, we used two different mouse models. First, we crossed *Dclk1*^{CreERT2} mice to *APC*$^{f/f}$ mice and treated the resulting *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice with tamoxifen to induce APC-loss specifically in Dclk1-expressing tuft cells. We then administered dextran sodium sulfate (DSS) in the drinking water for 5 days to induce colitis. Concurrent with DSS, mice were treated with low-dose Aspirin (25mg/kg) three times during DSS and every day thereafter until the experimental endpoint at 16 weeks post-tamoxifen (**Figure 3.1a**). As the established mechanism of action of Aspirin is inhibition of both COX-1 and COX-2 (Vane and Botting, 2003), we further sought to determine whether the chemopreventative effect was due to COX-1 and/or COX-2 inhibition. To do this, we compared the effects of Aspirin to the COX-2-selective inhibitors celecoxib (6mg/kg) and rofecoxib (5mg/kg), the COX-1-selective inhibitor SC-560 (10mg/kg), and the non-selective COX-inhibitor indomethacin (1mg/kg) (**Figure 3.1a**). Treatment with Aspirin significantly reduced tumor number but had no effect on tumor size (**Figure 3.1b,c**). The COX-2 inhibitors, in contrast, had no effect on tumor number, while SC-560 and indomethacin both significantly reduced tumor number, suggesting that COX-1 inhibition was important for chemoprevention. Neither COX-2 inhibitor, SC-560, nor indomethacin had an effect on tumor size (**Figure 3.1c**). Importantly, we further tested the effects of higher (anti-inflammatory) doses of COX-2 inhibitors on tumorigenesis, which are known to have a chemopreventative effect in sporadic CRC. However, this resulted in mortality in 50% of mice and worsened histological damage (**Appendix 1a,b**), suggesting that high dose COX-2 inhibitors are not feasible for chemoprevention in colitis. In contrast, mice treated with Aspirin had 100% survival and no change in body weight when compared to the vehicle or other NSAID-treated groups (**Figure 3.1d**, **Appendix 1c**).

To validate the effects we observed with COX-1 and -2 inhibition, we further analyzed the effects of low-dose NSAIDs in the well-characterized AOM/DSS model of colitis-associated cancer (Tanaka et al., 2003). Wild-type mice were administered the carcinogen azoxymethane (AOM) (10mg/kg i.p.), followed by 2.5% DSS in the drinking water. NSAIDs were administered (daily, p.o) three times during DSS and every day thereafter until the experimental endpoint at 20 weeks post-AOM (**Figure 3.2a**). Analogous to the *Dclk1*^{CreERT2}*;APC*$^{f/f}$ model, Aspirin, SC-560, and indomethacin, but not celecoxib or rofecoxib, significantly reduced colonic tumor number in the AOM/DSS model (**Figure 3.2b**). Similarly, there was no difference in tumor size, survival, or body weight observed amongst any of the treatment groups (**Figure 3.2c**, **Appendix 1d**). These data suggest that inhibition of COX-1, as opposed to COX-2, is most likely responsible for inhibition of colitis-induced tumorigenesis.

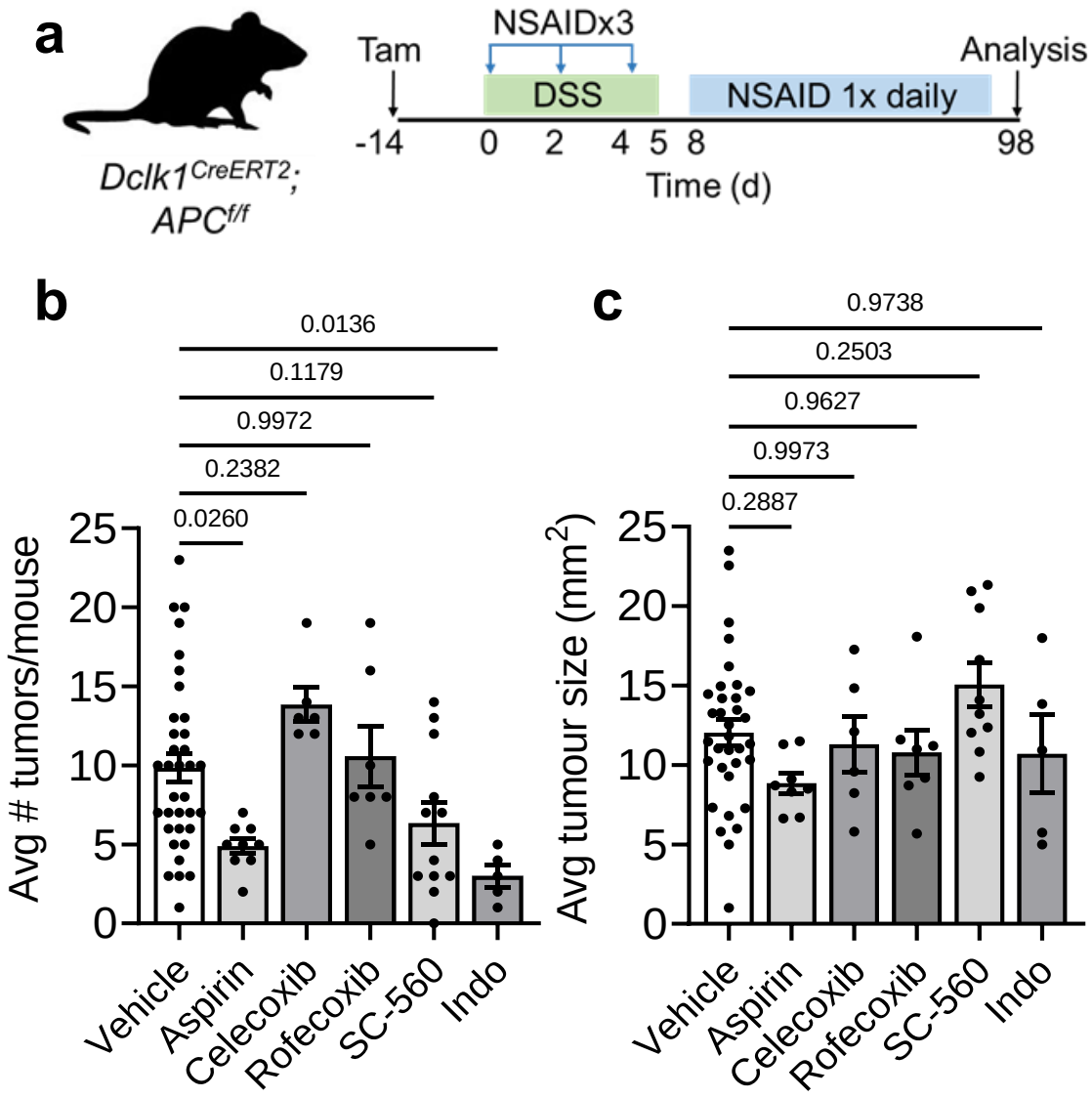

a
Dclk1CreERT2; APCf/f
Tam
NSAIDx3
DSS
NSAID 1x daily
Analysis
-14
0
2
4
5
8
98
Time (d)
b
Avg # tumors/mouse
0.0136
0.1179
0.9972
0.2382
0.0260
Vehicle
Aspirin
Celecoxib
Rofecoxib
SC-560
Indo
c
Avg tumour size (mm2)
0.9738
0.2503
0.9627
0.9973
0.2887

Figure 3.1 – Low-dose Aspirin, but not COX-2 inhibitors, prevents Dclk1+ cell-derived colitis-associated cancer initiation.

(**a**) Schematic illustration of the treatment of *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice with tamoxifen (3 doses q.a.d), 2.5% DSS for 5 days, and COX-inhibition by daily oral gavage of low-dose NSAIDs (Aspirin, 25mg/kg; celecoxib, 6mg/kg; rofecoxib, 5mg/kg; SC-560, 10mg/kg; indomethacin, 1mg/kg). (**b**) Average colonic tumor number of *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice treated with vehicle or NSAIDs (control, n = 35; Aspirin, n = 9; celecoxib, n = 6; rofecoxib, n = 7; SC-560, n = 12; indomethacin, n = 5). (**c**) Average colonic tumor size of *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice treated with vehicle or NSAIDs. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n = 34; Aspirin, n = 8; celecoxib, n = 6; rofecoxib, n = 7; SC-560, n = 10; indomethacin, n = 5). Statistical significance was determined by one-way ANOVA with Dunnett post-hoc test.

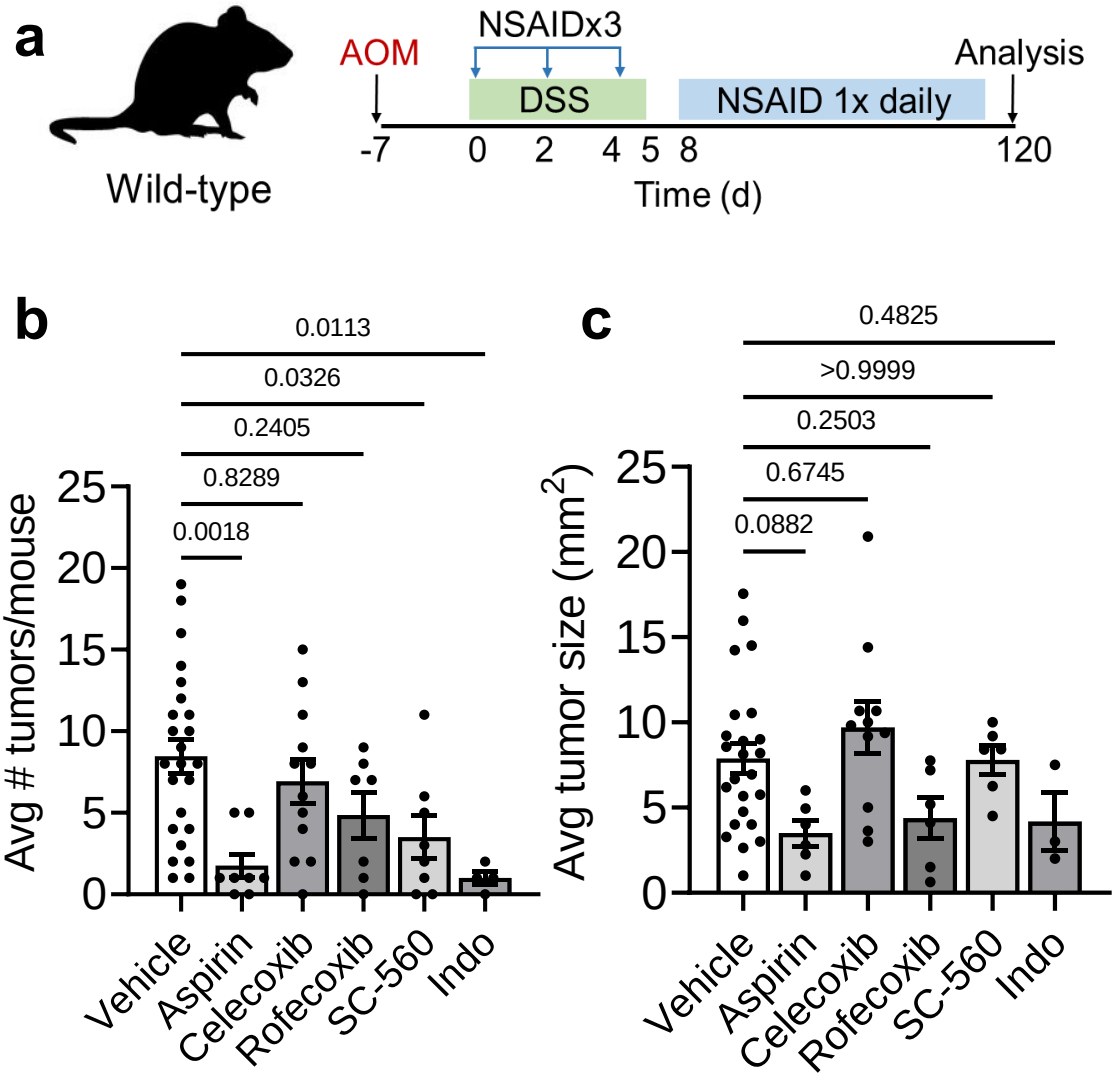

a
Wild-type
AOM
NSAIDx3
DSS
NSAID 1x daily
Analysis
-7
0
2
4
5
8
120
Time (d)
b
Avg # tumors/mouse
0.0113
0.0326
0.2405
0.8289
0.0018
Vehicle
Aspirin
Celecoxib
Rofecoxib
SC-560
Indo
c
Avg tumor size (mm2)
0.4825
>0.9999
0.2503
0.6745
0.0882

Figure 3.2 – Low-dose Aspirin, but not COX-2 inhibitors, prevents AOM/DSS-derived colitis-associated cancer initiation.

(**a**) Schematic illustration of the treatment of C57Bl/6 mice with AOM/DSS and COX-inhibition by daily oral gavage of low-dose NSAIDs (Aspirin, 25mg/kg; celecoxib, 6mg/kg; rofecoxib, 5mg/kg; SC-560, 10mg/kg; indomethacin (indo), 1mg/kg). (**b**) Average colonic tumor number of wild-type mice treated with vehicle or NSAIDs (control, n = 24; Aspirin, n = 8; celecoxib, n = 12; rofecoxib, n = 7; SC-560, n = 8; indomethacin, n = 4) (**c**) Average colonic tumor size of wild-type mice treated with vehicle or NSAIDs. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n = 24; Aspirin, n = 6; celecoxib, n = 11; rofecoxib, n = 6; SC-560, n = 6; indomethacin, n = 3). Statistical significance was determined by one-way ANOVA with Dunnett post-hoc test.

3.3.2 *Low-dose Aspirin prevents CAC through COX-1 inhibition.*

Our pharmacological studies using NSAIDs suggested that COX-1 may be the most important COX isoform for the initiation of CAC. To confirm that COX-1 inhibition prevents the initiation of CAC, we generated *K19$^{Cre(BAC)}$;COX-1$^{f/f}$* mice that have constitutive loss of COX-1 in all intestinal epithelial cells. *K19$^{Cre(BAC)}$;COX-1$^{f/f}$* mice treated with AOM/DSS had significantly reduced colonic tumor number compared to their Cre-negative littermates (**Figure 3.3a**). No change in tumor size was observed between groups (**Figure 3.3b**). These data support our observations that low-dose Aspirin prevents the initiation of CAC through COX-1 inhibition and highlights the importance of IEC-specific COX-1 in the initiation of CAC.

To investigate whether the epithelial source of COX-1 contributing to tumorigenesis was the Dclk1+ tuft cell, we assessed whether COX-1 in Dclk1+ cells was required for CAC initiation. To do this, we crossed *Dclk1^{CreERT2};APC$^{f/f}$* mice to *COX-1$^{f/f}$* mice to allow for a tamoxifen-inducible loss of both APC and COX-1 specifically in Dclk1-expressing cells. To specifically assess the effects of COX-1 loss in the tuft cell on tumor initiation, we analyzed these mice acutely at day 21 after tamoxifen and DSS treatment (**Figure 3.4a**). *COX-1$^{f/f}$* mice showed a significant reduction in the number of Dclk1+ lineage traced dysplastic lesions as compared to *COX-1$^{+/+}$* mice (**Figure 3.4b**), indicating that COX-1 specifically in Dclk1+ cells is important for the initiation of CAC.

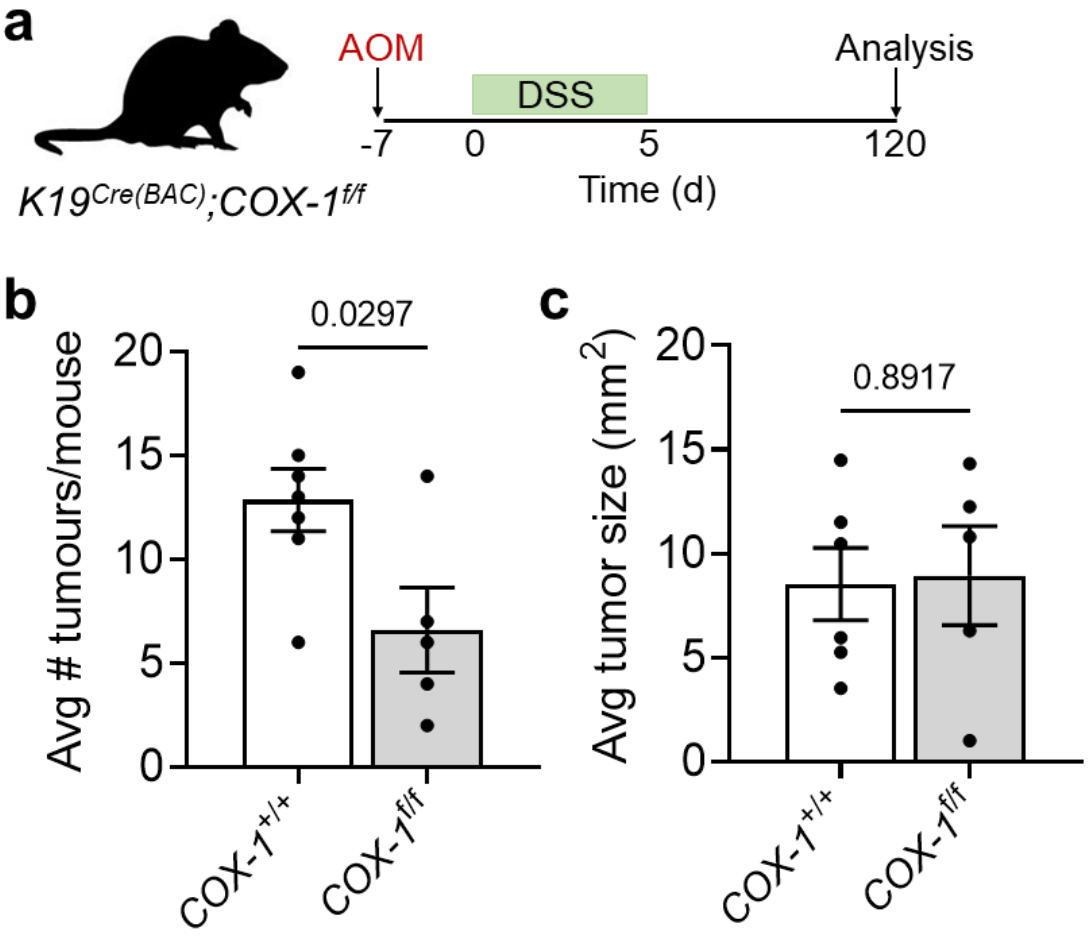

a
AOM
DSS
Analysis
-7
0
5
120
Time (d)
K19Cre(BAC);COX-1f/f
b
Avg # tumours/mouse
0.0297
20
15
10
5
0
COX-1+/+
COX-1f/f
c
Avg tumor size (mm2)
0.8917
20
15
10
5
0
COX-1+/+
COX-1f/f

Figure 3.3 – COX-1 loss in intestinal epithelial cells prevents AOM/DSS-derived colitis-associated cancer.

(**a**) Schematic illustration of the treatment of *K19$^{Cre(BAC)}$;COX-1$^{f/f}$* mice with AOM/DSS. (**b**) Colonic tumor number of *K19$^{Cre(BAC)}$;COX-1$^{f/f}$* mice relative to Cre-negative (*COX-1$^{+/+}$*) littermates (*COX-1$^{+/+}$*, n = 7; *COX-1$^{f/f}$*, n=5). (**c**) Colonic tumor size of *K19$^{Cre(BAC)}$;COX-1$^{f/f}$* mice relative to Cre-negative (*COX-1$^{+/+}$*) littermates. Data are presented as mean ± SEM and dots represent biologically independent animals (*COX-1$^{+/+}$*, n = 6; *COX-1$^{f/f}$*, n=5). Statistical significance was determined using unpaired Student's t-test.

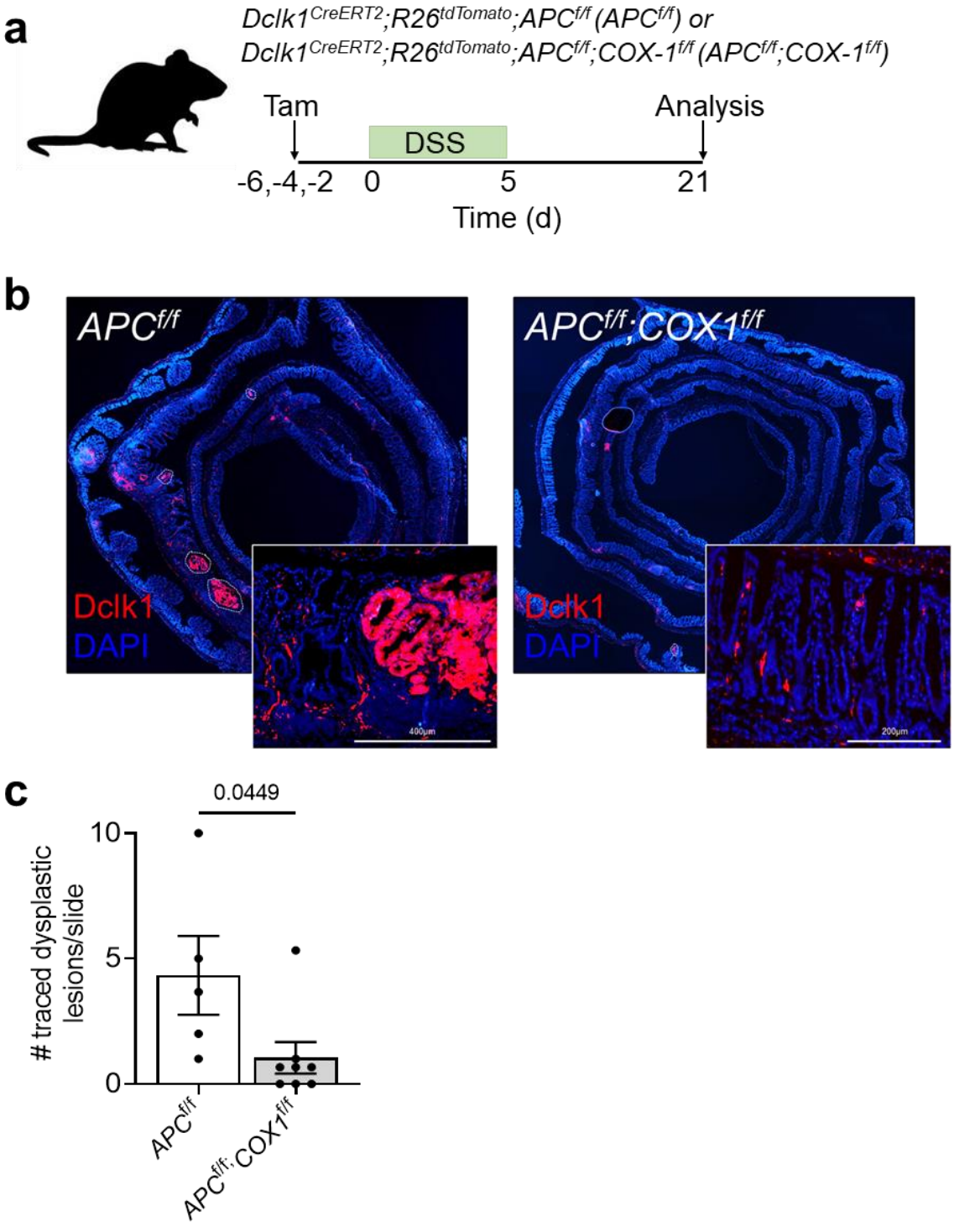

a
Dclk1CreERT2;R26tdTomato;APCf/f (APCf/f) or
Dclk1CreERT2;R26tdTomato;APCf/f;COX-1f/f (APCf/f;COX-1f/f)
Tam
Analysis
DSS
-6,-4,-2
0
5
21
Time (d)
b
APCf/f
Dclk1
DAPI
APCf/f;COX1f/f
Dclk1
DAPI
400μm
200μm
c
0.0449
traced dysplastic lesions/slide
10
5
0
APCf/f
APCf/f;COX1f/f

Figure 3.4 – COX-1 loss in Dclk1+ cells prevents the initiation of Dclk1+ cell-derived dysplastic lesions.

(**a**) Schematic illustration for the treatment of *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26*^{tdTomato} (*APC*$^{f/f}$) or *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26*^{tdTomato}*;COX-1*$^{f/f}$ (*APC*$^{f/f}$*;COX-1*$^{f/f}$) mice with tamoxifen (3 doses q.a.d.) and 1.5% DSS for 5 days. (**b**) Representative images of colonic tissue sections with tdTomato-traced Dclk1+ cell-derived dysplastic lesions (dotted white outlines). (**c**) Quantification of the number of tdTomato-traced dysplastic lesions for *APC*$^{f/f}$ and *APC*$^{f/f}$*;COX-1*$^{f/f}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (*APC*$^{f/f}$, n=5; *APC*$^{f/f}$*;COX-1*$^{f/f}$, n=8). Statistical significance was determined using unpaired Student's t-test. Scale bars = 200μm or 400μm, as indicated.

3.3.3 *Low-dose Aspirin does not exacerbate colitis severity.*

Given the risk of colitis-associated cancer correlates with the severity and duration of inflammation (Rutter et al., 2004), and to assess the safety of low-dose NSAID use during colitis, we next examined whether low-dose Aspirin alters the severity of DSS-colitis during the peak of inflammation (**Figure 3.5a**). Three days post-DSS, Aspirin-treated mice had no significant change in histologic damage, cell proliferation, myeloperoxidase activity, or levels of colonic inflammatory cytokines as compared to vehicle-treated controls (**Figure 3.5b-h**). Similarly, Aspirin had no significant effect on histologic damage or myeloperoxidase activity during the regenerative phase of DSS-colitis at day 19 (**Figure 3.6a-d**). Importantly, mice treated with both DSS and Aspirin showed significantly reduced weight loss at the peak of inflammation as compared to mice treated with DSS alone (**Figure 3.6e**). Taken together, these data suggest that low-dose Aspirin is safe to use in the setting of colitis for the prevention of CAC.

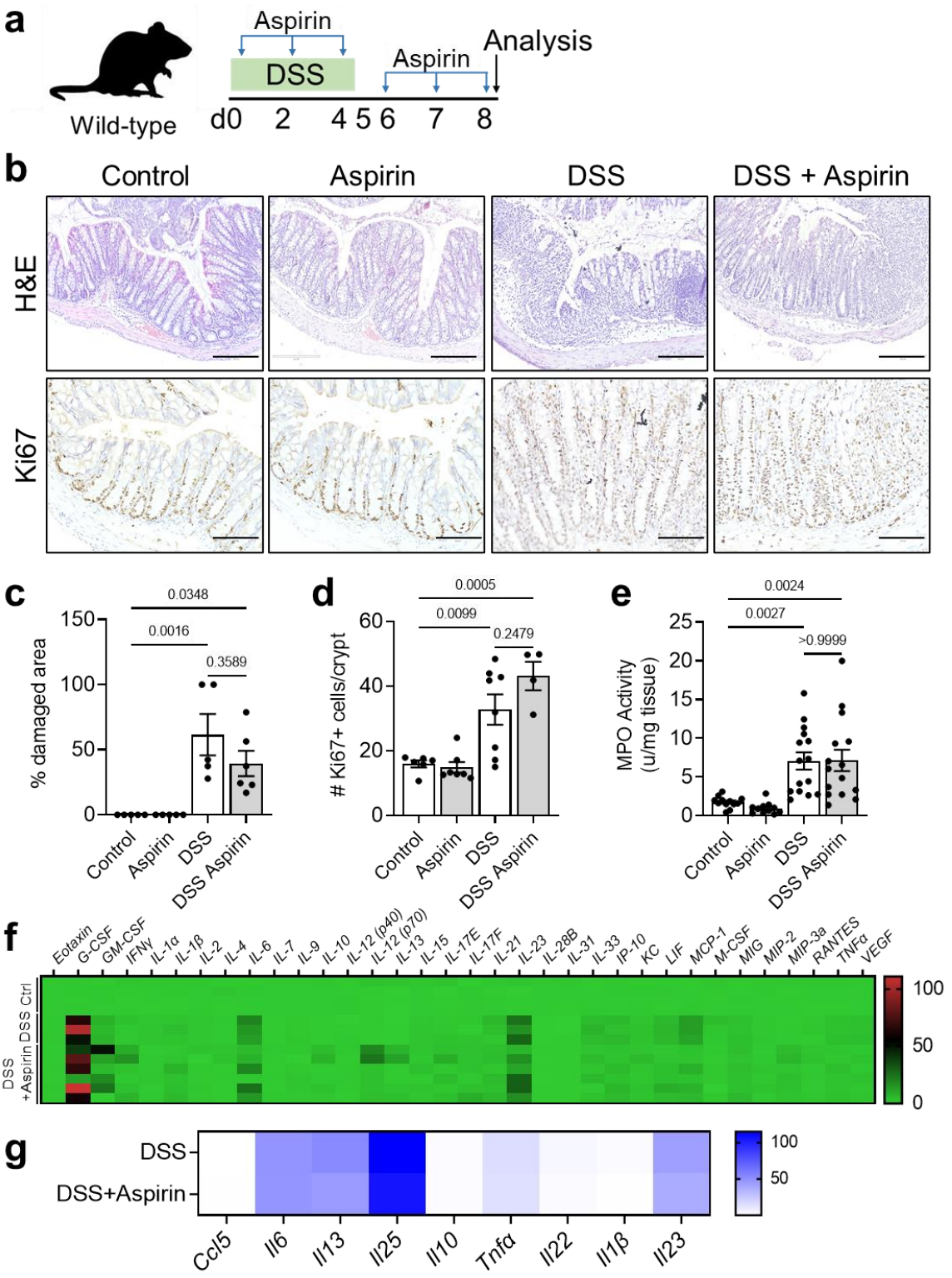

a
Aspirin
DSS
Aspirin
Analysis
Wild-type
d0 2 4 5 6 7 8
b
Control
Aspirin
DSS
DSS + Aspirin
H&E
Ki67
c
% damaged area
0.0348
0.0016
0.3589
d
Ki67+ cells/crypt
0.0005
0.0099
0.2479
e
MPO Activity (u/mg tissue)
0.0024
0.0027
>0.9999
Control
Aspirin
DSS
DSS Aspirin
f
Eotaxin
G-CSF
GM-CSF
IFNγ
IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-7
IL-9
IL-10
IL-12 (p40)
IL-12 (p70)
IL-13
IL-15
IL-17E
IL-17F
IL-21
IL-23
IL-28B
IL-31
IL-33
IP-10
KC
LIF
MCP-1
M-CSF
MIG
MIP-2
MIP-3a
RANTES
TNFα
VEGF
Ctrl
DSS
DSS +Aspirin
g
DSS
DSS+Aspirin
Ccl5
Il6
Il13
Il25
Il10
Tnfα
Il22
Il1β
Il23

Figure 3.5 – Low-dose Aspirin does not exacerbate the degree of acute DSS-colitis.

(**a**) Schematic illustration of the DSS colitis model with low-dose Aspirin treatment. C57Bl/6 mice were treated with Aspirin during and post-DSS and analyzed on day 8. (**b**) Representative images of hematoxylin and eosin staining (top; scale bars = 200μm) and Ki67+ cell staining (bottom; scale bars = 100μm) for mice treated with DSS and/or Aspirin at day 8. (**c**) Quantification of the percentage of damaged histological area in mice treated with DSS and/or Aspirin. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=5; Aspirin, n=5; DSS, n=5; DSS+Aspirin, n=6). (**d**) Quantification of the number of Ki67+ cells per colonic crypt (right) in mice treated with DSS and/or Aspirin. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=6; Aspirin, n=7; DSS, n=8; DSS+Aspirin, n=4). (**e**) Measurement of myeloperoxidase (MPO) activity in colonic tissue of mice treated with DSS and/or Aspirin. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=12; Aspirin, n=11; DSS, n=15; DSS+Aspirin, n=15). (**f**) Heatmap showing the relative protein levels of inflammatory cytokines and chemokines in colonic tissue of mice treated with DSS or DSS+Aspirin compared to control. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=5; DSS, n=3; DSS+Aspirin, n=6). (**g**) Heatmap showing the relative mRNA levels of inflammatory cytokines by qPCR in colonic tissue of mice treated with DSS or DSS+Aspirin as compared to control (control, n=5; DSS, n=4-6; DSS+Aspirin, n=4-6). Statistical significance was determined by one-way ANOVA with Tukey post-hoc test.

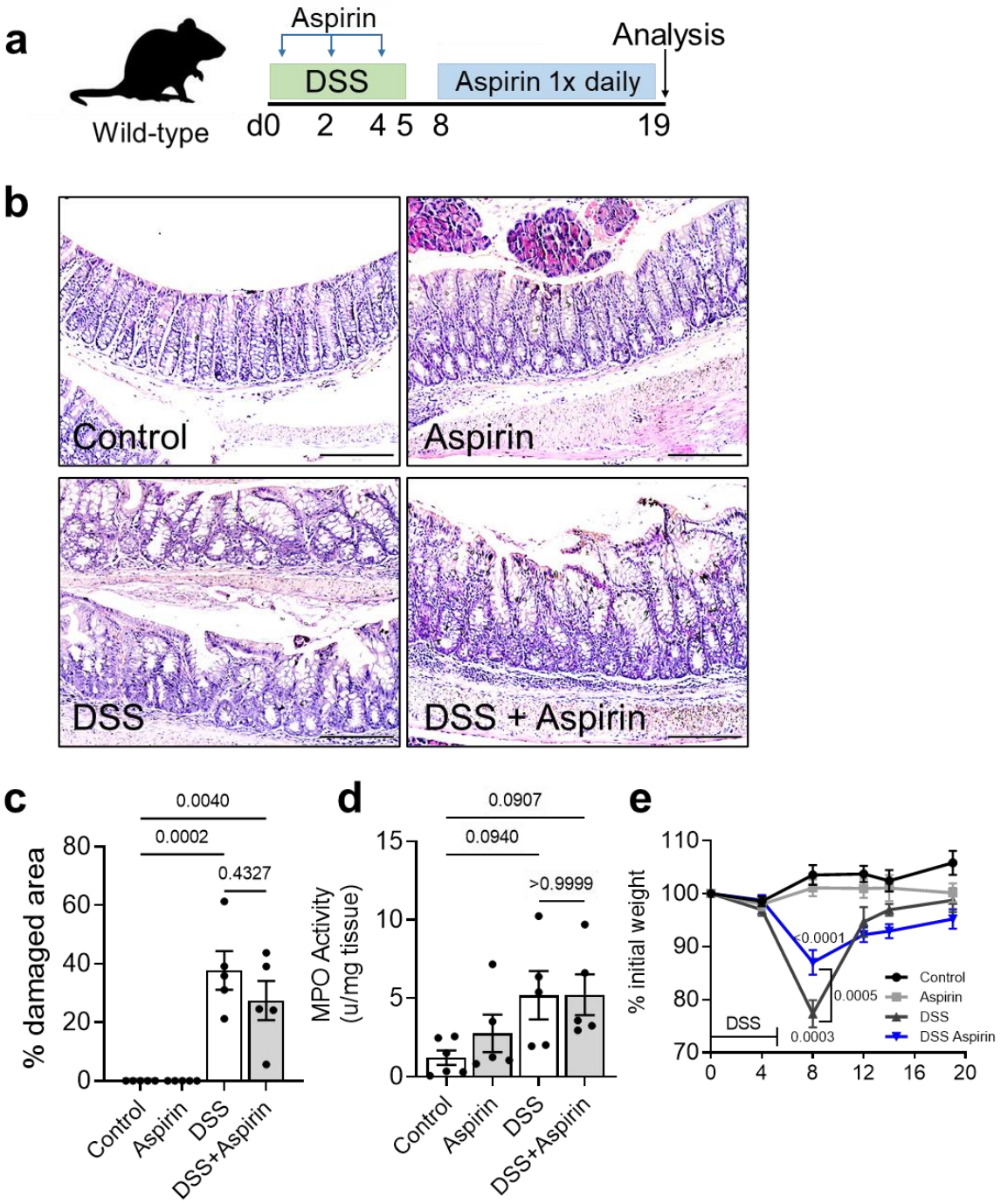

a
Aspirin
Analysis
DSS
Aspirin 1x daily
Wild-type
d0 2 4 5 8 19
b
Control
Aspirin
DSS
DSS + Aspirin
c
0.0040
0.0002
0.4327
% damaged area
Control
Aspirin
DSS
DSS+Aspirin
d
0.0907
0.0940
>0.9999
MPO Activity (u/mg tissue)
Control
Aspirin
DSS
DSS+Aspirin
e
% initial weight
<0.0001
0.0005
0.0003
DSS
Control
Aspirin
DSS
DSS Aspirin

Figure 3.6 – Low-dose Aspirin does not impair colonic regeneration after DSS-colitis.

