University of Alberta

Role of RASSF1A in Intestinal Inflammation

by

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Dedication

To My Parents

ABSTRACT

Ras association domain family 1 A (RASSF1A) is an important tumor suppressor, which expression is frequently lost in various cancers due to promoter hypermethylation. Chronic inflammation, such as inflammatory bowel disease (IBD), can increase the risk of developing cancer. Rassf1a conventional knockout studies suggest its essential role in protection against dextran sulphate sodium (DSS)-induced colitis. In this study, we further explored the role of Rassf1a in intestinal inflammation by utilizing an intestinal epithelial cell (IEC) specific knockout (*Rassf1a*$^{IEC\text{-}KO}$) mouse model. We found that the *Rassf1a*$^{IEC\text{-}KO}$ mice are more susceptible to DSS-induced colitis. Central to the pro-inflammatory signaling is the nuclear factor kappa B (NF-κB) transcription factor. We observed increased NF-κB DNA-binding activity in bone marrow cells and crypt cells in the *Rassf1a*$^{IEC\text{-}KO}$ mice. Our investigation demonstrates that the intestinal epithelial expression of Rassf1a is essential to protect the mice from DSS-induced intestinal inflammation, through the negative regulation of NF-κB activity.

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List of Abbreviations

AP-1	activator protein-1
APC	anaphase-promoting complex
Apc	adenomatous polyposis coli
ATM	ataxia telangiectasia mutated
BCL-3	B-cell lymphoma-3
C1	protein Kinase C conserved region 1
C19ORF5	chromosome 19 open reading frame 5 or MAP1S
CD	Crohn's disease
Cdc20	cell division cycle 20
COX-2	cyclooxygenase-2
DAG	diacylglycerol/phorbol ester
DNMT	DNA methyltransferases
DSS	dextran sulphate sodium
ECM	extracellular matrix
EGF	epidermal growth factor
ELAM	endothelial–leukocyte adhesion molecule 1

FITC	fluorescein isothiocyanate
FGF	fibroblast growth factor
GPCR	G-protein coupled receptor
HA	hyaluronic acid
H&E	hematoxylin and eosin
HIF	hypoxia-inducible factor
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule 1
IEC	intestinal epithelial cell
IFNγ	interferon γ
IκB	inhibitor of kappa light chain gene enhancer in B cells
IKK	IκB kinase
IL-6	interleukin-6
IP-10	interferon gamma-induced protein 10
IRAK	interleukin-1 receptor-associated kinase 1
IRF	IFN regulatory factors
KC	karatinocyte chemoattractant

KGF	keratinocyte growth factor
LPA	lysophosphatidic acid
LPS	lipopolysaccharides
PI3-K	phosphatidylinositol 3-kinase
MAP1B	microtubule-associated protein 1B
MCP-1	monocyte chemoattractant protein 1
MHC	major histocompatibility complex
MIP-2	macrophage inflammatory protein 2
MMP	metalloproteinase
MOAP-1	modulator of apoptosis 1
MPO	myeloperoxidase
MST1/2	mammalian ste20-like kinase 1/2
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB-inducing kinase
NEMO	NF-κB essential modulator
NLR	NOD-like receptors

NOD	nucleotide-binding oligomerization-domain protein
NOS	nitric oxide synthase
PAMP	pathogen-associated molecular pattern
PDGF	platelet-derived growth factor
PCNA	proliferating cell nuclear antigen
PMCA4	plasma membrane calcium ATPase 4
PRR	pattern recognition receptors
RA	Ras association
RalGDS	Ral guanosine nucleotide-exchange factor
RASSF	Ras association domain family
RBD	Ras-binding domains
RHD	REL-homology domain
RIP1	receptor interacting protein 1
ROS	reactive oxygen species
SARAH	Salvador/Rassf/Hippo
SIGIRR	single Ig IL-1 receptor-related molecule
TAD	transactivation domains

TAK1	transforming growth factor-β activated kinase 1
TCR	T cell receptor
TFF	trefoil factor
TGF-β	transforming growth factor-β
T_H1	T helper 1
T_H2	T helper 2
TLR	Toll-like receptors
TRADD	TNF-R-associated death domain protein
TRAF	TNF receptor associated factor
T_{Reg}	regulatory T cell
TSG	tumor suppressor gene
UC	ulcerative colitis
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial cell growth factor

