Role of Notch1 in acute colitis and colitis associated cancer

Adani Pujada Alcala
ROLE OF NOTCH1 IN ACUTE COLITIS AND COLITIS ASSOCIATED CANCER

by

ADANI PUJADA ALCALA

Under the Direction of Didier Merlin, PhD

ABSTRACT

Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) that causes chronic inflammation and ulcers affecting the innermost layer of the colon. Almost 1 million people suffer from UC in the United States. UC patients have an increased risk of colitis associated cancer (CAC). It is driven by continuous exposure to inflammation and progresses through “dysplasia-carcinoma axis”. Notch1 signaling is a conserved intracellular signaling pathway. It regulates cell fate, proliferation, differentiation, and death. However, Notch1 acts as a mediator or suppressor depending on the cellular context and disease stage. Aim of this study is to understand the mechanistic role of Notch1 in acute colitis and CAC. We used WT mice (control group) and crossed them with villin-cre mice to generate NCre, which cannot express Notch1 in colon epithelium. Acute colitis was induced with dextran sodium sulfate (DSS) for five days and
In recovery period, mice were given water after the completion of DSS cycle. CAC was induced by intraperitoneal injection of azoxymethane and two cycles of DSS. Enteroids were isolated from six weeks old NCre and WT mice to study proliferation and differentiation. Inhibition of Notch1 compromised epithelial crypt architecture, led to higher number of goblet cells, and compromised intestinal barrier function at the basal level. Notch1 mediated inflammation in DSS-induced acute colitis by increasing inflammatory cytokine levels and activating NF-kB pathway. In the acute colitis model following a recovery period, Notch1 promoted wound healing. In CAC, NCre mice showed higher susceptibility to inflammation as indicated by histology, higher number of polyps, and defective barrier function compared to WT mice. Western blot data showed increased p53 and cleaved caspase-3 expressions while decreased expressions of γ-H2AX and MLH1. Notch1 mediates acute colitis by cross-talking with NF-kB pathway and promoting abnormal proliferation. However, during the recovery period after acute colitis, Notch1 promotes wound healing. Inhibition of Notch1 exacerbates CAC condition due to lower levels of non-mutated p53 expression. Therefore, this study suggests that Notch1 inhibitors might be a better option as therapeutics only for acute colitis patients and induction of Notch1 signaling as a therapy, can protect against chronic colitis/CAC.

INDEX WORDS: Ulcerative colitis, Notch1 signaling, Colitis associated cancer, Tumor suppressor, Acute inflammation, Chronic inflammation
ROLE OF NOTCH1 IN ACUTE COLITIS AND COLITIS ASSOCIATED CANCER

by

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DEDICATION

To my parents, Daniel Matias Pujada and Gina Maribel Sernaque, who sacrificed so much to give my siblings and I a better future in this country.

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To the future women in STEM, be brave, you are more than a stereotype and gender. Be confident and don’t limit yourself from accomplishing your goals.
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LIST OF ABBREVIATIONS

4',6-diamidino-2-phenylindole (DAPI)
Azoxy methane (AOM)
Colitis Associated Cancer (CAC)
Sporadic colon cancer (CRC)
Crohn’s Disease (CD)
Ulcerative colitis (UC)
Dextran Sodium Sulfate (DSS)
Gastrointestinal tract (GI)
Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)
Hematoxylin & Eosin (H&E)
Human colon cancer cells (HCT116)
Notch intracellular domain (NICD)
Reactive Oxygen Species (ROS)
Delta-like 1,3,4 (Dl1,3,4)
Jagged-1 (Jag1)
Intestinal stem cells (ISCs)
G protein coupled receptor 5 gene (LGR5)
Mucin 2 (MUC 2)
Tight junctions (TJs)
Toll like receptors (TLRs)

Fluorescein isothiocyanate (FITC)

cyclooxygenase-2 (COX-2)

Nitric oxide synthase (iNOS)

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)

Proliferating cell nuclear antigen (PCNA)

Trefoil factor 3 (TFF3)

Damage-associated molecular pattern molecules (DAMPs)

Damage DNA response (DDR)

Mismatch Repair pathway (MMR)
1 GENERAL BACKGROUND

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is an incurable chronic inflammatory condition that affects the gastrointestinal (GI) tract [1, 2]. Around 3.1 million adults, in the United States, have being diagnosed with IBD [3]. This disease affects the quality of life, bringing substantial morbidity and complications that require hospitalizations and surgeries [4]. The exact cause of this disease is yet not known but studies appoint it to be hereditary, to environmental factors, or to abnormal immune responses [5]. There are two major forms of IBD: Crohn’s disease (CD) and Ulcerative colitis (UC). Both share similar symptoms; however, they also have remarkable differences in the location of inflammation and progression in the GI tract. CD commonly affects the small intestine, proximal colon, and all the layers of the GI tract, while UC is characterized by continuous inflammation of the rectal and colonic mucosal layers of the GI tract.

1.1.1 Ulcerative colitis

UC leads to epithelial barrier dysfunction affecting major components of the GI tract such as epithelial cells, goblet cells, immune cells, and commensal flora [1, 6]. In UC, due to inflammation, epithelial cell integrity is compromised resulting in the continuous stimulation of the mucosal immune system and the dysregulation of colonic permeability [7]. Permeability dysregulation allows the influx of pathogenic foreign substances and the efflux of immune cells leading to the development of chronic inflammation and then ulceration [8]. Due to this dysfunction, inflammatory responses inhibit the repair of tissue damage to fix mucosal homeostasis. Inflammation in short term can be a beneficial response; however, prolong inflammation can contribute to create a potential tumorigenic microenvironment [9].
1.2 Colitis associated cancer

Patients with chronically active UC have a 15-40% risk of developing colon associated cancer (CAC) due to the long duration of the inflammation in the colon [4]. CAC is a subtype of colorectal cancer, but several features make CAC specific and distinct from sporadic colorectal cancer (CRC) [10, 11]. While CRC develops from adenoma to carcinoma, CAC develops from dysplastic tissues among chronically active UC patients (Figure 1) [12]. CAC also presents a higher number of polyps in the distal colon region compared to CRC [8]. In CAC, chronic inflammation nurtures the tumor microenvironment and initiates carcinogenesis. Indeed, chronic inflammation favors the secretion of proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6, which promote the accumulation of reactive oxygen species (ROS) and maintains a pro-inflammatory environment favorable for oncogene activation and tumor suppressor gene mutations, activation of abnormal proliferation and differentiated anti-apoptotic pathways [10]. This disrupts the colonic epithelial homeostasis exposing the epithelial mucosa and adjacent cells.

Figure 1. CAC progression (above) vs. CRC progression (below).
CAC progresses from dysplasia to adenocarcinoma, while CRC develops from adenoma to carcinoma.
to inflammatory luminal content. This is followed by the alteration of immune responses, DNA damage, and mutations in DNA damage response genes [13-15].

1.3 Notch signaling

1.3.1 Notch family receptors

The Notch gene was first identified in *Drosophila melanogaster* and encodes a 300kD type I transmembrane receptor [16, 17]. Notch signaling is a conserved mechanism that controls a variety of cell fates and developmental pathways in different organisms that range from sea urchins to humans [18, 19]. In mammals, Notch proteins comprise a family of four receptors (Notch1-4) whose activity is controlled by Delta-like 1 (Dll1), Dll3, Dll4 and Jagged 1 (Jag1) and Jag2 family ligands [20-22]. The mutation of the Notch receptor has resulted in developmental abnormalities and in human pathologies as well [23, 24].

1.3.2 Notch1 structure and activation

*Figure 2. Notch1 protein domain structure.*

**Abbreviations used are:** ANK, ankyrin repeats; c, cleavage site at site 1,2 and 3; EGF, epidermal growth factor; HD, heterodimerization domain; LNR, Lin-Notch repeats; NRR, negative regulatory region; PEST, proline (P), glutamic acid (E), serine (S) and threonine (T) degradation domain; RAM, Rbp-associated molecule domain; SP, signal peptide; TAD, transactivation domain; TM, transmembrane domain. Adapted from (Jan Mašek and Andersson, 2017).

Notch1 contains four regions: a) extracellular epidermal growth factor (EGF)-like domains b) the negative regulatory region composed by three cysteine-Lin repeats and the
heterodimerization domain, c) the transmembrane domain, d) Notch intracellular domain (NICD) which contains a RBPjk association module (RAM) domain, seven ankyrin repeats, a transcription activation domain, and a transactivation domain containing degrons which regulate the stability of NICD (Figure 2).

Successful interaction of Notch1 receptor with the ligand, present in the neighboring cells, is mediated by 11-12 EGF repeats (trans interaction), while inhibitory interaction with the ligand, expressed in the same cell, is mediated by 24-29 EGF repeats (cis interaction) [25]. The Notch pathway can be activated canonically and non-canonically [26, 27]. The canonical signaling is well known to have a role during the early steps of organ development and the maintenance of cells of the organ [28, 29]. In the colon, Notch1 is required for the maintenance and differentiation of intestinal stem cells in the colon and maintains the balance between absorptive (epithelial cells) and secretory lineages (goblet, Paneth, tuft, and enteroendocrine cells) [30, 31]. Canonical activation of Notch1 results of three cleavages, two extracellular and one intracellular (Figure 3). The canonically activation starts when any Delta or Jag family ligand binds to extracellular Notch receptors located on the cell surface. This binding will trigger a first extracellular cleavage, mediated by furin-like proteases such as convertase, in the trans-Golgi network. The second extracellular cleavage is mediated by serine proteases or metalloproteases. Lastly, a third intracellular cleavage is mediated by intracellular γ-secretase generating the active Notch1 intracellular domain (NICD). NICD translocates to the nucleus where it forms a complex with transcription regulatory factors (CSL/RBP-J). This complex then allows the displacement of co-repressors [32] and the recruitment of co-activators [33] that activate the transcription of target genes such as c-Myc, p53, ARF, and Hes [20, 34, 35].
Very recently, the non-canonically activation of Notch1 has also been identified and is found to be involved with physiological and pathological cellular processes [36], such as cancer [37] and activation of the immune system. This differs from the regular cellular processes driven by the canonical Notch1 pathway. The non-canonical activation is RBP-Jk independent and plays an important role in the oncogenesis process in certain cell types. Some of the mechanisms that can activate Notch1 pathway crosstalk with any pathways such as β-catenin/Wnt signaling, MAPK, mTOR, etc [26].