(**a**) Schematic illustration of the DSS colitis model with low-dose Aspirin treatment. C57Bl/6 mice were treated with Aspirin during and post-DSS and analyzed on day 19. (**b**) Representative images of hematoxylin and eosin staining of colonic tissue of mice treated with DSS and/or Aspirin at day 19. (**c**) Quantification of the percentage of damaged histological area in mice treated with DSS and/or Aspirin. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=5; Aspirin, n=5; DSS, n=5; DSS+Aspirin, n=5). (**d**) Measurement of myeloperoxidase (MPO) activity in colonic tissue of mice treated with DSS and/or Aspirin. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=6; Aspirin, n=5; DSS, n=5; DSS+Aspirin, n=5). (**e**) Body weight changes for mice treated with vehicle, Aspirin, DSS, or DSS+Aspirin (control, n=5; Aspirin, n=5; DSS, n=5; DSS+Aspirin, n=5). Statistical significance was determined by one-way ANOVA (for panel c,d) or two-way ANOVA (for panel e) with Tukey post-hoc tests. Scale bars = 200µm.

3.3.4 *COX and Akt signaling pathways are upregulated in active inflammation.*

To examine the mechanism by which NSAIDs inhibit colitis-associated tumorigenesis, we focused on the role of COX and COX-derived prostaglandins in the acute phase of colitis. Correlating with the peak of inflammation, we detected an increase in COX-1 and COX-2 mRNA expression in the colonic tissue of mice with DSS-colitis (**Figure 3.7a,b**). To confirm that increased COX expression correlated with increased COX activity, we further assessed the levels of prostaglandins in DSS by liquid chromatography-mass spectrometry (LC-MS). Our data revealed that COX-derived lipid mediators were significantly increased in the colonic tissue of mice with DSS-colitis relative to untreated controls (**Figure 3.7c**). To next determine the role for COX-1 and/or -2 in prostaglandin synthesis during colitis, we assessed the effect of various NSAIDs on prostaglandin levels during DSS-colitis. Aspirin and SC-560 both reduced prostaglandin levels back to control (non-colitis) levels, whereas prostaglandins were only moderately affected by celecoxib treatment (**Figure 3.8a,b**). As the proportion of prostaglandins downregulated by NSAID treatment was largely due to inhibition of COX-1 (**Figure 3.8c**), this suggests that COX-1 is the predominant source of colonic prostaglandins in DSS-colitis. Given that both Aspirin and SC-560 inhibited colonic tumor formation and celecoxib did not, we further sought to identify which prostaglandins were differentially downregulated amongst the NSAIDs during colitis. We found that nine prostaglandins were differentially downregulated by Aspirin and SC-560 compared to celecoxib (**Figure 3.8d**). Of these, we focused on PGE_2, which has been previously shown to promote colonic tumorigenesis (Castellone et al., 2005; Mutoh et al., 2002; Wang et al., 2004) and is the most upregulated prostaglandin in human CRC (Rigas, 1993). As shown by our LC-MS data, PGE_2 levels were upregulated in colonic tissues of DSS-treated mice compared to controls, while the PGE_2 metabolites, 15-keto PGE_2 and 13,14-dihydro-15k-PGE_2, were unchanged or downregulated, respectively (**Figure 3.7c**). These data suggest that during inflammation, PGE_2 levels are increased while the metabolism of PGE_2 is reduced, allowing for a prolonged duration of activity.

PGE_2 has been shown to activate the cancer-related Akt pathway by increasing levels of phospho-Akt (Castellone et al., 2005; Peng et al., 2017; Wang et al., 2004). Therefore, we assessed whether Akt activation was also upregulated during DSS-colitis (**Figure 3.9a**). In alignment with previous studies, we detected increased levels of phospho-Akt in DSS-treated mice, indicating upregulated activity of the Akt pathway in colitis. However, in the setting of COX inhibition with Aspirin, DSS did not lead to upregulated phospho-Akt levels (**Figure 3.9b,c**), suggesting that COX activity may contribute to the activation of Akt signaling during inflammation. Using publicly available data we additionally confirmed that patients with active ulcerative colitis also had significantly increased expression of *COX-1*, *COX-2*, *PTGES* and *AKT3* relative to both control patients and patients with inactive UC (**Figure 3.10**). Taken together, these data suggest that the activity and/or expression of Cox and Akt signaling pathways are upregulated during active colitis.

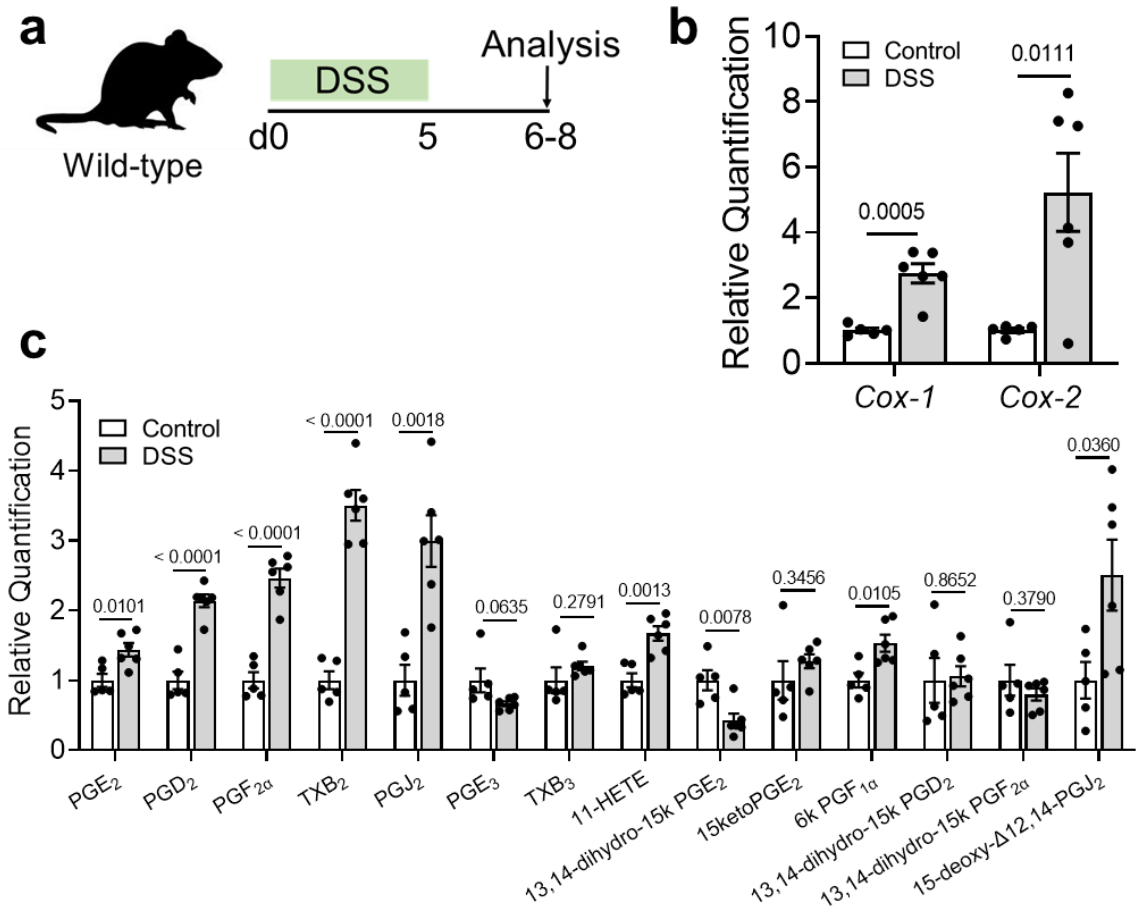


Figure 3.7 – COX expression and prostaglandin levels are upregulated in DSS-colitis.

(**a**) Schematic illustration of the DSS-colitis model. C57Bl/6 mice were administered 2.5% DSS in the drinking water for 5 days and analyzed on day 8. (**b**) Relative mRNA expression of *Cox-1* and *Cox-2* in DSS-treated mice as analyzed by qPCR. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=5; DSS, n=6). (**c**) Relative levels of COX-derived prostaglandins in DSS-treated mice as analyzed by LC-MS. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=5; DSS, n=6). Statistical significance was determined using unpaired Student's t-test.

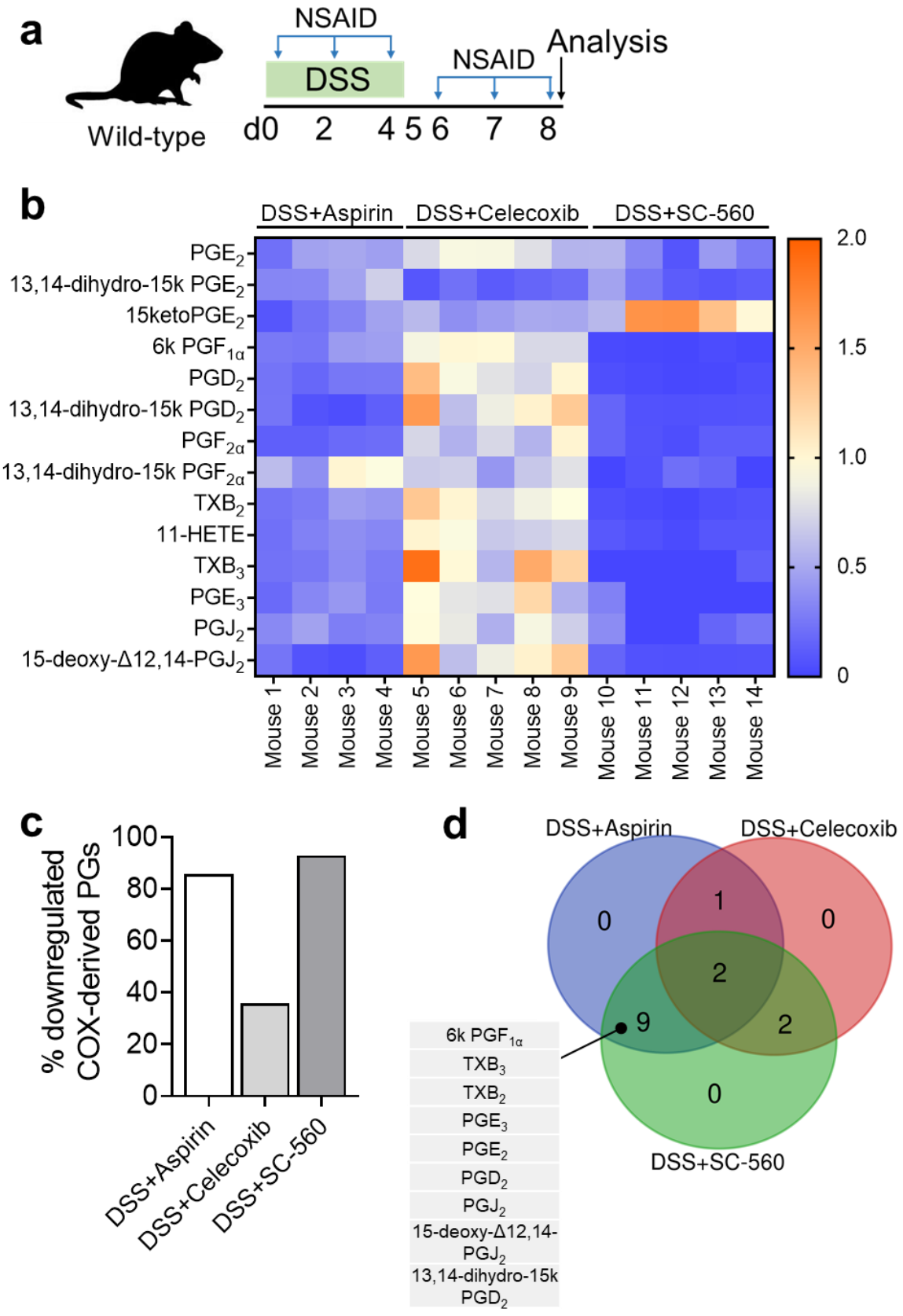

a
NSAID
DSS
NSAID
Analysis
Wild-type
d0 2 4 5 6 7 8
b
DSS+Aspirin
DSS+Celecoxib
DSS+SC-560
PGE2
13,14-dihydro-15k PGE2
15ketoPGE2
6k PGF1α
PGD2
13,14-dihydro-15k PGD2
PGF2α
13,14-dihydro-15k PGF2α
TXB2
11-HETE
TXB3
PGE3
PGJ2
15-deoxy-Δ12,14-PGJ2
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6
Mouse 7
Mouse 8
Mouse 9
Mouse 10
Mouse 11
Mouse 12
Mouse 13
Mouse 14
2.0
1.5
1.0
0.5
0
c
% downregulated COX-derived PGs
100
80
60
40
20
0
DSS+Aspirin
DSS+Celecoxib
DSS+SC-560
d
DSS+Aspirin
DSS+Celecoxib
DSS+SC-560
0
1
0
2
9
2
0
6k PGF1α
TXB3
TXB2
PGE3
PGE2
PGD2
PGJ2
15-deoxy-Δ12,14-PGJ2
13,14-dihydro-15k PGD2

Figure 3.8 – COX-1 inhibition downregulates prostaglandins during DSS-colitis.

(**a**) Schematic illustration of the treatment of C57Bl/6 mice with 2.5% DSS and COX-inhibition by NSAIDs. (**b**) Heatmap showing the relative levels of COX-derived prostaglandins in DSS-colitis upon treatment with Aspirin (non-selective COX inhibitor; n=4), celecoxib (COX-2 inhibitor; n=5), or SC-560 (COX-1 inhibitor; n=5). (**c**) Quantification of the percentage of prostaglandins significantly downregulated upon treatment with each NSAID during DSS-colitis relative to DSS+vehicle-treated controls. (**d**) Venn diagram showing the absolute number of prostaglandins downregulated during DSS-colitis upon treatment with each NSAID. Identification of prostaglandins downregulated upon treatment with Aspirin and SC-560, but not celecoxib, during DSS relative to DSS alone. Statistical significance was determined using unpaired Student's t-test.

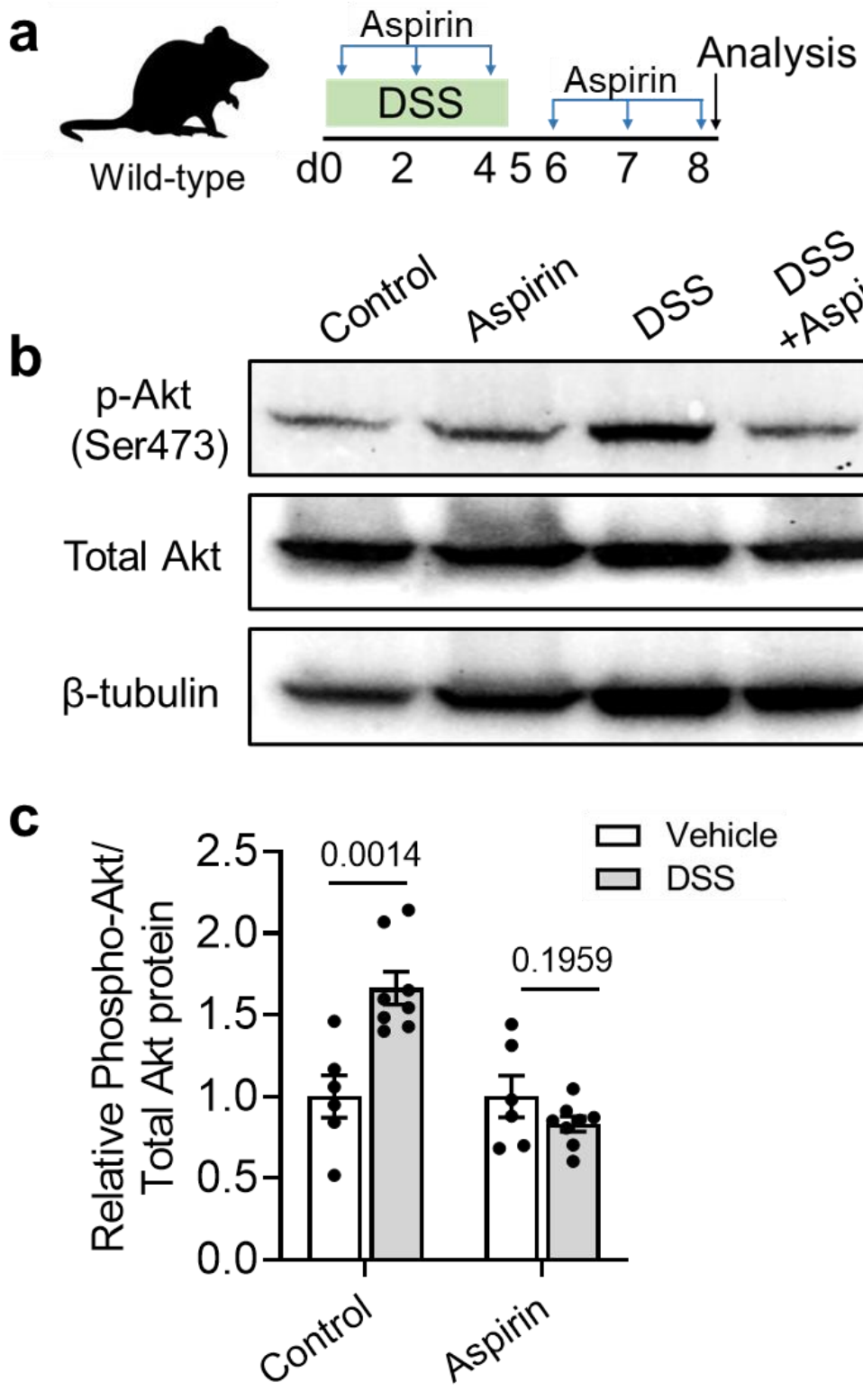

a
Aspirin
DSS
Aspirin
Analysis
Wild-type
d0 2 4 5 6 7 8
b
Control
Aspirin
DSS
DSS +Aspirin
p-Akt (Ser473)
Total Akt
β-tubulin
c
Vehicle
DSS
Relative Phospho-Akt/ Total Akt protein
2.5
2.0
1.5
1.0
0.5
0.0
0.0014
0.1959
Control
Aspirin

Figure 3.9 – Akt activation is upregulated in DSS-colitis.

(**a**) Schematic illustration of the treatment of C57Bl/6 mice with 2.5% DSS and COX-inhibition with Aspirin. (**b**) Western Blot showing phospho-Akt and total Akt protein levels in DSS-colitis and upon COX inhibition with Aspirin. (**c**) Quantification of the relative protein levels of phospho-Akt as compared to total Akt in DSS-colitis with and without COX inhibition by Aspirin. Data are normalized to vehicle-treated mice (no DSS) in the setting of both control and COX inhibition by Aspirin. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=6; DSS, n=8; Aspirin, n=6; DSS+Aspirin, n=8). Statistical significance was determined using unpaired Student's t-test.

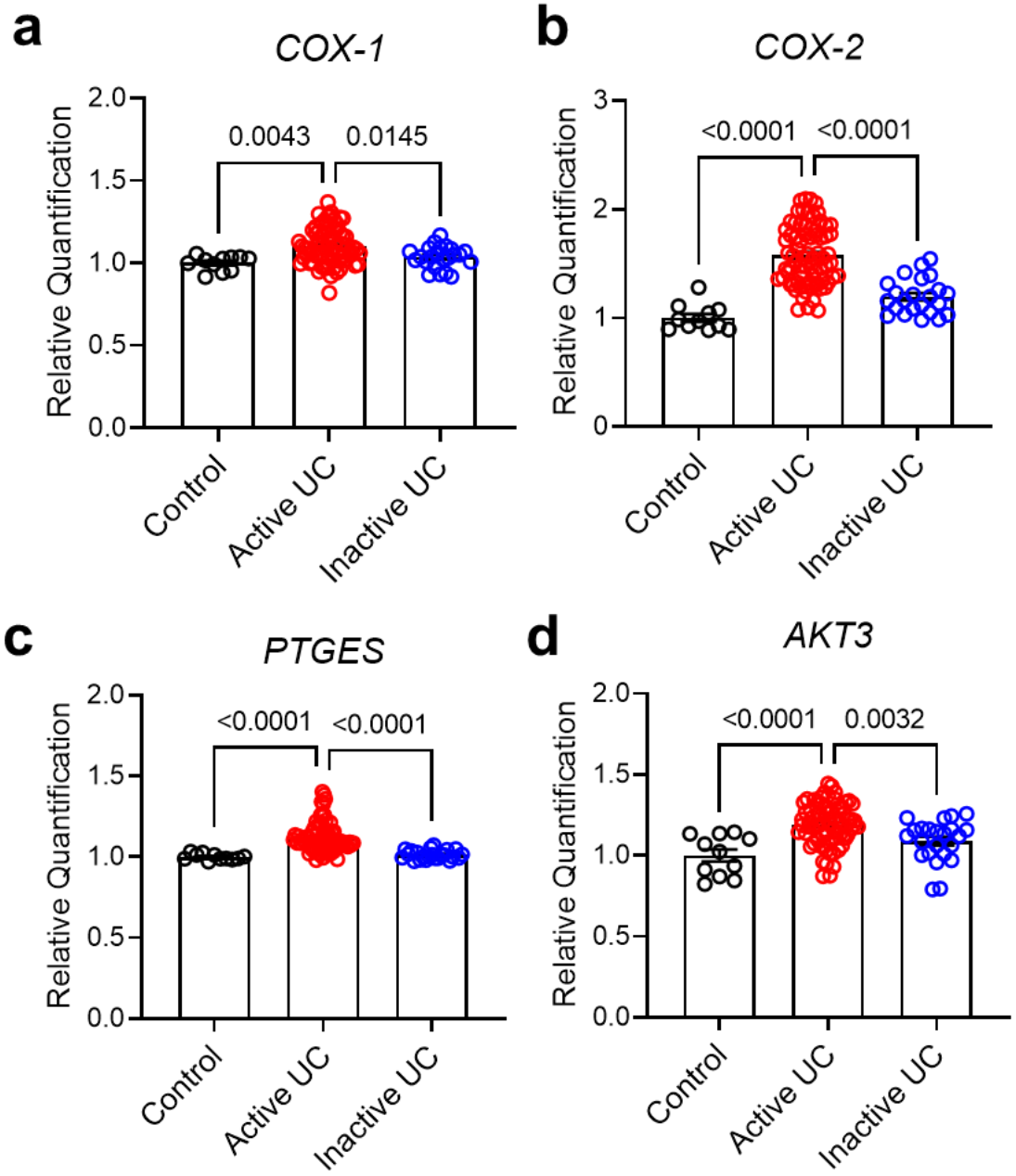

a
COX-1
Relative Quantification
2.0
1.5
1.0
0.5
0.0
0.0043
0.0145
Control
Active UC
Inactive UC
b
COX-2
Relative Quantification
3
2
1
0
<0.0001
<0.0001
Control
Active UC
Inactive UC
c
PTGES
Relative Quantification
2.0
1.5
1.0
0.5
0.0
<0.0001
<0.0001
Control
Active UC
Inactive UC
d
AKT3
Relative Quantification
2.0
1.5
1.0
0.5
0.0
<0.0001
0.0032
Control
Active UC
Inactive UC

Figure 3.10 – Active UC is associated with increased COX and Akt expression.

(**a-d**) Relative mRNA expression of *COX-1*, *COX-2*, *PTGES*, and *AKT3* in the colonic tissue from control subjects (n=11), patients with active UC (n=74), and patients with inactive UC (n=23). Data were derived from Gene Expression Omnibus (GEO) data set GSE75214 and normalized to control samples. Data are presented as mean ± SEM and dots represent biologically independent subjects. Statistical significance was assessed by ordinary one-way ANOVA with Tukey's post-hoc test.

3.3.5 *PGE_2 and Akt activity promote the stemness of mature epithelial tuft cells.*

Given that Cox and Akt were upregulated in active colitis, we next sought to determine whether PGE_2 and/or activation of Akt signaling have an effect on Dclk1+ cells. To do this, we capitalized on our *Dclk1^{CreERT2};R26^{mTmG};APC$^{f/f}$* (*APC$^{f/f}$*) transgenic mouse model which allows us to map the fate of mature Dclk1-expressing epithelial tuft cells using the Rosa26-mTmG reporter. We analyzed the effect of Misprostol (PGE analogue) and SC79 (an Akt activator (Jo et al., 2012)), not to be confused with the earlier mentioned COX-1 inhibitor SC-560, on Dclk1+ cell-derived lineage tracing. *Dclk1^{CreERT2};R26^{mTmG}* (*APC$^{+/+}$*) or *Dclk1^{CreERT2};R26^{mTmG};APC$^{f/f}$* (*APC$^{f/f}$*) mice were given tamoxifen and subsequently treated with Misoprostol and/or SC79 (**Figure 3.11a**). Importantly, no lineage tracing from Dclk1+ cells was observed in either *APC$^{+/+}$* or *APC$^{f/f}$* vehicle-treated mice. Furthermore, we detected exceedingly rare lineage tracing of crypts in *APC$^{+/+}$* (wildtype) mice treated with Misoprostol and/or SC79 (i.e. only 1 traced crypt was detected in 14-20 sections analyzed per group in 25-33% of mice). In contrast, SC79 and Misoprostol treatment of *APC$^{f/f}$* mice resulted in significantly increased Dclk1+ cell derived lineage tracing (i.e. 1 traced crypt was detected every 2-5 sections analyzed per group and in 86-100% of mice) (**Figure 3.11b-d**). These data suggest that Misoprostol and SC79 can promote the stemness of mature Dclk1+ tuft cells, and this is enhanced upon APC-loss. Interestingly, we observed no significant difference in the frequency of Dclk1+ cell lineage tracing amongst SC79, Misoprostol, or combination (SC79 plus Misoprostol)-treated mice (**Figure 3.11d**). To validate our observations that Akt activation and PGE_2 induce stemness in Apc-deficient Dclk1+ cells, we additionally treated *Dclk1^{CreERT2};APC$^{f/f}$;R26^{tdTomato}* enteroids with PGE_2 and/or SC79 *in vitro* (**Figure 3.12a**). Indeed, treatment with PGE2 and SC79 resulted in TdTomato+ lineage tracing from Apc-deficient Dclk1+ cells, an effect that was not observed in vehicle-treated enteroids (**Figure 3.12b,c**). Taken together, these data demonstrate that quiescent Apc-deficient Dclk1+ cells can be stimulated to proliferate and give rise to the entire crypt upon direct exposure to PGE_2 and activation of Akt signaling.

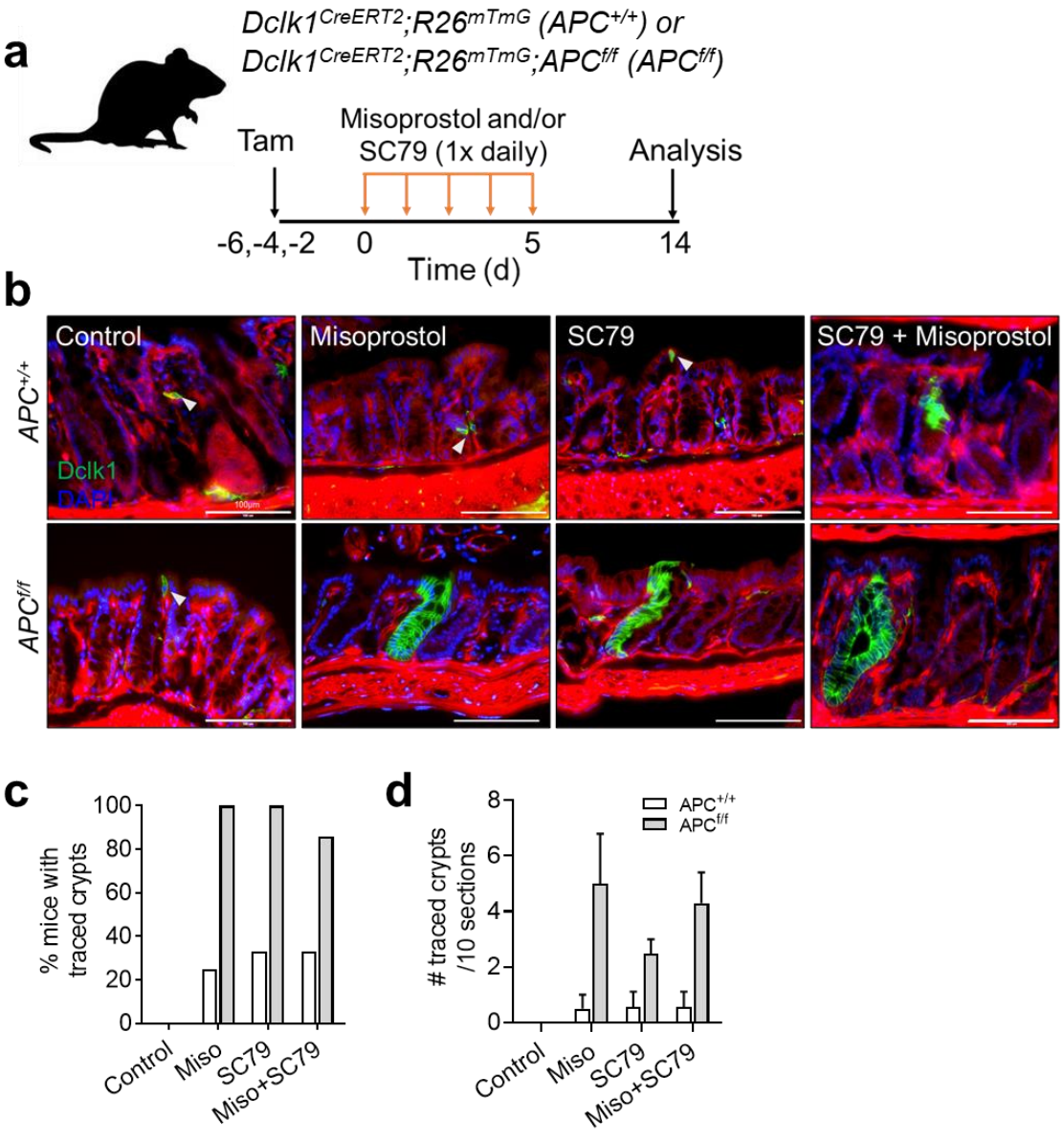
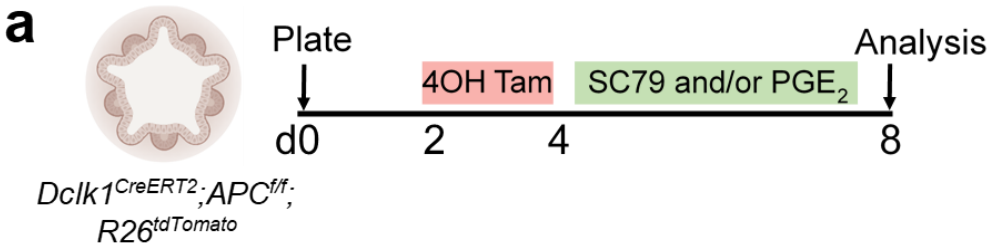
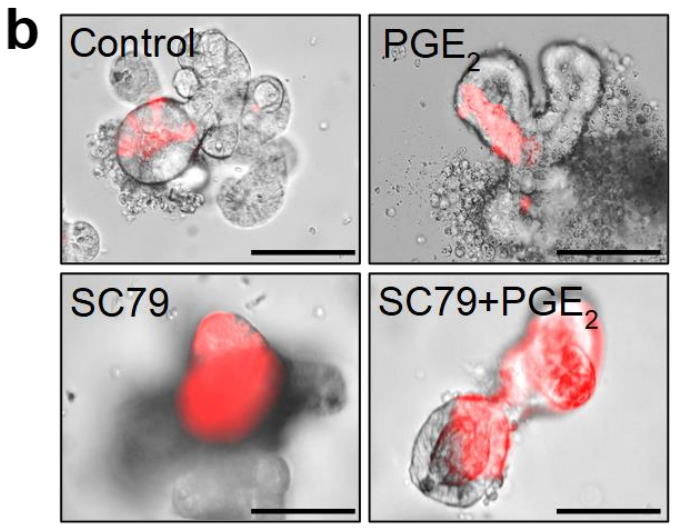

a
Dclk1CreERT2;R26mTmG (APC+/+) or
Dclk1CreERT2;R26mTmG;APCf/f (APCf/f)
Tam
Misoprostol and/or
SC79 (1x daily)
Analysis
-6,-4,-2
0
5
14
Time (d)
b
Control
Misoprostol
SC79
SC79 + Misoprostol
APC+/+
APCf/f
Dclk1
DAPI
c
% mice with traced crypts
100
80
60
40
20
0
Control
Miso
SC79
Miso+SC79
d
traced crypts /10 sections
8
6
4
2
0
APC+/+
APCf/f
Control
Miso
SC79
Miso+SC79

Figure 3.11 – Prostaglandin E2 and Akt signaling promote epithelial tuft cell stemness *in vivo*.

(**a**) Schematic illustration of the treatment of *Dclk1*^{CreERT2}*;R26*^{mTmG} (*APC*$^{+/+}$) or *Dclk1*^{CreERT2}*;R26*^{mTmG}*;APC*$^{f/f}$ (*APC*$^{f/f}$) mice with Misoprostol (PGE analogue) and/or SC79 (Akt activator). (**b**) Representative fluorescence microscopy images of the colonic epithelium with single Dclk1+ cells (white arrowheads) or Dclk1+ cell-derived lineage tracing (green). Scale bars = 100µm. (**c**) Quantification of the percentage of *APC*$^{+/+}$ or *APC*$^{f/f}$ mice with Dclk1+ cell-derived traced crypts upon treatment with SC79 and/or Misoprostol. (**d**) Quantification of the number of Dclk1+ cell-derived traced crypts per 10 sections analyzed in *APC*$^{+/+}$ or *APC*$^{f/f}$ mice (*APC*$^{+/+}$: control, n=3; Misoprostol, n=4; SC79, n=3; SC79+Misoprostol, n=3; *APC*$^{f/f}$: control, n=6; Misoprostol, n=4; SC79, n=6; SC79+Misoprostol, n=7). Data are presented as mean +SEM.


a
Plate
4OH Tam
SC79 and/or PGE2
Analysis
d0
2
4
8
Dclk1CreERT2;APCf/f;
R26tdTomato


b
Control
PGE2
SC79
SC79+PGE2

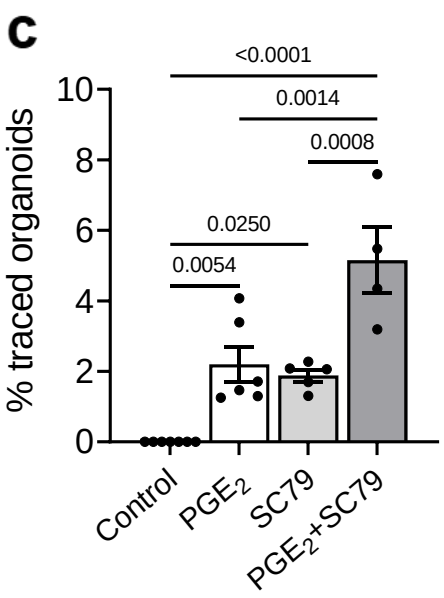
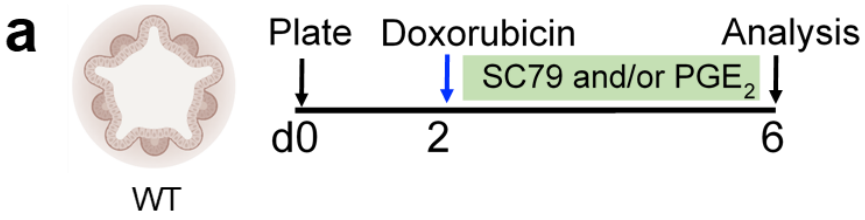

c
% traced organoids
<0.0001
0.0014
0.0008
0.0250
0.0054
10
8
6
4
2
0
Control
PGE2
SC79
PGE2+SC79

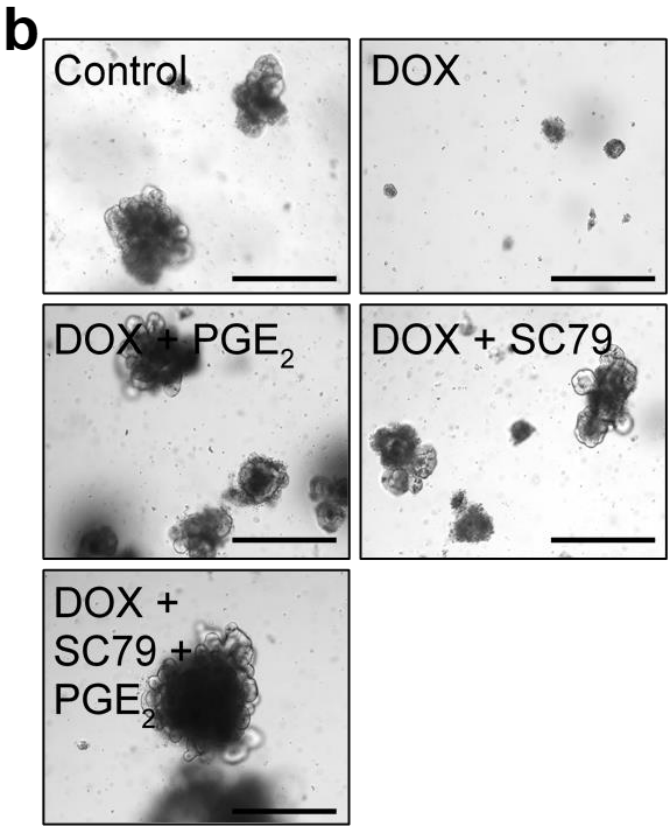
Figure 3.12 – Prostaglandin E_2 and Akt signaling promote epithelial tuft cell stemness *in vitro*.