Chapter 1

Introduction

1.1. RASSF1A

1.1.1. RASSFs Overview

The Ras association domain family (RASSF) tumor suppressor proteins are composed of ten members, RASSF1-10, with the Ras association (RA) domain as a characteristic feature of all family members. The classic RASSF1-6 proteins contain a C-terminal RA domain, whereas the newly joined members RASSF7-10 contain an N-terminal RA domain.[1-8] The C-terminus of RASSF1 is highly homologous (55% identity) with the novel RAS effector protein,Nore1A (later noted as RASSF5A) and contains a RA domain.[1]

The *RASSF1* gene is located on chromosome 3p21.3. Loss of heterozygosity studies in lung, breast, and kidney tumors identified several loci on chromosome 3p that were likely to harbour one or more tumor suppressor genes (TSGs). An important TSG was suspected to be located on 3p21.3 due to instability of this region.[9-16] Overlapping homozygous deletions in lung and breast cancer cell lines reduced the critical region in 3p21.3 to 120 kb residing 8 genes, among which the *RASSF1* gene is found to be frequently inactivated in most cancers and intensively studied.[12, 17, 18]

1.1.2. *RASSF1* Gene and Protein Primary Schematic Structure

The *RASSF1* gene is located on chromosome 3p21.3 and encodes for eight exons (1α, 1β, 2αβ, 2γ, 3, 4, 5 and 6) as shown in Figure 1.1. Seven *RASSF1* transcripts (*RASSF1A* to *RASSF1G*) are generated by differential usage of two promoters and through alternative splicing. Exon 1α is only present in *RASSF1A* isoform which

encodes for the N-terminus of RASSF1A.[19] RASSF1A and RASSF1C are the two major isoforms of RASSF1 that are ubiquitously expressed in human tissues, whereas RASSF1B is expressed predominantly in haematopoietic cells.[1] The remaining four isoforms (RASSF1D-G) are all splice variants of RASSF1A, among which the RASSF1D transcript is expressed specifically in cardiac cells and the RASSF1E transcript is expressed specifically in pancreatic cells.[20] The biological role of RASSF1B, D-H isoforms are uncertain and the mRNA stability of these isoforms has not been explored. RASSF1D-G isoforms share the same promoter region as RASSF1A and thereby are missing in various tumors as well.[7]

The RASSF1A isoform is frequently inactivated by promoter specific methylation in numerous cancers. In addition, numerous somatic polymorphisms of RASSF1A have been identified in various types of cancers. The second major isoform RASSF1C utilizes a different promoter region than RASSF1A. In contrast, no hypermethylation of the promoter has been discovered with RASSF1C.[1, 17, 20] Studies of the potential tumor suppressor properties of RASSF1C have been contradictory. The majority of publications report that RASSF1A, but not RASSF1C, has tumor suppressor function.[1, 20-23] However, Vos *et al.* and Li *et al.* suggest RASSF1C over-expression demonstrated growth inhibition in breast cancer cells.[24]

1.1.2.1. *RASSF1A* promoter hypermethylation

Promoter methylation plays an important role in epigenetic regulation of gene expression. The methylation reaction is catalyzed by DNA methyltransferases

(DNMTs) and involves the addition of a methyl group onto the carbon 5 position of the cytosine ring on the sequence 5'-CG-3'(a CpG dinucleotide). CpG islands are clusters of CpG repeats that are commonly found in the promoter region of genes. Hypermethylation of the CpG islands in the promoter region of a gene correlates transcriptional repression of this gene.[25] Both promoter regions of *RASSF1A* and *RASSF1C* contain CpG islands. However, only the CpG islands of *RASSF1A* promoter are frequently modified by hypermethylation in more than 40 types of cancers resulting in decreased or abolished expression of RASSF1A protein.[1] For example, 88% of small cell lung cancer, 81-95% of breast cancer, 20-52% of colorectal cancer, 99% of prostate cancer, 56-91% of renal cancer, 75% of hepatocellular cancer, 63% of pancreatic cancer, 83% of neuroblastoma, and 79% of medulloblastoma patients have shown *RASSF1A* promoter hypermethylation.[26-34] Additionally, RASSF1A expression is also progressively reduced as the tumor stage increases. The expression level was higher in well and moderately differentiated tumor groups than in poorly differentiated tumor group.[35]