![Notch1 signaling canonical activation](image)

**Figure 3. Notch1 signaling canonical activation.**

Interaction between Notch1 receptor and Notch ligands (Jagged/Delta) triggers two consecutive proteolytic cleavages by a metalloprotease and the γ-secretase complex. This generates NICD, which enters the nucleus and displaces corepressors (SMRT and CtBP1) and recruits the coactivator MAML1 and the acetyltransferase p300, resulting in the transcription of genes bound to the NICD-RBP-J complex, such as Hes. Adapted schematics (Lobry et al., 2014; Ranganathan et al., 2011)

### 1.3.3 Notch1 expression in the colon

Notch1 is highly expressed at the basal crypt of the colon and in the colon lamina propria cells [38, 39]. Notch1 signaling also orchestrates different biological functions such as cell
differentiation, proliferation, apoptosis, cell-fate, etc. controlling the organ morphogenesis as well as tissue homeostasis [20, 21] [40, 41]. Notch1 can activate Hes transcription factor implicated in the maintenance of progenitor and stem cells; thus, promoting epithelial cell differentiation in the colon [39]. Because Notch1 plays a critical role in these essential processes in the colon tissue, the dysregulation of Notch1 can contribute to multiple abnormalities that range from developmental disorders [42] [43] to inflammation and then cancer [44].

2 THE ROLE OF NOTCH1 IN ACUTE COLITIS

2.1 Introduction

UC is characterized by continuous inflammation of the rectal and colonic mucosal layers which extends proximally [4]. UC leads to epithelial cell barrier dysfunction affecting GI tract major components such as epithelial cells, goblet cells, immune cells, and commensal flora [1, 6]. Because of this alteration, inflammatory responses arise to help repair colonic injury and fix tissue homeostasis.

Intestinal homeostatic renewal of the damaged epithelial monolayer is mediated by a pool of intestinal stem cells (ISCs) called crypt base columnar cells (CBCs), which are constantly generating new progenitors. CBCs are found intercalated with Paneth like cells at the crypt of the colon highly expressing the G protein coupled receptor 5 gene (Lgr5) [45]. Notch1 receptor is expressed in CBCs promoting active Notch1 signaling in these cells to allow their differentiation into epithelial cells [46] while also serving as a marker for Lgr5 positive cells.

In UC, goblet cells are decreased or depleted [47-49]. Goblet cells main role is the secretion of Mucin 2 (MUC2), a component of colonic mucus, which protects the mucus layer, against luminal stimulants and pathogens. Differentiation of ISCs into goblet cells is facilitated
by Math1, an antagonistic of Notch1 transcription factor-Hes 1, which promotes the differentiation of secretory cells into goblet, Paneth, and enteroendocrine cells [50, 51].

The colonic epithelium barrier also acts as a line of defense against foreign antigens such as toxins and microorganisms [8, 52]. Colonic epithelial integrity is defined by apical junctions comprising tight junctions (TJs), adherent junctions (AJs), and adjacent colonic epithelial cells [53, 54]. TJs are apical protein complexes formed by transmembrane proteins, claudins, occludin and tricellulin. All three are connected to the actin cytoskeleton via zona occludens (ZO). In UC, epithelial integrity is compromised allowing the influx of pathogenic foreign substances which are recognized by toll like receptors (TLRs) from macrophages and dendritic cells leading to the activation of pro-inflammatory cytokines such as TNF-α, interlukin-6 (IL-6), and IL-1β. Notch1 receptor activation has been confirmed in studies done by the induction of synthetic TLRs using bacterial pathogens suggesting that TLRs can indirectly promote the activation of Notch1 [55].

The immediate repair response to an injured intestinal epithelial layer is the restitution process of epithelial cells. During this process, local epithelial cells redistribute around the wound to reseal and restore the continuity of this layer but most importantly to preserve colonic homeostasis. This first step does not require cell proliferation [56], but it does require other factors such as cytokines [57, 58], cell adhesion molecules [59], and trefoil factors [56]. Cell proliferation is required after restitution to replenish the decreased pool of epithelial cells. Epithelial cell proliferation is induced by the secretion of growth factors [60] from local epithelial cells and mesenchymal cells close to the injury. Hes-1, a downstream target of Notch1 signaling, drives progenitor cells to differentiate into absorptive epithelial cells.

The pathogenesis of UC tells us that there is loss of the mucosal immune system and epithelial cell integrity [61], linking the regenerative process disturbance directly with Notch1
functions in the colon. Therefore, understanding the role of Notch1 in UC can assist in understating the mechanism by which Notch1 mediates inflammation in UC.

2.2 Materials and Methods

2.2.1 Animal models

All animal procedures were performed in accordance with the Guide for the Care of Use of Laboratory Animals as described previously [58]. Eight-weeks old, both female and male, Villin\textsuperscript{cre/+}; Notch\textsuperscript{loxP/lox} (NCre) and wild-type (WT) C57/B6 background were used in these studies.

To investigate the role of Notch1 in acute colitis and CAC, epithelial cell-specific deletion of Notch1 was done using the Cre/Lox system. Mice harboring a floxed promoter region of the Notch1 gene (Notch1\textsuperscript{loxP/+}) were purchased from The Jackson laboratory. The Notch1\textsuperscript{loxP/+} were bred to generate Notch1\textsuperscript{loxP/loxP} and were monitored for healthy development and fertility until maturity. Notch1\textsuperscript{loxP/loxP} were bred with Villin-cre in which ‘cre’, under the control of the villin promoter, is specifically expressed in epithelial cells to generate Villin\textsuperscript{cre/+}; Notch1\textsuperscript{loxP/lox} (NCre) mice. The Notch1 suppression among NCre mice was identified by using PCR genotyping using Notch forward primer (5′-GCAGTTGTGCTCTAGAA-3′) and reverse primer (5′-CGGGCCGACAGGAA-3′).

2.2.2 Acute colitis (AC) induction and recovery phase

Eight weeks-old, both female and male, NCre and WT mice were induced with acute colitis by one cycle of 2% Dextran sodium sulfate (DSS) (MP Biomedicals, Solon, OH) by oral administration through their drinking water for 5 days (Figure 4). To study the role of Notch1 in recovery period after acute colitis, another set of mice were treated with one cycle of 2% DSS for five days and then they were allowed to recover for 7 days. Mice was euthanized on day 12.
(Figure 4). Control group mice, NCre and WT, were given regular water for the same amount of days. Body weight and stool consistency for all the mice were recorded during both, DSS and recovery phase.

**Figure 4. DSS-induced acute colitis model and DSS-induce colitis recovery model.**

*Abbreviations used are:* D-Day and DSS-Dextran sodium sulfate

### 2.2.3 Cell culture and wound healing assay

HCT116 cells, colon human carcinoma cells, were cultured with DMEM medium (GIBCO, Invitrogen) supplemented with 10% FBS (Atlanta Biologicals), and 100 units/ml penicillin, and 100 mg/ml streptomycin (GIBCO) at 37°C in 5% CO₂.

Cells (5 x 10⁵) were seeded in 6 well plates and incubated for 24 hrs. When cells were 70-90% confluent, cells were then transfected with or without NICD DNA construct (a kind gift from Dr. R. Kopan, University of St. Louis). 24 h after transfection, monolayer was wounded by scratching method with a sterile pipette tip and images were taken at 0, 24, and 48 hrs by using Keyence BZ-X700 microscope. Transfection efficiency was done by collecting cell lysates at the mentioned time points and were probed with anti-NICD (Cell Signaling).

### 2.2.4 Protein extraction and Western Blot (WB) analysis

Colon mucosal stripping was obtained from NCre and WT mice with and without acute colitis. Antibodies used were anti-NICD (Cell Signaling), anti-COX-2 (Cayman chemical), anti-
NFkB (Cell Signaling), anti-iNOS (Millipore), and anti-Cyclin D1 (Santa Cruz Biotechnology). Goat anti-mouse secondary antibody (Bio-Rad) or goat anti-rabbit secondary antibody (Abcam) were used. β-tubulin (Sigma-Aldrich) was used as loading control.

### 2.2.5 Mouse colonoscopy

NCre and WT mice with and without acute colitis were subjected to colonoscopy to assess the mucosal layer thickness due to inflammation and dysplastic lesions. This was performed using the colonoscope (Xenon Nova 47S, STORZ).

### 2.2.6 Hematoxylin and Eosin (H&E) staining and histological score evaluation

Formalin fixed and paraffin embedded colon Swiss rolls from NCre and WT mice with and without acute colitis were used. Histological scores were evaluated based on inflammation-infiltration of white blood cells, crypt damage, and foci of ulceration in the entire colon [62, 63]. Images were taken using Keyence BZ-X700 microscope at X20 and X40 magnification.

### 2.2.7 Alcian Blue-Periodic Acid Schiff (AB-PAS) staining

Formalin fixed and paraffin embedded colon Swiss rolls from NCre and WT mice with and without acute colitis were used. Sections were hydrated and incubated with acetic acid for 3 min. Next, sections were stained with 3% Alcian blue (Sigma-Aldrich). After washing, sections were incubated with 0.5% periodic acid and then with Schiff reagent (Sigma-Aldrich) at room temperature. Sections were then hydrated with alcohol, cleared with xylene, and sealed. Images were taken using Keyence BZ-X700 microscope at X20 magnification.

### 2.2.8 In-vivo permeability assay

Eight weeks-old age, both female and male, NCre and WT mice were gavaged with permeability tracer (60 mg/100g body weight of FITC-labeled dextran) (Sigma-Aldrich). Serum was collected retro-orbitally 4 h after gavage and fluorescence intensity of each sample was
measured at $\lambda_{ex}=492$ nm; $\lambda_{em}= 525$ nm by using Synergy 2; BioTek. FITC-dextran concentrations were determined from standard curves generated by serial dilutions of FITC-dextran and permeability was calculated by linear regression of sample fluorescence [64, 65].

2.2.9 Myeloperoxidase (MPO) assay

NCre and WT with and without AC colon tissue samples were homogenized in cold potassium phosphate buffer (50 mmol/l K2HPO4 and 50 mmol/l KH2PO4, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich). The homogenates were sonicated, freeze-thawed three times, and centrifuged. MPO activity in the samples was determined and compared to MPO standard after measuring the absorbance at 450nm [64].

2.2.10 Colonoid extraction

Crypts were isolated from six weeks NCre and WT mice. Crypts were grown to colonoids [66] on Matrigel (Corning, New York, NY) in a 24 well plate. Then, the growth of colonoids on the plates, were observed every day and images were taken at X10 and X20 magnification with ZEISS Primover AxioCam MRm microscope for days 2, 4, 7, and 9.