(**a**) Schematic illustration of the treatment of *Dclk1*^{CreERT2};*R26*^{tdTomato};*APC*$^{f/f}$ enteroids with 4-hydoxytamoxifen (4OH-Tam), SC79 and/or PGE_2. Organoid image from Biorender.com. (**b**) Representative brightfield and fluorescent images of *Dclk1*^{CreERT2};*R26*^{tdTomato};*APC*$^{f/f}$ enteroids treated with SC79 and/or PGE_2. Scale bars = 100µm. (**c**) Quantification of the percentage of *Dclk1*^{CreERT2};*R26*^{tdTomato};*APC*$^{f/f}$ enteroids showing Dclk1+ cell-derived lineage tracing (red) upon treatment with SC79 and/or PGE_2. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=7; PGE_2, n=6; SC79, n=5; PGE_2+SC79, n=4). Statistical significance was determined by one-way ANOVA and Tukey post-hoc test.

3.3.6 *PGE_2 and Akt activation promote epithelial regeneration after injury.*

To examine the role of PGE_2 and Akt during intestinal injury, we next assessed the effects of PGE_2 and SC79 on intestinal regeneration. We induced epithelial injury *in vitro* by treating enteroids with the chemotherapeutic agent doxorubicin, and then cultured the enteroids in the presence of PGE_2 and/or SC79 (**Figure 3.13a**). As previously reported, doxorubicin caused epithelial injury and reduced enteroid size when compared to untreated controls. Treatment with PGE_2 or SC79 after injury, however, prevented doxorubicin-induced reduction in enteroid size. Interestingly, the combination of both PGE_2 and SC79 resulted in even larger enteroids when compared to untreated control enteroids or enteroids treated with PGE_2 or SC79 alone (**Figure 3.13b**). This was also true in the absence of doxorubicin-induced injury (**Appendix 2**). These data suggest that both PGE_2 and SC79 both promote enteroid growth after injury, an effect that is enhanced when administered in combination. To analyze whether this effect was in part mediated by the dedifferentiation of mature cell types, we determined the effect of Akt activation and PGE_2 on Dclk1+ cell expansion in the setting of epithelial injury. $Dclk1^{CreERT2};APC^{f/f};R26^{tdTomato}$ enteroids were treated with doxorubicin (to induce injury) and subsequently cultured in the presence of SC79 and/or PGE_2 (**Figure 3.14a**). SC79 and PGE2 treatment of doxorubicin-treated organoids increased Dclk1+ cell-derived lineage tracing when compared to vehicle-treated controls, proving that SC79 and PGE_2 directly act on the intestinal epithelium to stimulate Dclk1+ cell stemness even in the setting of injury (**Figure 3.14b,c**). Taken together, these data prove that activation of PGE_2 and Akt signaling pathways can stimulate the stem cell activity of differentiated and mature tuft cells, particularly in the setting of Apc-loss, suggesting that this may be mediated through activation of Wnt signaling. As the effect of SC79 and PGE_2 on Dclk1+ lineage tracing persists even in the setting of epithelial damage, this suggests that the upregulation of Akt and PGE_2 in inflammation may contribute to the ability of non-stem cells to dedifferentiate and repopulate the colonic crypt when other cell populations are lost during injury.


a
Plate
Doxorubicin
Analysis
SC79 and/or PGE2
d0
2
6
WT


b
Control
DOX
DOX + PGE2
DOX + SC79
DOX + SC79 + PGE2

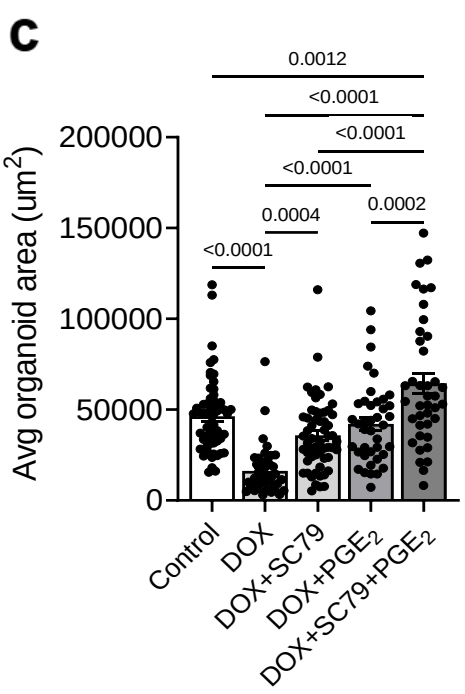

c
Avg organoid area (um2)
200000
150000
100000
50000
0
0.0012
<0.0001
<0.0001
<0.0001
0.0004
0.0002
<0.0001
Control
DOX
DOX+SC79
DOX+PGE2
DOX+SC79+PGE2

Figure 3.13 – PGE_2 and Akt activation enhance intestinal organoid regeneration after injury.

(**a**) Schematic illustration of the treatment of WT enteroids treated with doxorubicin (DOX) plus SC79 and/or PGE_2. Organoid image from Biorender.com. (**b**) Representative brightfield images of WT enteroids treated with doxorubicin plus SC79 and/or PGE_2. Scale bars = 200μm. (**c**) Quantification of the average organoid area of WT enteroids treated with doxorubicin plus SC79 and/or PGE_2. Data are presented as mean ± SEM and dots represent individual organoids pooled from n=3 biologically independent animals. Statistical significance was determined by one-way ANOVA and Tukey post-hoc test.

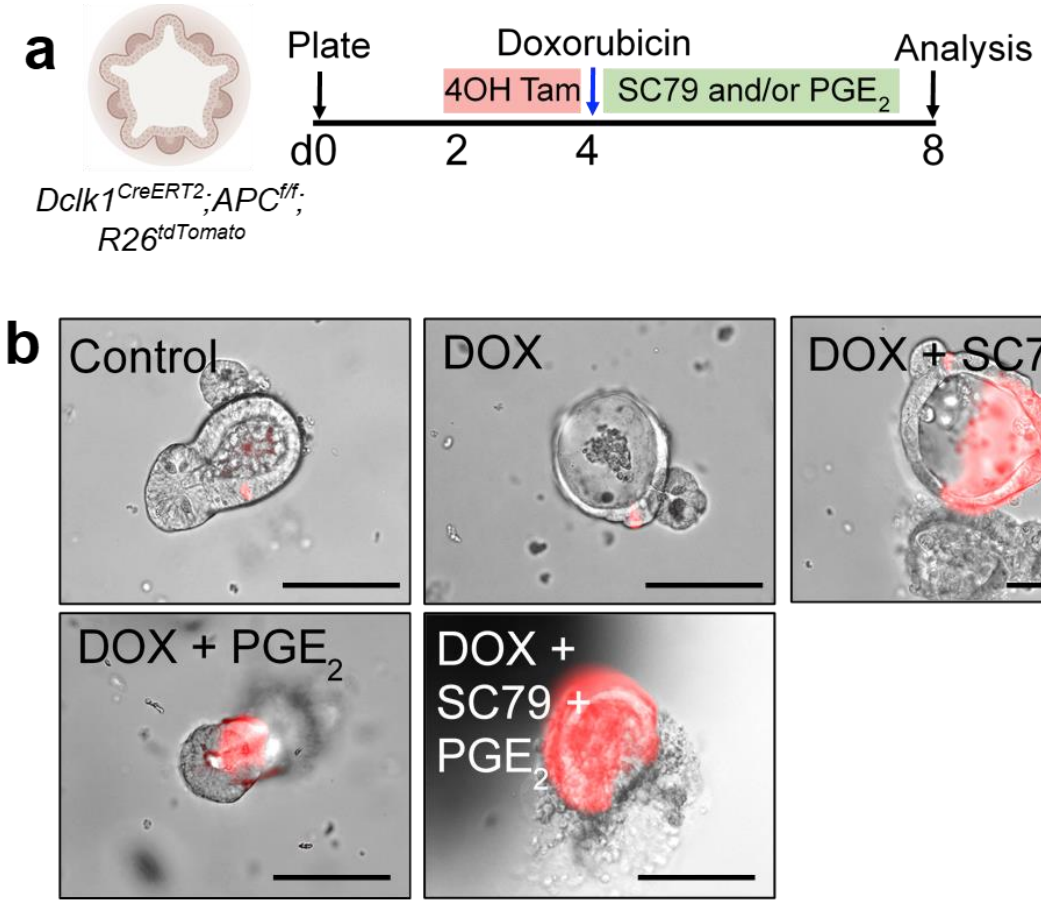
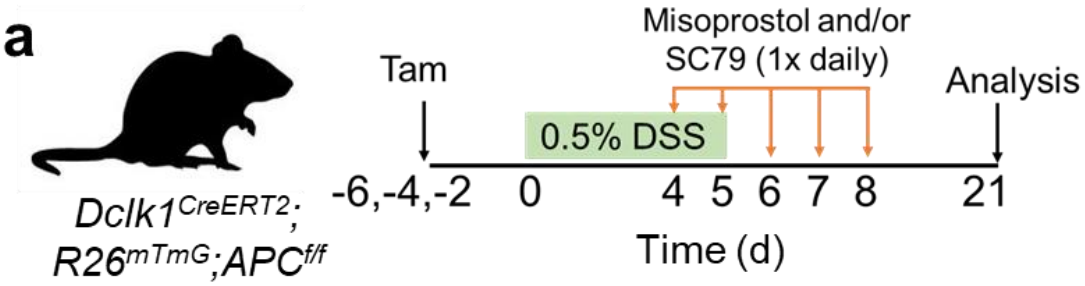

a
Plate
Doxorubicin
Analysis
4OH Tam
SC79 and/or PGE2
d0
2
4
8
Dclk1CreERT2;APCf/f;
R26tdTomato
b
Control
DOX
DOX + SC79
DOX + PGE2
DOX +
SC79 +
PGE2
c
% traced organoids
0.0004
10
8
6
4
2
0
Control
Dox
Dox+PGE2
Dox+SC79
Dox+PGE2+SC79

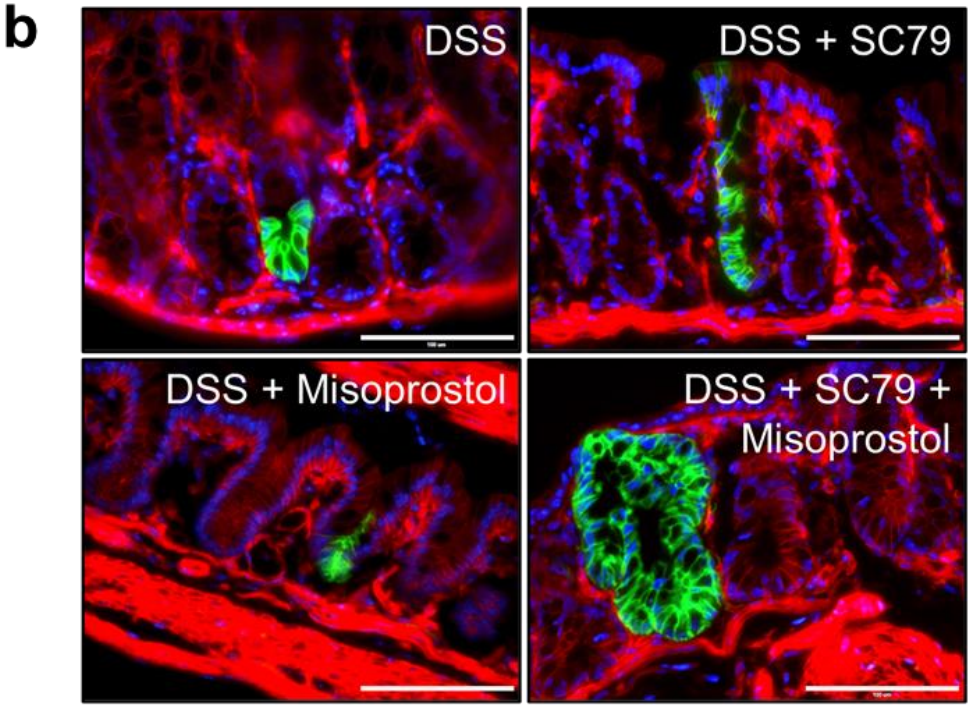
Figure 3.14 – PGE_2 and Akt activation stimulate Dclk1+ cell stemness after intestinal injury.

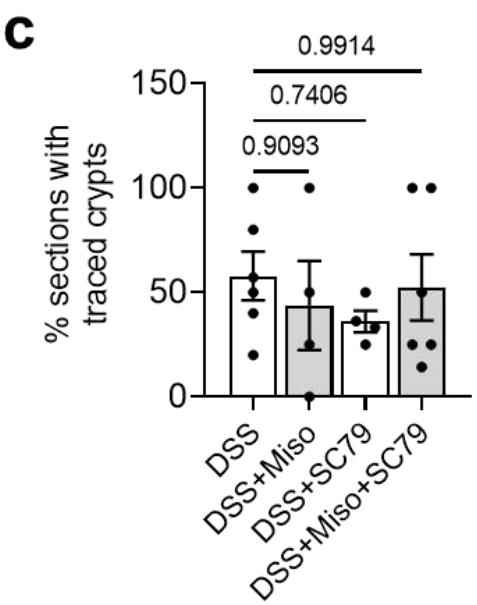
(**a**) Schematic illustration of the treatment of *Dclk1*^{CreERT2};*R26*^{tdTomato};*APC*$^{f/f}$ enteroids with doxorubicin plus SC79 and/or PGE_2. Organoid image from Biorender.com. (**b**) Representative brightfield and fluorescent images of *Dclk1*^{CreERT2};*R26*^{tdTomato};*APC*$^{f/f}$ enteroids treated with doxorubicin plus SC79 and/or PGE_2. Scale bars = 100µm. (**c**) Quantification of the percentage of *Dclk1*^{CreERT2};*R26*^{tdTomato};*APC*$^{f/f}$ enteroids showing Dclk1+ cell-derived lineage tracing (red) upon treatment with doxorubicin plus SC79 and/or PGE_2. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=5; DOX, n=5; DOX+SC79, n=5; DOX+PGE_2, n=3; DOX+PGE_2+SC79, n=4). Statistical significance was determined by one-way ANOVA and Tukey post-hoc test.

3.3.7 *PGE_2 and Akt promote Dclk1+ cell-derived colonic tumorigenesis upon epithelial injury.*

Previous studies have shown that non-stem cells likely initiate tumorigenesis in the setting of injury through dedifferentiation (Schwitalla et al., 2013). As we observed the ability of PGE_2 and Akt activation to stimulate the stemness of Dclk1+ cells and promote intestinal regeneration in the setting of injury, we next sough to examine whether activation of PGE_2 and/or Akt signaling pathways contribute to colitis-associated cancer initiation. To examine the effects of PGE_2 and Akt activation on Dclk1+ cell-derived tumorigenesis, we treated *$Dclk1^{CreERT2};APC^{f/f};R26^{mTmG}$* mice with Misoprostol and/or SC79 during DSS colitis. We first titrated the dose of DSS to a level that allowed us to induce Dclk1+ cell-derived lineage tracing without inducing dysplasia (i.e. less than 10% of mice analyzed showed dysplasia using 0.5% DSS) (**Figure 3.15a**, **Appendix 3**). Administration of either SC79 or Misoprostol during exposure to 0.5% DSS had no effect on the frequency of lineage tracing or dysplasia detected when compared to DSS alone. However, concurrent treatment with both Misoprostol and SC79 similarly did not increase the frequency of lineage tracing events, but led to increased Dclk1+ cell-derived dysplasia when compared to the vehicle-treated controls (i.e. dysplasia was observed in 80% of mice treated with Misoprostol plus SC79 versus 10% of mice treated with vehicle) (**Figure 3.15b,c**). Importantly, we did not observe a change in colonic histology or body weight loss upon treatment with Misoprostol and/or SC79 in the setting of low-dose DSS-colitis (**Appendix 4**). These data suggest that the simultaneous presence of PGE_2 and activation of Akt can promote the transformation of Dclk1+ cells to initiate tumorigenesis. The clinical relevance of our findings was further confirmed using data from patients with IBD. We found that relative to healthy controls, *COX-1*, *COX-2*, *AKT-3*, and *PTGES* were significantly upregulated in colonic tissue of patients with UC and neoplasia, versus those with UC, but no neoplasia (**Figure 3.16**), suggesting a critical role for both PGE_2 and Akt signaling pathways in CAC initiation.


a
Misoprostol and/or
SC79 (1x daily)
Tam
Analysis
0.5% DSS
-6,-4,-2
0
4 5 6 7 8
21
Time (d)
Dclk1CreERT2;
R26mTmG;APCf/f


b
DSS
DSS + SC79
DSS + Misoprostol
DSS + SC79 +
Misoprostol


c
0.9914
0.7406
0.9093
% sections with
traced crypts
150
100
50
0
DSS
DSS+Miso
DSS+SC79
DSS+Miso+SC79

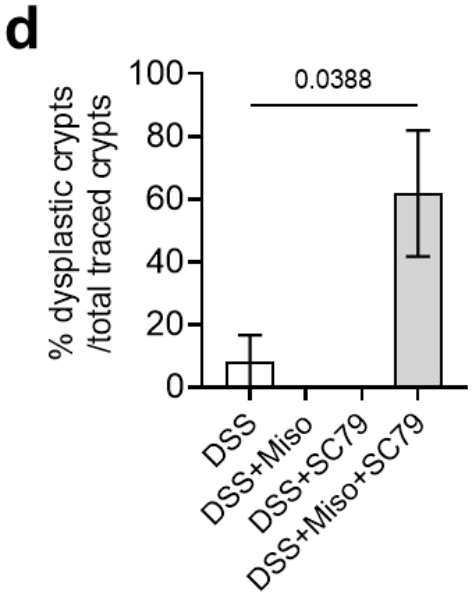

d
0.0388
% dysplastic crypts
/total traced crypts
100
80
60
40
20
0
DSS
DSS+Miso
DSS+SC79
DSS+Miso+SC79

Figure 3.15 – Simultaneous activation of PGE_2 and Akt signaling drives the initiation of Dclk1+ cell-derived dysplastic lesions.

(**a**) Schematic illustration of the treatment of *Dclk1*^{CreERT2}*;R26*^{tmTmG}*;APC*$^{f/f}$ mice with 0.5% DSS plus SC79 and/or Misoprostol. (**b**) Representative fluorescence microscopy images of the colonic epithelium with Dclk1+ cell-derived lineage traced crypts (green) in *Dclk1*^{CreERT2}*;R26*^{tmTmG}*;APC*$^{f/f}$ mice upon treatment with DSS plus SC79 or Misoprostol, or Dclk1+ cell-derived lineage traced dysplastic lesions upon treatment with DSS plus SC79 and Misoprostol. Scale bars = 100µm. (**c**) Quantification of the percentage of sections with Dclk1+ cell-derived traced crypts upon treatment with DSS plus Misoprostol and/or SC79. Data are presented as mean ± SEM and dots represent biologically independent animals (DSS, n=6; DSS+Miso, n=4; DSS+SC79, n=4; DSS+Miso+SC79, n=6). (**d**) Quantification of the percentage of Dclk1+ cell-derived lineage traced dysplastic lesions over the total number of tracing events upon treatment with DSS plus Misoprostol and/or SC79. Statistical significance was determined by one-way ANOVA and Tukey post-hoc test.

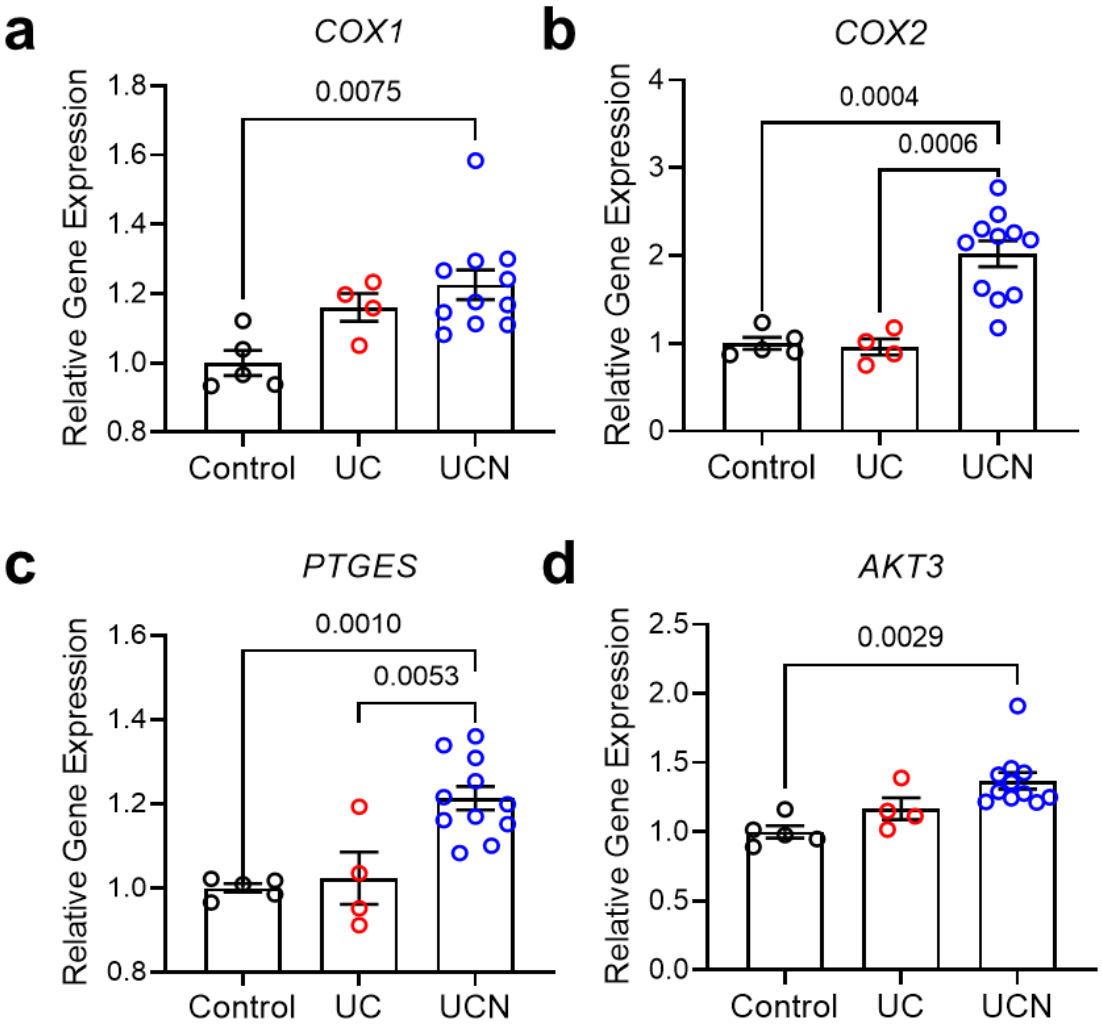

a
COX1
0.0075
Relative Gene Expression
1.8
1.6
1.4
1.2
1.0
0.8
Control
UC
UCN
b
COX2
0.0004
0.0006
Relative Gene Expression
4
3
2
1
0
Control
UC
UCN
c
PTGES
0.0010
0.0053
Relative Gene Expression
1.6
1.4
1.2
1.0
0.8
Control
UC
UCN
d
AKT3
0.0029
Relative Gene Expression
2.5
2.0
1.5
1.0
0.5
0.0
Control
UC
UCN

Figure 3.16 – Cox and Akt signaling pathways are associated with UC progression to neoplasia.

(**a-d**) Relative mRNA expression of *COX-1*, *COX-2*, *PTGES*, and *AKT3* in colonic tissue from healthy control subjects (n=5), patients with quiescent ulcerative colitis (UC) (n=4), or patients with quiescent UC and UC-associated neoplasia (n=11). Data were derived from Gene Expression Omnibus (GEO) data set GSE37283 and normalized to control samples. Data are presented as mean ± SEM and dots represent biologically independent subjects. Statistical significance was assessed by ordinary one-way ANOVA with Tukey's post-hoc test.

3.3.8 *Canonical Wnt signaling is upregulated in colitis-associated cancer and is preceded by Cox and Akt signaling.*

Although APC-loss in Lgr5+ stem cells results in the rapid nuclear localization of β-catenin and the initiation of tumorigenesis (Barker et al., 2009), our previous data suggested that APC-loss in Dclk1+ cells was not sufficient to drive the nuclear localization of β-catenin (Westphalen et al., 2014). We confirmed this finding by performing immunofluorescence staining for β-catenin and RFP in colonic sections from tamoxifen-treated *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26*^{TdTomato} mice. In all Apc-deficient TdTomato+ Dclk1+ cells examined, β-catenin was localized to the membrane (**Figure 3.17a**). However, β-catenin was detected in the cytoplasm and nucleus of both Dclk1+ cell-derived and AOM/DSS-derived colitis-associated tumors (**Figure 3.17b**). This finding is analogous to that previously reported for both sporadic and colitis-associated CRC (Fujita et al., 2017; Han et al., 2013; Kameyama et al., 2018; Tanaka et al., 2003). Active Wnt signaling in CAC was further confirmed by the detection of upregulated Wnt target genes in AOM/DSS-derived tumors compared to control tissues in a publicly available dataset (**Figure 3.17c**). These data suggest that canonical Wnt signaling is active in murine colitis-associated cancer, which may be stimulated as a result of DSS-induced intestinal injury. To assess whether Wnt target genes are upregulated during colitis, we analyzed the expression profiles of patients with active UC. Interestingly, in contrast to the upregulation of Cox and Akt-related mediators we detected in active colitis (**Figure 3.10**), we did not observe an increase in Wnt target genes in patients with active UC relative to control or inactive UC patient samples (**Figure 3.18a**). To better characterize the timeline of Wnt, Cox and Akt-related gene expression in colitis and CAC, we analyzed a publicly available database of gene expression at various timepoints throughout the AOM/DSS model of CAC. Consistent with gene expression profiles of patients with active UC, we found that Cox and Akt expression were elevated at early timepoints following colitis, and this correlated with reduced Wnt target gene expression (**Figure 3.18b**). However, elevation of most Wnt target genes occurred later in disease progression (**Figure 3.18b**). Taken together, these data suggest that the observed increase in Cox and Akt expression in colitis precedes canonical Wnt activation during the pathogenesis of CAC.

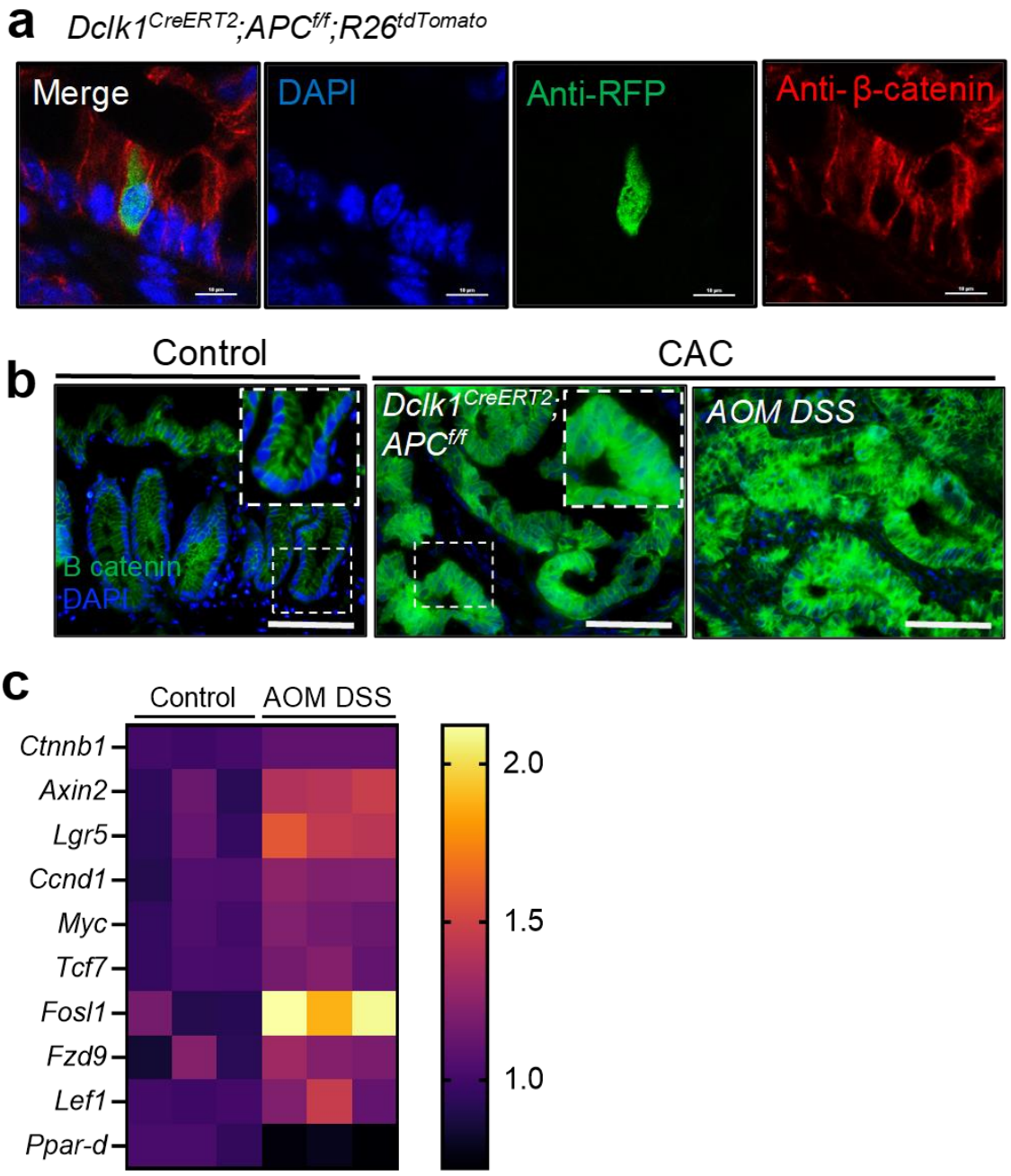

a
Dclk1CreERT2;APCf/f;R26tdTomato
Merge
DAPI
Anti-RFP
Anti- β-catenin
b
Control
CAC
B catenin
DAPI
Dclk1CreERT2;
APCf/f
AOM DSS
c
Control
AOM DSS
Ctnnb1
Axin2
Lgr5
Ccnd1
Myc
Tcf7
Fosl1
Fzd9
Lef1
Ppar-d
2.0
1.5
1.0

Figure 3.17 – Canonical Wnt signaling is active in mouse models of colitis-associated cancer.

(**a**) *Dclk1*^{CreERT2}*;R26*^{tdTomato}*;APC*$^{f/f}$ mice were treated with tamoxifen and analyzed at 48 hours. Representative immunofluorescent images for Dclk1+ cells (anti-RFP, green) and associated β-catenin immunostaining (red). Scale bars = 10μm. (**b**) Representative immunofluorescent images of β-catenin immunostaining (green) in sections of control colonic tissue, *Dclk1*^{CreERT2}*;APC*$^{f/f}$ colonic tumors, and AOM/DSS-derived colonic tumors. Scale bars = 100μm. (**c**) Heatmap showing the relative mRNA expression of Wnt target genes in the colonic tissue of control mice (n=3) and distal colonic tissue of mice with AOM/DSS-derived colonic tumors (n=3). Data were derived from Gene Expression Omnibus (GEO) data set GSE44904 and normalized to control samples.

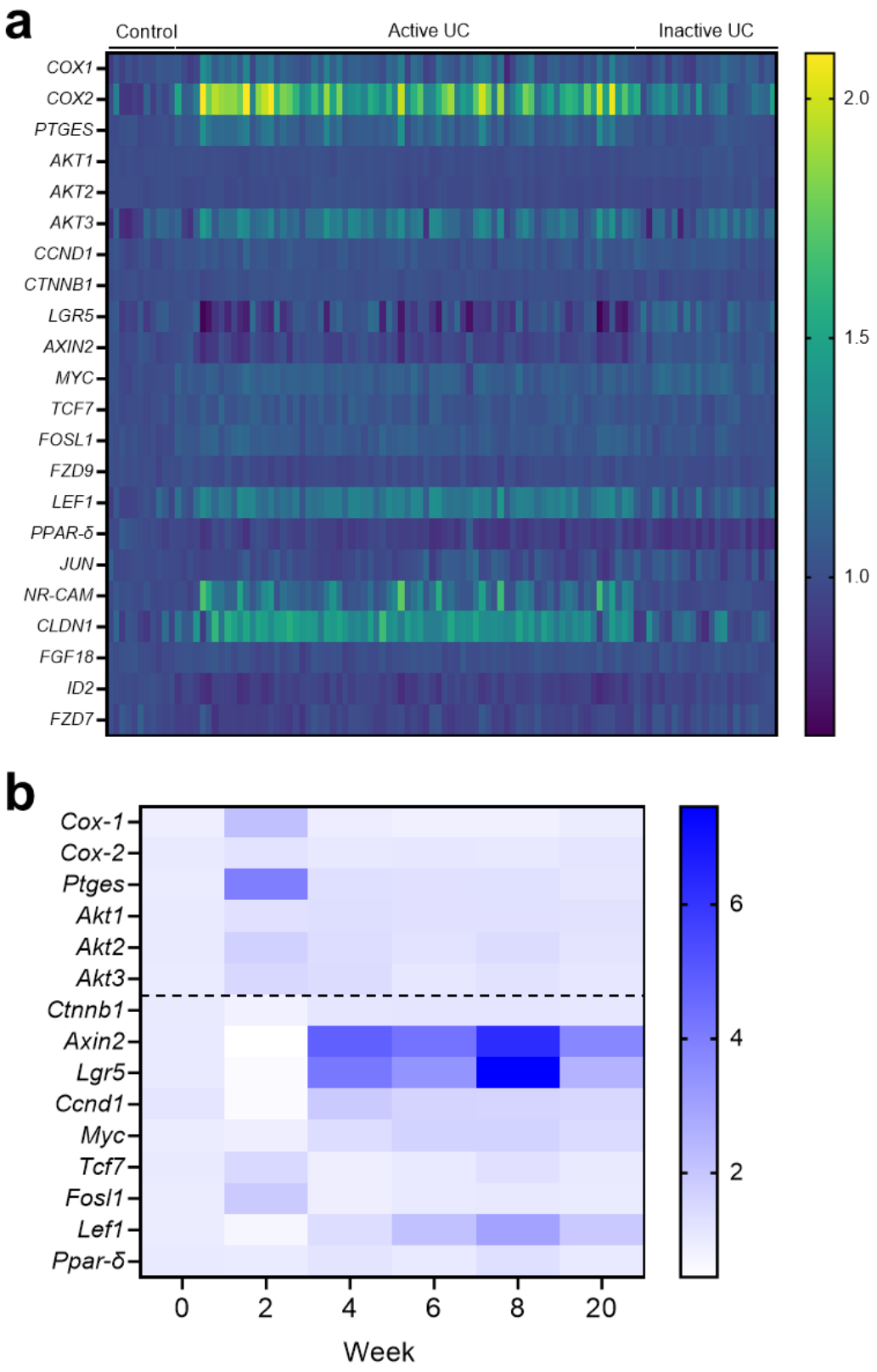

a
Control
Active UC
Inactive UC
COX1
COX2
PTGES
AKT1
AKT2
AKT3
CCND1
CTNNB1
LGR5
AXIN2
MYC
TCF7
FOSL1
FZD9
LEF1
PPAR-δ
JUN
NR-CAM
CLDN1
FGF18
ID2
FZD7
2.0
1.5
1.0
b
Cox-1
Cox-2
Ptges
Akt1
Akt2
Akt3
Ctnnb1
Axin2
Lgr5
Ccnd1
Myc
Tcf7
Fosl1
Lef1
Ppar-δ
6
4
2
0
2
4
6
8
20
Week

Figure 3.18 – Canonical Wnt signaling proceeds Cox and Akt activation in colitis and CAC.