Although loss of RASSF1A expression is largely attributed to promoter hypermethylation, and germline mutations of RASSF1A are uncommon. However, numerous somatic polymorphisms have been identified in lung, breast, kidney and nasopharyngeal carcinomas and cell lines, many of which are located in the functional domains of RASSF1A.[36] Some of the polymorphisms have proven to encode a functionally impaired mutant RASSF1A and possibly play an important role in tumorigenesis.[7] For example, A133S or S131F RASSF1A mutants cannot induce cell cycle arrest by blocking cyclin D1 accumulation.[23] The S131F

polymorphism conveys resistance to DNA-damaging agents possibly through blocking the phosphorylation site of RASSF1A by the DNA damage kinase ataxia telangiectasia mutated (ATM) kinase.[37] C65R, R257 and A133S mutants have reduced microtubule association and increased nuclear localization resulting in tumorigenesis.[38-40] Therefore, functional studies of these polymorphisms are important to understand the tumor suppressor properties of RASSF1A.

1.1.2.2. RASSF1A protein domains

RASSF1A contains 340 amino acids and has a molecular weight of 39 kDa. The protein is comprised of four major domains, the N-terminal C1 (protein kinase C conserved region 1) domain, followed by the ATM domain, the RA domain and the C-terminal SARAH (Salvador/Rassf/Hippo) domain (Figure 1.2). The RA domain is the characteristic feature for Ras effectors and all RASSFs that potentially can associate with the GTP-bound form of Ras.[41] The RA domain containing Ras effectors share a conserved motif, namely the Ral guanosine nucleotide-exchange factor (RalGDS)/AF6 domain. This is defined by sequence homologies between the Ras effectors RalGDS (involved in Ras-induced transformation) and the ALL-1 fusion partner from chromosome 6 (AF6; involved in regulating cell adhesion).[41, 42] Different from the RA domain discovered in the RASSF family members, another domain that is frequently found in the Ras effectors is the Ras-binding domains (RBD), which binds to a different region on Ras than RA domain. Two of the best-studied Ras effectors containing RBDs are Raf, and phosphatidylinositol 3-kinase (PI3-K). Raf is a serine-threonine kinase that activates the MEK-ERK pathway and promotes cellular proliferation. The

PI3-K is required for activation of the protein kinase B (Akt) in order to inhibit apoptosis.[43, 44] Although RASSF family members can potentially associate with GTP-bound form of Ras, a direct association with activated Ras has only been observed with RASSF2, 4, 5, 6 and 9.[2, 6, 45] Direct association of RASSF1 to Ras has not been consistently observed. RASSF1A is thought to indirectly associate with activated Ras through heterodimerization with RASSF5 and can potentially modulate the function of RASSF1A.[22]

The C1 domain is named after its high homology with a cysteine-rich diacylglycerol (DAG)/phorbol ester-binding domain.[46] Binding of DAG/phorbol ester to the C1 domain of protein kinase C (PKC) can activate the kinase activity of PKC. Overlapping the C1 domain is a putative zinc-binding domain.[47] This cysteine rich domain of RASSF1A has been demonstrated to associate with tumor necrosis factor receptor 1 (TNF-R1) complex, which is important for TNF-R1 induced cell death.[48] The ATM domain corresponds to a phosphorylation site that can be phosphorylated by ATM kinase at S131 of RASSF1A upon DNA damage *in vitro*.[23, 37] The ATM kinase is of central importance for the regulation of cell cycle checkpoints that lead to DNA damage repair and apoptosis.[49] However, a functional relevance of this ATM domain has not been confirmed *in vivo* yet. Another characteristic feature for RASSF1-6 is the SARAH domain.[50] SARAH domain is a protein interaction domain that is named from the proapoptotic pathway Salvador/Rassf/Hippo in *Drosophila melanogaster*. The human orthologues of Salvador, Rassf and Hippo are WW45, RASSF and the human proapoptotic kinase MST1.[51] The possible roles of RASSF5 and RASSF1A to

inhibit proliferation through MST1/2 have been suggested, while the interacting mechanisms are not clearly understood and need further investigation.[52]