2.2.11 RNA extraction and QPCR analysis

Total RNA was extracted from colonic tissues using the RNeasy mini Kit (Qiagen) according to the manufacturer’s instructions. Then, complementary DNA was generated from the earlier-described total RNA isolated using the Maxima first-strand complementary DNA synthesis kit (ThermoFisher Scientific). mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction using Maxima SYBR green quantitative polymerase chain reaction Master Mix (ThermoFisher Scientific). The following sense and antisense primers (Table 1) were used: *IL-6*, *IL-1β*, *TNF-α*, *IFN-γ*, *IL-17*, *and 16SrRNA*. Real-
time PCR data is presented using the DDCT method and using 36B4 gene levels as the internal standard.

Table 1. Sequences of the QPCR primers used in the study.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Nucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18</td>
<td>F: 5'-TCGCTCAGGGGTCACACAAGAAA-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CATCAGAGGCAAGGAGAAAAAC-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: 5'-AGGGCTGCCCCGACTACGT-3'</td>
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<tr>
<td></td>
<td>R: 5'-GACTTTTCTCTGGGTATGAGATAGCAAAA-3'</td>
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<td>F: 5'-ACAAAGTGGAGGGCTTAATTACACAT-3'</td>
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<tr>
<td></td>
<td>R: 5'-TTGCCATTGGCAACAACCTCTTTTC-3'</td>
</tr>
<tr>
<td>IL-17</td>
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<tr>
<td></td>
<td>R: 5'-CTTTCCCCCTCCGCATTGACA-3'</td>
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<td>IFN-γ</td>
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<tr>
<td>16SrRNA</td>
<td>F: 5'-AGAGTTTGATCTGGCCTACAG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AAGGAGGTGWTCCTCCACCC-3'</td>
</tr>
<tr>
<td>36B4</td>
<td>F: 5'-TCCAGGGGTGGGCACTCA-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTTTATCAGCTGACATCAGTCAGA-3'</td>
</tr>
</tbody>
</table>

Abbreviations used are: F: forward, R: reverse
2.2.12 Immunofluorescent staining of colon Swiss rolls and colonoids

NCre and WT colon Swiss rolls were formalin fixed and paraffin embedded for immunofluorescence staining. Colon swiss rolls were deparaffinized and tissue was hydrated with graded concentration of ethanol. For antigen retrieval, proteinase K (Denville Scientific Inc.) was used for 1 hr at 37°C. Slides were washed with 0.01% TPBS 3 times for 5 mins. Endogenous peroxidase activity block and immunostaining was performed according manufacture’s protocol using the Alexa Fluor™ 488 Tyramide SuperBoost™ Kit, goat anti-rabbit IgG. Primary antibody used was anti-Lgr5 (Abcam). Colon Swiss rolls were inoculated with Hoechst 3342, Trihydrochloride, Trihydrate in 1X PBS for 30 mins, washed with 1X PBS, and finally inoculated with TRITC-phalloidin diluted with 1X PBS for 1 h [67]. Images were taken with Keyence BZ-X700 microscope at X40.

NCre and WT colonoids were grown on Matrigel for 4 days in Tissuetek chambered slides (Thermo-Fischer) for immunofluorescence staining. Colonoids were fixed with 4% paraformaldehyde solution for 2h, washed with 1X PBS, and inoculated with FocusClearTM for 2 nights [68]. Colonoids or Swiss rolls were permeabilized with 2% TritonX100 solution and washed with IF buffer (0.2% TritonX100, 0.05% Tween 20 in 1X PBS). Blocking buffer (2% TritonX100, 10% goat serum, and 0.02% sodium azide in 1XPBS) was used and then, colonoids were inoculated with primary antibody anti-TFF3 (Millipore Sigma), and anti-PCNA (Abcam) in dilution buffer (0.25% TritonX100, 1% normal serum goat, 0.02% sodium azide in 1X PBS) overnight. Colonoids were washed with IF buffer and inoculated later with secondary antibody anti-rabbit Alexa Fluor 488- green (ThermoFisher) for 2 nights. After washing, colonoids were inoculated with Hoechst 3342, Trihydrochloride, Trihydrate in dilution buffer for 1h, washed
again with IF buffer, and finally inoculated with TRITC-phalloidin in dilution buffer for 2 h. Images were taken with Keyence BZ-X700 microscope at X40.

2.2.13 Blood biochemical analysis

Blood samples (100µL) were collected retro-orbitally from, both female and male, NCre and WT mice. Biochemical analyses were obtained by loading the blood sample in VetScan rotor spins and measuring the chemical parameters by the biochemical analyzer VetScan VS2, Abaxis.

2.2.14 Statistical analysis

All data was expressed as means ± SEM and were compared by Student’s t-test [69]. P values <0.05 was considered statistically significant.

2.3 Results

2.3.1 Characterization of NCre mice

To study the role of Notch1 in acute versus chronic inflammation in colon, NCre mice was generated by crossing Notch1loxP/loxP and Villin cre mice (C57/B6 background) in which “cre” (under the control of the villin promoter, is expressed in colon epithelial cells). Eight weeks old, both female and male, NCre mice along with their wild type WT mice were used for these studies. The Notch1 deletion among NCre mice was identified by using PCR genotyping. Figure 5A-i shows the genotyping result where lanes 2-4 are homozygous NCre mice expressing only one band (281 bp) while lanes 6, 8, and 9 are heterozygous mice, expressing two bands, the mutant and the WT (281 and 231 bp, respectively). To confirm Notch1 deletion at protein level, protein lysates were prepared from NCre and WT mice vital organs such as colon, small intestine, and liver. Figure 5A ii-iii show the western blot analysis from colon and small intestine respectively indicating the lack of NICD (Notch1 intracellular domain- cleaved or active Notch1)
presence in colon (lanes 4-6) and small intestine (lanes 3-4), among NCre mice compared to WT mice. However, Figure 5A- iv shows presence of Notch1 signaling in the liver of NCre mice (lanes 4-6).

To assess the histological changes in the colon due to the conditional deletion of Notch1, H&E staining was performed as described in the methods section. Figure 5B shows the H&E images indicating disrupted crypt architecture, increased neutrophil infiltration, and a few foci of ulceration among NCre mice compared to WT mice. Figure 5C is a bar graph representation of the histological score calculated on three parameters as described in the methods section. The bar graph shows that NCre mice had a significantly higher histological score (2.6 ± 0.9) compared to WT mice (0.7 ± 0.6). Notch1 mediates enterocyte population against goblet cells, mucus secretory cells, during stem cell differentiation in colon [38]. To assess the goblet cell hyperplasia due to the conditional deletion of Notch1 signaling, AB-PAS staining was performed. Figure 5D shows AB-PAS images indicating that NCre had more goblet cells as indicated by red arrows compared to WT mice. Figure 5E is the bar graph representation of the quantification of goblet cells indicating that NCre mice had a significantly increased percentage of goblet cells (41.49% ± 1.97) compared to WT mice (25.93% ± 4.84).

To assess the role of Notch1 in maintaining epithelial integrity, 4kD FITC–dextran uptake assay was performed. Figure 5F shows that epithelial barrier was compromised as NCre mice showed a higher number of FITC units (33.0 ± 7.8) compared to WT mice (11.8 ± 3.9). These results together indicate that inhibition of Notch1 signaling compromises epithelial cell integrity due to higher number of goblet cells and impaired intestinal barrier function.
Figure 5. Conditional deletion on Notch1 signaling in mice colon leads to the loss of epithelial crypt architecture, increased goblet cell population, and a defective intestinal barrier function.

NCre mice were generated by using a conditional knockout method of ‘Villin-cre’ system and WT mice were used as a control. (A) (A-i) Qualitative PCR of the DNA extracted from mice
tails with the primers designed by the supplier (Jackson Laboratories). Homozygous NCcre mice has one mutant band at 281 bp while heterozygous mice have two bands, the mutant at 281 and the WT at 231 bp. Lane 1 is 100 bp ladder. Each lane shows blots that were performed loading (25µg/lane) using whole organ cell lysate of (A-ii) colon (A-iii) small intestine (A-iv) liver probed with anti-NICD. GAPDH was used as a loading control. (B) H&E staining of NCcre and WT colon Swiss rolls (n=5 per group). H&E images were taken at X20. Magnified view of the squared area is shown in the right panel of the original picture at X40. (C) Bar graph presentation of the histological score calculated on three parameters- infiltration of neutrophils, loss of crypt architecture, and foci of ulceration. (D) NCcre and WT colon swiss rolls (n=5 per group) were stained with AB-PAS. Images were taken at X20 magnification. Red arrows show goblet cell population. (E) Quantification of goblet cell percentage was obtained by counting goblet cells per epithelial cell crypt (12 crypts/mice). (F) In vivo permeability was performed by gavaging NCcre and WT mice with 4kD FITC-dextran in PBS (60mg/100g body weight). Then, serum via retro-orbitally was collected after 4 hrs., and the fluorescence within the blood was measured at λex=492 nm; λem= 525. Each bar represents mean ± S.E., *p<0.05, ***p<0.001.

2.3.2 Conditional deletion of Notch1 in colonic epithelium compromises epithelial cell differentiation and proliferation in the colon

To investigate the effects of the absence of Notch1 signaling on stem cell differentiation, immunofluorescence staining of stem cell marker, Lgr5, was performed using colon Swiss rolls from NCcre and WT mice. Figure 6A shows a higher expression of Lgr5, as indicated by white arrows, among WT compared to NCcre colon crypts. To further see the effect of Notch1 silencing in colonic stem cell differentiation and proliferation, three dimensional colonoids isolated from
NCre and WT mice were used as *ex vivo* model. Figure 6B i-iv shows the images of crypts growing into colonoids monitored for days 2,4,7, and 9, respectively. Figure 6-iv shows that crypts isolated from NCre mice differentiated slowly as the colonoids look smaller and less branched compared to the colonoids from WT mice. Figure 6B-v shows a line graph representing the measured area of organoids per day and it indicates that NCre colonoids had significantly decreased area on the days 2,4,7, and 9 compared to WT colonoids. Additionally, immunofluorescent staining of 4 days old colonoids was performed by probing fixed NCre and WT colonoids with PCNA (proliferation marker) and TFF3 (goblet cell differentiation marker). Figure 6C-i shows PCNA staining images indicating less expression among the NCre derived colonoids compared to WT derived colonoids. Figure 6C-ii shows TFF3 staining images indicating high expression (as a goblet cell marker) among NCre derived colonoids compared to WT derived colonoids. Together these data imply that Notch1 signaling plays a critical role in maintaining the stem cell differentiation and proliferation of epithelial cells in the colon.
Figure 6. Conditional deletion of Notch1 compromises colonic epithelial cell differentiation and proliferation.