(**a**) Heatmap showing the relative mRNA expression levels of Cox, Akt, and Wnt target genes in the colonic tissue from control subjects (n=11), patients with active UC (n=74), and patients with inactive UC (n=23). Data were derived from Gene Expression Omnibus (GEO) data set GSE75214 and normalized to control samples. (**b**) Relative mRNA expression levels of Cox, Akt, and Wnt target genes in the colonic tissue of mice at week 2, 4, 6, 8, and 20 of disease progression in the AOM/DSS model of CAC (n=3). Data were derived from Gene Expression Omnibus (GEO) data set GSE31106 and normalized to baseline (time = 0) control samples.

3.3.9 *Simultaneous activation of PGE2 and Akt stimulate the nuclear localization of β-catenin.*

Given our findings that Cox and Akt signaling precede Wnt activation, and that PGE_2 and phospho-Akt are upregulated in DSS-colitis, which together promote Dclk1+ cell-derived dysplasia upon APC loss, we next analyzed whether Wnt was activated in dysplastic lesions indued by SC79 and Misoprostol treatment during low-dose DSS. Indeed, we confirmed that β-catenin was localized to the nucleus upon treatment with SC79 and Misoprostol during low-dose intestinal injury (**Figure 3.19a**). We next tested whether PGE_2 and/or Akt activation is sufficient to drive the nuclear localization of β-catenin in Apc-deficient Dclk1-expressing cells in the absence of injury. Colonic crypts lineage traced by Apc-deficient Dclk1+ cells upon Misoprostol and/or SC79 treatment were further analyzed with respect to the cellular localization of β-catenin. Misoprostol or SC79 alone induced Apc-deficient Dclk1+ cell-derived lineage tracing, but β-catenin remained localized to the membrane within these crypts. Interestingly, the combination of Misoprostol plus SC79 resulted in Dclk1+ cell-derived lineage tracing associated with nuclear localization of β-catenin (observed in ~60% of crypts analyzed) (**Figure 3.19b,c**). Consistent with this observation, SC79 plus Misoprostol treatment resulted in significantly increased mRNA expression of several Wnt target genes in colonic tissue (*Lgr5*, *C-myc*, *Ppar-δ*, and *Ccnd1*) compared to control (**Figure 3.19d**). Taken together, these data suggest that the upregulation of both PGE_2 and Akt in colitis is associated with localization of β-catenin to the nucleus to drive tumorigenesis.

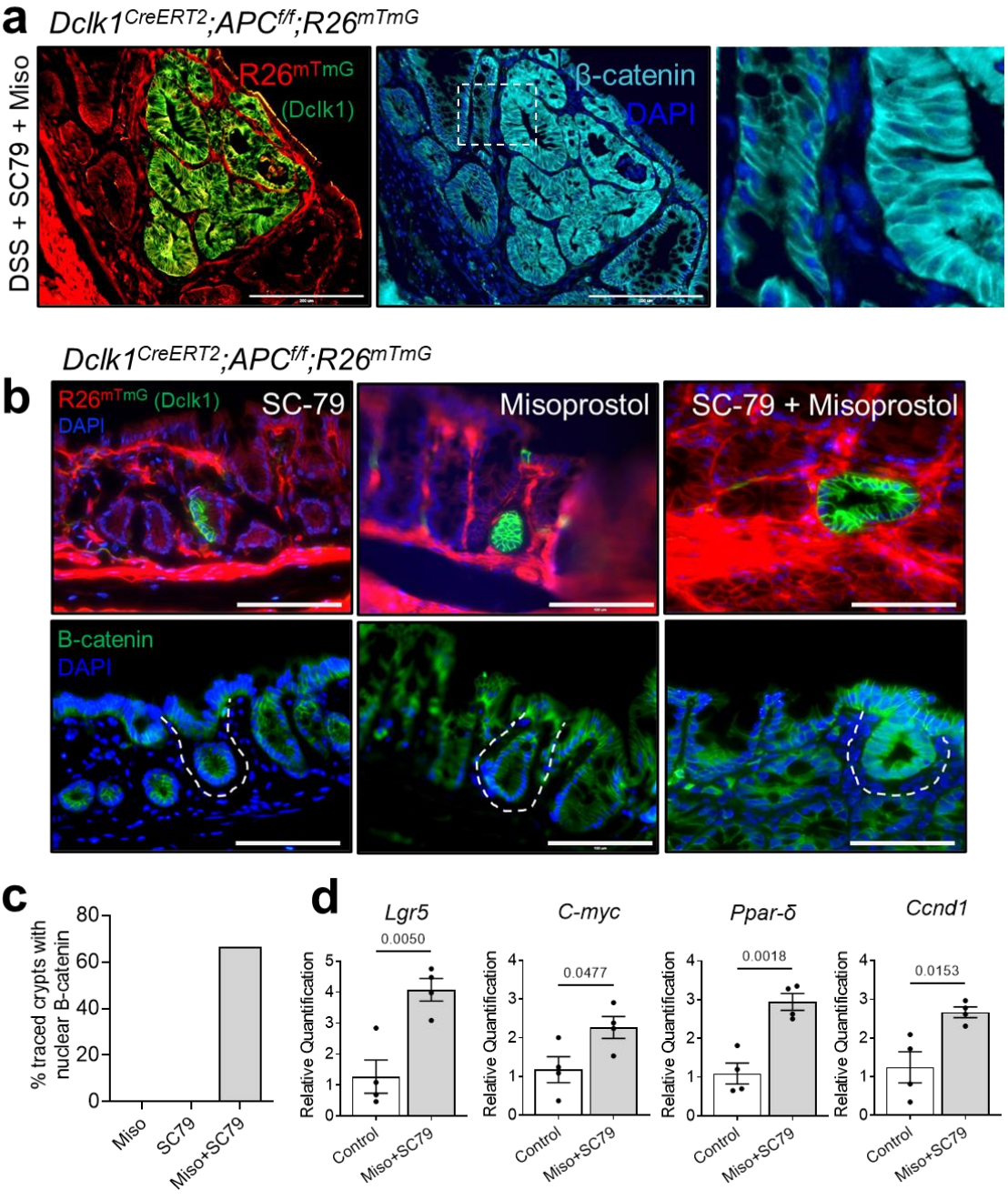

a
Dclk1CreERT2;APCf/f;R26mTmG
DSS + SC79 + Miso
R26mTmG
(Dclk1)
β-catenin
DAPI
b
Dclk1CreERT2;APCf/f;R26mTmG
R26mTmG (Dclk1)
DAPI
SC-79
Misoprostol
SC-79 + Misoprostol
B-catenin
DAPI
c
% traced crypts with nuclear B-catenin
Miso
SC79
Miso+SC79
d
Lgr5
C-myc
Ppar-δ
Ccnd1
Relative Quantification
0.0050
0.0477
0.0018
0.0153
Control
Miso+SC79

Figure 3.19 – Co-activation of PGE_2 and Akt drives the activation of canonical Wnt signaling.

(**a**) Representative immunofluorescent images of β-catenin immunostaining (right, cyan) of Dclk1+ cell-derived traced dysplastic lesions (left, green) upon treatment with DSS plus SC79 and Misoprostol. Scale bars = 200μm. (**b**) Representative immunofluorescent images of β-catenin immunostaining (bottom, green) of Dclk1+ cell-derived traced colonic crypts in *Dclk1*^{CreERT2};*R26*^{tmTmG};*APC*$^{f/f}$ mice (top, green) induced by treatment of SC79 and/or Misoprostol. Scale bars = 100μm. mice (Misoprostol, 4 crypts, n=3; SC79, 4 crypts, n=3; SC79+Misoprostol, 9 crypts, n=4). (**c**) Quantification of the percentage of Dclk1+ cell-derived traced colonic crypts with nuclear β-catenin localization. (**d**) Relative mRNA expression of Wnt target genes in colonic tissue of mice treated with Misoprostol and SC79 as analyzed by qPCR. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n=4; Miso+SC79, n=4). Statistical significance was determined using unpaired Student's t-test.

3.3.10 *Low-dose Aspirin reduces Dclk1+ cell number*

To investigate another potential mechanism by which COX inhibition may influence Dclk1+ cell-derived tumorigenesis, we assessed the effect of NSAIDs on Dclk1+ cell number. We treated wild-type mice with 3 doses of low-dose Aspirin and harvested colonic tissue on day 6 (**Figure 3.20a**). The number of tuft cells in the colonic epithelium was assessed by immunofluorescent staining. Mice treated with Aspirin showed a significant reduction in the number of Dclk1+ cells in the colonic epithelium as compared to vehicle-treated controls (**Figure 3.20b,c**). We next assessed whether this reduction of tuft cell number upon Aspirin treated was also observed in the setting of DSS-colitis. We treated wild-type mice with 2.5% DSS for 5 days with concurrent Aspirin or vehicle treatment 3 times during DSS and every day thereafter until day 19 (**Figure 3.20d**). We confirmed that Aspirin treatment led to a reduction in Dclk1+ cells in the colonic epithelium after DSS-induced intestinal injury (**Figure 3.20e,f**). Overall, these findings show that Aspirin reduces Dclk1+ cell number, suggesting that COX activity may be essential for tuft cell viability. To assess whether low-dose Aspirin reduces tuft cell number through inhibition of COX-1 and/or COX-2, we analyzed the number of Dclk1+ cells in the colonic epithelium upon treatment with rofecoxib, celecoxib, or SC-560 (**Figure 3.21a**). We did not observe a significant reduction in Dclk1+ cells with either of the COX-selective NSAIDs (**Figure 3.21b,c**), indicating that inhibition of both COX-1 and COX-2 may be required to reduce Dclk1+ cell number.

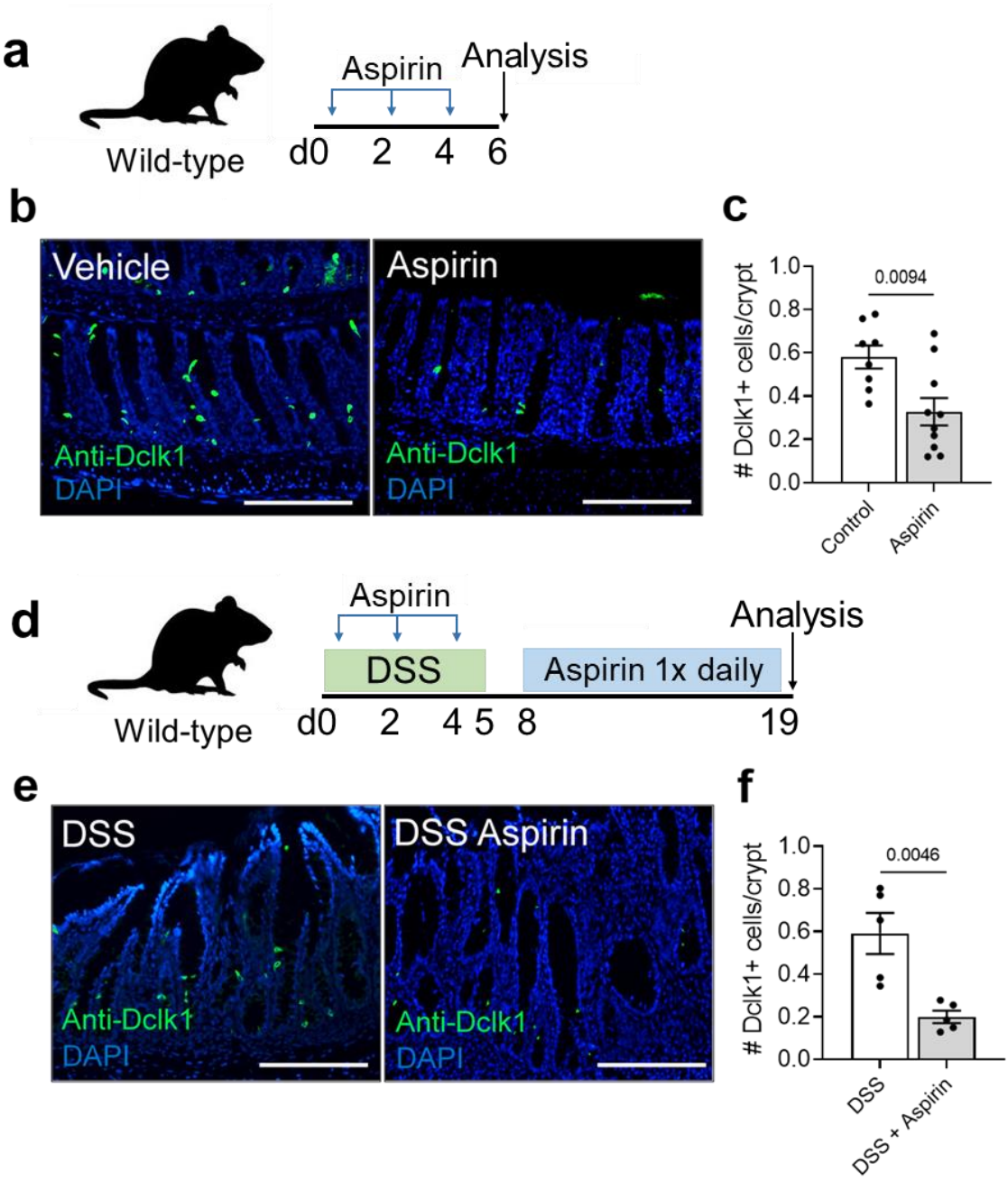

a
Wild-type
Aspirin
Analysis
d0 2 4 6
b
Vehicle
Anti-Dclk1
DAPI
Aspirin
Anti-Dclk1
DAPI
c
Dclk1+ cells/crypt
1.0
0.8
0.6
0.4
0.2
0.0
0.0094
Control
Aspirin
d
Wild-type
Aspirin
DSS
Aspirin 1x daily
Analysis
d0 2 4 5 8 19
e
DSS
Anti-Dclk1
DAPI
DSS Aspirin
Anti-Dclk1
DAPI
f
Dclk1+ cells/crypt
1.0
0.8
0.6
0.4
0.2
0.0
0.0046
DSS
DSS + Aspirin

Figure 3.20 – Low-dose Aspirin reduces colonic Dclk1+ cell number during colonic homeostasis and after DSS-injury.

(**a**) Schematic illustration of the treatment of C57Bl/6 mice with aspirin. (**b**) Representative fluorescent images of anti-Dclk1+ immunofluorescent staining (green) of the colonic epithelium in Aspirin and vehicle-treated mice. Scale bars = 200µm. (**c**) Quantification of the number of Dclk1+ cells per colonic crypt in the epithelium of Aspirin or vehicle-treated mice. Data are presented as mean ± SEM and dots represent biologically independent animals (vehicle, n=8; Aspirin, n=10). (**d**) Schematic illustration of the treatment of C57Bl/6 mice with DSS and Aspirin or vehicle. (**e**) Representative fluorescent images of anti-Dclk1+ immunofluorescent staining (green) of the colonic epithelium in DSS and DSS+Aspirin-treated mice. (**f**) Quantification of the number of Dclk1+ cells per colonic crypt in the epithelium of DSS and DSS+Aspirin-treated mice. Data are presented as mean ± SEM and dots represent biologically independent animals (DSS, n=5; DSS+Aspirin, n=5). Scale bars = 200µm. Statistical significance was determined using unpaired Student's t-test.

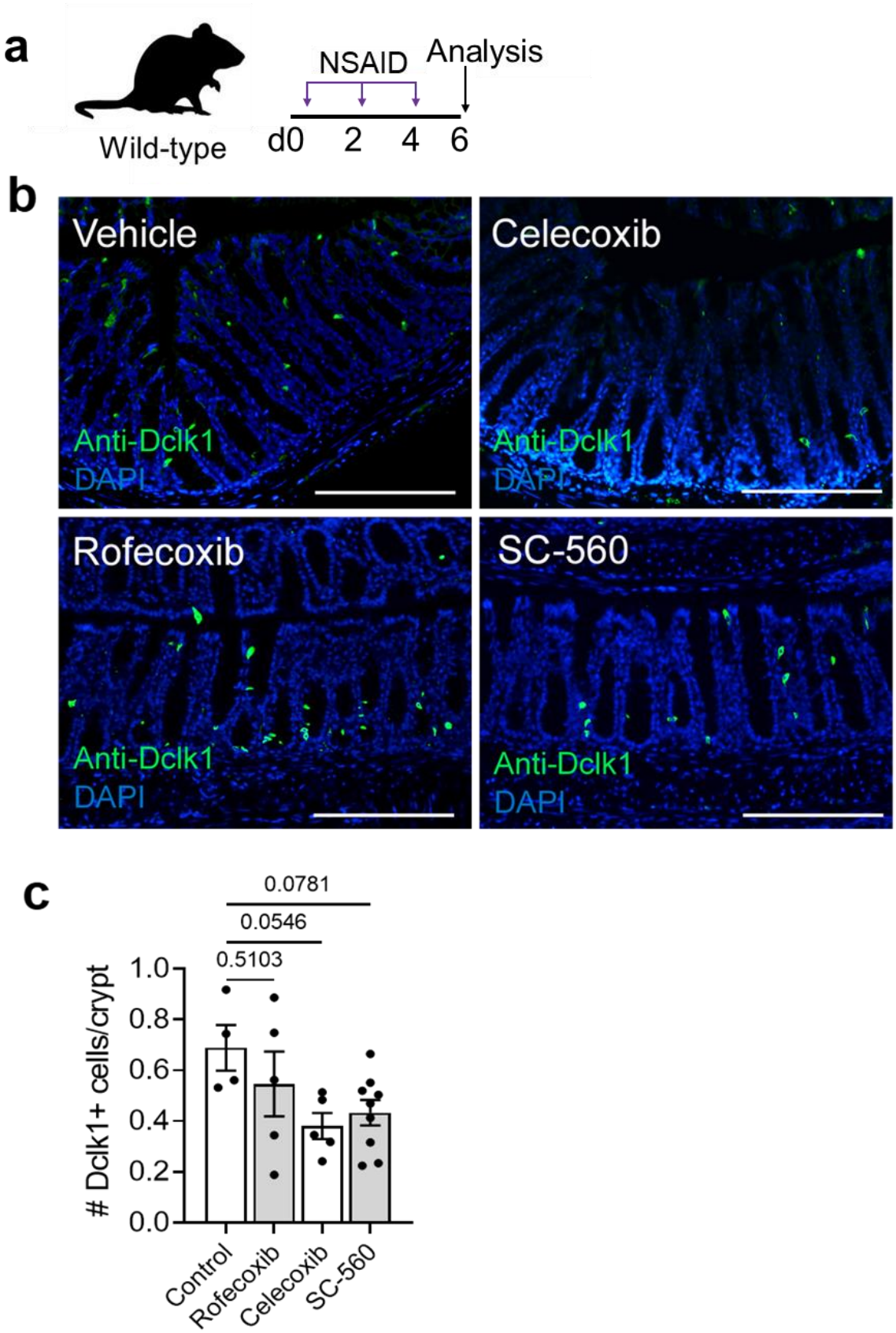

a
Wild-type
NSAID
Analysis
d0 2 4 6
b
Vehicle
Anti-Dclk1
DAPI
Celecoxib
Anti-Dclk1
DAPI
Rofecoxib
Anti-Dclk1
DAPI
SC-560
Anti-Dclk1
DAPI
c
0.0781
0.0546
0.5103
Dclk1+ cells/crypt
1.0
0.8
0.6
0.4
0.2
0.0
Control
Rofecoxib
Celecoxib
SC-560

Figure 3.21 – COX-selective NSAIDs do not affect colonic Dclk1+ cell number.

(**a**) Schematic illustration of the treatment of C57Bl/6 mice with NSAIDs (rofecoxib, celecoxib, SC-560). (**b**) Representative fluorescent images of anti-Dclk1+ immunofluorescent staining (green) of the colonic epithelium in NSAID and vehicle-treated mice. Scale bars = 200μm. (**c**) Quantification of the number of Dclk1+ cells per colonic crypt in the epithelium of rofecoxib, celecoxib, SC-560, or vehicle treated-mice. Data are presented as mean ± SEM and dots represent biologically independent animals (vehicle, n=4; rofecoxib, n=5; celecoxib, n=5; SC-560, n=9). Statistical significance was determined by one-way ANOVA and Dunnet post-hoc test.

3.3.11 *COX-1 loss in intestinal epithelial cells reduces Dclk1+ cell number.*

To further assess whether inhibition of COX-1 alters Dclk1+ cell number, we utilized our *K19$^{Cre(BAC)}$;COX1$^{f/f}$* mice in which COX-1 is constitutively lost from all intestinal epithelial cells. Upon immunofluorescent staining for Dclk1+ cells, we detected a slight yet significant reduction in tuft cell number in the colonic epithelium of *COX-1$^{f/f}$* mice as compared to Cre-negative controls (**Figure 3.22a,b**). This finding was also observed in *K19$^{Cre(BAC)}$;COX1$^{f/f}$* mice treated with DSS when analyzed 2 weeks-post DSS (**Figure 3.22c-e**). These data show that in contrast to pharmacological inhibition of COX-1, genetic loss of COX-1 in the colonic epithelium does result in reduced Dclk1+ cell number. This discrepancy may be due to the insufficient duration of exposure of the colonic epithelium to SC-560 in previous experiments. Taken together, these findings suggest that COX-1 inhibition reduces the number of Dclk1+ cells within the colonic epithelium, which may contribute to the reduced incidence of colitis-associated cancer we observed within our mouse models.

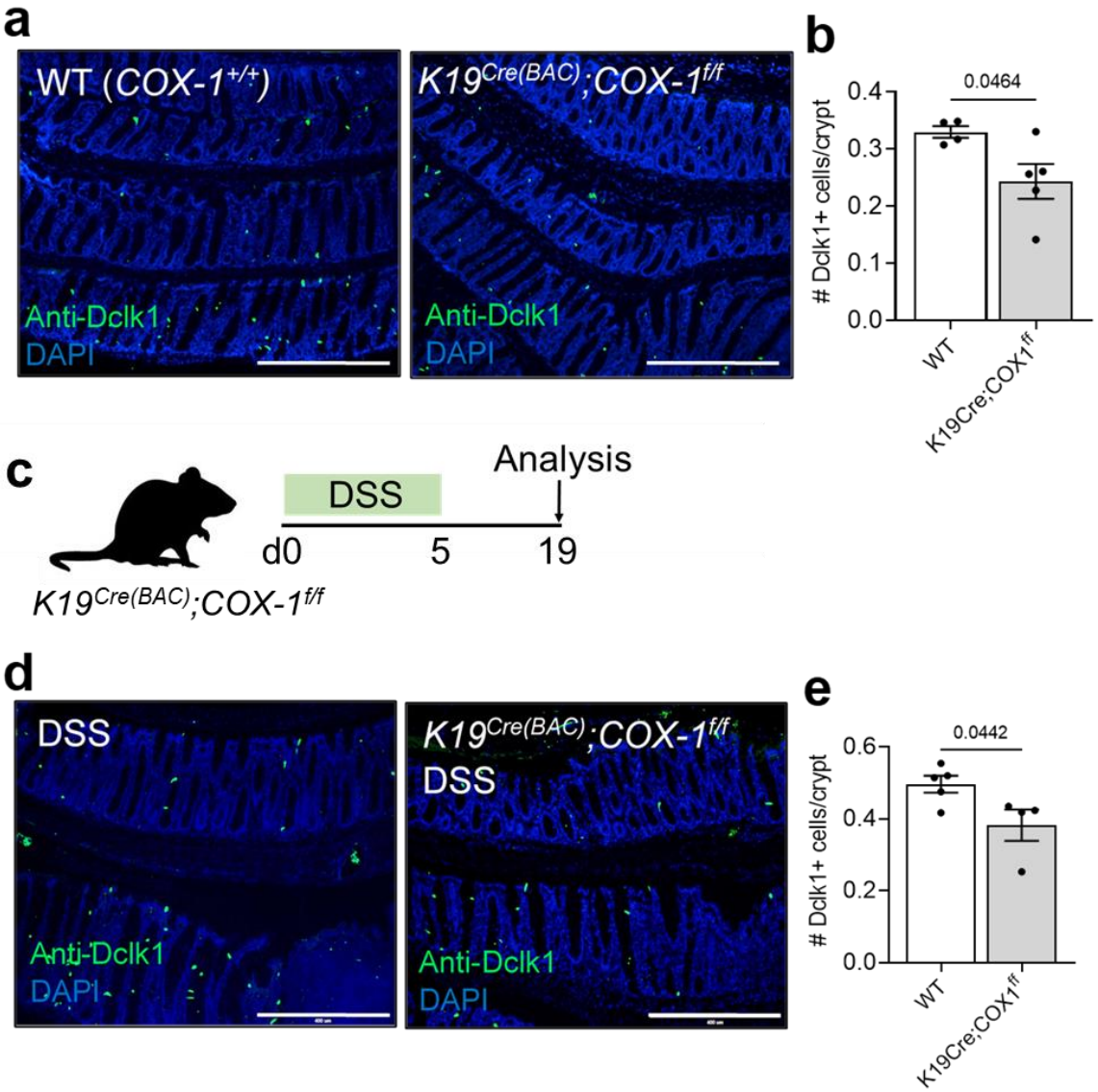

a
WT (COX-1+/+)
Anti-Dclk1
DAPI
K19Cre(BAC);COX-1f/f
Anti-Dclk1
DAPI
b
0.0464
Dclk1+ cells/crypt
0.4
0.3
0.2
0.1
0.0
WT
K19Cre;COX1ff
c
Analysis
DSS
d0
5
19
K19Cre(BAC);COX-1f/f
d
DSS
Anti-Dclk1
DAPI
K19Cre(BAC);COX-1f/f
DSS
Anti-Dclk1
DAPI
e
0.0442
Dclk1+ cells/crypt
0.6
0.4
0.2
0.0
WT
K19Cre;COX1ff

Figure 3.22 – COX-1-loss in intestinal epithelial cells reduces Dclk1+ cell number.

(**a**) Representative fluorescent images of anti-Dclk1+ immunofluorescent staining (green) of the colonic epithelium in wild-type (*COX-1*$^{+/+}$) or *K19*$^{Cre(BAC)}$*;COX-1*$^{f/f}$ mice. Scale bars = 400µm. (**b**) Quantification of the number of Dclk1+ cells per colonic crypt in the epithelium of wild-type (*COX-1*$^{+/+}$) or *K19*$^{Cre(BAC)}$*;COX-1*$^{f/f}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (*COX-1*$^{+/+}$, n=4; *K19*$^{Cre(BAC)}$*;COX-1*$^{f/f}$, n=5). (**c**) Schematic illustration of DSS treatment of wild-type (*COX-1*$^{+/+}$) or *K19*$^{Cre(BAC)}$*;COX-1*$^{f/f}$ mice. (**d**) Representative fluorescent images of anti-Dclk1+ immunofluorescent staining of the colonic epithelium (green) in DSS-treated wild-type (*COX-1*$^{+/+}$) or *K19*$^{Cre(BAC)}$*;COX-1*$^{f/f}$ mice at day 19. Scale bars = 400µm. (**d**) Quantification of the number of Dclk1+ cells per colonic crypt in the epithelium of DSS-treated wild-type (*COX-1*$^{+/+}$) or *K19*$^{Cre(BAC)}$*;COX-1*$^{f/f}$ mice at day 19. Data are presented as mean ± SEM and dots represent biologically independent animals (*COX-1*$^{+/+}$, n=5; *K19*$^{Cre(BAC)}$*;COX-1*$^{f/f}$, n=4). Statistical significance was determined using unpaired Student's t-test.

3.3.12 *Aspirin prevents Dclk1+ cell-derived tumor organoid growth through inhibition of COX-1.*

We next sought to investigate a role for COX in the growth of colitis-associated tumor organoids. To do this, we assessed the effect of various NSAIDs on the growth of tumor organoids cultured from Dclk1+ cell-derived colonic tumors over 7 days. Treatment with Aspirin and SC-560 resulted in a dose-dependent inhibitory effect on tumor organoid growth over time (**Figure 3.23a,b**). However, no effect was observed on tumor organoid growth upon treatment with either low or high doses of rofecoxib (**Figure 3.23c**). We did observe a reduction in organoid area at some time points with high doses of celecoxib (**Figure 3.23d**), however this may be due to loss of COX-2 selectivity. Taken together, these data suggest that COX-1 plays an important role in Dclk1+ cell-derived tumor organoid growth. To assess whether inhibition of organoid growth upon COX-1 inhibition may be due to downregulation of PGE_2, we tested the effects of exogenous PGE_2 administration on tumor organoid growth (**Figure 3.24a**). We detected a significant increase in tumor organoid size over time upon treatment with PGE_2 (**Figure 3.24b,c**), further supporting a role for PGE_2 in the pathogenesis of colitis-associated cancer.

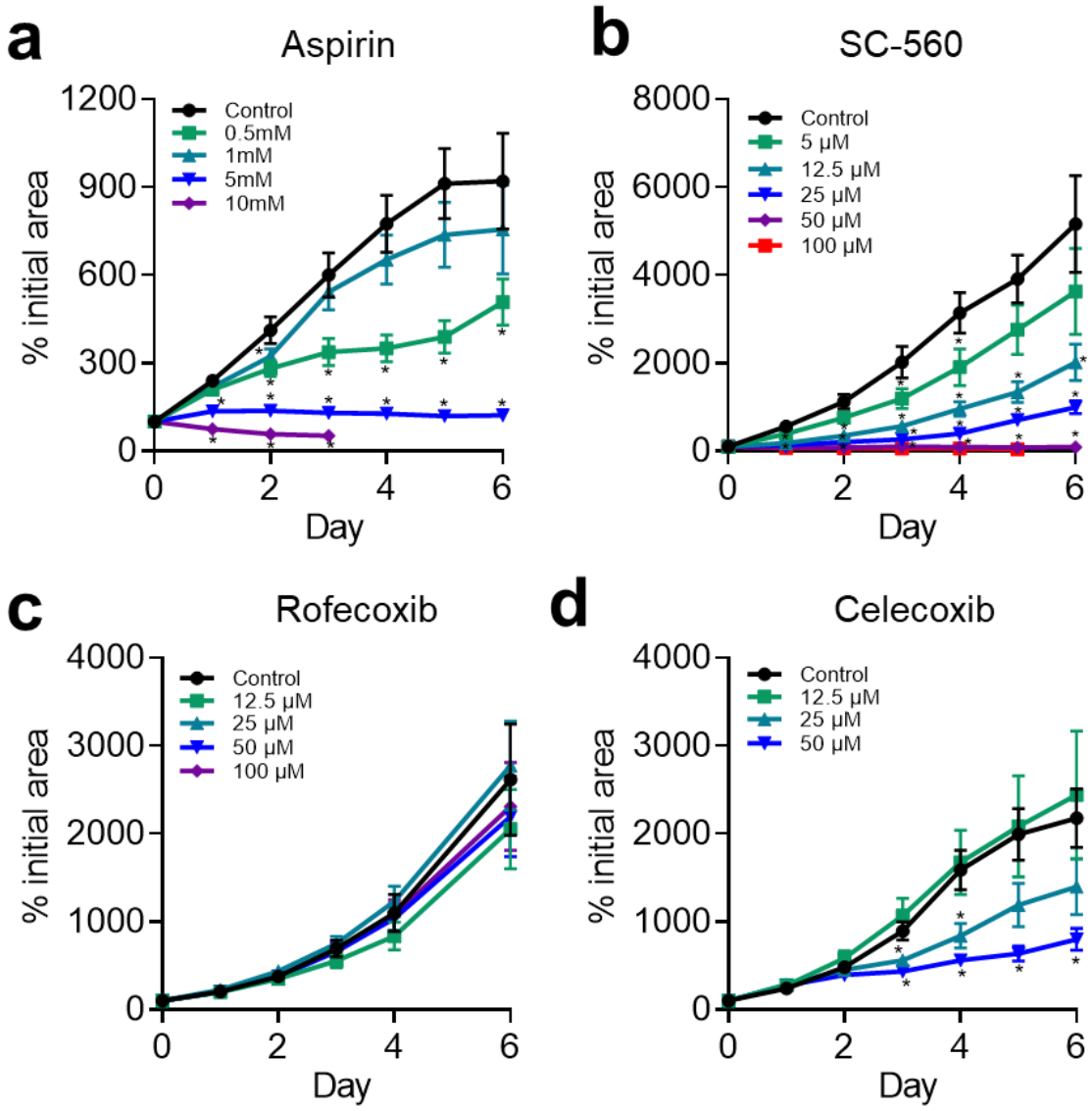
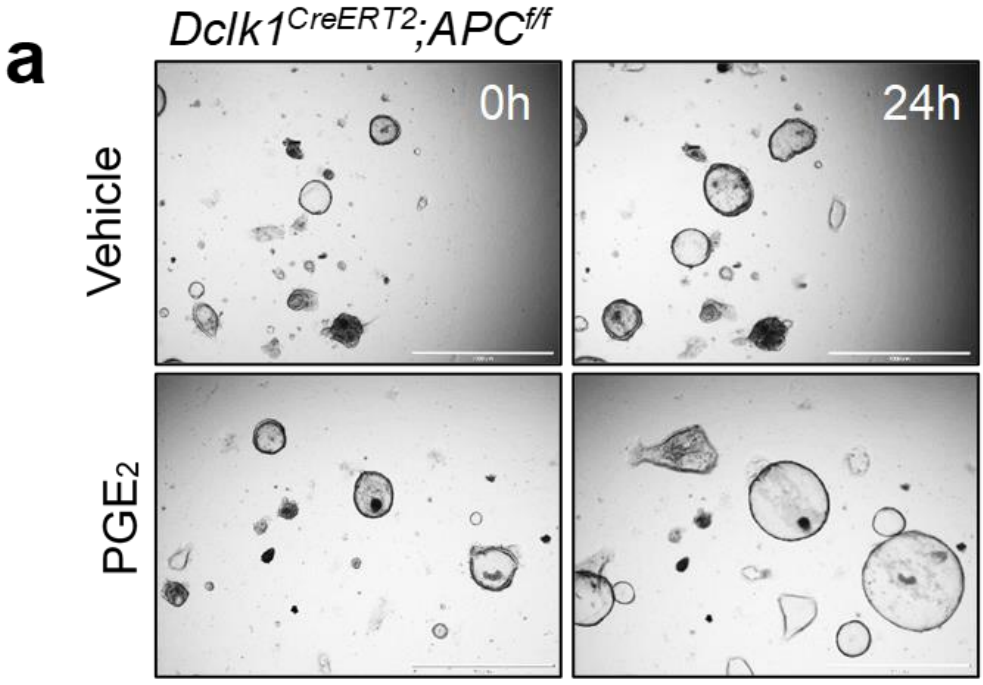

a
Aspirin
% initial area
Day
Control
0.5mM
1mM
5mM
10mM
b
SC-560
% initial area
Day
Control
5 μM
12.5 μM
25 μM
50 μM
100 μM
c
Rofecoxib
% initial area
Day
Control
12.5 μM
25 μM
50 μM
100 μM
d
Celecoxib
% initial area
Day
Control
12.5 μM
25 μM
50 μM

Figure 3.23 – COX-1 inhibition prevents Dclk1+ cell-derived tumor organoid growth.