(A) NCre and WT colon Swiss rolls were probed with anti-Lgr5. FITC as green fluorescence was used as secondary antibody. (B) Crypts isolated from NCre and WT mice colons were grown on
Matrigel to form colonoids in 24 well plates. Colonoid formation was observed and images were captured on (B-i) day 2 and (B-ii) day 4, at X20 magnification, and (B-iii) day 7 and (B-iv) day 9 at X10 magnification. (B-v) Organoid area was measured (6 organoids/ phenotype) by using ImageJ program and values are shown in X 10^4 cm^2. (C) Immunofluorescence staining of the 4 days old NCre and WT derived colonoids, fixed in chambered slides (n=4 wells per group), were probed with (C-i) anti-PCNA and (C-ii) anti-TTF3. FITC as green fluorescence was used as secondary antibody. Images are the Z-sections at X40 magnification. DAPI was used to counterstain nuclei with blue fluorescence and red fluorescence is for actin-phalloidin. Each bar represents mean ± S.E., *p<0.05, **p<0.01.

2.3.3 NCre mice presents increased levels of biochemical parameters

To evaluate the alteration of biochemical parameters due to the conditional deletion of Notch1 signaling in vivo, VetScan VS2, a chemistry blood analyzer machine, was used. Blood samples (100 µL) from NCre and WT mice were collected for biochemical analysis. As shown on Table 1, this analysis revealed a higher number of the following functional parameters: ALB (3.6 ± 0.1; 3.96 ± 0.2), ALT (28.8 ± 2.3; 34.4 ± 2.5), GLU (180.8 ± 37; 287.4 ± 37.3), TP (4.52 ± 0.2 4.8 ± 0.1), and AMY (543.8 ± 94.6; 665.2 ± 47.3) among NCre mice compared to WT mice, respectively. Together this data indicates that the lack of Notch1 expression in the colon, leads to increased levels of ALB and GLU indicating the inflammation in liver.
Table 2. Biochemical analysis of NCre and WT mice.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>WT</th>
<th>NCre</th>
</tr>
</thead>
<tbody>
<tr>
<td>** ALB (2.5-3.0 g/dL)</td>
<td>3.6 ±0.1</td>
<td>3.96 ± 0.2</td>
</tr>
<tr>
<td>* TP (N/A g/dL)</td>
<td>4.52 ± 0.2</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>GLOB (N/A g/dL)</td>
<td>0.9 ± 0.2</td>
<td>0.86 ± 0.2</td>
</tr>
<tr>
<td>ALP (N/A U/L)</td>
<td>192.6 ± 17.4</td>
<td>162.4 ± 24.9</td>
</tr>
<tr>
<td>** ALT (17-77 U/L)</td>
<td>28.8 ± 2.3</td>
<td>34.4 ± 2.5</td>
</tr>
<tr>
<td>* AMY (N/A U/L)</td>
<td>543.8 ± 94.6</td>
<td>665.2 ± 47.3</td>
</tr>
<tr>
<td>TBIL (0-1 mg/dL)</td>
<td>0.38 ± 0.1</td>
<td>0.34 ± 0.1</td>
</tr>
<tr>
<td>BUN (8-33.0 mg/dL)</td>
<td>25.6 ± 1.1</td>
<td>27.4 ± 5.6</td>
</tr>
<tr>
<td>CA (7.1-10.1 mg/dL)</td>
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<td>9.96 ± 0.2</td>
</tr>
<tr>
<td>PHOS (4.2-8.5 mg/dL)</td>
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<td>6.72 ± 0.4</td>
</tr>
<tr>
<td>CRE (0.2-0.9 mg/dL)</td>
<td>0.38 ± 0.1</td>
<td>0.28 ± 0.1</td>
</tr>
<tr>
<td>** GLU (62-175 mg/dL)</td>
<td>180.8 ± 37</td>
<td>287.4 ± 37.3</td>
</tr>
<tr>
<td>NA+ (140-160 mmol/L)</td>
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<td>146 ± 2.5</td>
</tr>
<tr>
<td>K+ (4.5-7.5 mmol/L)</td>
<td>4.64 ± 0.5</td>
<td>4.74 ± 0.4</td>
</tr>
</tbody>
</table>

Biochemistry parameters: ALB: Albumin; TP: Total protein; GLOB: Globulin; ALP: Alkaline; ALT: Alanine aminotransferase Phosphatase; AMY: Amylase; TBIL: Total bilirubin; BUN: Blood urea nitrogen; CA: Calcium; PHOS: Phosphorus; CRE: Creatinine; GLU: Glucose. (mean ± standard deviation, n=5 mice/group).*p<0.05, **p<0.01.
2.3.4 *Noct1* signaling mediates inflammation in DSS-induced acute colitis

Eight weeks old, both female and male, NCre mice and WT mice were induced with acute colitis by DSS as described in the methods section. Body weight of mice was monitored throughout the experiment and mice were euthanized at day 5. Figure 7A shows the body weight change in percentage indicating a significant increase in body weight among NCre mice compared to WT mice at day 5 of DSS-induced with colitis. Figure 7B shows colonoscopy images indicating the thickening of mucosa and bleeding among WT mice compared to NCre mice. To assess inflammation among NCre and WT mice in acute colitis, MPO assay was performed as described in the methods section. Figure 7C is the bar graph representation of the MPO activity of the colon indicating that NCre mice had significantly lower (3.63 ± 0.17) MPO activity compared to WT mice (7.14 ± 1.18) in acute colitis. To assess the severity of inflammation in NCre mice induced with colitis, H&E staining was also performed. Figure 7D shows H&E images indicating crypt architecture damage, infiltration of neutrophils, and loci of ulceration among WT mice compared to NCre mice in acute colitis. Figure 7E shows AB-PAS images indicating that NCre had a decreased number of goblet cells but non-significant, as indicated by red arrows, compared to WT mice in acute colitis. Figure 7F is the bar graph quantification of goblet cells indicating there was no significant difference in goblet cell population between NCre mice (37.5 ± 5.3) and WT mice (38.2 ± 3.35) in acute colitis.
NCre and WT mice (n=5 each group) were treated with one cycle of 2% DSS for 5 days. Body weight was monitored every day and mice were euthanized at day 5. (A) Line graph representation of body weight change in percentage of NCre and WT mice with DSS-induced acute colitis. (B) Colonoscopy images from WT mice (top panel) and NCre mice (bottom panel).

Figure 7. Notch1 signaling mediates inflammation DSS-acute colitis

NCre and WT mice (n=5 each group) were treated with one cycle of 2% DSS for 5 days. Body weight was monitored every day and mice were euthanized at day 5. (A) Line graph representation of body weight change in percentage of NCre and WT mice with DSS-induced acute colitis. (B) Colonoscopy images from WT mice (top panel) and NCre mice (bottom panel).
with or without induced colitis. (C) Bar graph representation of MPO activity. (D) H&E staining of colonic Swiss rolls from NCre and WT mice in acute colitis at X20. (E) AB-PAS staining of colon Swiss rolls from NCre and WT mice with acute colitis. Images were taken at X20 magnification. (F) Quantification of goblet cell was obtained by counting goblet cells per epithelial cell crypt (12 crypts/mice). Each bar represents mean ± S.E., *p< 0.05, **p< 0.01, ***p< 0.001.

2.3.5 Notch1 signaling modulates cytokines levels in acute colitis

To assess the inflammatory environment, mRNA levels of cytokines that are known to mediate acute inflammatory progression to acute colitis were measured by qPCR as described in the methods section. Interestingly, the mRNA levels of IL-6 were significantly low among NCre mice (7.48 ± 1.4-fold) compared to WT mice (15.08 ± 1.80-fold) (Figure 8A). Figure 8B-E shows significantly increased mRNA levels of IL-1β (3.27 ± 0.53-fold), TNF-α (2.89 ± 1.10-fold), IFN-γ (9.19 ± 2.39-fold), and IL-17 (9.58 ± 0.4.82-fold) among NCre mice compared to WT mice (1.10 ± 1.04, 1.37 ± 0.42, 1.50 ± 1.36; 1.62 ± 1.57-fold). These results together indicate that Notch1 signaling mediates DSS-induced colitis and is associated with upregulation of pro-inflammatory and downregulation of anti-inflammatory cytokines.
Figure 8. Notch1 signaling modulates inflammatory cytokines levels in acute colitis.

Bar graph representation of qPCR analyses representing mRNA levels of cytokines in colonic mucosal stripping from NCre and WT mice with acute colitis. Relative change in mRNA levels
of the cytokines (A) IL-6 (B) IL-1β (C) TNF-α (D) IFN-γ and (E) IL-17 are shown. Each bar represents mean ± S.E., *p< 0.05, **p< 0.01.

**Notch1 signaling mediates NF-κβ activation in acute colitis**

To assess the mechanistic pathway by which Notch1 signaling mediates acute colitis, western blot (WB) analysis was performed to identify the inflammatory signaling pathways. Figure 9A shows no significant difference in COX-2 protein levels among both groups. Figure 9B shows negligible levels of i-NOS protein in both groups. Figure 9C and 9D shows decreased protein levels of NF-kB and its downstream target, Cyclin D1, respectively among NCre mice (lanes 4-6) compared to WT mice (lanes 1-3). NF-kB is a pro-inflammatory signaling pathway, regulating the secretion of inflammatory cytokine levels [70] and Cyclin D1 regulates cell cycle progression [71]. Together these data indicate that Notch1 activates NF-kB inflammatory pathway promoting abnormal cell proliferation and thereby favoring inflammation in acute colitis.

<table>
<thead>
<tr>
<th>Acute colitis</th>
<th>WT</th>
<th>NCre</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td><img src="image" alt="COX-2" /></td>
<td><img src="image" alt="COX-2" /></td>
</tr>
<tr>
<td>i-NOS</td>
<td><img src="image" alt="i-NOS" /></td>
<td><img src="image" alt="i-NOS" /></td>
</tr>
<tr>
<td>NF-kB</td>
<td><img src="image" alt="NF-kB" /></td>
<td><img src="image" alt="NF-kB" /></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td><img src="image" alt="Cyclin D1" /></td>
<td><img src="image" alt="Cyclin D1" /></td>
</tr>
<tr>
<td>β-tubulin</td>
<td><img src="image" alt="β-tubulin" /></td>
<td><img src="image" alt="β-tubulin" /></td>
</tr>
</tbody>
</table>

**Figure 9.** Notch1 signaling is associated with increased expression of NF-kB and Cyclin D1 in acute colitis.
WB analyses of whole cell protein lysates (25µg/lane) using colon mucosal stripping from NCre and WT mice with acute colitis. Blots were probed with (A) anti-COX-2 (B) anti-iNOS (C) anti-NF-κB and (D) anti-Cyclin-D1. β-tubulin was used as loading control.