(**a-d**) Quantification of the percent change in area of *Dclk1*^{CreERT2}*;APC*$^{f/f}$ tumor organoids treated with various doses of NSAIDs (Aspirin, SC-560, rofecoxib, or celecoxib) relative to vehicle-treated controls over time. Data are presented as mean ± SEM. (n=8-60 individual organoids per group across 3 technical replicates). Statistical significance was determined using one-way ANOVA with Tukey's post-hoc test. *$p<0.05$.


a
Dclk1CreERT2;APCf/f
0h
24h
Vehicle
PGE2

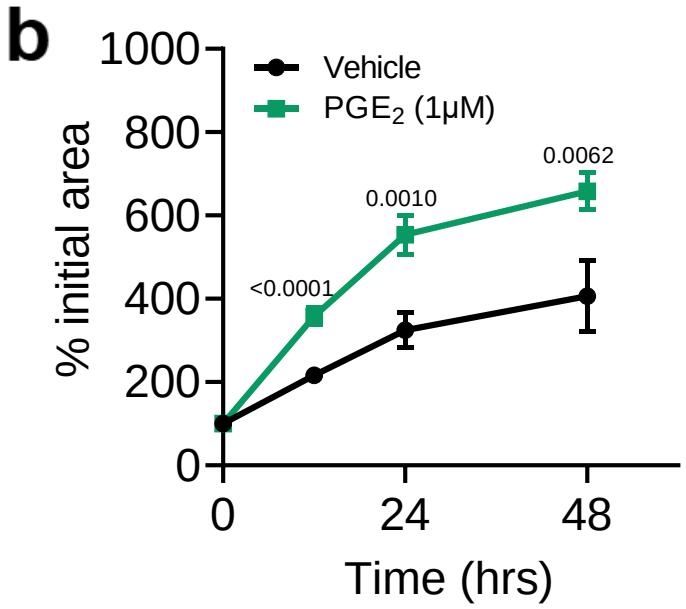

b
1000
800
600
400
200
0
% initial area
Vehicle
PGE2 (1μM)
0.0062
0.0010
<0.0001
0
24
48
Time (hrs)

Figure 3.24 – PGE_2 promotes Dclk1+ cell-derived tumor organoid growth.

(**a**) Representative brightfield images of *Dclk1*^{CreERT2}*;APC*$^{f/f}$ colonic tumor organoids treated with vehicle or PGE_2 (1µM). Scale bars = 1000µm. (**b**) Quantification of the percentage change in area of *Dclk1*^{CreERT2}*;APC*$^{f/f}$ tumor organoids treated with vehicle or PGE_2. Data are presented as mean ± SEM (vehicle, n=24; PGE_2; n=26 individual organoids across 3 technical replicates). Statistical significance was determined using unpaired Student's t-test.

3.4 Discussion

There are currently no effective chemopreventative strategies available for colitis-associated cancer. Numerous epidemiological studies have established that low-dose Aspirin is chemopreventative for sporadic colorectal cancer (Baron et al., 2003; Cole et al., 2009; Flossmann and Rothwell, 2007). However, the efficacy of Aspirin, particularly at low doses, has not been clearly evaluated in CAC. This has been largely due to limitations to NSAID use in patients with colitis attributable to safety concerns of high anti-inflammatory doses of Aspirin (Evans et al., 1997; Meyer et al., 2006; Allison et al., 1992). Here, we show that low-dose Aspirin is not only effective for the prevention of colitis-associated cancer, but also safe to use in IBD as it does not exacerbate colitis severity. This finding is consistent with a recent study showing that daily low-dose Aspirin does not negatively impact clinical outcomes in patients with IBD (Patel et al., 2021).

Previous studies have identified COX-2 as the most important COX isoform for the prevention of sporadic CRC (Gupta and DuBois, 2001; Oshima et al., 1996; Phillips et al., 2002). Accordingly, the chemopreventative effects of Aspirin in sporadic CRC, which is non-selective for COX-1 versus COX-2 (Vane and Botting, 2003), are attributed to its inhibition of COX-2 (Chan et al., 2007; Lee et al., 2020). In this study, we investigated whether the mechanism by which low-dose Aspirin prevents CAC is through inhibition of COX-1 and/or COX-2. Here, were we report the novel observation that the inhibition of COX-1, and not COX-2, results in prevention of inflammation-driven tumorigenesis. This result was confirmed in two different mouse models of CAC using both a pharmacologic and genetic approach. We report that while low-dose COX-2 inhibitors did not prevent the initiation of CAC, high (anti-inflammatory equivalent) doses of COX-2 inhibitors led to increased mortality in mice with colitis. The increased mortality observed in association with COX-2 inhibition in colitis is consistent with previous literature reporting that COX-2 inhibitors exacerbate colitis (Biancone et al., 2003; Hegazi et al., 2003; Ishikawa et al., 2011; Matuk et al., 2004; Zhang et al., 2008) and confirms that pharmacological inhibition of COX-2 is not feasible for the prevention of inflammation-driven tumorigenesis. Correlating with our findings, other studies have reported a critical role for COX-1 in

intestinal tumorigenesis, particularly in initiation and early polyp growth (Chulada et al., 2000; Oshima et al., 1996).

To investigate whether inhibition of COX-1 prevented colitis-associated tumorigenesis by downregulating prostaglandins during inflammation, we analyzed the effect of NSAIDs on COX-derived prostaglandins during acute colitis. Although COX-2 has been attributed to being the dominant source of inflammatory prostaglandins from studies using the air pouch model of granulomatous inflammation (Futaki et al., 1993; Vane et al., 1994), other studies have suggested an important role for COX-1 in inflammation and colitis (McAdam et al., 2000; Wallace et al., 1998). Here, we confirm that prostaglandins are upregulated in DSS-colitis and report that COX-1, as opposed to COX-2, is the predominant source of these prostaglandins during DSS-injury. These findings are consistent with those of Sacco et al., 2019 who also reported a critical role for COX-1 in prostaglandin synthesis during colitis (Sacco et al., 2019). Of the specific prostaglandins upregulated in DSS-colitis that also correlated with tumorigenesis, we focused on PGE_2, given the strong link between PGE_2 and colorectal cancer (see **Section 1.4.3**). However, we do recognize that the other COX-derived prostaglandins upregulated in DSS may also play a role in the pathogenesis of inflammation-associated cancer and should be investigated further in studies beyond the scope of this thesis.

Given that PGE_2 directly activates the cancer-related Akt pathway (Castellone et al., 2005; Peng et al., 2017; Wang et al., 2004), and that Akt is highly expressed in Dclk1+ cells (Chandrakesan et al., 2015), we next examined the role of both PGE_2 and Akt in colitis and cancer. Our data clearly shows that both PGE_2 and p-Akt are elevated in colitis, and that *COX*, *PTGES*, and *AKT3* expression are increased in patients with IBD, correlating with the presence of active colitis and the progression to neoplasia. Furthermore, our fate mapping studies of Dclk1+ cells demonstrate that PGE_2 and/or activation of Akt stimulate normally quiescent tuft cells to transform into facultative stem cells capable of lineage tracing the entire colonic crypt. This novel observation highlights the significance of these pathways for epithelial plasticity following injury, in both non-mutated and Apc-deficient tuft cells. This corresponds with studies by Cohn et al., 1997 which showed that in the setting of injury, PGE_2 that is derived from COX-1, and not COX-2, promotes epithelial

stem cell proliferation (Cohn et al., 1997). Our findings suggest that PGE_2 and Akt-induced epithelial plasticity was detected predominantly when Dclk1+ cells were APC-deficient, suggesting that PGE_2 and Akt act in combination with or enhance Wnt signaling to promote Dclk1+ cell proliferation.

In this study, we report the novel finding that the simultaneous presence of PGE_2 and Akt activation promoted transformation of Dclk1+ cells to initiate cancer in the setting of epithelial injury. These data suggest that PGE_2 and Akt activation during colitis may be the "second-hit" required to initiate tumorigenesis from a mutated epithelial tuft cell. Interestingly, previous studies have shown that both PGE_2 and phospho-Akt can lead to the downstream nuclear translocation of β-catenin and subsequent activation of Wnt signaling (Castellone et al., 2005; Goessling et al., 2009), which is known to be aberrantly upregulated in both sporadic and colitis-associated cancers. Interestingly, we identified that the upregulation of COX and Akt related genes preceded aberrant Wnt activation in the pathogenesis of CAC. Furthermore, Hayakawa et al., 2017 showed that GPCR signaling was required to stimulate nuclear β-catenin in Apc-deficient cells (Hayakawa et al., 2017). As PGE_2 acts through binding to one of its four G-protein coupled receptors (GPCRs), EP1-4, we investigated whether PGE_2 and Akt contribute to tumorigenesis by driving canonical Wnt signaling. Indeed, we found that the combination of PGE_2 and Akt activation drives nuclear localization of β-catenin in Apc-deficient Dclk1+ cell lineage traced crypts, supporting the notion that PGE_2 and Akt act together to enhance Wnt signaling. Previous data has shown that EP4 is a predominant receptor expressed in the epithelium of human (Olsen Hult et al., 2011) and mice (Lejeune et al., 2010; Morimoto et al., 1997), particularly in the setting of colitis. Therefore, we propose a novel mechanism by which epithelial COX-1-derived PGE_2 acts in a paracrine manner in the setting of concurrent Akt activation to initiate tumorigenesis from an Apc-deficient epithelial cell during colitis. Interestingly, we further identified that PGE_2 promotes the growth of colitis-associated tumor organoids, and this growth is prevented by COX-1 inhibition. This suggests that COX-1-derived PGE_2 may also contribute to tumor growth in the pathogenesis of CAC.

Roulis et al., 2020 previously reported that fibroblast derived PGE_2 contributes to sporadic colorectal cancer (Roulis et al., 2020). Our data now proves that PGE_2 also plays a critical role in the initiation of colitis-associated tumorigenesis. In contrast to Roulis et al., however, our data point to PGE_2 derived from epithelial COX-1, rather than fibroblast COX-2, as being most important for CAC. Thus, we have identified critical differences in the pathogenesis of colitis-associated cancer compared to sporadic colon cancer, with differing cellular and enzymatic sources of PGE_2 contributing to tumorigenesis.

We further identified that Aspirin treatment or genetic loss of epithelial COX-1 led to a reduced number of Dclk1+ cells in the colonic epithelium. This suggests that Aspirin may also contribute to chemoprevention by reducing the number of tumor-initiating cells available for tumorigenesis. These findings suggest that COX-1 may be required for tuft cell viability or the differentiation of progenitor cells towards the tuft cell lineage, however further experiments are required to definitively determine the role for COX in tuft cell homeostasis.

Taken together, our data clearly demonstrate that low-dose Aspirin is an effective chemopreventative strategy for colitis-associated cancer. Furthermore, we have identified a novel mechanism for colitis-associated tumorigenesis in which COX-1-derived PGE_2 and activation of Akt signaling co-operate to promote colonic tumor initiation. Interestingly, this is consistent with the observation that regular Aspirin use in patients with CRC increases survival and improves prognosis, particularly in a subset of patients with mutations in the Akt signaling pathway (Liao et al., 2012). The upregulation of both COX-1-derived PGE_2 and p-Akt in colitis leads to the activation and dedifferentiation of some Apc-deficient Dclk1+ tuft cells to a stem cell state, leading to tumor initiation. We propose that this process is mediated, in part, through the promotion of nuclear translocation of β-catenin in tuft cells and activation of canonical Wnt signaling. Our novel findings prove that Aspirin prevents colitis-associated cancer by inhibiting the production of COX-1-derived PGE_2 in colitis. Most importantly, we highlight that this effect of low-dose Aspirin does not exacerbate colitis, pointing to the safety of this effective chemopreventative strategy in patients with IBD.

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Chapter 4

4 NF-κB Signaling in Dclk1+ Tuft Cells Affects Colitis Severity and Colitis-Associated Cancer

The text and figures from this chapter have been adapted from the following manuscript to conform to the format of this thesis:

Good HJ*, Shin AE*, Zhang L, and Asfaha S. NF-κB signaling in Dclk1+ cells is protective against colitis and colitis-associated cancer. (In preparation). *HJG and AES are co-first authors on this manuscript.

*Experiments in Figures 4.1, Figure 4.2, and Appendix 5 were performed by AES. DNA PCR assay in Appendix 6 was performed by LZ.

4.1 Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death in Canada (Brenner et al., 2020). A major risk factor for CRC is chronic inflammation, which is primarily seen in patients with Inflammatory Bowel Disease (IBD). Therefore, patients with IBD, such as Crohn's Disease or Ulcerative Colitis, are at an increased risk for colitis-associated cancer (CAC) (Eaden et al., 2001; Ekbom et al., 1990; Kraus and Arber, 2009; Rutter et al., 2004; Shanahan, 2001). Despite the strong association between inflammation and cancer, the mechanism by which colitis leads to CAC remains largely unknown. A key signaling pathway that has been shown to play a major role in both colitis and CRC is the NF-κB pathway.

The NF-κB signaling pathway is an evolutionarily conserved pathway that triggers and coordinates inflammatory, immune, and anti-apoptotic responses (Karin and Lin, 2002). Signaling in this pathway is driven by a series of homo- and heterodimer transcription factors consisting of the p65 (RelA), p50 (p105), p52 (p100), c-Rel, and/or RelB subunits. These dimers are inactivated and retained in the cytoplasm by IκB inhibitory proteins. Upon stimulation of the NF-κB signaling pathway, IκB inhibitor proteins are tagged for proteasomal degradation by the IKK-complex allowing for the NF-κB dimers to translocate to the nucleus (Pasparakis, 2009). The IKK-complex consists of the catalytic IKKα and IKKβ subunits, and the regulatory NEMO/IKKγ subunit. Canonical NF-κB signaling is activated by various extracellular pro-inflammatory signals such as luminal bacteria-producing LPS or inflammatory cytokines (e.g. TNF-α, IL-1β), and results in IKKβ-mediated degradation of IκBα and the nuclear accumulation of p50, p65, and c-Rel dimers (Dejardin et al., 2002; Pasparakis, 2009; Senftleben et al., 2001). Canonical NF-κB signaling leads to the upregulation of a wide range of genes involved in inflammation, immunity, cell adhesion, antioxidant processes, cell survival, and cell proliferation (Ghosh and Karin, 2002).

Aberrant canonical NF-κB activation has been detected in colonic tissue samples from patients with active CD or UC (Ellis et al., 1998; Rogler et al., 1998; Schreiber et al., 1998) and the degree of NF-κB activation has been shown to correlate with disease severity (Han

et al., 2017). Furthermore, constitutive activation of canonical NF-κB signaling has been detected in human colorectal cancer (Hardwick et al., 2001; Lind et al., 2001; Voboril and Weberova-Voborilova, 2006), and is associated with tumor progression and poor prognosis in patients with CRC (Kojima et al., 2004; Lin et al., 2012). These data suggest that NF-κB signaling serves as a key link between inflammation and cancer. The first study examining this relationship in the gut was by Greten et al., 2004 who showed that inhibition of canonical NF-κB signaling through intestinal epithelial cell-specific IKKβ-loss resulted in fewer colonic tumors in a mouse model of CAC (Greten et al., 2004). A major limitation, however, was the lack of identification of the intestinal epithelial cell responsible for this effect.

In sporadic CRC, it was previously shown that Lgr5+ stem cells can serve as the cell of origin upon loss of the tumor suppressor and negative Wnt regulator, APC (Barker et al., 2009). In the case of CAC, however, a cell-of-origin has not been identified. Importantly, Lgr5+ cells are sensitive to injury and are dispensable for gut regeneration (Tian et al., 2011), while non-stem cells can display cellular plasticity and acquire stemness to regenerate the epithelium during intestinal damage (Asfaha et al., 2015; Ayyaz et al., 2019; Castillo-Azofeifa et al., 2019; Murata et al., 2020; Yui et al., 2018). These observations suggest that, in the setting of inflammatory or intestinal injury, non-stem cells harbouring mutations can potentially serve as the cellular origin of cancer. Our previous work examined the role of long-lived, mature quiescent tuft cells that are found throughout the gastrointestinal epithelium and marked by expression of *Dclk1*. We found that these normally quiescent post-mitotic Dclk1+ tuft cells can act as facultative stem cells and initiate tumor formation upon APC-loss and colitis injury (Westphalen et al., 2014). Interestingly, Schwitalla et al., 2013 further showed that the ability of non-stem cells to initiate tumors was dependent on NF-κB activation (Schwitalla et al., 2013). However, the role for NF-κB signaling in tuft cells and whether this pathway contributes to the initiation of Dclk1+ cell-derived colitis-associated cancer has not been examined. Thus, the aim of this study was to investigate the role of canonical NF-κB signaling in Dclk1+ cell-derived colitis-associated cancer.

Specifically, we generated two separate transgenic mouse models in order to activate or inhibit NF-κB signaling. In the first model, IKKβ was constitutively activated in Dclk1+ tuft cells, whereas, in the second model, NF-κB signaling was inhibited in Dclk1+ cells. Our findings demonstrate that constitutive activation of IKKβ in tuft cells is protective against both colitis and CAC, while IKKβ inhibition promotes colonic inflammation and the initiation of CAC. Our findings highlight the dual pro- and anti-inflammatory roles for NF-κB signaling in the intestine and demonstrate a critical role for Dclk1+ tuft cells in the regulation of inflammatory responses and initiation of CAC.

4.2 Materials & Methods

4.2.1 *Experimental Mouse Models*

Dclk1^{CreERT2} mice crossed to *APC*$^{f/f}$ and *Rosa26*^{tdTomato} strains as previously described (Westphalen et al., 2014). *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice were further crossed to either *R26*$^{IKK2ca\text{-}eGFP}$ (JAX#008242) (Sasaki et al., 2006) or *IKKβ*$^{f/f}$ mice (Park et al., 2002). Tamoxifen was administered by oral gavage (3 doses of 6mg q.a.d) to induce Cre recombinase activity in *Dclk1*-expressing cells. Mice were housed in 12hr-light/12hr-dark cycles with controlled temperature and humidity. All animal procedures were performed in accordance with the Animal Care and Use Committee at The University of Western Ontario. Further information on these mouse models is provided in **Section 2.2**.

4.2.2 *DSS Colitis Model*

To induce experimental colitis, 6-8-week old mice were treated with 2-2.5% (wt/vol) DSS (molecular weight 36 000 – 50 000 kDa, Gojira) in the drinking water for 5 days (see

Section 2.4 for further information). Mice were analyzed 3 days post-DSS for acute studies and 14 weeks post-DSS for tumor experiments.

4.2.3 *Myeloperoxidase (MPO) Assay*

Colonic tissue was collected for measurement of MPO activity as previously described (Kim, 2012; Krawisz, 1984). Briefly, fresh colonic tissue was collected, weighed, and homogenized using the Bullet Blender 5 Storm (Next Advance) in hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM potassium phosphate buffer, pH 6.0). Tissue homogenates were spun down at 13,400 rpm for 6 minutes at 4°C. Supernatant was collected and 7µL of each sample was loaded in triplicate to a 96-well plate. Hydrogen peroxide (0.0005%) was added to freshly made *O*-dianisidine dihydrochloride solution buffer (0.167mg/mL o-dianisidine in 50mM potassium phosphate buffer, pH 6.0), and was added to each well containing sample. Immediately, the Perkin Elmer Wallac 1420 Victor2 Microplate Reader (GMI) was used to measure absorbance at 450nm. MPO activity was calculated as units of MPO activity per mg of tissue. See **Section 2.8** for further information.

4.2.4 *RNA Extraction & qRT-PCR*

Colonic tissue was collected and lysed in TRIzol (Life Technologies) using the Bullet Blender 5 Storm (Next Advance) and 0.5mm RNase Free Stainless Steel Beads (Next Advance). RNA was isolated according to the manufacturer's instructions and purified with 8M lithium chloride as described previously (Viennois et al., 2013). RNA concentration was determined using the NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). iScript cDNA Synthesis Kit (Bio-Rad) was used to synthesize complementary DNA (cDNA) and qRT-PCR was performed in triplicate using PowerUp SYBR Green Master Mix (Thermo Fisher) and ViiA QuantStudio 5 (Thermo Fisher). Relative expression values were determined using the delta delta CT

method and were normalized to the housekeeping gene *Gapdh*. See **Table 2.5** for complete list of primers used and **Section 2.7** for further information.

4.2.5 *Histology, Immunohistochemistry, & Immunofluorescence*

Colonic tissue was collected and fixed in 4% paraformaldehyde for 6-8 hours at 4°C. Tissues were cryoprotected using 30% sucrose in 1xPBS for 24 hours at 4°C and embedded in OCT compound (Sakura) in dry ice. Tissues were sectioned at 5µm using the Leica CM3050 cryostat (Leica Biosystems). For endogenous fluorescence, tissue sections were rehydrated for 5 minutes in PBS and mounted using Vectashield Mounting Medium containing DAPI (Vector Laboratories). For paraffin embedded tissues, tissues were fixed in 10% formalin solution (Fisher) overnight at 4°C, embedded in paraffin, and sectioned at 5µm onto glass slides. For H&E, tissue sections were deparaffinized, rehydrated, and stained using CAT hematoxylin (Biocare Medical) and Eosin Y (Sigma). Stained tissues were rehydrated and mounted using Permount (Thermo Fisher Scientific). For immunofluorescence staining using FFPE sections, antigen retrieval was performed by boiling slides in Tris-EDTA buffer (pH 9.0) in the microwave for 12 minutes. Slides were rinsed in PBS, incubated with blocking solution (5% normal goat serum, Vector Laboratories) for 30 minutes, followed by incubation with primary antibodies overnight at 4°C. Secondary antibodies were applied for 1 hour at room temperature. Slides were rinsed with PBS and mounted using Vectashield Mounting Medium containing DAPI (Vector Laboratories). For immunohistochemistry using FFPE sections, antigen retrieval was performed as described above, followed by incubation in 3% hydrogen peroxide to quench endogenous peroxidase activity. Staining was performed according to the manufacturer's instructions for the R.T.U. Vectastain Universal Elite ABC Kit (Vector Laboratories) and ImmPACT DAB Substrate Kit (Vector Laboratories). Slides were counter-stained using CAT hematoxylin, rehydrated, and mounted using Permount. See **Table 2.4** for complete list of antibodies used and refer to **Section 2.5** and **2.6** for further information.

4.2.6 *Intestinal Organoid Cultures*

Intestinal organoids were cultured as previously described (Sato et al., 2009). Detailed protocol description can be found in **Section 2.9**. Organoids were grown in Matrigel (Corning) and allowed to polymerize at 37°C before adding media. Media used was Dulbecco's Modified Eagle's Medium/F12 containing N2 supplement (1x**;** Gibco), B27 supplement (1x; Thermo Fisher Scientific), *N*-acetylcysteine (1 µM; Sigma), Glutamax (1x; Thermo Fisher), HEPES (10µM; Gibco), and penicillin/streptomycin (500 µg/mL, Thermo Fisher Scientific), which was refreshed every 4-5 days. Growth factors were added to the media every 2 days: EGF (50 ng/ml, Thermo Fisher Scientific), mNoggin (100 ng/ml, Peprotech), and R-Spondin (1 µg/ml). For experiments, organoids were treated with 4-hydroxytamoxifen (100nM; Sigma) which was added to conditioned media for 48 hours. Following 4-hydroxytamoxifen treatment, organoids were washed in warm PBS, followed by replacement with fresh conditioned media and growth factors.

4.2.7 *DNA PCR Assay*

Colonic tumor tissue was collected and snap frozen if not processed immediately. Genomic DNA (gDNA) was extracted from 25mg of colonic tissue using the DNeasy Blood & Tissue Kit (Qiagen, 69504) following manufacturer's instructions. Template gDNA (400ng) was added to 20µL of 1xPCR Taq FroggaMix (FroggaBio, FBTAQM) and amplified using a thermocycler. PCR product (12.5µL) was run on 2% agarose gel containing ethidium bromide. Gels were visualized using a GelDoc UV Trans Illuminator (Bio-Rad). DNA sequencing was performed to confirm PCR bands.

4.2.8 *Imaging*

Imaging was performed using the EVOS FL Auto Imaging System (Thermo Fisher).

4.2.9 *Statistics*

All data are presented as ± SEM, unless indicated otherwise. GraphPad Prism, version 9.0 (GraphPad Software, Inc.) as use for statistical analysis. Unpaired Student's t-test was used when comparing 2 groups, one-way ANOVA was used when comparing 3 or more groups, and two-way ANOVA when comparing groups with 2 independent variables. Data was considered statistically significant when $p < 0.05$.

4.3 Results

4.3.1 *Tuft cell-specific NF-κB activation prevents CAC.*

To examine the role of NF-κB in Dclk1+ cell-derived colitis associated cancer, we first determined the effects of tuft cell-specific NF-κB activation on tumorigenesis. Our previously generated *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice were crossed to *R26*$^{IKK2ca\text{-}eGFP}$ mice. Resulting *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26*$^{IKK2ca\text{-}eGFP}$ mice allowed for selective and sustained activation of NF-κB canonical signaling in Dclk1+ cells through constitutive activation of the IKKβ (inhibitor of IκB kinase 2) (Sasaki et al., 2006) and corresponding eGFP expression. To confirm successful recombination and activation of NF-κB signaling in Dclk1+ cells, tamoxifen-treated *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26*$^{IKK2ca\text{-}eGFP}$ mice expressing 2 copies of the IKK2ca-eGFP allele (*IKK2ca*$^{mut/mut}$) were analyzed by immunofluorescence for the detection of epithelial GFP+ cells. We identified the presence of GFP+ cells in the colonic crypt with analogous localization and morphology to tuft cells labelled in *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26*^{tdTomato} mice (**Figure 4.1a**). To next analyze the effects of NF-κB activation in Dclk1+ cells on colitis-associated tumorigenesis, we subjected *IKK2ca*$^{mut/mut}$ mice to our previously established model of CAC. *Dclk1*^{CreERT2}*;APC*$^{f/f}$ (*IKK2*$^{+/+}$), *IKK2ca*$^{+/mut}$, and *IKK2ca*$^{mut/mut}$ mice were treated with 2.5% dextran sodium sulfate (DSS) in the drinking water for 5 days to induce experimental colitis and tumorigenesis (**Figure 4.1b**). As previously described, colonic tumors were detected 16-weeks post-DSS in 100% of *IKK2*$^{+/+}$ mice. However, we observed a significant reduction in colonic tumor number in both *IKK2ca*$^{+/mut}$ and *IKK2ca*$^{mut/mut}$ mice relative to *IKK2*$^{+/+}$ controls (**Figure 4.1c**). There was no difference in tumor size, colon length, or body weight amongst any of the groups (**Figure 4.1d, Appendix 5**). Taken together, these data suggest that canonical NF-κB signaling in Dclk1+ cell is protective against colitis-associated tumorigenesis.

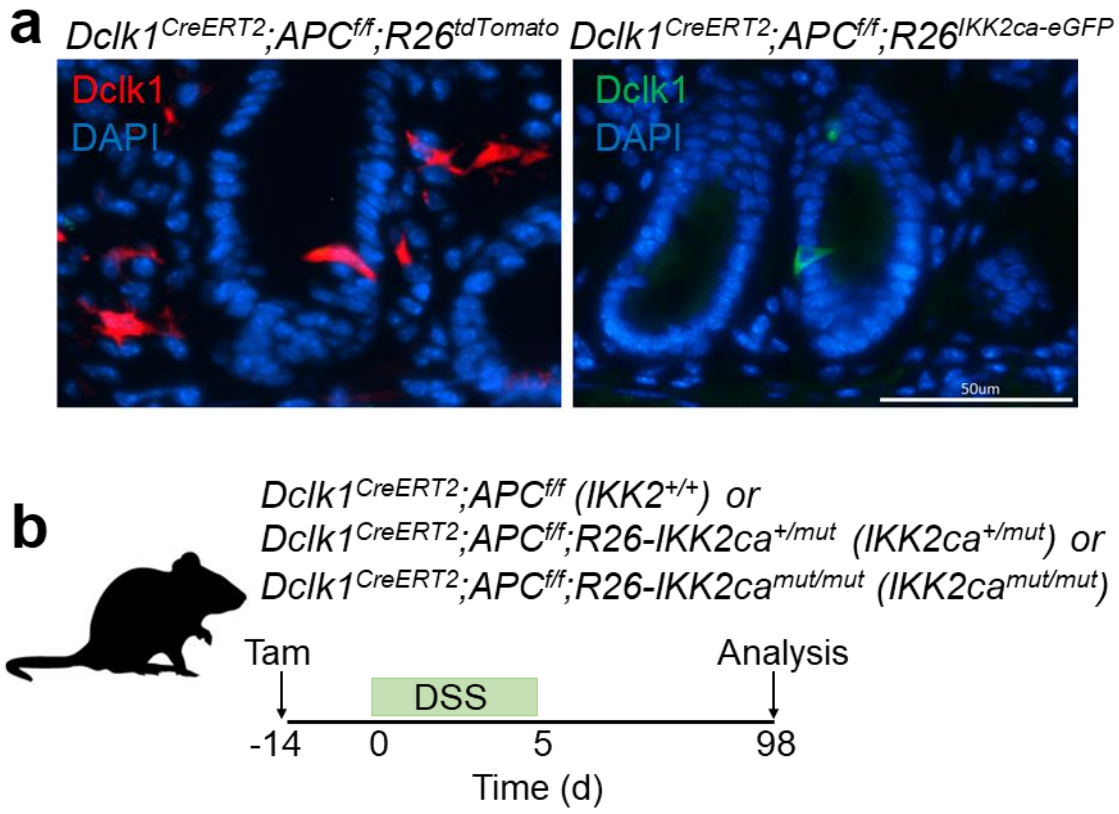

a
Dclk1CreERT2;APCf/f;R26tdTomato
Dclk1CreERT2;APCf/f;R26IKK2ca-eGFP
Dclk1
DAPI
Dclk1
DAPI
50um
b
Dclk1CreERT2;APCf/f (IKK2+/+) or
Dclk1CreERT2;APCf/f;R26-IKK2ca+/mut (IKK2ca+/mut) or
Dclk1CreERT2;APCf/f;R26-IKK2camut/mut (IKK2camut/mut)
Tam
Analysis
DSS
-14
0
5
98
Time (d)
c
# of tumors/mouse
0.0001
0.0443
IKK2+/+
IKK2ca+/mut
IKK2camut/mut
d
Avg tumor size (mm2)
0.9559
IKK2+/+
IKK2ca+/mut
IKK2camut/mut

Figure 4.1 – Constitutive IKKβ activation in tuft cells prevents CAC initiation.

(**a**) Representative immunofluorescent images of labeled Dclk1-expressing tuft cells in the colonic epithelium of *Dclk1*^{CreERT2}*;Rosa26*^{tdTomato}*;APC*$^{f/f}$ (left, red) and *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26*^{IKK2ca}-eGFP (right, green) mice at 48 hours post-tamoxifen. (**b**) Schematic illustration of the treatment of *Dclk1*^{CreERT2}*;APC*$^{f/f}$ *(IKK2*$^{+/+}$*),* *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26-IKK2ca*$^{+/mut}$ (*IKK2ca*$^{+/mut}$) or *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26-IKK2ca*$^{mut/mut}$ *(IKK2ca*$^{mut/mut}$*)* with DSS followed by analysis at day 98. (**c**) Average number of colonic tumors in *IKK2*$^{+/+}$, *IKK2ca*$^{+/mut}$, and *IKK2ca*$^{mut/mut}$ mice after DSS-treatment. Data are presented as mean ± SEM and dots represent biologically independent animals (*IKK2*$^{+/+}$, n = 6; *IKK2ca*$^{+/mut}$, n = 4; *IKK2ca*$^{mut/mut}$, n = 18). (**d**) Average colonic tumor size of *IKK2*$^{+/+}$, *IKK2ca*$^{+/mut}$, and *IKK2ca*$^{mut/mut}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (*IKK2*$^{+/+}$, n = 7; *IKK2ca*$^{+/mut}$, n = 2; *IKK2ca*$^{mut/mut}$, n = 5). Statistical significance was determined by one-way ANOVA with Tukey post-hoc test.

4.3.2 *Activation of NF-κB in tuft cells reduces DSS-colitis severity.*

As the risk of CAC correlates with the severity and duration of inflammation (Rutter et al., 2004), and given NF-κB is an inflammation-associated pathway, we next sought to determine whether Dclk1+ cell-specific NF-κB activation affected the degree of acute DSS-colitis. To do this, *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26*$^{IKK2ca\text{-}eGFP}$ (*IKK2ca*$^{mut/mut}$) mice were treated with DSS for 5 days and analyzed at the peak of inflammation on day 8 (**Figure 4.2a**). Consistent with the presence of colitis, both *Dclk1*^{CreERT2}*;APC*$^{f/f}$ (*IKK2*$^{+/+}$) and *IKK2ca*$^{mut/mut}$ mice treated with DSS showed reduced colon length, reduced body weight, increased MPO activity, and increased histological damaged relative to untreated controls (**Figure 4.2b-f**). However, DSS-treated *IKK2ca*$^{mut/mut}$ mice showed significantly reduced MPO activity and histologic damage as compared to DSS-treated *IKK2*$^{+/+}$ mice (**Figure 4.2b-f**), indicating that constitutive NF-κB activation in tuft cells results in reduced colitis severity. These data suggest that Dclk1+ cell-specific NF-κB signaling is protective against DSS-colitis.

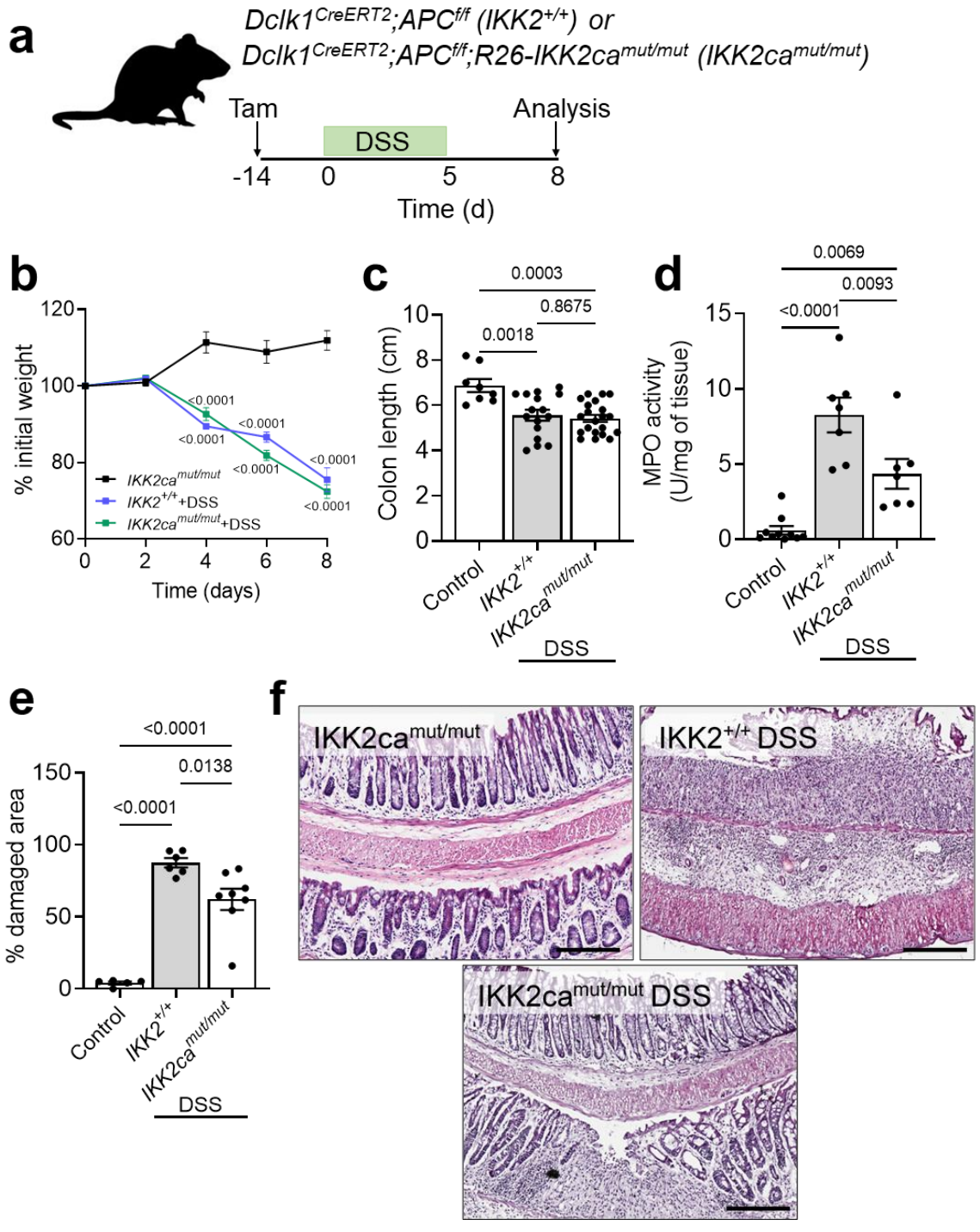

a
Dclk1CreERT2;APCf/f (IKK2+/+) or
Dclk1CreERT2;APCf/f;R26-IKK2camut/mut (IKK2camut/mut)
Tam
Analysis
DSS
-14
0
5
8
Time (d)
b
% initial weight
Time (days)
IKK2camut/mut
IKK2+/+DSS
IKK2camut/mut DSS
<0.0001
c
Colon length (cm)
0.0003
0.8675
0.0018
Control
IKK2+/+
IKK2camut/mut
DSS
d
MPO activity (U/mg of tissue)
0.0069
0.0093
<0.0001
e
% damaged area
<0.0001
0.0138
f
IKK2camut/mut
IKK2+/+ DSS
IKK2camut/mut DSS

Figure 4.2 – Constitutive IKKβ activation in tuft cells reduces DSS-colitis severity.