2.3.6 Notch1 promotes wound healing

Eight weeks old, both female and male, NCre mice and WT mice induced with acute colitis by DSS were allowed to recover for 7 days as described in the methods section. Their body weight was monitored throughout the experiment and mice were euthanized on day 12. Figure 10A shows that at the end of DSS phase NCre mice had significantly body weight loss compared to WT mice. However, during recovery phase, NCre mice were unable to recover compared to WT mice. Figure 10B shows colonoscopy images indicating the increased mucosa thickening, bleeding, and inflammation among NCre mice (indicated by red arrows) compared to WT mice after 7 days of recovery. Figure 10C-i shows the histological images indicating that NCre mice presented increased loss of crypt architecture, neutrophil infiltration, and foci of ulceration compared to WT mice. Figure 10C-ii is the bar graph representation of the histological inflammatory score based on three parameters mentioned in the methods section indicating that NCre mice had a significantly higher histological score compared to WT mice after the recovery phase. Furthermore, to test the hypothesis that Notch1 signaling indeed promotes colonic wound healing, colon carcinoma human cell line HCT116 was used and transiently transfected to overexpress NICD as in vitro model. Wound healing assay was performed as described in the methods section. Figure 10D-i indicates that NICD overexpression promoted cell migration and proliferation to heal the wound compared to control cells. Figure 10D-ii shows the quantification of wound closure indicating that HCT116 cells overexpressing NICD initiated wound closure by
24 hours (87.3%) compared to control cells (91.5 %). Together these data indicate that Notch1 signaling mediates wound healing at recovery phase following acute colitis.

**Figure 10. Notch1 promotes wound healing in the recovery phase following acute colitis.**

NCre and WT mice (n=5 each group) were treated with one cycle of 2% DSS for 5 days and went under a recovery phase for 7 days. Body weight was monitored every day and mice were
euthanized at day 12. (A) Line graph representation of body weight change in percentage of NCre and WT mice during one cycle of DSS and one recovery phase. (B) Colonoscopy images from WT mice (top panel) water, acute colitis, and recovery phase, respectively. The bottom panel shows the same treatments for NCre mice. Arrows indicate signs of inflammation and thickening of mucosa layer. (C) H&E staining of colon cross sections from NCre and WT mice completed a recovery phase. (C-i) Magnified view of the squared area is shown in the right side of the original picture at X40 indicating the disrupted architecture of crypts and infiltration of neutrophils. (C-ii) Bar graph representation of histological score calculated on three parameters: infiltration of neutrophils, loss of crypt architecture, and foci of ulceration. (D) Wound healing assay of human colon carcinoma HCT116 cells transfected to overexpressed NICD. (D-i) Cell proliferation and migration was monitored and images were taken at 0hrs, 24hrs, and 48 hrs. (D-ii) Quantification of wound closure was performed by calculating the wound area using ImageJ program and it is represented as percent initial wound. Data represent the mean ± S.E., *p< 0.05.

2.4 Discussion

UC is a form of IBD causing inflammation and ulcers in the colon either in segments or completely. The main symptom of active disease is usually constant diarrhea mixed with blood. UC as its name suggests, affects only the colon and rectum, leaving the rest of the GI tract unaffected, hence is very different from CD (another form of IBD). Although UC has no known cause, presumed genetic component and susceptibility to western diet and lifestyle increases the prevalence of this disease. UC is triggered in a susceptible person by environmental factors, although dietary modifications may reduce the discomfort of a person with the disease. Recent Studies of migrant populations as well as populations
of developing countries have demonstrated a slow but steady increase in the incidence of UC [72]. The incidence of UC is higher than CD incidence in the United States [73]. UC is characterized by continuous inflammation throughout the colon affecting the mucosal layer of the GI tract compromising epithelial cells integrity and increasing the paracellular permeability allowing the entrance of foreign antigens and microorganisms to the lamina propria [8, 52].

Notch1 signaling is a highly conserved intercellular signal transduction pathway which is induced upon environmental cues. It is associated with colonic inflammation [26] and colon cancer [74]. Notch1 is a key player in the differentiation of intestinal stem cells to epithelial /enterocyte lineage [75]. Notch1 activation requires three cleavages: two extracellular and one intracellular [35]. Translocation of Notch1 intercellular domain (NICD) to the nucleus activates downstream targets such as Hes, c-Myc. These downstream targets are directly involved with differentiation, inflammation, or carcinogenesis pathways [35].

Inflammatory mediators modulate Notch1 signaling and have been documented in acute colitis. However, the precise mechanistic role of Notch1 signaling in acute colitis is not well understood. Therefore, in the present study, a conditional Notch1 knockout mice (NCre) lacking Notch1 expression in colon epithelial cells was generated to identify the role and mechanism of Notch1 in acute colitis.

We observed that the conditional deletion of Notch1 in colonic epithelial cells resulted in higher histological score, increased goblet cells population, defective barrier function, compromised epithelial cell differentiation and proliferation, and liver inflammation at the basal level. Interestingly, we also observed that silencing of Notch1 signaling attenuated inflammation in the in vivo DSS-induced acute colitis model as implied by colonoscopy, decreased MPO levels, and higher histological score. These data together showed less severity of colonic
inflammation among NCre mice compared to WT mice. Although, in acute colitis the conditional deletion of Notch1 signaling did not cause goblet cell hyperplasia among NCre mice compared to WT mice.

Recent studies have found the activation of Notch1 during inflammation. Dysregulated Notch1 signaling has been associated with inflammatory conditions such as rheumatoid arthritis [76], systemic lupus erythematosus [77], pre-term labor [78], bacterial infection [79]. Although, one can also reason that inflammatory cells modulate Notch1 through canonical and non-canonical signaling pathways [80]. Inflammation can stimulate the Notch1 pathway through an extrinsic stimulus like recognition of pathogen infection toll-like receptors (TLRs) activating innate immune cells or through an intrinsic stimulus such as immune signaling molecules (cytokines) [26]. Our data shows that interestingly the conditional deletion of Notch1 significantly decreased mRNA levels of pro-inflammatory cytokine, IL-6, increased mRNA levels of the pro-inflammatory cytokines: IL-1β, IFN-γ, and TNF-α, while it decreased anti-inflammatory cytokines: IFN-γ and IL-17 compared to Notch+/+ mice with acute colitis. Increased levels of pro-inflammatory cytokine IL-6 among Notch+/+ mice with acute colitis might be due to the possible non-canonical activation of Notch1 signaling in the cytoplasm to intrinsically upregulate IL-6 signaling mediating inflammation in acute colitis compared to WT mice in acute colitis.

Additionally, there was no difference in the expression of COX-2, an inflammatory enzyme mediator, in both NCre and WT mice. Several of the transcription factors regulate COX-2 are known to be activated by inflammatory cytokines [81], which explains a low but constant expression in both NCre and WT mice induced with acute colitis. There was also negligible expression of i-NOS, which is known to produce nitric oxide that dysregulates inflammatory
processes and causes tissue injury [82]. However, we observed a significant decrease in NF-kB protein levels as well as its downstream target cyclin D1 among NCre mice compared to WT in acute colitis. NF-kB is an inflammatory pathway [83] which regulates the expression of Cyclin D1. Cyclin D1 is a cell cycle progression marker and regulates cell cycle progression and cell proliferation [71]. Therefore, this study elucidates that Notch1 signaling mediates acute colitis by activating NF-kB signaling pathway. Activation of NF-kB promotes the proliferation of inflamed epithelial cells via its downstream target Cyclin D1 as well as IL-6 secretion (Figure 11).

The epithelial lining of the GI tract continually renews throughout life [84]. This layer has the capacity of regenerating whenever there is a damage [85]. During UC, the mature epithelial cell migration (restitution process) and regeneration process (proliferation process) gets disturbed by the unmanageable progression of inflammation on the mucosal layer. The restitution process involving local epithelial cells redistribute around the wound to reseal and restore the continuity of this layer but most importantly to preserve homeostasis of the tissue and it is mediated by cytokines [57] [58], cell adhesion molecules [59], trefoil factors[56], etc. Cell proliferation is required after restitution to replenish the decreased pool of progenitor cells that will later differentiate into mature epithelial cells. This lineage decision and maturation of epithelial cells is mediated by Notch1 signaling in the GI tract [28, 86]. Although, Notch1 signaling seems to mediate inflammation in DSS-induced acute colitis, we sought to further investigate its role in the colonic epithelia restitution and regeneration system, in a UC completed recovery phase model.

Interestingly, we observed an opposite role played by Notch1 signaling during recovery after acute colitis phase. Notch1 signaling mediated wound healing during recovery phase as indicated by gain of body weight, and lower historical score among WT mice compared to NCre
mice. Additionally, our *in vitro* data suggested that the overexpression of NICD in HCT116 cells facilitated in the restoration of the scratched-wounded area by the first 24 hrs. Therefore, these data together imply that Notch1 plays a contrasting role in recovery phase following acute colitis by enhancing wound healing in the colon.

Our study highlights that Notch1 is critical in the maintenance of epithelial cell integrity, goblet cell homeostasis, efficient barrier function, epithelial cell differentiation and proliferation under normal conditions. We also observed the contrasting role of Notch1 in acute colitis versus a completed recovery phase. Notch1 signaling worsens acute colitis but mediates wound healing during recovery phase of acute inflammation. Overall, the modulation of Notch1 signaling in this study confirms the differing role of Notch1 signaling in different settings.

In acute colitis, inflammation causes epithelial-mucosal injury and disrupts the defensive barrier against pathogenic luminal contents. Therefore, the delicate equilibrium between healthy epithelial-mucosal restitution versus the removal of inflamed epithelium is lost due to inflammatory mediators such as cytokines, chemokines, and growth factors. Consequently, epithelial cells fail to exit the inflammatory stage. Loss of healthy epithelium is the major challenge in preventing acute colitis to chronic inflammation progression. Therefore, as long-term cure, therapeutics pertinent to epithelial-mucosal restitution are very critical for UC patients. The beneficial role of Notch1 in wound healing could be translated as an efficient targeted therapy for UC patients.
Figure 11. Notch1 signaling mediates inflammation in acute colitis by activating NF-κB signaling pathway.

Activation of NF-κB signaling pathway promotes the proliferation of inflamed epithelial cells via its downstream target Cyclin D1 as well as IL-6 cytokine secretion. **Abbreviations used are:** NICD- Notch1 intracellular domain; S-2,3-Site 2,3.
3 THE ROLE OF NOTCH1 IN COLITIS ASSOCIATED CANCER

3.1 Introduction

Although acute or short term inflammation may be self-healing, repeated flares of acute inflammation can cause chronic inflammation and contribute to create a tumorigenic microenvironment [9] as seen in colitis associates cancer (CAC). Chronic inflammation leads to the secretion of proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6, which promote the accumulation of reactive oxygen species (ROS) maintaining a pro-inflammatory environment favorable for mutations, activation of proliferation and anti-apoptotic pathways [10]. This causes a disruption of the intestinal homeostasis, inhibition of the proper communication between the epithelial mucosa and adjacent tissues, an altered immune response, DNA damage, and an accumulation of mutations in DNA repair genes involved in DNA damage response [13, 14, 87].

DNA damage response (DDR) signaling is triggered by DNA double-strand breaks (DSBs) and it is initiated by phosphatidylinositol-3-like protein kinase ataxia-telangiectasia mutated (ATM). Then, a complex known as MRN (MRE11, RAD50, and NBS1) is formed and localized to the DSBs together with ATM. After this recruitment, a lysine acetyltransferase 5 (Tip60) binds ATM directly which induces its activity and the acetylation of ATM, an early step of ATM activation. The phosphorylation of histone H2AX is critical for signal amplification and recruitment of DDR factors at DNA lesions. This signal is further amplified by the engagement of downstream checkpoint kinases, CHK1 and CHK2, cell-cycle checkpoint components [88]. FOXO3a has also been proposed to bind to ATM upon DNA damage and to be necessary for its activation [89]. DDR signaling comprises different pathways and one of them is the mismatch repair (MMR) pathway. MMR role is to repair base-base mismatches and insertion/deletions loops arising from replication errors. Two key proteins in this system are the MutS and MutL families.
MutS is comprised by MSH2 and MSH6 proteins and MutL is comprised by MLH1 and PMS1 proteins. Complexes (MutS/MutL) interact with each other to orchestrate a recruitment of downstream repair proteins, such as proliferating cell nuclear antigen (PCNA), to execute DNA repair [90].

Complete remission of CAC would require the elimination of inflammation as well as the repair of damaged epithelium. Epithelial repair can be done by organized steps of restitution, proliferation, and differentiation of epithelial cells. Recent studies have focused in Notch1 signaling because of its role in cell proliferation, differentiation, and self-destruction processes. Studies have shown that disruption of transcription factors downstream of Notch1 signaling, such as Hes1, in mice results in the depletion of intestinal epithelial cells [30, 50, 91]. Administration of a pharmacological Notch1 inhibitor results in an increase number of goblet cells in mice intestine, providing the evidence that Notch1 regulates the lineage decision of epithelial cells [92, 93]. However, this regulation in a chronic inflammation environment has yet to be studied.

Notch1 signaling function has been found to be context dependent in different cell types and tissue context, at different developmental stages, and under normal versus pathological conditions. Notch1 can be oncogenic or tumor suppressive, this means that it can promote irregular differentiation in some tissues [94] or it can help maintain stem cells homeostasis in others [95]. Its oncogenic role was first illustrated in 1991 where Notch1 was suspected to be related to the development of T-cell acute lymphocytic leukemia (T-ALL)/lymphoma [96]. Studies have shown that activated Notch1 can induce neoplastic transformation in mouse mammary and salivary glands and in rat kidney cells in vitro [97-99]. Another study mentions that Notch signaling is required for the neoplastic transformation in Ras-transformed human breast cancer cells [98]. Studies in
mice have shown that Notch1 signaling in sporadic colon cancer (CRC) is also oncogenic and it is required for adenoma formation due to the elevated Wnt signaling that occurs in adenomatous polyposis coli/multiple intestinal neoplasia (Apc\textsuperscript{Min}) mice model of colon cancer [28, 44, 100, 101]. Moreover, activated Notch1, via β-catenin, has the oncogenic role in promoting primary melanoma cells progression [102].

However, different studies have shown that Notch1 is not exclusively oncogenic, it can also act as a tumor suppressor depending on the inflammatory stimulus and context. For example, Liu et al have shown that deletion of Notch1 in one allele leads to vascular tumors and massive hemorrhage in the liver and it is associated with decreased mice survival suggesting a Notch1 tumor suppressive function [95]. Another study using Notch1-deficient mice, in epidermis and corneal epithelium, developed basal-cell-carcinoma like tumors after being chemical-induced for carcinogenesis, again suggesting a tumor suppressor role in the mammalian skin [103]. Notch1 also functions as a tumor suppressor by inhibiting the tumor growth of mouse prostate cancer cells [104] and small cell lung cancer cells [105]. It was previously shown that inhibition of Notch1 by the γ-secretase inhibitor DAPT (N- (N-(3,5 difluorophenacetyl)-1-alanyl 1]-S-phenylglycine t-butyl ester) reduces apoptosis and promotes CAC. As previously mentioned, different groups have also shown that Notch1 has a protective role in a variety of malignancies such as as prostate cancer [104], cervical cancer [106, 107] and skin cancer [103]. However, the precise mechanism of action of Notch1 signaling in CAC remains unclear. Therefore, understanding the mechanistic role of Notch1 would assist in the identification of key proteins and signaling pathways that are critical to treat CAC development. Additionally, this could also bring a direct treatment to prevent cancer in UC patients.
3.2 Materials and Methods

3.2.1 Colitis associated cancer (CAC) induction

Ten weeks old, both female and male, NCre and WT mice were injected intraperitoneally with Azoxymethane (AOM) (7.6 mg/kg) (Sigma-Aldrich), a cancer inducer, on day 0. On day 7, both mice groups were exposed to 2% DSS (w/v) (MP Biomedicals, Solon, OH) by oral administration through their drinking water for 7 days. On day 14, their water was changed to regular drinking water. On day 28, a second cycle of 2% DSS was administered to both groups and after a week DSS was changed to regular drinking water and mice were euthanized on day 56 (Figure 12). Body weight and stool consistency of all mice was monitored during DSS and recovery phase.

![Colitis associated cancer model](image)

**Figure 12.** AOM/DSS-induced colitis associated cancer (CAC) model.

Abbreviations used are: D-Day, AOM- Azoxymethane, and DSS-Dextran sodium sulfate.

3.2.2 Protein extraction and Western blot (WB) analysis

Colon mucosal stripping was obtained from NCre and WT mice with and without CAC. Antibodies used were anti-NICD (Cell signaling), anti-p53 (Cell Signaling), anti-Cleaved caspase 3, (Cell Signaling), anti-p21\(^{WAF1/CIP1}\) (BD Biosciences), anti-\(\gamma\)H2AX (Abcam), and anti-MLH1 (Abcam). Goat anti-mouse secondary antibody (Bio-Rad, Hercules, CA) or goat anti-rabbit secondary antibody (Abcam) were use. \(\beta\)-tubulin (Sigma-Aldrich) or GAPDH (Abcam) were used as loading control.
3.2.3 **Mouse colonoscopy**

NCre and WT mice with and without CAC were subjected to colonoscopy to assess the mucosal layer thickness due to inflammation and dysplastic lesions. This was performed using the colonoscope (Xenon Nova 47S, STORZ).

3.2.4 **Hematoxylin and Eosin (H&E) staining and histological score evaluation**

Formalin fixed and paraffin embedded colon Swiss rolls from NCre and WT mice with and without CAC were used. Histological scores were evaluated based on inflammation-infiltration of white blood cells, crypt damage, and foci of ulceration in the entire colon. Images were taken using a Keyence BZ-X700 microscope at X20 and X40 magnification.

3.2.5 **Alcian Blue- Periodic Acid Schiff (AB-PAS) staining**

Formalin fixed and paraffin embedded colon Swiss rolls from NCre and WT mice with and without CAC were used. Sections were hydrated and incubated with acetic acid for 3 min. Next, sections were stained with 3% Alcian blue (Sigma-Aldrich). After washing, sections were incubated with 0.5% periodic acid and then with Schiff reagent (Sigma-Aldrich) at room temperature. Sections were then hydrated with alcohol, cleared with xylene, and sealed. Images were taken using a Keyence BZ-X700 microscope at X20 magnification.

3.2.6 **Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining**

Paraffin sections of colons were deparaffinized and apoptotic cells were identified by immunofluorescent terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining using In Situ Cell Death Detection Kit (Millipore-Sigma), Fluorescein according to the manufacturer’s instructions. Quantification of apoptosis was performed by counting the number of apoptotic cells in a crypt and was shown as percentage.
3.2.7 In vivo-permeability assay

Ten weeks-old age, both female and male, NCre and WT mice treated with or without CAC were gavaged with permeability tracer (60 mg/100g body weight of FITC-labeled dextran) (Sigma-Aldrich). Serum was collected retro-orbitally 4 h after gavage and fluorescence intensity of each sample was measured at λex=492 nm; λem= 525 nm by using Synergy 2; BioTek. FITC-dextran concentrations were determined from standard curves generated by serial dilutions of FITC-dextran and permeability was calculated by linear regression of sample fluorescence.

3.2.8 Myeloperoxidase (MPO) assay

NCre and WT with and without CAC colon tissue samples were homogenized in cold potassium phosphate buffer (50 mmol/l K2HPO4 and 50 mmol/l KH2PO4, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma-Aldrich). The homogenates were sonicated (40% amplitude), freeze-thawed three times and centrifuged (4°C at 14,000 rpm). MPO activity in the samples was determined and compared to MPO standard (Sigma-Aldrich) after measuring the absorbance at 450nm.

3.2.9 RNA extraction and QPCR

Total RNA was extracted from colonic tissues using the RNeasy mini Kit (Qiagen) according to the manufacturer’s instructions. Then, complementary DNA was generated from the earlier-described total RNA isolated using the Maxima first-strand complementary DNA synthesis kit (ThermoFisher Scientific). mRNA expression was quantified by quantitative real-time reverse-transcription polymerase chain reaction using Maxima SYBR green quantitative polymerase chain reaction Master Mix (ThermoFisher Scientific). The following sense and antisense primers (Table 1) were used: TNF-a, S100A8, S100A9, and 16SrRNA. Real-time PCR data is presented using the DDCT method and using 36B4 gene levels as the internal standard.
3.2.10 Statistical analysis

All data was expressed as means ± SEM and were compared by Student’s t-test [69]. P values <0.05 was considered statistically significant.