(a) Schematic illustration of the treatment of *Dclk1*^{CreERT2};*APC*$^{f/f}$ *(IKK2*$^{+/+}$*)* and *Dclk1*^{CreERT2};*APC*$^{f/f}$;*R26-IKK2ca*$^{mut/mut}$ *(IKK2ca*$^{mut/mut}$*)* with DSS followed by analysis at day 8. (**b**) Body weight changes of *IKK2*$^{+/+}$ and *IKK2ca*$^{mut/mut}$ mice during acute DSS-colitis as compared to untreated *IKK2ca*$^{mut/mut}$ mice. Data are presented as mean ± SEM (*IKK2ca*$^{mut/mut}$, n = 6; *IKK2*$^{+/+}$+ DSS, n = 7; *IKK2ca*$^{mut/mut}$ + DSS, n = 8). (**c**) Average colon length of DSS-treated *IKK2*$^{+/+}$ or *IKK2ca*$^{mut/mut}$ mice at day 8. Data are presented as mean ± SEM and dots represent biologically independent animals (control; n = 8; *IKK2*$^{+/+}$, n = 16; *IKK2ca*$^{mut/mut}$, n = 21). (**d**) Measurement of myeloperoxidase (MPO) activity in colonic tissue of DSS-treated *IKK2*$^{+/+}$ or *IKK2ca*$^{mut/mut}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (control; n = 10; *IKK2*$^{+/+}$, n = 7; *IKK2ca*$^{mut/mut}$, n = 7). (**e**) Quantification of the percentage of damaged histological area in DSS-treated *IKK2*$^{+/+}$ or *IKK2ca*$^{mut/mut}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (control; n = 5; *IKK2*$^{+/+}$, n = 6; *IKK2ca*$^{mut/mut}$, n = 8). (**f**) Representative images of hematoxylin and eosin staining of colonic tissue of untreated *IKK2ca*$^{mut/mut}$ control mice, DSS-treated *IKK2*$^{+/+}$ mice, and DSS-treated *IKK2ca*$^{mut/mut}$ mice during acute colitis. Scale bars = 200µm. Statistical significance was determined by one-way ANOVA (for panel c-e) or two-way ANOVA (for panel b) with Tukey post-hoc tests.

4.3.3 *Tuft cell-specific NF-κB inhibition promotes CAC*

To further investigate the role of NF-κB signaling in colitis-associated cancer, we next sought to examine the effect of NF-κB inhibition in tuft cells on tumorigenesis. We crossed *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice to *IKKβ*$^{f/f}$ mice to generate resultant *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;IKKβ*$^{f/f}$ (*IKKβ*$^{f/f}$) pups. These *IKKβ*$^{f/f}$ mice allow for tuft cell-specific loss of IKKβ, which thereby prevents the phosphorylation of IκB and subsequently results in inhibition of canonical NF-κB signaling in tuft cells (Greten et al., 2004). *Dclk1*^{CreERT2}*;APC*$^{f/f}$ (*IKKβ*$^{+/+}$) and *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;IKKβ*$^{+/f}$ (*IKKβ*$^{+/f}$) mice were used as controls harboring wild-type IKKβ and heterozygous loss of IKKβ, respectively. Mice were administered 2 to 2.5% DSS in the drinking water for 5 days to induce experimental colitis and tumorigenesis (**Figure 4.3a**). At 14-weeks post-DSS, homozygous loss of IKKβ in Dclk1+ cells significantly increased the number of Dclk1+ cell-derived colonic tumors as compared to *IKKβ*$^{+/+}$ mice (**Figure 4.3b**). There was no significant change in tumor size or survival across any of the groups (**Figure 4.3c,d**). Interestingly, *IKKβ*$^{f/f}$ and *IKKβ*$^{+/f}$ mice showed reduced colon length and a trending increase in colonic MPO activity as compared to *IKKβ*$^{+/+}$ controls upon analysis at 16 weeks (**Figure 4.3e,f**), suggesting that there may be sustained colonic inflammation post-DSS in mice with tuft cell IKKβ loss. To confirm loss of IKKβ in our mouse model, colonic tumor DNA was collected from each group and was analyzed by PCR to confirm the presence and/or absence of the floxed exon 3 of *Ikbkb* (gene encoding IKKβ). Indeed, the knockout PCR product was detected in tumor DNA from mice homozygous for IKKβ loss (**Appendix 6)**. We further detected a significant reduction in *Ikbkb* mRNA expression in colonic tumors derived from *IKKβ*$^{f/f}$ mice relative to tumors derived from *IKKβ*$^{+/+}$ mice (**Appendix 6**). Taken together, these data prove that IKKβ in Dclk1+ tuft cells is protective against colitis-associated tumorigenesis.

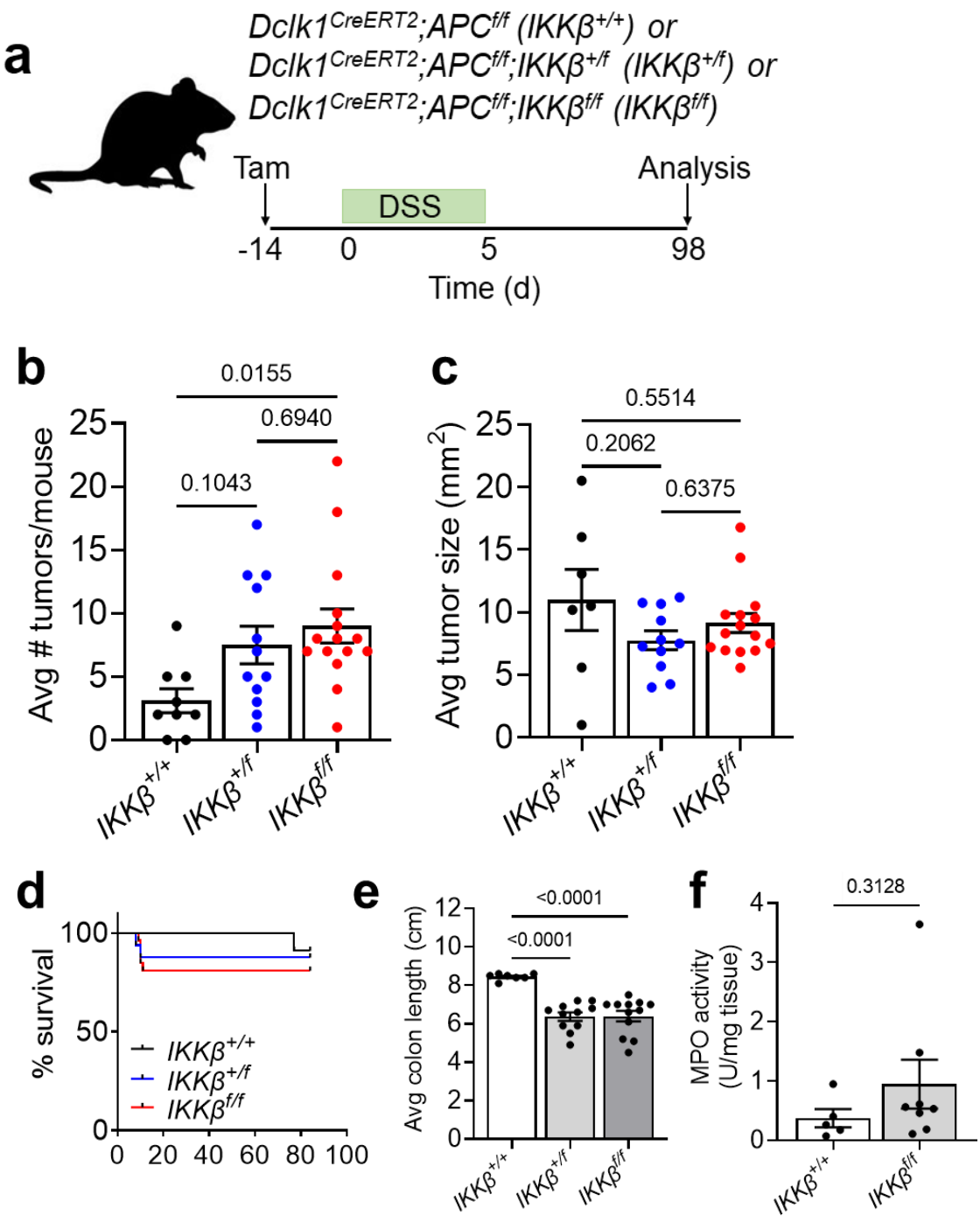
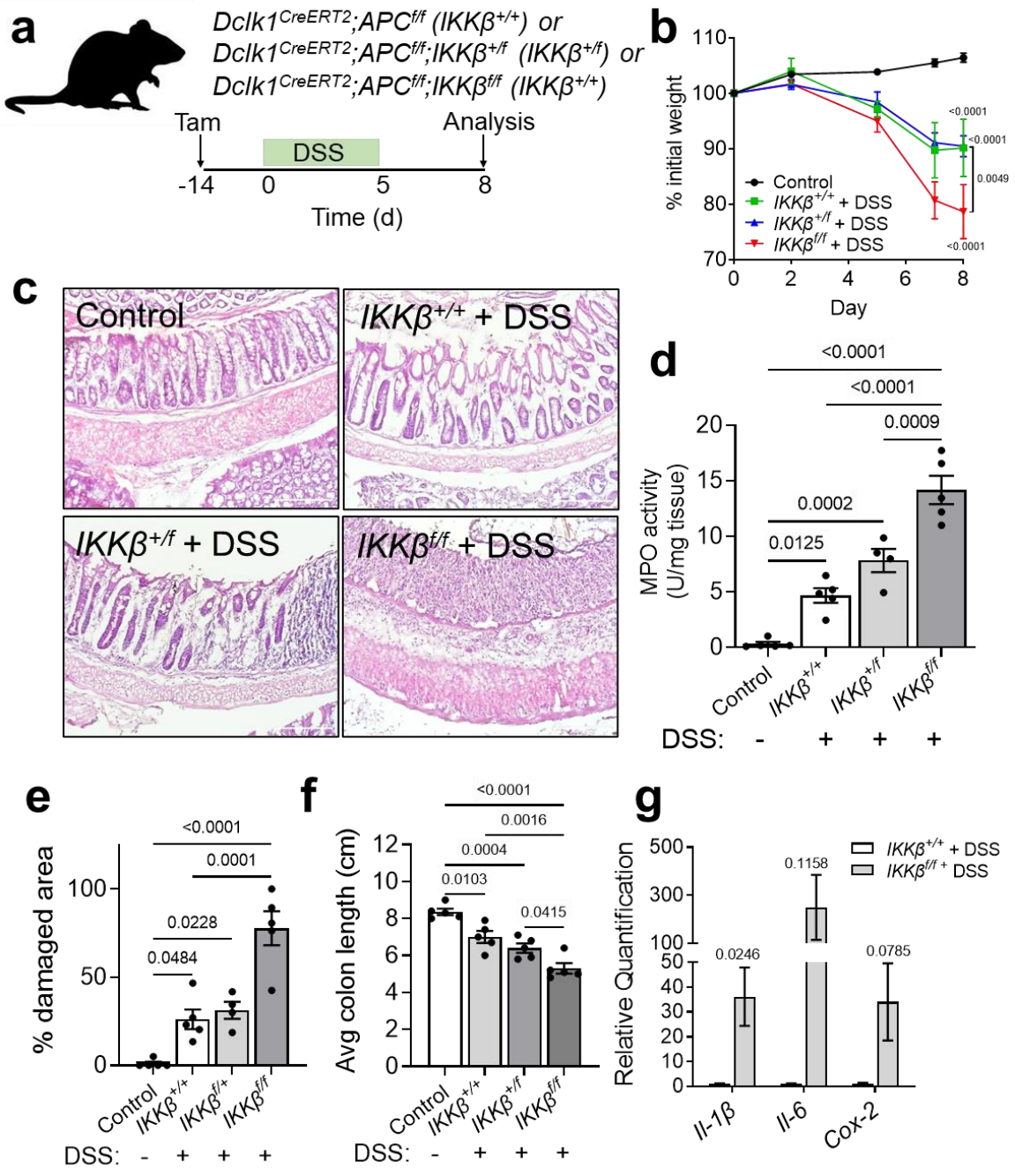

a
Dclk1CreERT2;APCf/f (IKKβ+/+) or
Dclk1CreERT2;APCf/f;IKKβ+/f (IKKβ+/f) or
Dclk1CreERT2;APCf/f;IKKβf/f (IKKβf/f)
Tam
Analysis
DSS
-14
0
5
98
Time (d)
b
Avg # tumors/mouse
0.0155
0.6940
0.1043
IKKβ+/+
IKKβ+/f
IKKβf/f
c
Avg tumor size (mm2)
0.5514
0.2062
0.6375
d
% survival
e
Avg colon length (cm)
<0.0001
<0.0001
f
MPO activity (U/mg tissue)
0.3128

Figure 4.3 – Loss of IKKβ in tuft cells promotes CAC initiation.

(**a**) Schematic illustration of the treatment of *Dclk1*^{CreERT2};*APC*$^{f/f}$ *(IKKβ*$^{+/+}$*)*, *Dclk1*^{CreERT2};*APC*$^{f/f}$;*IKKβ*$^{+/f}$ (*IKKβ*$^{+/f}$) or *Dclk1*^{CreERT2};*APC*$^{f/f}$;*IKKβ*$^{f/f}$ (*IKKβ*$^{f/f}$) mice with DSS followed by analysis at day 98. (**b**) Average number of colonic tumors in *IKKβ*$^{+/+}$, *IKKβ*$^{+/f}$, and *IKKβ*$^{f/f}$ mice after treatment with DSS. Data are presented as mean ± SEM and dots represent biologically independent animals (*IKKβ*$^{+/+}$, n = 9; *IKKβ*$^{+/f}$, n = 12; *IKKβ*$^{f/f}$, n = 15). (**c**) Average colonic tumor size of *IKKβ*$^{+/+}$, *IKKβ*$^{+/f}$, and *IKKβ*$^{f/f}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (*IKKβ*$^{+/+}$, n = 7; *IKKβ*$^{+/f}$, n = 11; *IKKβ*$^{f/f}$, n = 15). Statistical significance was determined by one-way ANOVA with Tukey post-hoc test. (**d**) Survival curve of *IKKβ*$^{+/+}$, *IKKβ*$^{+/f}$, and *IKKβ*$^{f/f}$ mice during and post-DSS and throughout tumorigenesis (*IKKβ*$^{+/+}$, n =11; *IKKβ*$^{+/f}$, n = 16; *IKKβ*$^{f/f}$, n = 26). (**e**) Average colon length of *IKKβ*$^{+/+}$, *IKKβ*$^{+/f}$, and *IKKβ*$^{f/f}$ mice at 98 days post-DSS treatment. Data are presented as mean ± SEM and dots represent biologically independent animals (*IKKβ*$^{+/+}$, n = 7; *IKKβ*$^{+/f}$, n = 11; *IKKβ*$^{f/f}$, n = 12). (**f**) Measurement of myeloperoxidase (MPO) activity in colonic tissue of *IKKβ*$^{+/+}$ or *IKKβ*$^{f/f}$ mice at 98 days post-DSS treatment. Data are presented as mean ± SEM and dots represent biologically independent animals (*IKKβ*$^{+/+}$, n = 5; *IKKβ*$^{f/f}$, n = 8). Statistical significance was determined by one-way ANOVA with Tukey post-hoc test (for panel b, c, e) or unpaired Student's t-test (for panel f).

4.3.4 *Inhibition of NF-κB in tuft cells exacerbates the severity of DSS-colitis.*

As the incidence of colitis-associated cancer is associated with severity and duration of IBD (Rutter et al., 2004), and given we observed reduced colitis severity upon constitutive IKKβ activation in tuft cells, we next sought to determine whether Dclk1+ cell specific IKKβ loss might also affect the severity of acute colitis. To assess this, $IKK\beta^{f/f}$ mice were treated with 2-2.5% DSS for 5 days and analyzed at the peak of inflammation at day 8 (**Figure 4.4a**). $IKK\beta^{+/+}$, $IKK\beta^{f/+}$, and $IKK\beta^{f/f}$ mice showed significantly reduced body weight at the peak of DSS when compared to untreated control mice, however, $IKK\beta^{f/f}$ mice lost significantly more weight relative to $IKK\beta^{+/+}$ mice (**Figure 4.4b**). $IKK\beta^{f/f}$ mice also showed increased histological damage, increased body weight loss, increased MPO activity, and reduced colon length relative to $IKK\beta^{+/+}$ and $IKK\beta^{f/+}$ mice (**Figure 4.4c-f**). This suggests that inhibition of canonical NF-κB signaling in tuft cells exacerbates DSS-colitis severity. Colonic tissue was also collected for analysis of gene expression changes between DSS-treated $IKK\beta^{+/+}$ and $IKK\beta^{f/f}$ mice. We detected a substantial increase in expression of the pro-inflammatory cytokines IL-1β and IL-6, and enzyme COX-2 in $IKK\beta^{f/f}$ mice as compared to $IKK\beta^{+/+}$ mice in the setting of colitis (**Figure 4.4g**). As these mediators have been implicated in colitis and colitis-associated tumorigenesis (Grivennikov et al., 2009; Wang et al., 2014; Wang and DuBois, 2010), IKKβ loss in Dclk1+ cells may promote CAC as a result of increased colitis severity and upregulated levels of pro-inflammatory mediators.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Dclk1CreERT2;APCf/f (IKKβ+/+) or
Dclk1CreERT2;APCf/f;IKKβ+/f (IKKβ+/f) or
Dclk1CreERT2;APCf/f;IKKβf/f (IKKβ+/+)
Tam
Analysis
DSS
-14
0
5
8
Time (d)
b
% initial weight
Day
Control
IKKβ+/+ + DSS
IKKβ+/f + DSS
IKKβf/f + DSS
<0.0001
<0.0001
0.0049
<0.0001
c
Control
IKKβ+/+ + DSS
IKKβ+/f + DSS
IKKβf/f + DSS
d
MPO activity (U/mg tissue)
<0.0001
<0.0001
0.0009
0.0002
0.0125
Control
IKKβ+/+
IKKβ+/f
IKKβf/f
DSS: - + + +
e
% damaged area
<0.0001
0.0001
0.0228
0.0484
Control
IKKβ+/+
IKKβf/+
IKKβf/f
DSS: - + + +
f
Avg colon length (cm)
<0.0001
0.0016
0.0004
0.0103
0.0415
Control
IKKβ+/+
IKKβ+/f
IKKβf/f
DSS: - + + +
g
Relative Quantification
IKKβ+/+ + DSS
IKKβf/f + DSS
0.0246
0.1158
0.0785
Il-1β
Il-6
Cox-2

Figure 4.4 – Loss of IKKβ in tuft cells exacerbates DSS-colitis severity.

(**a**) Schematic illustration of the treatment of *Dclk1*^{CreERT2};*APC*$^{f/f}$ *(IKKβ*$^{+/+}$*)*, *Dclk1*^{CreERT2};*APC*$^{f/f}$;*IKKβ*$^{+/f}$ (*IKKβ*$^{+/f}$) or *Dclk1*^{CreERT2};*APC*$^{f/f}$;*IKKβ*$^{f/f}$ (*IKKβ*$^{f/f}$) mice with DSS followed by analysis at day 8. (**b**) Body weight changes of *IKKβ*$^{+/+}$, *IKKβ*$^{f/+}$, and *IKKβ*$^{f/f}$ mice during acute DSS-colitis as compared to untreated controls. Data are presented as mean ± SEM (control, n= 5; *IKKβ*$^{+/+}$+ DSS, n = 5; *IKKβ*$^{+/f}$ + DSS, n = 5; *IKKβ*$^{f/f}$ + DSS, n = 5). (**c**) Representative images of hematoxylin and eosin staining of colonic tissue of *IKKβ*$^{+/+}$, *IKKβ*$^{f/+}$, and *IKKβ*$^{f/f}$ mice during acute DSS-colitis. Scale bars = 200μm. (**d**) Measurement of myeloperoxidase (MPO) activity in colonic tissue of control and DSS-treated *IKKβ*$^{+/+}$, *IKKβ*$^{f/+}$, and *IKKβ*$^{f/f}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n= 5; *IKKβ*$^{+/+}$+ DSS, n = 5; *IKKβ*$^{+/f}$ + DSS, n = 4; *IKKβ*$^{f/f}$ + DSS, n = 5). (**e**) Quantification of the percentage of damaged histological area in control and DSS-treated *IKKβ*$^{+/+}$, *IKKβ*$^{f/+}$, and *IKKβ*$^{f/f}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n= 5; *IKKβ*$^{+/+}$+ DSS, n = 5; *IKKβ*$^{+/f}$ + DSS, n = 4; *IKKβ*$^{f/f}$ + DSS, n = 5). (**g**) Average colon length of control and DSS-treated *IKKβ*$^{+/+}$, *IKKβ*$^{f/+}$, and *IKKβ*$^{f/f}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (control, n= 5; *IKKβ*$^{+/+}$+ DSS, n = 5; *IKKβ*$^{+/f}$ + DSS, n = 5; *IKKβ*$^{f/f}$ + DSS, n = 5). (**f**) Relative quantification of *Il-1β*, *Il-6*, and *Cox-2* mRNA levels by qPCR in the colonic tissue of DSS-treated *IKKβ*$^{+/+}$and *IKKβ*$^{f/f}$ mice. Data are presented as mean ± SEM (*IKKβ*$^{+/+}$, n = 4; *IKKβ*$^{f/f}$, n = 4). Statistical significance was determined by one-way ANOVA (for panel e-g) or two-way ANOVA (for panel b) with Tukey post-hoc tests.

4.3.5 *Tuft cell-specific inhibition of NF-κB leads to basal colonic inflammation and crypt hyperplasia.*

Given our findings that IKKβ loss in tuft cells exacerbates DSS-colitis severity, we next analyzed the effect of NF-κB inhibition in Dclk1+ cells on colonic homeostasis. *IKKβ$^{f/f}$* mice were treated with tamoxifen and analyzed at day 22 (**Figure 4.5a**). Compared to wild-type mice, *IKKβ$^{f/f}$* mice showed increased crypt height (**Figure 4.5b**), suggestive of a hyperproliferative colonic epithelium. We observed no change in crypt density in *IKKβ$^{f/f}$* sections suggesting that IKKβ-loss in tuft cells has no effect on crypt fission (**Figure 4.5d**). Further analysis of mRNA expression in colonic tissue of *IKKβ$^{f/f}$* mice by qPCR revealed a significant increase in the expression of the inflammatory mediators IL-1β, IL-6, TNF-α, and IL-25 as compared to WT controls, suggestive of an inflammatory response promoted by NF-κB inhibition in tuft cells (**Figure 4.5e**). To further validate that crypt hyperplasia was due to NF-κB pathway inhibition in epithelial tuft cells, we utilized intestinal organoids. Treatment of IKKβ organoids with 4-hydroxytamoxifen to induce IKKβ loss in tuft cells resulted in a significantly increased number of buds per organoid and increased organoid area as compared to IKKβ$^{+/+}$ organoids (**Figure 4.6a,b**). Given that the intestinal organoid model is solely limited to epithelial cells, these data suggest that an epithelial cell autonomous mechanism is responsible for the induction of proliferation upon IKKβ loss in tuft cells. Taken together, these data prove that IKKβ loss in Dclk1+ cells leads to basal colonic inflammation and hyperproliferation of the epithelium.

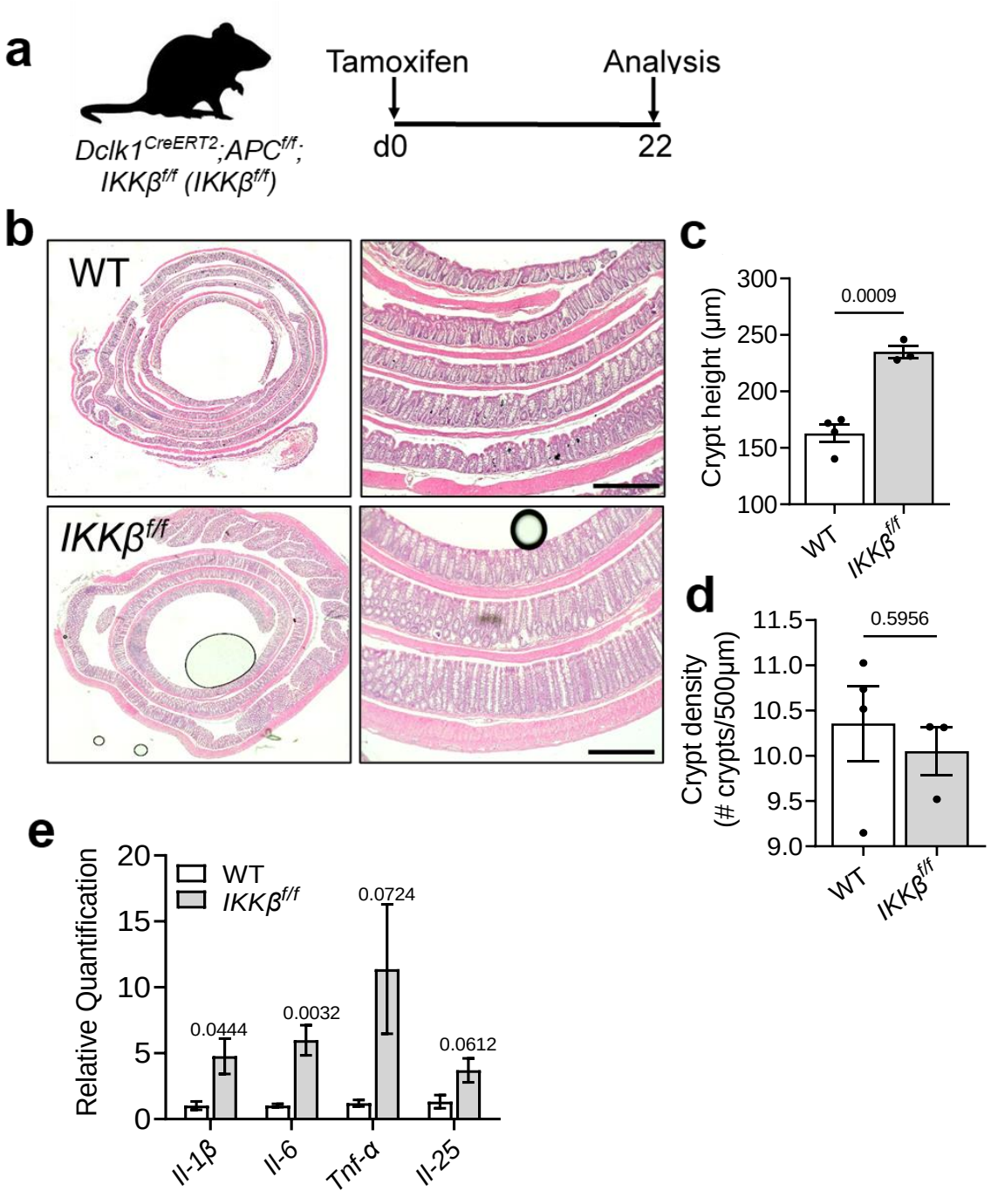
a
Tamoxifen
Analysis
d0
22
Dclk1CreERT2;APCf/f;
IKKβf/f (IKKβf/f)
b
WT
IKKβf/f
c
Crypt height (μm)
300
250
200
150
100
0.0009
WT
IKKβf/f
d
Crypt density
(# crypts/500μm)
11.5
11.0
10.5
10.0
9.5
9.0
0.5956
WT
IKKβf/f
e
Relative Quantification
20
15
10
5
0
WT
IKKβf/f
0.0444
0.0032
0.0724
0.0612
Il-1β
Il-6
Tnf-α
Il-25

Figure 4.5 – Loss of IKKβ in tuft cells induces basal colonic inflammation.

(**a**) Schematic illustration of the treatment of wild-type or *IKKβ*$^{f/f}$ mice with tamoxifen followed by analysis at day 22. (**b**) Representative images of hematoxylin and eosin staining of colonic tissue of WT and *IKKβ*$^{f/f}$ mice at day 22. Scale bars = 400μm. (**c**) Quantification of average crypt height in WT and *IKKβ*$^{f/f}$ mice at day 22. Data are presented as mean ± SEM and dots represent biologically independent animals (WT, n= 4; *IKKβ*$^{f/f}$, n = 3). (**d**) Quantification of average crypt density in WT and *IKKβ*$^{f/f}$ mice at day 22. Data are presented as mean ± SEM and dots represent biologically independent animals (WT, n= 4; *IKKβ*$^{f/f}$, n = 3). (**e**) Relative quantification of mRNA expression of *Il-1β*, *Il-6*, *Tnf-α*, and *Il-25* by qPCR in colonic tissue of WT and *IKKβ*$^{f/f}$ mice. Data are presented as mean ± SEM (WT, n = 5; *IKKβ*$^{f/f}$, n = 7). Statistical significance was determined by unpaired Student's t-test.

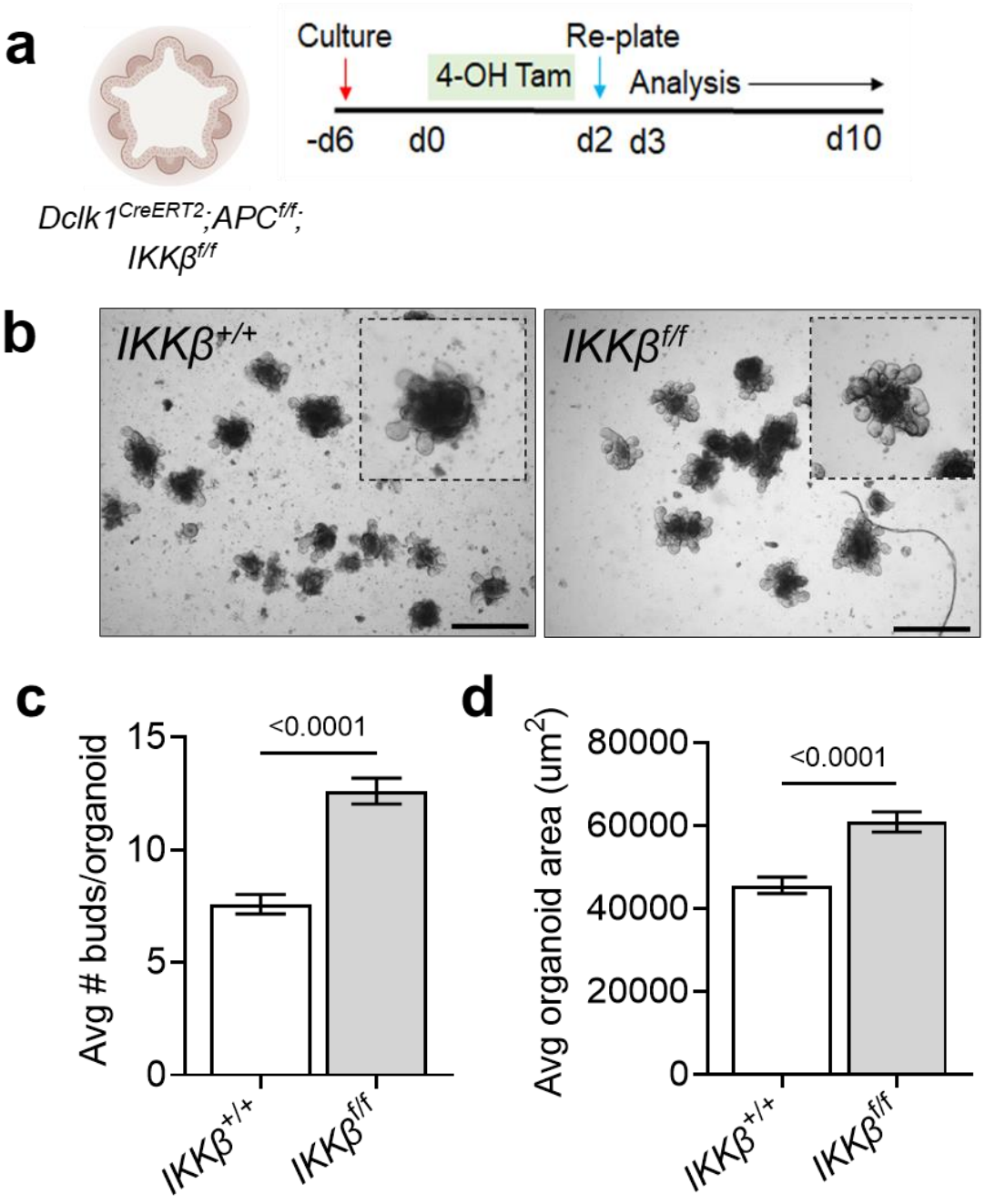
a
Culture
Re-plate
4-OH Tam
Analysis
-d6
d0
d2
d3
d10
Dclk1CreERT2;APCf/f;
IKKβf/f
b
IKKβ+/+
IKKβf/f
c
Avg # buds/organoid
<0.0001
15
10
5
0
IKKβ+/+
IKKβf/f
d
Avg organoid area (um2)
<0.0001
80000
60000
40000
20000
0
IKKβ+/+
IKKβf/f

Figure 4.6 – Loss of IKKβ in tuft cells increases intestinal organoid budding and growth.

(**a**) Schematic illustration of the treatment of *IKKβ*$^{+/+}$ or *IKKβ*$^{f/f}$ intestinal organoids with 4-hydroxytamoxifen (4-OH Tam). Organoid image from Biorender.com. (**b**) Representative brightfield images of intestinal organoids from *IKKβ*$^{+/+}$ or *IKKβ*$^{f/f}$ mice after 4OH-Tam treatment. Scale bars = 200μm. (**c**) Quantification of the average number of buds per organoid in intestinal organoids from *IKKβ*$^{+/+}$ or *IKKβ*$^{f/f}$ mice. Data are presented as mean ± SEM (*IKKβ*$^{+/+}$, n = 58; *IKKβ*$^{f/f}$, n = 63 individual organoids across 3 technical replicates). (**d**) Quantification of the average size of intestinal organoids from *IKKβ*$^{+/+}$ or *IKKβ*$^{f/f}$ mice. Data are presented as mean ± SEM (*IKKβ*$^{+/+}$, n = 86; *IKKβ*$^{f/f}$, n = 85 individual organoids across 3 technical replicates). Statistical significance was

4.3.6 *Tuft cell-specific inhibition of NF-κB leads to increased Dclk1+ cell viability.*

Given the role of NF-κB in the regulation of apoptosis, and to further investigate the mechanism by which NF-κB signaling in tuft cells affects tumorigenesis, we analyzed the effect of IKKβ loss in tuft cells on Dclk1+ cell number. $IKK\beta^{f/f}$ mice were treated with tamoxifen and analyzed by anti-Dclk1 immunofluorescence staining at day 22 (**Figure 4.7a**). Compared to wild-type mice, $IKK\beta^{f/f}$ mice showed a significant increase in the number of Dclk1+ cells per colonic crypt (**Figure 4.7b**). To determine whether this increase in tuft cell number was a result of prolonged tuft cell viability, we further assessed for changes in Dclk1+ cell number by crossing our $Dclk1^{CreERT2};APC^{f/f};IKK\beta^{f/f}$ ($IKK\beta^{f/f}$) mice to $R26^{tdTomato}$ reporter mice, generating $Dclk1^{CreERT2};APC^{f/f};IKK\beta^{f/f};R26^{tdTomato}$ pups. Resulting $Dclk1^{CreERT2};APC^{f/f};IKK\beta^{f/f};R26^{tdTomato}$ mice allow for genetic labeling of Dclk1+ cells and their progeny with expression of tdTomato (RFP). $Dclk1^{CreERT2};APC^{f/f};R26^{tdTomato}$ ($IKK\beta^{+/+}$) and $Dclk1^{CreERT2};APC^{f/f};IKK\beta^{f/f};R26^{tdTomato}$ ($IKK\beta^{f/f}$) mice were treated with tamoxifen and analyzed on day 12 (**Figure 4.7c**). The number of tuft cells was quantified based on the number of endogenous tdTomato+ cells within the colonic epithelium by fluorescence microscopy. We observed a significant increase in the number of tdTomato+ Dclk1+ cells within the epithelium of $IKK\beta^{f/f}$ mice relative to $IKK\beta^{+/+}$ controls (**Figure 4.7d**). Taken together, these data suggest that IKKβ loss in tuft cells increases tuft cell number, likely due to increased longevity of these cells.