3.3 Results

3.3.1 Conditional deletion of Notch1 in colonic epithelium increases CAC susceptibility

Ten weeks old, both male and female, NCre and WT mice were induced with CAC as described in the methods section. Body weight was monitored throughout the experiment and mice were euthanized at day 56. Figure 13A shows the body weight change in percentage indicating that NCre mice had a significant decrease in body weight at the end of week 5 and week 6 compared to WT mice in CAC. Figure 13B shows a bar graph representation of colon length indicating that there was a significant decrease in colon length among the NCre mice compared to WT mice in CAC. Figure 13C shows colonoscopy images indicating that NCre mice presented flat polyps, thickened mucosa, and inflammation compared to WT mice in CAC. Figure 13D shows the quantification of the polyps indicating that there was indeed a significant number of polyps on NCre mice (7.2 ± 1.75) compared to WT mice (4.0 ± 0.82) in CAC. Figure 13E shows the histological images of colon Swiss rolls indicating increased infiltration of inflammatory cells, an extensive loss of crypt architecture, increased foci of ulceration, and dysplasia among NCre mice compared to WT mice in CAC. Figure 13F is the bar graph representation of the histological score based on the criteria as mentioned in the methods section. The bar graph indicated that NCre mice presented significantly higher histological score (9.4 ±
1.38) compared to WT mice (7.29 ± 1.11) in CAC. Together these data indicate that deletion of Notch1 signaling in the colon exacerbates CAC condition.

Figure 13. Conditional deletion of Notch1 in colonic epithelium results in increased susceptibility to CAC.
NCre and WT mice (n=7 each group) were treated with one AOM intraperitoneal injection and 2 cycles of 2% DSS to induce CAC. Body weight were monitored throughout the experiment and mice were euthanized at day 56. (A) Line graph representation of body weight change of NCre and WT (B) Bar graph representation of colon length among NCre mice and WT mice in CAC. (C) Colonoscopy images from WT mice (top panel) and NCre mice (bottom panel). Arrows indicate polyps, the thickening of mucosa, and inflammation. (D) Bar graph representation of number of polyps among NCre mice and WT mice in CAC. (E) H&E staining of colon Swiss rolls from NCre mice and WT mice in CAC. Left panel shows images at X10 and right panel at X20. (F) Bar graph presentation of the histological score calculated on three parameters-infiltration of neutrophils, loss of crypt architecture and foci of ulceration. Each bar represents mean ± S.E., *p< 0.05, **p<0.01, ***p<0.001.

3.3.2 Notch1 signaling modulates cytokine levels, maintains epithelial cell integrity and barrier function, and maintains the microbiome population

Chronic inflammation favors the secretion of pro-inflammatory cytokines and damaged-associated molecular pattern molecules (DAMPs) nurturing the tumor microenvironment that can progress to carcinogenesis and metastasis [108, 109]. The mRNA expression level of pro-inflammatory cytokine, TNF-α, and damage-associated molecular pattern (DAMP) molecules, such as S100A8 and S100A9, were measured by performing QPCR. Figure 14A shows the significantly increased levels of TNF-α among NCre mice (2.36 ± 1.13-fold) compared to WT mice (0.64 ± 0.09-fold) in CAC. Figure 14B interestingly shows no significant difference in mRNA levels of S100A8 in both NCre mice (4.11 ± 2.78-fold) compared to WT mice (1.34 ± 0.47-fold) in CAC. However, Figure 14C shows significantly decreased levels of S100A9 among
NCre mice (1.22 ± 0.60-fold) compared to WT mice (2.59 ± 0.65) in CAC. In the setting of chronic inflammation, the epithelial barrier function is compromised which worsens CAC conditions, resulting in the downregulation of the microbial population. Epithelial barrier function was assessed by gavaging mice with 4kD FITC-dextran. Figure 14D shows significantly increased number of FITC units among NCre mice (39.5 ± 8.6) compared to WT mice (23.9 ± 3.12) in CAC. Figure 14E shows decreased levels of 16SrRNA (universal bacteria primer) among NCre mice (1.73 ± 0.8-fold) compared to WT mice (3.21 ± 0.24-fold) in CAC. Together these data indicate that conditional deletion of Notch1 promotes the secretion of pro-inflammatory cytokine, TNF-α, and impairs epithelial cell integrity and microbiota population in CAC.
Figure 14. Conditional deletion of Notch1 increases cytokine levels and disrupts colonic epithelia cell barrier function in CAC.

NCre and WT mice (n=7 each group) were treated with one AOM intraperitoneal injection and 2 cycles of 2% DSS to induce CAC. Bar graph representation of qPCR analyses representing
mRNA levels of cytokine and DAMP molecules, in colonic mucosal stripping from NCre and WT mice in CAC, respectively. (A) TNF-α (B) S100A8 and (C) S100A9. (E) In vivo permeability was performed by gavaging NCre and WT mice with 4kD FITC-dextran in PBS (60mg/100g body weight). Then, serum via retro-orbitally was collected after 4 hrs, and the fluorescence within the blood was measured at λex=492 nm; λem= 525. (D) Bar graph representation of qPCR analysis representing mRNA levels of 16SrRNA. Each bar represents mean ± S.E., *p< 0.05, **p< 0.01, NS= non-significant.

3.3.3 Notch1 signaling activates tumor suppressor p53 and downstream target p21WAF1/Cip1 increasing apoptosis and decreasing DDR and MMR proteins in CAC

The secretion of pro-inflammatory cytokines, under chronic inflammation, promotes the accumulation of reactive oxygen species (ROS), promoting a pro-inflammatory environment favorable for oncogene activation and tumor suppressor gene mutations. NICD, the cleaved cytoplasmic domain of Notch1, can translocate to the nucleus and activate tumor suppressor transcription factors, such as p53 which also happens to be the first gene mutation to appear in CAC. Therefore, to identify the mechanistic pathway by which Notch1 signaling acts as a tumor suppressor in CAC, protein lysates from the colon mucosal stripping were used for western blot analysis as described in the methods section. Figure 15A-i confirms the lack of NICD protein expression (lanes 4-6) among NCre mice compared to WT mice (lanes 1-3) in CAC. Figure 15A-ii indicates the absence of p53 protein among NCre mice (lanes 4-6) compared to WT mice (lanes 1-3) in CAC. ROS accumulation, due to chronic inflammation, can produce DNA damage, accelerate cancer cell growth, and alter anti-apoptotic pathways increasing mutations in DNA response (DDR) and mismatch repair (MMR) genes, which are two important cellular events for
the maintenance of healthy epithelium [10, 13-15, 110]. To assess apoptosis in CAC, TUNEL staining was performed in the colonic epithelium. Figure 15B shows the overlay image indicating that there was a significant decrease in apoptosis, as indicated by white arrows, among NCre mice compared to WT mice in CAC. Further, to confirm the activation of apoptosis associated proteins, Caspase-3 and p21\textsuperscript{WAF1/Cip1}, in CAC, WB analysis was performed. Figure 15C shows decreased protein level of cleaved caspase-3 among NCre mice (lanes 3-5) compared to WT mice (lanes 1-2) in CAC. Figure 15D-ii also shows the decreased protein level of p21\textsuperscript{WAF1/Cip1} among NCre mice (lanes 4-5) compared to NCre mice (lanes 1-3) in CAC. To assess the effects of conditional deletion of Notch1 on DDR and MMR proteins in CAC, WB analyses of γ-H2AX and MLH1 were performed. Figure 15D-i and 14D-iii show higher protein levels of γ-H2AX and MLH1 among NCre mice (lanes 4-5) compared to WT mice (lanes 1-3) in CAC. Together these data indicate that conditional deletion of Notch1 in colonic epithelium is associated with lower levels of p53, p21, and apoptosis. Therefore, absence of Notch1 signaling leads to increase DNA damage and impaired MMR pathway.
Figure 15. Notch1 exhibits a tumor suppressor role by increasing apoptosis and DDR and MMR proteins in CAC.
NCre and WT mice (n=7 each group) were treated with one AOM intraperitoneal injection and 2 cycles of 2% DSS to induce CAC. Western blots of protein from colon mucosal stripping from NCre and WT mice induced with CAC were used and probed with (A-i) anti-NICD (A-ii) antip53 and (C) anti-Cleaved caspase-3. (B) The overlay images of green-yellow nuclei apoptotic cells as indicated by white arrows. Magnification at X20. (D) WB of protein from colon mucosal stripping from NCre and WT mice induced with CAC were used and probed with (D-i) anti-γ-H2AX (D-ii) anti-p21WAF1/Cip1 and (D-iii) anti-MLH1. β-actin or GAPDH was used as a loading control and each lane shows (25ug/lane) from an individual mouse.

3.4 Discussion

Chronic inflammation increases the risk for developing cancer. It is known that patients with chronically active UC have a 15-40% risk of developing CAC due to the long duration of the inflammation in the colon [4]. CAC progresses from dysplastic tissue to adenoma and it is different from colorectal cancer (CRC) progression. CRC progression starts from adenoma to carcinoma stage. Chronic inflammation environment is a complex network involving different cell types, inflammatory cytokines, signaling molecules, extracellular matrices, etc. that work together to start carcinogenesis. Indeed, chronic inflammation favors to the secretion of pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6, which promote the accumulation of reactive oxygen species (ROS) and maintains a pro-inflammatory environment favorable for oncogene activation and tumor suppressor gene mutations, activation of abnormal proliferation and differentiated anti-apoptotic pathways [10]. This disrupts the colonic epithelial homeostasis and ruptures the normal communication between the epithelial mucosa and adjacent tissues, alters immune responses, leads to DNA damage, and mutations in DNA response (DDR) genes worsening CAC condition [13-15].
The mammalian receptor, Notch1, which is highly expressed at the basal crypt of the human colon [38], is known for stem cell renewal, progenitor cell-fate determination, terminal differentiation of proliferating cells, and tissue homeostasis in the healthy colon [20, 21, 38, 40]. Notch1 activation leads to the transcription of downstream target genes, such as hairy enhancer of split 1 (Hes1), which is critical in the lineage decision for progenitor cells to differentiate in colonic epithelial cells [39, 111]. Studies have defined Notch1 role to be context dependent, it can be oncogenic [96, 97] or tumor suppressive [112], therefore, it can promote irregular differentiation in some tissues [94] or it can help maintain stem cells homeostasis in others [95].