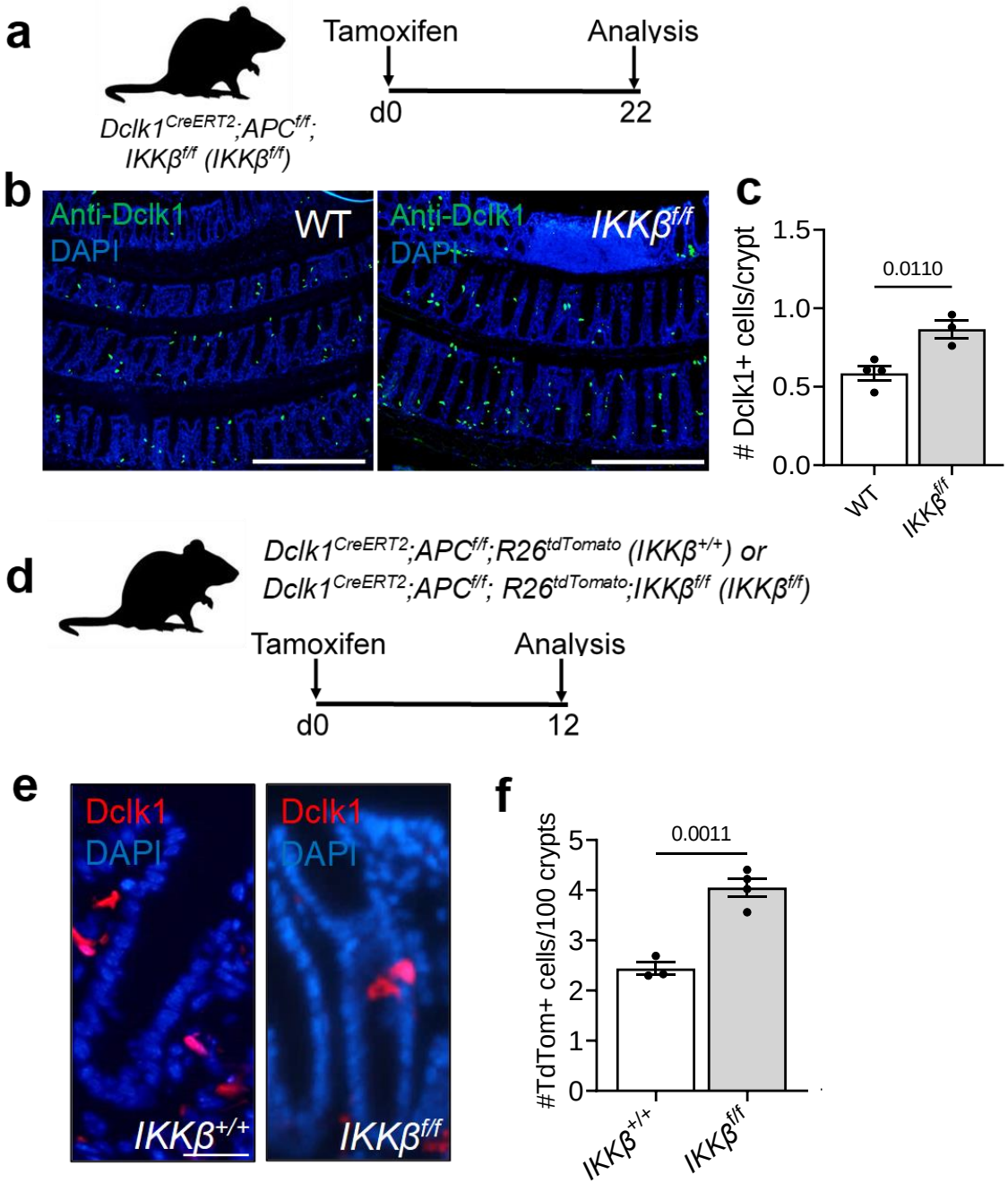
a
Tamoxifen
Analysis
d0
22
Dclk1CreERT2;APCf/f;
IKKβf/f (IKKβf/f)
b
Anti-Dclk1
DAPI
WT
Anti-Dclk1
DAPI
IKKβf/f
c
Dclk1+ cells/crypt
1.5
1.0
0.5
0.0
0.0110
WT
IKKβf/f
d
Dclk1CreERT2;APCf/f;R26tdTomato (IKKβ+/+) or
Dclk1CreERT2;APCf/f; R26tdTomato;IKKβf/f (IKKβf/f)
Tamoxifen
Analysis
d0
12
e
Dclk1
DAPI
IKKβ+/+
Dclk1
DAPI
IKKβf/f
f
#TdTom+ cells/100 crypts
5
4
3
2
1
0
0.0011
IKKβ+/+
IKKβf/f

Figure 4.7 – Loss of IKKβ in tuft cells increases colonic tuft cell number.

(**a**) Schematic illustration of the treatment of wild-type or *IKKβ*$^{f/f}$ mice with tamoxifen followed by analysis at day 22. (**b**) Representative fluorescent images of anti-Dclk1 immunofluorescence staining (green) in colonic tissue of wild-type or *IKKβ*$^{f/f}$ mice. Scale bars = 400μm. (**c**) Quantification of the number of Dclk1+ cells per colonic crypts by anti-Dclk1 immunofluorescence staining in colonic tissue of wild-type or *IKKβ*$^{f/f}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (WT, n= 4; *IKKβ*$^{f/f}$, n = 3). (**d**) Schematic illustration of the treatment of *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26*^{tdTomato} (*IKKβ*$^{+/+}$) and *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;IKKβ*$^{f/f}$*;R26*^{tdTomato} (*IKKβ*$^{f/f}$) mice with tamoxifen followed by analysis at day 12. (**e**) Representative fluorescence images of a colonic crypt from *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;R26*^{tdTomato} (*IKKβ*$^{+/+}$) or *Dclk1*^{CreERT2}*;APC*$^{f/f}$*;IKKβ*$^{f/f}$*;R26*^{tdTomato} (*IKKβ*$^{f/f}$) mice. Scale bar = 25μm. (**f**) Quantification of the number of tdTomato+ (Dclk1+) cells per 100 colonic crypts in *IKKβ*$^{+/+}$ and *IKKβ*$^{f/f}$ mice. Data are presented as mean ± SEM and dots represent biologically independent animals (WT, n= 3; *IKKβ*$^{f/f}$, n = 4). Statistical significance was determined by unpaired Student's t-test.

4.4 Discussion

The mechanism by which inflammation leads to colitis-associated cancer is not well understood. NF-κB signaling is upregulated in chronic inflammatory disease states, such as IBD, and has also been associated with CRC. Given this association of NF-κB with both colonic tumorigenesis and inflammation, it has been hypothesized that NF-κB may contribute to the pathogenesis of colitis-associated cancer (CAC).

The sensitivity of Lgr5+ stem cells to intestinal damage and inflammation has led to the discovery that non-stem cells are able to regenerate the intestinal epithelium in the setting of injury or Lgr5+ cell loss (Asfaha et al., 2015; Ayyaz et al., 2019; Castillo-Azofeifa et al., 2019; Murata et al., 2020; Yui et al., 2018). Therefore, studies have assessed whether inflammation-associated cancer may be of non-stem cell origin. Indeed, we have shown that fully differentiated Dclk1+ tuft cells can transform to facultative stem cells that serve as the cellular origin for CAC following injury (Westphalen et al., 2014). Schwitalla et al., 2013 similarly identified that non-stem cells initiate cancer in an NF-κB-dependent manner (Schwitalla et al., 2013). Thus, in this study we investigated the role of tuft cell-specific canonical NF-κB signaling in colitis-associated cancer.

IKKβ-loss in IECs has been shown to be preventative against CAC, whereas mice harbouring IEC-specific constitutive activation of IKKβ develop spontaneous colonic tumors (Greten et al., 2004; Vlantis et al., 2011). In light of these observations, we hypothesized that activated IKKβ signaling in epithelial tuft cells would promote colonic tumorigenesis. However, we found the surprising result that constitutive activation of IKKβ in Dclk1+ tuft cells strongly reduced, rather than promoted, CAC. Accordingly, we also found that inhibition of IKKβ in tuft cells increased the initiation of inflammation-associated tumors. These data prove that canonical NF- κB signaling in tuft cells is protective against CAC.

The risk of colonic tumorigenesis is thought to directly correlate with the severity and duration of inflammation (Rutter et al., 2004). Thus, we further investigated whether the effects of NF-κB activation and/or inhibition in Dclk1+ cell-derived CAC were due to

alterations in colitis severity. Activation of IKKβ in tuft cells reduced colitis severity, whereas loss of IKKβ exacerbated colitis. These data indicate that canonical NF-κB signaling in tuft cells may function to prevent CAC by reducing intestinal inflammation. Whilst NF-κB signaling is typically considered to be a pro-inflammatory pathway, the role for NF-κB in intestinal epithelial cells has been controversial. Indeed, both inhibition and over-activation of IKKβ in IECs have been reported to increase intestinal inflammation and worsen acute colitis severity (Eckmann et al., 2008; Greten et al., 2004; Guma et al., 2011; Vlantis et al., 2011). Thus, our finding that inhibition of NF-κB in Dclk1+ cells leads to increased intestinal inflammation is consistent with previous reports in the literature. Furthermore, it has been shown that IEC-specific deletion of various canonical NF-κB signaling mediators results in spontaneous intestinal inflammation (Nenci et al., 2007; Vlantis et al., 2016), increased susceptibility to gut ischemia-reperfusion or irradiation injury (Chen et al., 2003; Egan et al., 2004), increased inflammatory response to *C. difficile* or *T. muris* infection (Chae et al., 2006; Zaph et al., 2007), and exacerbated DSS-colitis severity (Steinbrecher et al., 2008). These protective effects of NF-κB have been attributed to the anti-apoptotic role of this pathway. Loss of NF-κB signaling in IECs has been reported to increase epithelial cell apoptosis and reduce the expression of antimicrobial peptides, leading to compromised barrier function and an enhanced inflammatory response (Chae et al., 2006; Eckmann et al., 2008; Egan et al., 2004; Greten et al., 2004; Nenci et al., 2007; Steinbrecher et al., 2008; Vlantis et al., 2016). However, Mikuda et al., 2020 reported that IEC-specific loss of IκBα, a key NF-κB inhibitor, also resulted in increased apoptosis and intestinal inflammation (Mikuda et al., 2020), highlighting the dual role of NF-κB signaling in contributing to both pro-inflammatory and protective functions during intestinal homeostasis and injury.

Tuft cells can serve as the predominant source of certain cytokines in the intestine, such as IL-25 and thymic stromal lymphopoietin (TSLP) (Haber et al., 2017; Schneider et al., 2019). During infections, TSLP is known to be induced by IKKβ-dependent NF-κB signaling to induce a protective Th2 cell response (Zaph et al., 2007). Indeed, reduced levels of TSLP have been observed in mice harbouring IEC-specific loss of IKKβ leading to a shift from a protective Th2 cell response to enhanced Th1 cell-mediated inflammation (Zaph et al., 2007). Thus, we speculate that this could be a potential mechanism by which

IKKβ loss in tuft cells promotes colitis severity, and by which constitutive IKKβ activity serves a protective function in colitis.

Interestingly, Nenci et al., 2007 demonstrated that NEMO/IKKγ loss in IECs leads to spontaneous intestinal inflammation (Nenci et al., 2007). This effect was consistent in mice lacking both IKKα and IKKβ subunits. However, mice lacking either the IKKα or IKKβ subunit alone did not develop spontaneous inflammation, suggesting a potential compensatory mechanism between these two subunits (Nenci et al., 2007). Previous reports have suggested that IKKα can activate canonical NF-κB signaling in the setting of IKKβ-loss (Lam et al., 2008; Luedde et al., 2005). For this reason, canonical NF-κB signaling may be sustained in IKKβ-deficient Dclk1+ cells through an IKKα-dependent IKKβ-independent mechanism. Alternatively, IKKα is known to facilitate the activation of non-canonical NF-κB signaling, which has also been shown to be aberrantly upregulated in patients with IBD and in colorectal cancer (Allen et al., 2017; Lauscher et al., 2010). Studies assessing the effects of genetic loss of *Nlrp12*, a negative regulator of non-canonical NF-κB signaling, have shown that this leads to increased tumorigenesis in a mouse model of CAC (Allen et al., 2012). This suggests that our observation of increased tumorigenesis upon IKKβ loss in tuft cells may be mediated through a non-canonical NF-κB signaling mechanism. Furthermore, IKKα, but not IKKβ, has been shown to stimulate the nuclear localization and transcriptional activity of β-catenin (Albanese et al., 2003; Lamberti et al., 2001). As the tumors in our Dclk1+ cell-derived model of CAC are driven by APC-loss and display nuclear accumulation of β-catenin (Westphalen et al., 2014), tuft cells deficient in IKKβ may harbor a compensatory upregulation of IKKα activity, and therefore, increased Wnt signaling to promote tumor initiation.

In summary, we have shown that loss of IKKβ in Dclk1+ cells induces basal colonic inflammation and crypt hyperplasia *in vivo*. These results are additionally supported by experiments done *in vitro* using intestinal organoids, that lack stromal or immune cells, suggesting that an epithelial cell-autonomous mechanism is likely mediating these effects. Furthermore, we detected upregulated expression of inflammatory cytokines, including IL-6 and TNF-α, in basal colonic tissue of $IKK\beta^{f/f}$ mice relative to WT controls. These cytokines have been shown to promote epithelial proliferation (Bradford et al., 2017;

Jeffery et al., 2017), suggesting that a tuft cell-mediated signaling pathway may exist to regulate the release of these cytokines from the epithelium in the setting of IKKβ-loss. The pro-proliferative function of these cytokines may further contribute to the increase in tumorigenesis that we observed in our model of CAC. This finding could be further explained by our detection of an increased number of colonic Dclk1+ cells in mice with a tuft cell-specific loss of IKKβ, suggesting that there may be an increased number of cells with the ability to initiate cancer in our model.

The described dual role for NF-κB signaling in intestinal inflammation and the complexity of this pathway provide several potential mechanisms for the results we observed in this study. These potential cellular mechanisms will be further examined in our ongoing and futures studies. Overall, this study highlights the importance for Dclk1+ cells in intestinal inflammation and homeostasis, and further proves that there is a key protective role for tuft cell-specific canonical NF-κB signaling in colitis and colitis-associated cancer.

4.5 References

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Chapter 5

5 General Discussion

5.1 Overview

Inflammation is one of the major hallmarks of cancer, yet the mechanism underling this transformation is not well-understood. Indeed, patients with Inflammatory Bowel Disease have a 20 to 30-fold greater risk of colorectal cancer as compared to the general population (Castaño-Milla et al., 2014). Given that there are currently few, if any, effective chemopreventative strategies for colitis-associated cancer (CAC), there is a pressing need to identify safe and effective strategies that reduce the risk of this disease. To do this, we must examine the mechanism by which colitis leads to CAC. The research presented in this thesis has focused on two major inflammatory pathways, cyclooxygenase (**Chapter 3**) and NF-κB (**Chapter 4**), and the role that these pathways play in the initiation of colitis-associated cancer. In summary, we have discovered two novel mechanisms by which inflammation leads to cancer and have elucidated how inflammatory mediators contribute to cellular plasticity within the intestinal epithelium. Importantly, we have also shown that Aspirin is a pharmacological agent that is safe and effective for the chemoprevention of CAC.

5.2 Summary of Findings

5.2.1 *Low-dose Aspirin prevents the initiation of colitis-associated cancer*

As outlined in **Chapter 3**, we first assessed the role of COX signaling in colitis-associated cancer by analyzing the effects of various NSAIDs in two mouse models of CAC. We utilized our previously established *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mouse model, in which tumors are derived from APC-mutated Dclk1+ cells, and the more widely used AOM/DSS model of

CAC. In both models, we identified that low-dose Aspirin is effective against the initiation of colitis-associated tumorigenesis. In contrast, COX-2-selective inhibitors had no effect on tumor initiation. Our studies assessing the effects of pharmacological and genetic inhibition of COX-1 revealed that low-dose Aspirin likely exerts its chemopreventative function through inhibition of COX-1. We further identified that COX-derived prostaglandins are upregulated in DSS-colitis and are repressed upon treatment with low-dose Aspirin or inhibition of COX-1. Importantly, analysis of the effects of Aspirin during the acute and regenerative phases of DSS-colitis revealed that low-dose Aspirin does not exacerbate colitis severity. Thus, we prove that low-dose Aspirin is both safe and effective for the prevention of CAC in colitis.

5.2.2 *PGE_2 and phospho-Akt promote the initiation of colitis-associated cancer by inducing the stemness of tuft cells.*

In the setting of injury, non-stem cells are able to acquire stem cell properties that allow them to contribute to regeneration the epithelium (Ayyaz et al., 2019; Buczacki et al., 2013; van Es et al., 2012; Schmitt et al., 2018; Schonhoff et al., 2004; Tetteh et al., 2016; Yan et al., 2017; Yu et al., 2018). If these non-stem cells harbour a mutation, however, they can also give rise to cancer (Davis et al., 2015; Schwitalla et al., 2013; Westphalen et al., 2014). We capitalized on our mouse model of CAC, in which the cellular origin of cancer is known to be the Dclk1+ tuft cell, to examine the mechanism by which non-stem cells initiate tumors in the setting of injury. Given the link between PGE_2 and cancer (Rigas et al., 1993), as well as PGE_2 and colitis (Sharon et al., 1978), we investigated how the upregulation of PGE_2 in colitis contributes to cancer. PGE_2 has been shown to induce stemness in various tissue types (Fan et al., 2014; Frisch et al., 2009; Goessling et al., 2009; Gupta et al., 2019; Hoggatt et al., 2009; Kuroda et al., 2018; Lee et al., 2016; North et al., 2007), and has been linked to the activation of Akt (Peng et al., 2017) which is known to be highly expressed in Dclk1+ cells (Chandrakesan et al., 2015). Thus, in **Chapter 3** we focused on the role of PGE_2 and Akt in the initiation of CAC from mature Dclk1+ tuft cells. First, we confirmed that phopsho-Akt levels are upregulated in DSS-colitis. We next assessed the effects of PGE_2 and Akt activation on tuft cells. Upon treatment with Misoprostol (PGE analogue)

and/or SC79 (Akt activator), we made the novel observation that PGE_2 and activation of Akt are able to stimulate tuft cells to display stem cell activity and lineage trace the entire colonic crypt. This finding is of critical importance as tuft cells are a fully differentiated, quiescent, and mature cell type that can only proliferate and give rise to tumors in the setting of DSS-colitis. Notably, the plasticity of tuft cells associated with PGE_2 and Akt occurred predominantly in the setting of APC-loss. This observation suggests that PGE_2 and Akt act in concert with Wnt signaling to promote Dclk1+ cell stemness. Moreover, only the combined activation of PGE_2 and Akt was able to stimulate Dclk1+ tuft cells to initiate dysplastic lesions in the setting of low-dose epithelial injury, suggesting that PGE_2 and Akt signaling pathways act in a cooperative manner to promote the initiation of CAC. To investigate whether PGE_2 and Akt induce tuft cell stemness in a Wnt-dependent manner, we assessed the localization of β-catenin in tuft cell-derived lineage traced crypts. We observed that the combined activation of both PGE_2 and Akt activation were required to stimulate the nuclear localization of β-catenin in colonic crypts, suggesting that the ability for PGE_2 and Akt to promote dysplasia may be mediated, in part, through canonical Wnt signaling. These findings identify a novel mechanism by which inflammation and/or intestinal injury leads to cellular plasticity and stimulates non-stem cells to repopulate the intestinal epithelial and initiate cancer. In **Chapter 3**, we demonstrated that low-dose Aspirin downregulated PGE_2 levels in colitis and thus, we conclude that Aspirin prevents the initiation of CAC by downregulating COX-1-derived PGE_2 levels.

5.2.3 *Canonical NF-κB signaling in tuft cells is protective against colitis-associated cancer.*

In **Chapter 4** we explored the role of canonical NF-κB signaling in colitis-associated cancer. We generated two novel transgenic mouse models that allowed us to either constitutively activate or inhibit the canonical NF-κB mediator IKKβ specifically in Dclk1+ tuft cells. Using these models, we found that activation of tuft cell IKKβ reduced colitis severity and prevented colonic tumorigenesis, whereas inhibition of IKKβ in tuft cells exacerbated colitis severity and increased the number of tumors in our model. Given that the risk for CAC correlates with the severity and duration of IBD (Rutter et al., 2004),

we speculate that the mechanism by which canonical NF-κB signaling promotes CAC is by modulating the severity of colitis. This may in part be mediated by the dual role for NF-κB signaling in pro-inflammatory and anti-apoptotic mechanisms as reported in previous studies (described in detail in **Section 1.7**). Our findings in **Chapter 4** highlight the importance of epithelial Dclk1+ tuft cells in regulating inflammatory responses, and identify a novel protective role for tuft cell NF-κB signaling in colitis and colitis-associated cancer.

5.3 Future Directions

5.3.1 *Chapter 3*

The results reported in **Chapter 3** strongly suggest that low-dose Aspirin is a safe and effective chemopreventative agent against CAC. Further clinical studies should be performed to confirm the tolerability and efficacy of this drug for the use in patients with IBD. It is important to note that the promotion of epithelial regeneration during colitis is a double-edged sword. Regeneration of the gut is critical for remission of IBD, but over-regeneration can lead to cancer. Our organoid data in **Chapter 3** demonstrate that the Akt activator, SC79, promotes intestinal regeneration, yet does not contribute to tumor initiation in absence of PGE_2. Thus, it is important to next investigate whether SC79 can promote intestinal regeneration during colitis *in vivo*, and determine whether the combination treatment of both low-dose Aspirin and SC79 reduces colitis severity as well as the risk of cancer.

Importantly, we report, for the first time, the ability of Misoprostol and/or SC79 to stimulate fully differentiated Dclk1+ cells to acquire stem cell capacity. This finding has provided us with important insight into how non-stem cells may be recruited to regenerate the epithelium and/or initiate cancer in the setting of inflammation. Our findings suggest that the combinted activation of Akt and PGE_2 is able to upregulate Wnt signaling and promote the initiation of dysplasia, but only in the setting of low-dose injury. These data suggest that additional mediators upregulated in DSS-colitis may also contribute to the

initiation of CAC. As a result, studies defining the exact mechanism by which DSS leads to tumor initiation in our models of CAC will need to be investiaged further.

Based on our findings, we propose that PGE_2 acts in an autocrine manner to stimulate tuft cell EP receptors and promote tuft cell stemness and the initiation of dysplasia. However, further experiments defining the expression of EP receptors in Dclk1+ tuft cells and assessing of the effects of inhibition and/or activation of these receptors will need to be performed to confirm this hypothesis and determine which EP receptors are most important for inducing the initiation of tumorigenesis.

Further results in **Chapter 3** show that low-dose Aspirin reduces the number of Dclk1+ cells within the colonic epithelium in a COX-1-dependent manner. As described in **Section 1.2.2.1**, recent reports have described a role for tuft cells in type 2 immunity against helminth or parasite infections (Gerbe et al., 2016; Howitt et al., 2016; von Moltke et al., 2016). Thus, in addition, it would be valuable to determine whether patients taking daily low-dose Aspirin display reduced intestinal tuft cell numbers and whether this may impact their response to helminth or protozoa infection.

5.3.2 *Chapter 4*

In **Chapter 4**, we report the novel finding that canonical NF-κB signaling, specifically in Dclk1+ tuft cells, has a dramatic effect on colitis severity and CAC. As our findings are in contrast to what we initially hypothesized, our next steps are to identify whether a compensatory pro-inflammatory pathway is upregulated in this model and to further investigate the mechanism by which this effect is mediated. For example, it would be helpful to assess the role of IKKα and non-canonical NF-κB signaling that may be upregulated in response to IKKβ-loss. The dual role for NF-κB signaling in both pro-inflammatory and protective functions of the intestinal epithelium suggests that feasible clinical intervention must target a mediator downstream of NF-κB. By determining the signaling cascade downstream of IKKβ that promotes inflammation and tumorigenesis, we may identify a druggable target for the treatment of colitis and prevention of CAC. As one

of the rarest cell types within the epithelium, our findings further highlight the importance of tuft cells in intestinal homeostasis and injury. The novel transgenic models generated as a part of this project may enable us to further elucidate the role of these cells within the uninflamed epithelium.

5.4 Conclusions

In summary, the findings reported in this thesis contribute greatly to our understanding of how colitis leads to cancer and how intestinal inflammation and/or injury can stimulate non-stem cells to initiate tumors in the setting of Lgr5+ stem cell loss. Importantly, we show that low-dose Aspirin prevents the initiation of colitis-associated cancer. Furthermore, we show the importance of Dclk1+ tuft cells in the colon and their role in inflammation and cancer initiation. Moving forward, the results of this thesis will provide rationale and support to further investigate and expand our knowledge of intestinal stem cells, colitis, and colorectal cancer.

5.5 References

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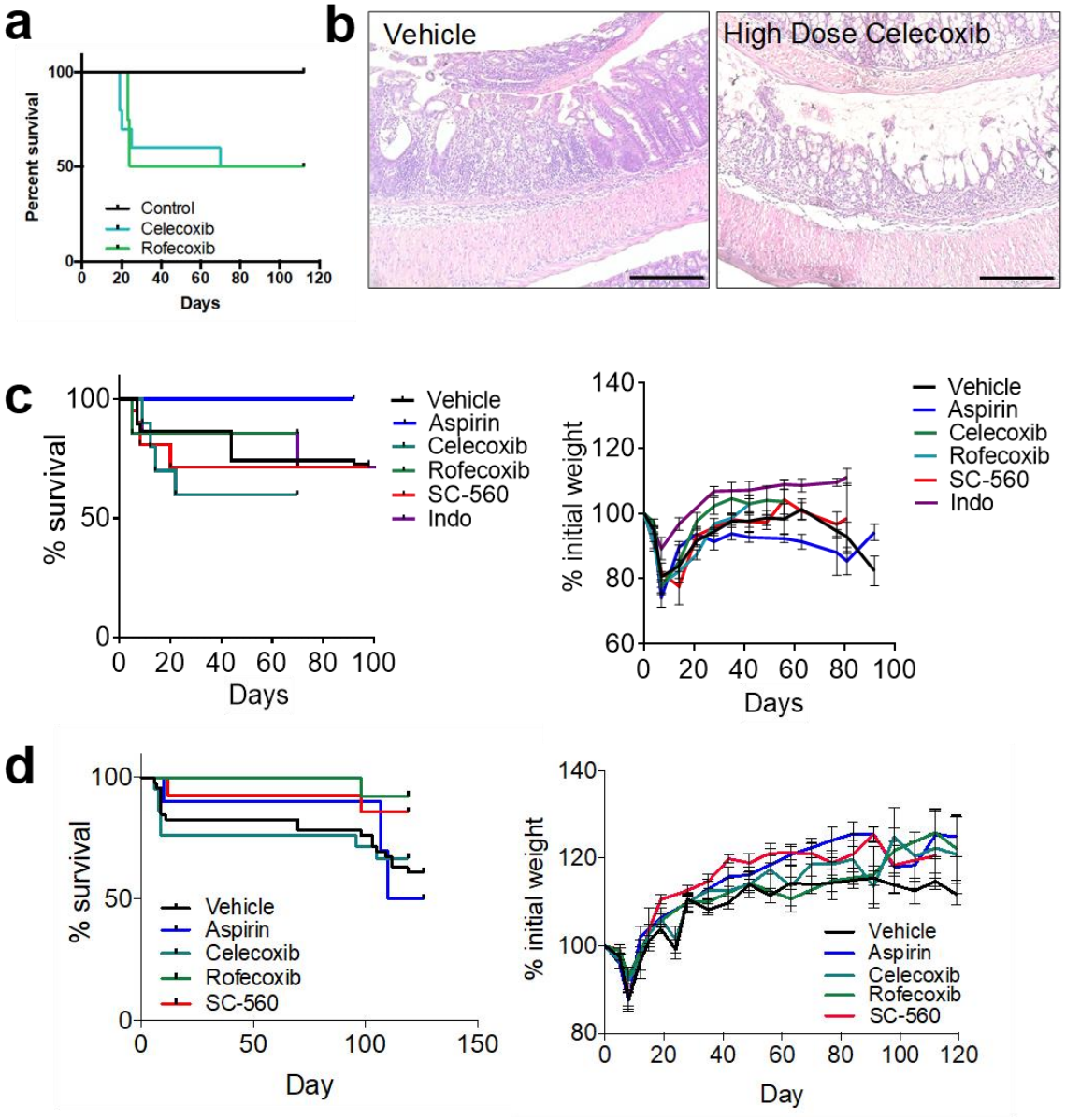
Appendices - Chapter 6

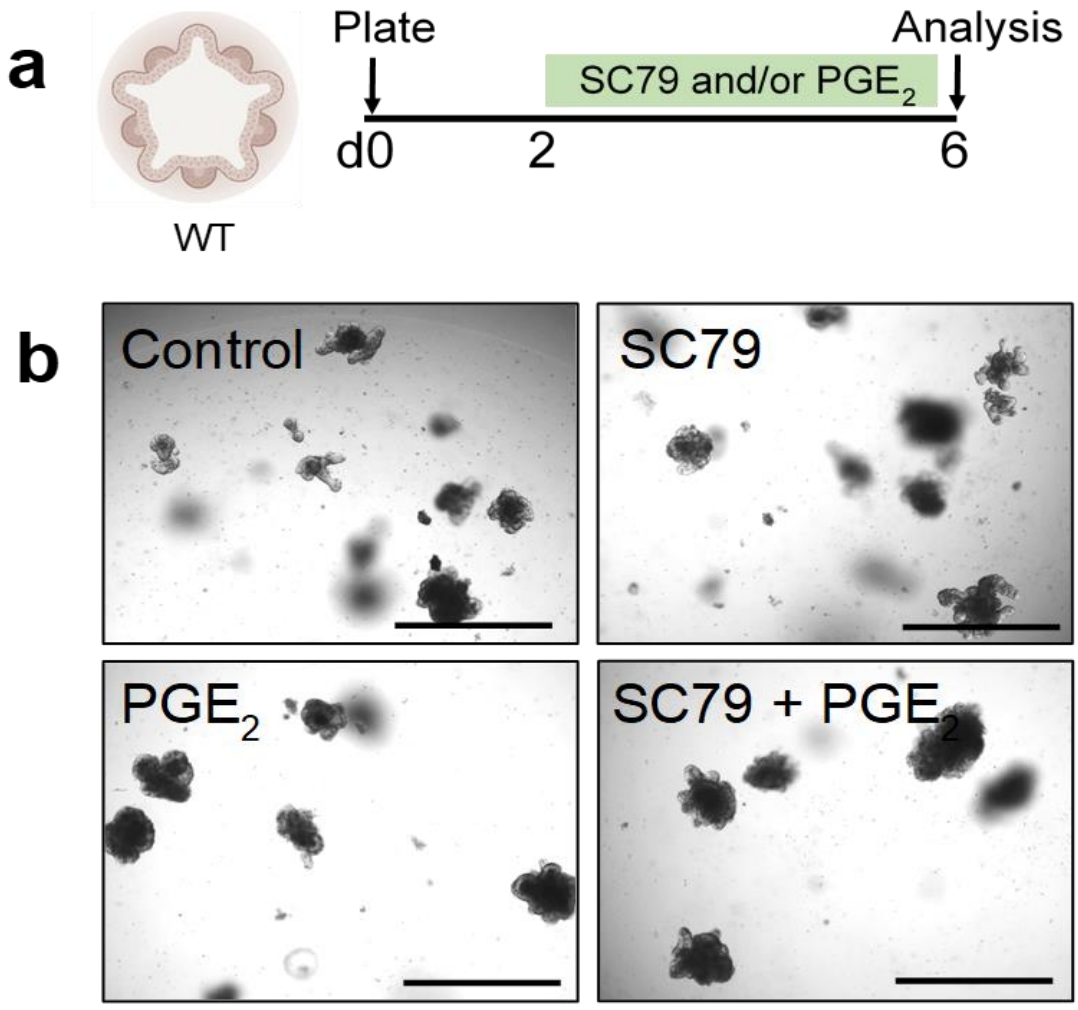
6 Appendices

6.1 Chapter 3 – Supplementary Data



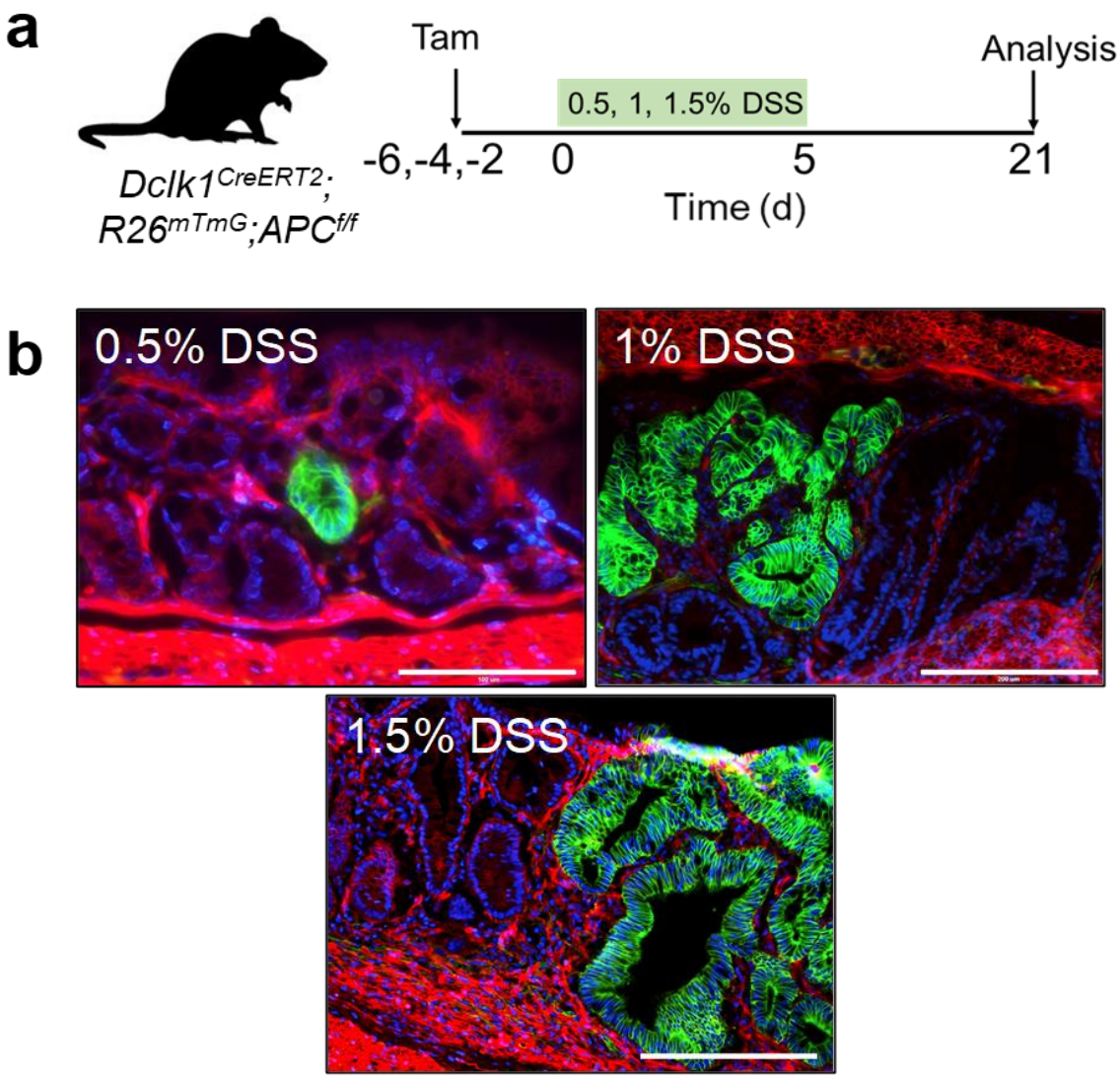
Appendix 1 – Survival and body weight for NSAID treated mice in mouse models of CAC.