Its oncogenic role was first illustrated in 1991 where Notch1 was suspected to be related to the development of T-cell acute lymphocytic leukemia (T-ALL)/lymphoma [96]. Studies have shown that activated Notch1 can induce neoplastic transformation in mouse mammary and salivary glands and in rat kidney cells in vitro [97-99]. Another study mentions that Notch signaling is required for the neoplastic transformation in Ras-transformed human breast cancer cells [98]. Studies in mice have shown that Notch1 signaling in CRC is also oncogenic and it is required for adenoma formation due to the elevated Wnt signaling that occurs in adenomatous polyposis coli/multiple intestinal neoplasia (Apc^{Min}) mice model of colon cancer [28, 44, 100, 101]. However, different studies have shown that Notch1 is not exclusively oncogenic, in fact it can also act as a tumor suppressor. Liu et al. have shown that chronic inhibition of Notch1 leads to vascular tumors in the liver and it is associated with decreased survival suggesting the tumor suppressive role of Notch1 [95]. Another study using Notch1-deficient mice, in epidermis and corneal epithelium, developed basal-cell-carcinoma like tumors after being chemical-induced for
carcinogenesis, again suggesting a tumor suppressor role in the mammalian skin [103]. Notch1 also functions as a tumor suppressor by inhibiting the growth of mouse prostate cancer cell [104].

It has been previously shown that inhibition of Notch1 by the γ-secretase inhibitor DAPT (N- [N-(3,5 difluorophenacetyl)-1-alanyl 1]-S-phenylglycine t-butyl ester) reduces apoptosis during CAC [113]. Although, in recent years, different studies have reported the protective role of Notch1 in various malignancies however, the underlying precise mechanism Notch1 signaling in CAC remains unclear. Understanding the role of Notch1 in CAC is critical and will assist in unraveling novel efficient therapeutics to prevent carcinogenesis in the chronically inflamed colon tissue. We hypothesized that Notch1 acts as a tumor suppressor in CAC by activating tumor suppressor p53 [114] and p21 (downstream target of p53) by regulating cell cycle progression for DNA repair of damaged cells. In this study, we observed that conditional deletion of Notch1 signaling exacerbated CAC conditions as indicated by the shortening of colon length, increased dysplastic lesions, mucosa thickening, increased number of polyps, loss of crypt architecture, increased infiltration of white blood cells, and higher histological score. These all represent severity of CAC under chronic inflammatory colonic conditions. Moreover, our data shows that the conditional deletion of Notch1 promoted the secretion of pro-inflammatory cytokine, TNF-α, which is one of the cytokines critical in nurturing tumor environment in CAC.

Damage-associated molecular pattern molecules (DAMP) contribute significantly in mediating inflammation, modulating tumor growth and metastasis [109]. Interestingly, we observed that mRNA level of the DAMP molecule, S100A8, was no different between both groups, however, S100A9 mRNA level was increased in Notch+/+ mice compared to NCre mice in CAC. Further, the reason why S100A9 might be high in Notch+/+ mice could be due to the recent discovery from LI et al. mentioning that S100A9 has several putative p53 binding sites
meaning that its overexpression can induce apoptosis in a p53 dependent manner [115]. This was also supported by the p53 WB analysis in our study indicating increased p53 protein expression among WT mice compared to NCre mice in CAC. Our data supports the fact that, conditional deletion of Notch1 in CAC, disrupts the colonic epithelial cell integrity compromising epithelial cell barrier as indicated by the increase number of FITC units compared to Notch\(^{+/+}\) mice. This impaired barrier function and epithelial integrity results in the decreased of microbiota population among NCre mice compared to WT mice. Therefore, our data elucidates that conditional deletion of Notch1 results in microbiota depletion causing an imbalance epithelial homeostasis and favoring chronic inflammation in CAC.

Disruption of the colonic epithelial homeostasis leads to the increase in DNA damage and for damaged epithelial cells to evade apoptosis. Critical cellular events, such as DRR and MMR, activate to the rescue to repair and replenish healthy epithelial cells in the damaged tissue. Our data shows that the conditional deletion of Notch1 is associated with lower levels of tumor suppressor- p53 and apoptosis associated protein- p21\(^{WAF1/Cip1}\) while it increases DDR sensors-\(\gamma\)H2AX and MMR protein-MLH1 indicating that Notch1 signaling absence increases DNA damage, decreases apoptosis, and compromises DDR and MMR pathways. In conclusion, we demonstrate that Nocth1 plays a tumor suppressor role in CAC, by activating p53 and p21 and by promoting DDR and MMR activity to maintain healthy colonic epithelial cells over inflamed/damaged ones (Figure 16).

Tissue specific deletion of Notch1 in colonic epithelium worsened CAC conditions. Both, epithelial injury and lack of epithelial healing, favor chronic inflammation progression to dysplasia. Epithelial injury is primarily caused due to the ROS generation during chronic inflammation. Therefore, as an efficient and long-term preventive strategy for CAC, therapeutic
interventions are required to limit the chronic inflammation at the initial stages. This window is very critical to avoid the extensive and irreversible damage caused by the inflammatory environment leading to cancer in CAC. Overall, activation of Notch1 in the early stages of chronic inflammation could be an efficient preventive therapeutic strategy for CAC progression.

Figure 16. *Notch1 plays a tumor suppressor role in CAC.*

Notch1 signaling plays a tumor suppressor role in CAC by activating p53 and downstream target p21 and by promoting DDR and MMR activity to maintain healthy colonic epithelial cells over inflamed/damaged ones. **Abbreviations used are:** NICD-Notch intracellular domain, S2-S3-Site2 and Site3; TJ-Tight junction; ROS- Reactive oxygen species; MMR-Mismatch Repair pathway
3.5 Conclusion

IBD is a chronic condition that involve dysregulated immune response and inflammation of the gastrointestinal (GI) tract [1, 6]. UC is a form of IBD that affects the rectum and inflammation also spreads throughout the colon affecting the inner layer of the colon as well as the mucosa. Patients with active UC have a 15-40% risk of developing colitis associated cancer (CAC) due to the longterm inflammation in the colon [4]. CAC is a subtype of colorectal cancer which develops from dysplasia to carcinoma axis among chronically active UC patients [10-12].

Notch1 is highly expressed at the basal crypt of the human colon [38] and it plays a role in stem cell renewal, progenitor cell-fate determination, terminal differentiation of proliferating cells, and tissue homeostasis in the colon [20, 21, 38, 40]. Notch1 activation can be multifaceted, depending on the severity of the disease, Notch1 can be protective or deleterious. Previous studies have found that Notch1 has a beneficial role in corneal and skin healing while it is deleterious in breast cancer [116-118]. Interestingly, Notch1 has been found to be upregulated in early stages of cervical cancer; whereas, it is downregulated in late stages of cervical cancer [119-121]. Notch1 signaling can activate cancer associated transcription factors such as, c-Myc, ARF and p53 [122]. It can also activate Hes transcription factor implicated in the maintenance of progenitor and stem cells; thus, promoting epithelial cell differentiation in the colon [39]. Therefore, Notch1 function is context dependent, it can be oncogenic or tumor suppressive. It was previously shown that pharmacological inhibition of Notch1 decreases apoptosis and promotes CAC progression during the disease [113]. In recent years, different groups have also shown that Notch1 has a protective role in a variety of malignancies such as prostate cancer [104], cervical cancer [106, 107] and skin cancer [103]. However, the precise mechanism of Notch1 signaling in acute colitis and CAC remains unclear.
In this study, we sought to explore the role of Notch1 through different stages of colitis, starting from acute inflammation to CAC. Understanding the role of Notch1 will assist in the improvement for treatments to prevent carcinogenesis in chronically inflamed colon tissues. We hypothesized that Notch1 activation mediates inflammation in acute colitis; however, it plays a protective role during CAC. This hypothesis was addressed by two aims which can potentially 1) target inflammatory pathways which mediate acute inflammation via Notch1 activation 2) bring a more specific treatment to prevent the development of cancer in IBD patients.

To study the role of Notch1 in acute colitis and CAC, we generated a conditional knockout mouse (NCre) using the Cre/Lox system. NCre mice do not express Notch1 in epithelial cells. The first section of this dissertation goes over the characterization of NCre mice. We showed that the conditional deletion of Notch1 leads to higher numbers of goblet cells and compromises epithelial crypt architecture and colonic barrier function. We also showed that the conditional deletion of Notch1 compromises epithelial cell differentiation and proliferation in the colon. In addition, biochemical blood analytes showed significantly higher among NCre mice indicating that there was inflammation in their livers compared to the control mice.

Next, we explored if Notch1 activation mediates inflammation in acute colitis. By using a DSS- induced colitis model, we showed that Notch1 mediates inflammation in acute colitis as it upregulates pro-inflammatory (IL-6) and downregulates anti-inflammatory (IFN-γ) cytokines. Notch1 activation in DSS-induced model also activated NF-kB-Cyclin D1 inflammatory pathway which promotes proliferation favoring inflammation in acute colitis. Notch1 is known for its role in colonic stem renewal, progenitor-cell fate determination, and tissue homeostasis; therefore, we also wanted to explore the role of Notch1 in wound healing in the recovery phase following acute colitis. By using a DSS-induced colitis recovery model and HCT116 cells
transfected to overexpress NICD, we showed that Notch1 indeed promotes wound healing in the recovery phase following acute colitis. Therefore, Notch1 signaling plays an opposite role as it protected the mice, during the recovery phase, from mediating inflammation.

In CAC, chronic inflammation leads to the secretion of proinflammatory cytokines, which promotes the accumulation of ROS and maintains a pro-inflammatory environment favorable for oncogenes and mutations in tumor suppressor genes. Chronic inflammation dysregulates the balance between proliferation and apoptotic pathways. Altogether, contribute to the disruption of intestinal homeostasis [10, 13, 14, 87]. To study the role of Notch1 in CAC, mice were injected with azoxymethane, a carcinogen, and treated with two cycles of DSS. In the second section of this dissertation, we showed that the conditional deletion of Notch1 in the colonic epithelium increases CAC susceptibility by disrupting epithelial cell integrity, barrier, and microbiota population. We also showed that the conditional deletion of Notch1 promoted the secretion of pro-inflammatory cytokine, TNF-α, as well as increased DNA damage and decrease in apoptosis. In addition, Notch1 conditional deletion in colon impaired DDR sensors and MMR pathway. Therefore, these data suggest that Notch1 acts as a tumor suppressor in chronic colitis.

In conclusion, these results imply that Notch1 activation is context dependent. Depending on the severity of the disease, in this case acute colitis, we showed that Notch1 is deleterious during acute inflammation. However, Notch1 activation can be protective during chronic colitis/CAC. These results could further be translated to a potential therapeutic solution for IBD patients. Notch1 target inhibitors should be avoided during acute inflammation and the induction of Notch1 signaling can be protective in CAC.
REFERENCES


62. Cooper, H.S., et al., Dysplasia and cancer in the dextran sulfate sodium mouse colitis model. Relevance to colitis-associated neoplasia in the human: a study of histopathology,