(**a**) Survival curve for *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice treated with high dose COX-2 inhibitors (celecoxib, 50mgkg; rofecoxib, 15mg/kg) during and after DSS in the *Dclk1*^{CreERT2}*;APC*$^{f/f}$ model of CAC. (**b**) Colonic histology by H&E staining showing epithelial damage during DSS-colitis upon treatment with vehicle or high dose celecoxib. Scale bars = 200μm. (**c**) Survival curve (left) and body weight (right) of *Dclk1*^{CreERT2}*;APC*$^{f/f}$ mice treated with low-dose NSAIDs during the *Dclk1*^{CreERT2}*;APC*$^{f/f}$ model of CAC. (**d**) Survival curve (left) and body weight (right) of C57Bl/6 wild-type mice treated with low-dose NSAIDs during the AOM/DSS model of CAC.


a
Plate
Analysis
SC79 and/or PGE2
d0
2
6
WT
b
Control
SC79
PGE2
SC79 + PGE2
c
Average organoid area (um2)
200000
150000
100000
50000
0
0.0032
0.0816
0.9690
Control
SC79
PGE2
SC79 + PGE2

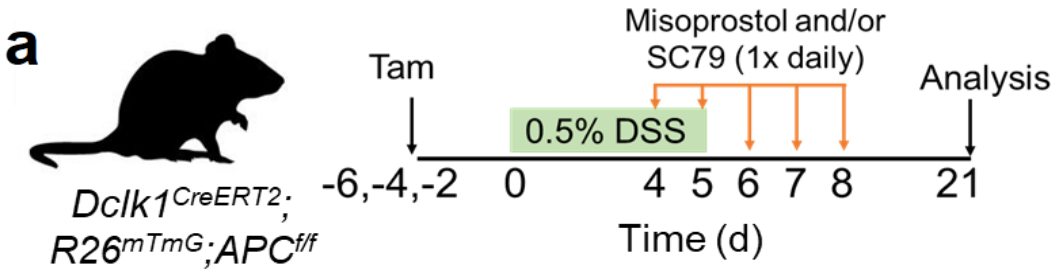
Appendix 2 – PGE_2 and Akt activation co-operate to enhance intestinal organoid growth.

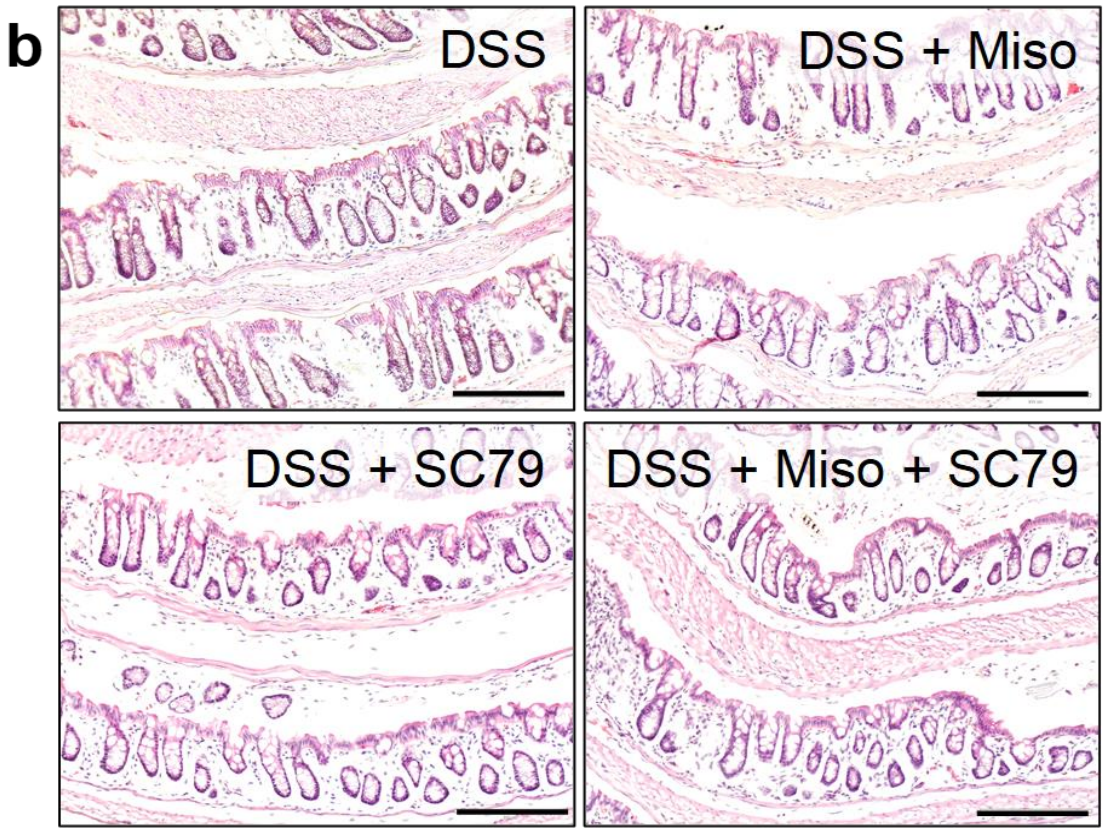
(**a**) Schematic illustration of the treatment of wild-type enteroids with SC79 and/or PGE_2. (**b**) Representative brightfield images of WT enteroids treated with SC79 and/or PGE_2. Scale bars = 400µm. (**c**) Quantification of the average organoid area upon treatment with SC79 and/or PGE_2. Data are presented as mean ± SEM and dots represent individual organoids from n=3 biologically independent animals. Statistical significance was determined by one-way ANOVA and Tukey post-hoc test.

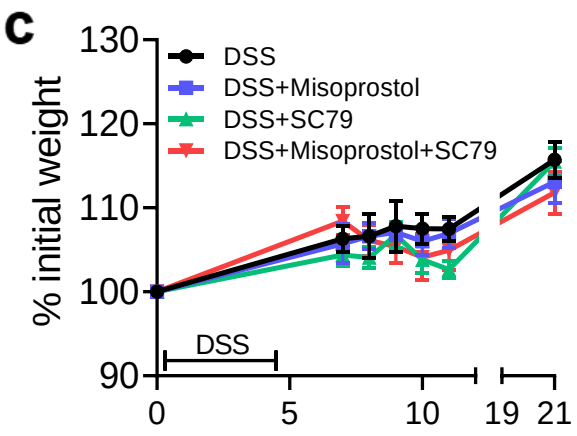


Appendix 3 – DSS dose titration on Dclk1+ cell-derived lineage tracing and progression to dysplasia.

(**a**) Schematic illustration of the treatment of *Dclk1*^{CreERT2}*;R26*^{mTmG}*;APC*$^{f/f}$ mice treated with 0.5%, 1% or 1.5% DSS for 5 days and analyzed on day 21. (**b**) Representative fluorescent images of *Dclk1*^{CreERT2}*;R26*^{mTmG}*;APC*$^{f/f}$ mice treated with 0.5%, 1% or 1.5% DSS. (0.5% DSS, n=4; 1% DSS, n=3; 1.5% DSS, n=4). Scale bars = 100µm (0.5% DSS) or 200µm (1% and 1.5% DSS).


a
Misoprostol and/or
SC79 (1x daily)
Tam
Analysis
0.5% DSS
-6,-4,-2 0 4 5 6 7 8 21
Time (d)
Dclk1CreERT2;
R26mTmG;APCf/f

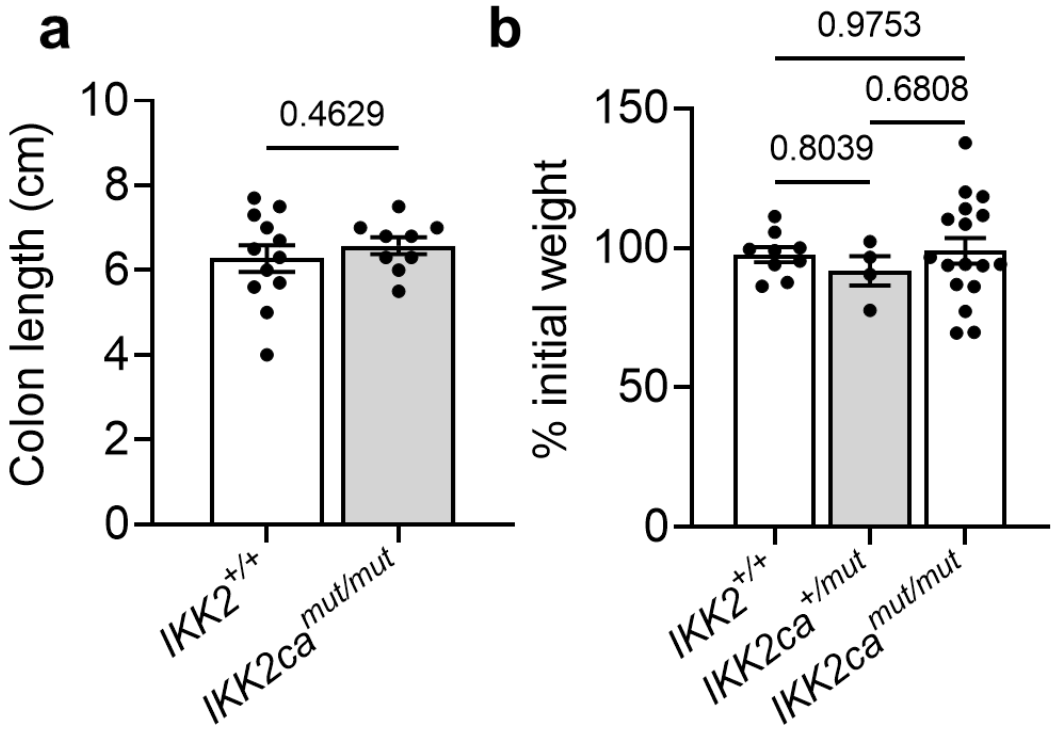

b
DSS
DSS + Miso
DSS + SC79
DSS + Miso + SC79


c
% initial weight
130
120
110
100
90
DSS
DSS+Misoprostol
DSS+SC79
DSS+Misoprostol+SC79
DSS
0
5
10
19
21

Appendix 4 – SC79 or Misoprostol does not alter colonic epithelial histology upon 0.5% DSS treatment.

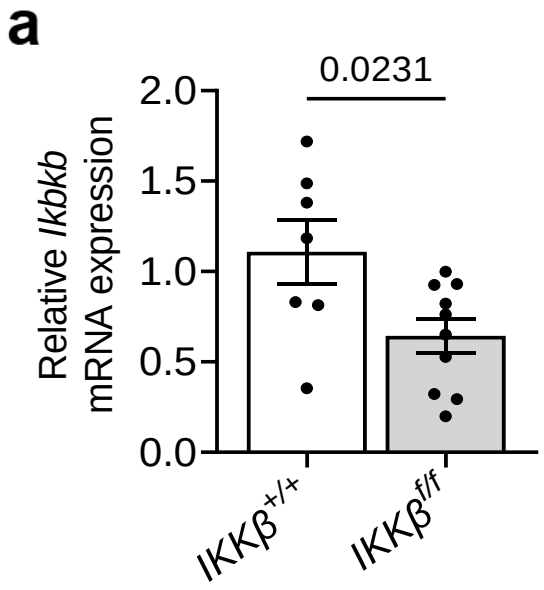
(**a**) Schematic illustration of the treatment of *Dclk1*^{CreERT2}*;R26*^{mTmG}*;APC*$^{f/f}$ mice with 0.5% DSS plus SC79 and/or Misoprostol. (**b**) Representative histological images of the colonic epithelium by H&E staining upon treatment with 0.5% DSS plus SC79 and/or Misoprostol (Miso). Scale bars = 100µm. (DSS, n=6; DSS+Miso, n=4; DSS+SC79, n=4; DSS+Miso+SC79, n=6). (**c**) Body weight changes for *Dclk1*^{CreERT2}*;R26*^{mTmG}*;APC*$^{f/f}$ mice treated with 0.5% DSS plus SC79 and/or Misoprostol. Statistical significance was determined by two-way ANOVA and Tukey's multiple comparisons test.

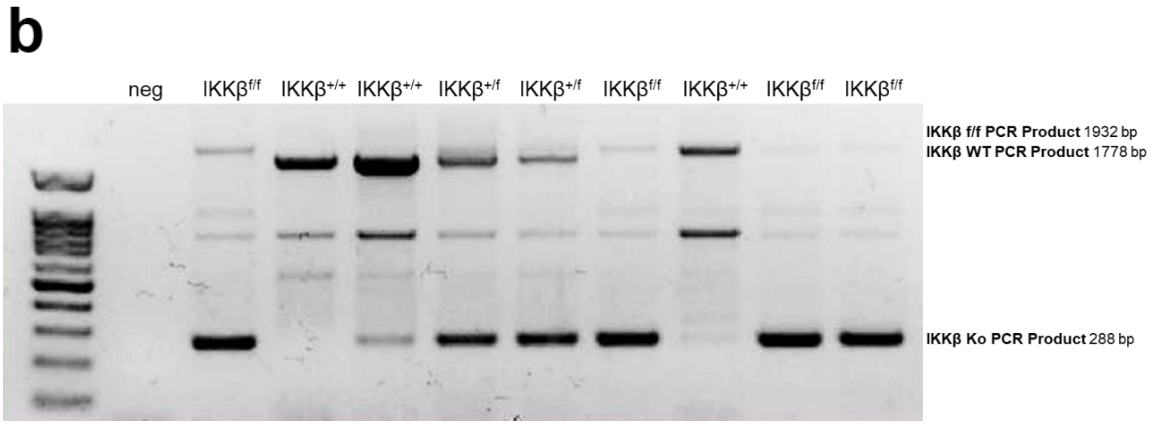
6.2 Chapter 4 – Supplementary Data



Appendix 5 – IKK2ca$^{mut/mut}$ mice display no change in colon length or body weight 16 weeks-post DSS.

(**a**) Average colon length of *IKK2*$^{+/+}$and *IKK2ca*$^{mut/mut}$ mice 98 days after DSS-treatment. Data are presented as mean ± SEM and dots represent biologically independent animals (*IKK2*$^{+/+}$, n = 12; *IKK2ca*$^{mut/mut}$, n = 9). (**b**) Percent change in initial weight of *IKK2*$^{+/+}$, *IKK2ca*$^{+/mut}$, and *IKK2ca*$^{mut/mut}$ mice after 98 days DSS-treatment. Data are presented as mean ± SEM and dots represent biologically independent animals (*IKK2*$^{+/+}$, n = 9; *IKK2ca*$^{+/mut}$, n = 4; *IKK2ca*$^{mut/mut}$, n = 17). Statistical significance was determined using unpaired Student's t-test or one-way ANOVA with Tukey's post-hoc test.


a
0.0231
2.0
1.5
1.0
0.5
0.0
Relative Ikbkb mRNA expression
IKKβ+/+
IKKβf/f


b
neg
IKKβf/f
IKKβ+/+
IKKβ+/+
IKKβ+/f
IKKβ+/f
IKKβf/f
IKKβ+/+
IKKβf/f
IKKβf/f
IKKβ f/f PCR Product 1932 bp
IKKβ WT PCR Product 1778 bp
IKKβ Ko PCR Product 288 bp

Appendix 6 – Confirmation of IKKβ loss in colonic tumors derived from *Dclk1^{CreERT2};APC$^{f/f}$;IKKβ$^{f/f}$* (*IKKβ$^{f/f}$*) mice.

(**a**) Relative mRNA expression of *Ikbkb* (gene encoding IKKβ) in colonic tumors of *IKKβ$^{+/+}$* and *IKKβ$^{f/f}$* mice as assessed by qPCR. Data are presented as mean ± SEM and dots represent biologically independent animals (*IKKβ$^{+/+}$*, n= 7; *IKKβ$^{f/f}$*, n = 10). (**b**) DNA PCR assay for detection of IKKβ WT (1778bp), IKKβ floxed (1932bp), and IKKβ-KO (288bp) alleles in colonic tumors of *IKKβ$^{+/+}$*, *IKKβ$^{f/+}$* and *IKKβ$^{f/f}$* mice (*IKKβ$^{+/+}$*, n= 3; *IKKβ$^{+/f}$*, n = 2; *IKKβ$^{f/f}$*, n = 4 individual tumors from biologically independent mice).

6.3 Animal Care & Biosafety Documentation

Appendix 7 – 2019 Animal Use Protocol (AUP)

Western

AUP Number: 2019-021
PI Name: Asfaha, Samuel
AUP Title: Breeding AUP for the Study of Gastrointestinal Stem Cells in Colitis and Gastrointestinal Cancers
Official Notification of ACC Approval: A MODIFICATION to Animal Use Protocol **2019-021** has been approved.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western's Senate MAPPs 7.12, 7.10, and 7.15 http://www.uwo.ca/univsec/policies_procedures/research.html
   b) University Council on Animal Care Policies and related Animal Care Committee procedures
   c) http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm
2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.http://uwo.ca/research/services/animalethics/animal_use_protocols.html
3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training ([training@uwo.ca]training@uwo.ca); and
   c) be overseen by me to ensure appropriate care and use of animals.
4) As per MAPP 7.15,
   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;

c) UCAC policies and related ACC procedures will be followed, including but not limited to:

i) Research Animal Procurement
ii) Animal Care and Use Records
iii) Sick Animal Response
iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance

the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets, http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

The University of Western Ontario
Animal Care Committee / University Council on Animal Care
London, Ontario Canada N6A 5C1
519-661-2111 x 88792 Fax 519-661-2028
[auspc@uwo.ca]auspc@uwo.ca ï¿½
http://www.uwo.ca/research/services/animalethics/index.html

Appendix 8 – Mouse Scoring Scheme for Colitis & Colorectal Cancer Models

Mouse Scoring Scheme – Colitis/Colon Cancer

Score	% Body Weight Loss	Diarrhea Score	Visible Fecal Blood	Appearance	Activity	Provoked Behaviour
0	Normal	Normal pellets	Normal	Normal	Normal – nesting; alert; active	Moves spontaneously with presence of hand in cage
1	5-10%	Slightly loose feces	Spotty blood	Lack of grooming	Activity decreased but still alert; responds to external stimuli; moves normally	Moves easily with gentle touch
2	10-15%	Loose feces and/or mild rectal prolapse	Slightly bloody	Pale and/or ruffled/unkempt coat; ocular or nasal discharge	Activity decreased; no evidence of nest building; movement is off	Sluggish to respond; requires a more forceful touch than above
3	15-20%	Watery diarrhea and/or moderate to severe rectal prolapse	Bloody	Pale; severe piloerection; dull eyes; discharge; skin cool to touch; hunching; eyes closed or squinting	Lethargic; not interested in surroundings or responding to external stimuli	Reluctant to move; requires force; moves only small distance

Criteria for early euthanasia:

- Total score is ≥ 9, greater than 20% of body weight loss*
- 3 mm rectal prolapse
- Lack of response to provoked behaviour
- Any condition where the veterinarian feels euthanasia is warranted

*colitis/colon cancer induced animals are allowed a higher weight loss (due to fluid loss) with the understanding the weight will rebound

Appendix 9 – Biosafety Permit

LAWSON
HEALTH RESEARCH INSTITUTE

Dear Dr. Asfaha,

Your biosafety approval number is **BSP-LHSC-0036.** This number is a requirement for your research grants, purchasing of biohazardous materials and Level 2 inspections. Please use this number on all correspondence with the Biosafety Officer (BSO).

This permit expires on **March 16, 2024**.

Research Grants

Your study's LBAPP number is required for any research grants involving biohazards. Please provide this number to Research Services when requested.

Purchasing Materials

Your LBAPP number must be included on purchase orders for all Risk Group 1 and Risk Group 2 pathogens and toxins. Please include your name as the Primary Investigator (PI) and your biosafety approval number on all purchase order through HMMS or on all University of Western Ontario purchases.

Annual Inspections

Your Containment Level 2 laboratory will be inspected every year by the BSO and Lawson Safety Analyst.

This permit allows you to work with Risk Group 1 and Risk Group 2 biohazardous agents.

To maintain your Biosafety Permit, you will need to:

- Have a complete, up to date Biohazardous Agents inventory (this will be checked annually);
- Ensure that the employees, students and researchers working in your laboratory are trained in Biosafety and all other required training modules;
- Ensure that your laboratory follows the requirements of the Lawson Biosafety Manual, relevant standard operating procedures (SOPs) and mitigation strategies on your Local Biosafety Risk Assessment; and
- Follow the guidance of the BSO and Lawson Safety Analyst on laboratory safety.

Please let me know if you have questions or comments.

Regards.

Charis Johnson-Antaran, MSc
Biosafety Officer
Lawson Health Research Institute
Ext 61456
Charis.johnsonantaran@lawsonresearch.com

LAWSON
HEALTH RESEARCH INSTITUTE

BIOSAFETY PERMIT

Permit Summary

Permit Holder	Dr. Samuel Asfaha		
Permit #	BSP-LHSC-0036	Containment Level	2+
Site/Zone/Floor/Room	A4-113, A4-128, A4-815, A4-108, A4-822		
Phone	519-685-8500	Extension	53293
E-mail	Samuel.asfaha@lhsc.on.ca		
Approval Date	March 17, 2021	Expiration Date	March 16, 2024
BSO Signature			
LBSC Chair Signature			

Approved Microorganisms	Lentivirus, Citrobacter Rodentium, E.coli DH5α, E.coli SW105
Approved Primary and Established Cell Lines	Primary human colonrectal tissue, rodent primary embryos and other organs, HEK293, HEK293T, HT-29, CACO2, HeLa, MKN28, MKN45, MKN74, Ct26.wt, L-WRN
Approved Human-source Material	Whole blood, colorectal tissues, colorectal tumors
Approved Genetic Modifications (plasmids/vectors/rDNA)	pLK0.1, LentiCRISPRv-2GFP, LentiCRISPRv2, Lenti dCAS-VP64_Blast, Lenti sgRNA(MS2)_puro optimized backbone, lentiMPHv2
Approved Use of Animals	*Mus Musculus*
Approved Biological Toxins and Hormones	Tamoxifen, Diphtheria Toxin
Approved Gene Therapy	
Approved Animal Source Material	
Approved Plants	
Approved Insects	

Curriculum Vitae

Hayley J. Good

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1. EDUCATION

2015 – 2021 **Doctor of Philosophy (PhD)**
Department of Pathology & Laboratory Medicine
Schulich School of Medicine & Dentistry
Western University, London ON

Project: The role of inflammation in colitis-associated cancer
Supervisor: Dr. Samuel Asfaha

2010 – 2015 **Bachelor of Medical Sciences (BMSc)**
Honours Double Major in Pathology and Medical Sciences
Schulich School of Medicine & Dentistry
Western University, London ON

2. HONOURS AND AWARDS

2.1 Scholarships & Travel Awards

2019 – 2022 **CIHR Doctoral Award** – Frederick Banting and Charles Best Canada Graduate Scholarship ($105,000), Canadian Health Research Institute

2020 **Dutkevich Memorial Foundation Award** ($1000), Dept. of Pathology & Laboratory Medicine, Schulich School of Medicine & Dentistry

2019 – 2020 **Ontario Graduate Scholarship (declined)** – Queen Elizabeth II Graduate Scholarship in Science and Technology, ($15,000)

2019 **Dr. Frederick Luney Graduate Scholarship in Pathology and Laboratory Medicine** ($5000), Dept. of Pathology & Laboratory Medicine, Schulich School of Medicine & Dentistry

2019 **Crohn's and Colitis Canada Student Research Prize** ($750), Canadian Digestive Diseases Week, Canadian Association of Gastroenterology

2018 – 2019	**Ontario Graduate Scholarship** – Queen Elizabeth II Graduate Scholarship in Science and Technology, ($15,000)
2018	**Dr. Frederick Luney Graduate Research Award** ($1600), Dept. of Pathology & Laboratory Medicine, Schulich School of Medicine & Dentistry
2016 – 2017	**Internal Research Fund Studentship** ($15,000), Lawson Health Research Institute
2015	**The University of Western Ontario Gold Medal**, highest standing in Major in Pathology, Western University

2.2 Presentation Awards

2021	**Best Oral Presentation Award** ($500), Department of Medicine Research Day, Schulich School of Medicine & Dentistry
2021	**Best Basic/Clinical Science Collaborative Poster Presentation** ($100), Pathology and Laboratory Medicine Research Day, Schulich School of Medicine & Dentistry
2019	**CIHR Silver Medal** ($250), Canadian Student Health Research Forum (CSHRF), University of Manitoba
2019	**Best Oral Presentation Award** ($500), Department of Medicine Research Day, Schulich School of Medicine & Dentistry
2018	**Certificate of Recognition as an Early Career Investigator**, Digestive Disease Week 2018, American Association of Gastroenterology
2018	**Best Poster Presentation Award** ($500), Department of Medicine Research Day, Schulich School of Medicine & Dentistry
2017	**Dr. M. Daria Haust Award for the Best Basic Science Oral Presentation** ($200), Pathology and Laboratory Medicine Research Day, Schulich School of Medicine & Dentistry

3. PUBLICATIONS

Castillo-Azofeifa D[*], Fazio EN[*], Nattiv R[*], **Good HJ**, Wald T, Pest MA, de Sauvage FJ, Klein OD, Asfaha S. (2019) Atoh1+ secretory progenitors possess renewal capacity independent of Lgr5+ cells during colonic regeneration. *The EMBO Journal*. DOI: 10.15252/embj.201899984
[*]Co-first authors

3.1 Conference Publications

Good HJ, Shin AE, Zhang L, Asfaha S. (2021) NF-κB signaling in DCLK1+ tuft cells affects colitis severity and colitis-associated cancer. *Gastroenterology.* 160(6):S-108.

Shin AE, **Good HJ**, Tesfagiorgis Y, Zhang L, Kerfoot SM, Sherman PM, Wang TC, Howlett CJ, Asfaha S. (2020) F4/80+Ly6Chi Macrophages contribute to cancer initiation in colitis. *Gastroenterology.* 160(6):S-623.

Shin AE, **Good HJ**, Tesfagiorgis Y, Zhang L, Kerfoot SM, Sherman PM, Wang TC, Howlett CJ, Asfaha S. (2020) F4/80+Ly6Chi Macrophages are key to cancer initiation in colitis. *Journal of the Canadian Association of Gastroenterology.* 4(suppl_1):1-2.

Good HJ, Shin AE, Zhang L, Asfaha S. (2020) Inhibition of NF-κB signaling in DCLK1+ cells promotes colitis and colitis-associated cancer. *Journal of the Canadian Association of Gastroenterology.* 3(suppl_1):37-38.

Iablokov V, **Good HJ**, Shin AE, Fazio EN, Loggie JW, Zhang L, Asfaha S. (2020) Hopx labels a colonic stem cell that contributes to colonic regeneration but not colonic tumors. *Journal of the Canadian Association of Gastroenterology.* 3(suppl_1):35-37.

Shin AE, **Good HJ**, Tesfagiorgis Y, Zhang L, Kerfoot SM, Sherman PM, Wang TC, Asfaha S. (2020) Role of myeloid cells in the initiation of colitis-associated colon cancer. *Journal of the Canadian Association of Gastroenterology.* 3(suppl_1):19-20.

Good HJ, Shin AE, Zhang L, Asfaha S. (2020) Inhibition of NF-κB signaling in DCLK1+ cells promotes colitis and colitis-associated cancer. *Gastroenterology.* 158(6):S-212-S-213.

Shin AE, **Good HJ**, Tesfagiorgis Y, Zhang L, Kerfoot SM, Sherman PM, Wang TC, Asfaha S. (2020) Role of myeloid cells in the initiation of colitis-associated colon cancer. *Gastroenterology.* 158(6):S-836.

Good HJ, Shin A, Zhang L, Fazio E, Meriwether D, Reddy S, and Asfaha S. (2019) The role of cyclooxygenase in colitis-associated cancer. *Gastroenterology.* 156(6):S-836.

Shin AE, **Good HJ**, Zhang L, Fazio EN, Sherman PM, Wang TC, Asfaha S. (2019) Role of LGR5 in Dclk1+ Cell-Derived Colitis-Associated Colon Cancer. *Gastroenterology*. 156(6):S-128.

Good HJ, Shin A, Zhang L, Fazio E, Meriwether D, Reddy S, and Asfaha S. (2019) The role of cyclooxygenase in colitis-associated cancer. *Journal of the Canadian Association of Gastroenterology.* 2(suppl_2):17-18.

Shin A, **Good HJ**, Zhang L, Fazio E, Sherman PM, Wang TC, and Asfaha S. (2019) Role of Lgr5 in Dclk1 positive cell-derived colitis-associated colon cancer. *Journal of the Canadian Association of Gastroenterology*. 2(suppl_2):36-37.

Good HJ, Shin A, Fazio E, Zhang L, and Asfaha S. (2018) The role of non-steroidal anti-inflammatory drugs in colitis-associated cancer. *Journal of the Canadian Association of Gastroenterology*. 1(suppl_2): 358.

Shin A, Fazio E, Zhang L, **Good HJ**, and Asfaha S. (2018) Role of doublcortin-like kinase 1 (Dclk1) tuft cells in colitis-associated colorectal cancer. *Journal of the Canadian Association of Gastroenterology*. 1(suppl_2): 359.

Good HJ, Fazio E, Shin A, Zhang L, and Asfaha S. (2018) The role of cyclooxygenase in colitis-associated cancer. *Gastroenterology*. 154(6)S-20-S-21.

Shin A, Fazio E, Zhang L, **Good HJ**, and Asfaha S. (2018) Role of doublcortin-like kinase 1 (Dclk1) tuft cells in colitis-associated colorectal cancer. *Gastroenterology*. 154(6)S23.

4. PRESENTATIONS

4.1 National & International Presentations

May 2021 — **Digestive Disease Week 2021**
Virtual Meeting (International)
Title: "*NF-κB signaling in Dclk1+ tuft cells affects colitis severity and colitis-associated cancer*"
Presentation: Platform

May 2020 — **Digestive Disease Week 2020** **Cancelled due to COVID-19**
Chicago IL, USA (International)
Title: "*Inhibition of NF-κB signaling in DCLK1+ cells promotes colitis and colitis-associated cancer*"
Presentation: Platform

February 2020 **Canadian Digestive Diseases Week 2020**
Montreal QC (National)
Title: "*Inhibition of NF-κB signaling in DCLK1+ cells promotes colitis and colitis-associated cancer*"
Presentation: Poster of distinction

June 2019 **Canadian Student Health Research Forum 2019***
University of Manitoba, Winnipeg MB (National)
Title: "*Aspirin inhibits the initiation of tuft cell-derived colitis-associated cancer"*
Presentation: Poster
**CIHR Silver Medal*

May 2019 **Digestive Diseases Week 2019**
San Diego CA, USA (International)
Title: "*The role of cyclooxygenase in colitis-associated cancer*"
Presentation: Poster of distinction

March 2019 **Canadian Digestive Diseases Week 2019***
Banff AB (National)
Title: "*The role of cyclooxygenase in colitis-associated cancer*"
Presentation: Platform
**CAG Student Research Prize*

June 2018 **Digestive Disease Week 2019**
Washington DC, USA (International)
Title: "*The role of cyclooxygenase in colitis-associated cancer*"
Presentation: Platform

February 2018 **Canadian Digestive Diseases Week 2018**
Toronto ON (National)
Title: "*The role of non-steroidal anti-inflammatory drugs in colitis-associated cancer*"
Presentation: Poster of distinction

4.2 Local and Institutional Presentations

May 2021 **Department of Medicine Research Day 2021**
Schulich School of Medicine & Dentistry, London ON
Title: "*Inhibition of NF-κB signaling in Dclk1+ cells promotes colonic inflammation and colitis-associated cancer"*
Presentation: Platform
**Best oral presentation*

March 2021 **Pathology & Laboratory Medicine Research Day 2021**
Schulich School of Medicine & Dentistry, London ON
Title: "*Aspirin prevents tuft cell-derived colitis-associated cancer in a PGE_2 and phopsho-Akt dependent manner"*
Presentation: Poster
**Best poster presentation*

May 2019 **Department of Medicine Research Day 2019***
Schulich School of Medicine & Dentistry, London ON
Title: "*Aspirin inhibits tuft cell-derived colitis-associated cancer*"
Presentation: Platform
**Best oral presentation*

April 2019 **London Health Research Day 2019**
Schulich School of Medicine & Dentistry, London ON
Title: "*The role of cyclooxygenase in the initiation of colitis-associated cancer*"
Presentation: Platform

March 2019 **Pathology & Laboratory Medicine Research Day 2019**
Schulich School of Medicine & Dentistry, London ON
Title: "*Role of cyclooxygenase in the initiation of colitis-associated cancer*"
Presentation: Poster

June 2018 **15th Annual Oncology Research & Education Day**
Schulich School of Medicine & Dentistry, London ON
Title: "*The role of cyclooxygenase in colitis-associated colon cancer*"
Presentation: Poster

May 2018 **London Health Research Day 2018**
Schulich School of Medicine & Dentistry, London ON
Title: "*The role of cyclooxygenase in the initiation of colitis-associated cancer*"
Presentation: Platform

May 2018 **Department of Medicine Research Day 2018***
Schulich School of Medicine & Dentistry, London ON
Title: "*The role of cyclooxygenase in the initiation of colitis-associated cancer*"
Presentation: Poster
**Best poster presentation*

April 2018 **Pathology & Laboratory Medicine Research Day 2018**
Schulich School of Medicine & Dentistry, London ON

Title: "*The role of cyclooxygenase in the initiation of colitis-associated cancer*"
Presentation: Poster

June 2017 **14th Annual Oncology Research & Education Day**
Schulich School of Medicine & Dentistry, London ON
Title: "*The effect of non-steroidal anti-inflammatory drugs on the initiation of Dclk1+ tuft cell-derived colorectal cancer*"
Presentation: Poster

March 2017 **Pathology & Laboratory Medicine Research Day 2017**
Schulich School of Medicine & Dentistry, London ON
Title: "*The effect of non-steroidal anti-inflammatory drugs on the initiation of Dclk1+ tuft cell-derived colitis-associated cancer*"
Presentation: Platform
**Best basic science platform presentation*

March 2017 **London Health Research Day 2017**
Schulich School of Medicine & Dentistry, London ON
Title: "*The role of Dclk1+ tuft cells in initiation of colitis-associated colorectal cancer*"
Presentation: Poster

June 2016 **13th Annual Oncology Research & Education Day**
Schulich School of Medicine & Dentistry, London ON
Title: "*The role of inflammation in Dclk1+ cell-derived colitis-associated cancer*"
Presentation: Poster

April 2016 **Pathology & Laboratory Medicine Research Day 2016**
Schulich School of Medicine & Dentistry, London ON
Title: "*Role of inflammation in transformation of Dclk1+ colon cancer initiating cells*"
Presentation: Poster

5. RELATED EXPERIENCE

2020 **Student Supervisor**, PEL Cooperative Education High School Student, London ON

2019 **Graduate Teaching Assistant**, *Pathology 4400B* – Current Concepts in the Pathogenesis of Human Diseases, Western University, London ON

2018 – 2019	**Student Supervisor**, *Biochemistry 4486E* – Cancer Biology Research Project, Western University, London ON Title: The role of COX inhibition on Dclk1+ cell-derived tumor organoids
2018 – 2019	**Student Supervisor**, PEL Cooperative Education High School Student, London ON
2018	**Student Supervisor**, CAG Summer Studentship Undergraduate Program
2018	**Graduate Teaching Assistant**, *Pathology 4400B* – Current Concepts in the Pathogenesis of Human Diseases, Western University, London ON
2017	**Graduate Teaching Assistant**, *Pathology 4200A* – Environmental Pathology, Western University, London ON