1.1.3. RASSF1A Biological Function

We now know that RASSF1A is involved in a number of biological functions, such as microtubule dynamics, apoptosis, cell cycle arrest and mitotic arrest (Figure 1.3).[53] Loss of function of RASSF1A in numerous human cancers suggests it plays a key role in tumor prevention. *Rassf1A* knockout mice have an increased incidence of spontaneous tumorigenesis as they age (predominantly gastrointestinal carcinomas and B cell lymphomas) compared with wild type mice.[54, 55] Consistent with this, over-expression of RASSF1A in various tumor cell lines results in cells that are less viable, growth suppressed, and less invasive, and exhibit reduced anchorage/substrate independence *in vivo* as well as drastically reduced tumorigenecity *in vitro*.[7, 21, 47]

1.1.3.1. RASSF1A and microtubule dynamics

RASSF1A associates with microtubule networks, specifically α-, β- and γ-tubulins, during interphase of cell cycle, whereas during mitosis, RASSF1A localizes to spindles and centrosomes.[40, 56-58] Overexpression of RASSF1A stabilizes polymerized microtubules and protects the cells from microtubule depolymerising agents, such as nocodazole.[56] The C65R polymorphism of RASSF1A has been demonstrated to lose microtubule association ability and nucleus localization resulting in microtubule instability and loss of RASSF1A tumor suppressor properties.[40] Meanwhile, RASSF1A has been reported to

interact with microtubule-associated proteins microtubule-associated protein (MAP) 1B and C19ORF5/MAP1S (chromosome 19 open reading frame 5 or microtubule-associated protein 1S).[39, 59] RASSF1A-C19ORF5 localization to the centrosomes is thought to be important in mitosis progression.[58] Recently, Verma *et al.* have demonstrated that RASSF1A is a substrate for the PKC and can be phosphorylated at serine 197 and 203 both *in vitro* and *in vivo* resulting in an inability to modulate microtubule organization.[60]

1.1.3.2.RASSF1A and apoptosis

Apoptosis is programmed cell death and is essential for development and tumorigenesis. Since RASSF1A is inactivated in most cancers and functions as a tumor suppressor, the role of RASSF1A in apoptosis is intensively studied. RASSF1A is involved in apoptosis by participating in the death receptor mediated cell death or the proapoptotic MST/RASSF/WW45 pathway (mammalian Hippo pathway). Baksh *et al.* have reported that RASSF1A is required for death receptor-mediated BAX conformational change and apoptosis.[61] Bax is a proapoptotic protein involved in the permeabilization of the mitochondrial outer membrane, leading to the release of cytochrome c and cell death. In unstimulated cells, RASSF1A may be sequestered by associating with 14-3-3, a phospho-serine/threonine binding protein involved in regulating cellular homeostasis.[62, 63] After receptor activation by TNFα or TRAIL (TNF-related apoptosis inducing ligand), RASSF1A is released from 14-3-3, allowing subsequent association with the proapoptotic protein modulator of apoptosis 1 (MOAP-1) and the receptor intracellular domains. MOAP-1 then becomes activated and undergoes

conformational change, resulting in association with Bax and activation of Bax mediated apoptosis.[48] Additionally, Vos *et al.,* have detected endogenous association of RASSF1A and MOAP-1 in stromal cells surrounding hepatocellularcarcinoma tissue, but not in normal liver tissue, and inconsistently in hepatocellularcarcinoma tissue. Over-expressed RASSF1A and MOAP-1 in 293T cells have weak association and this association can be enhanced by co-overexpressing activated K-Ras (K-Ras12V), possibly leading to K-Ras mediated apoptosis.[64] However, this result is controversial to most researches, whereas RASSF1A only associate with K-Ras very weakly.

Additionally, RASSF1A associates with the proapoptotic kinase MST1/2 (mammalian ste20-like kinase 1/2) through the SARAH interacting domain. Defects in the MST/RASSF/WW45 pathway can lead to abnormal mitosis and failure to induce apoptosis.[65] It is thought that RASSF1A expression is required for full activation of MST1-mediated apoptosis.[66] Another study shows that co-expression of RASSF1A, RASSF1C, RASSF5A and RASSF5C with MST1 markedly suppress MST1 activation. However, presence of oncogenic H-RAS (RAS-G12V) and RASSF5A results in increased MST1 activation than RASSF5A alone.[67] Binding of RASSF1A to MST2 leads to the transcription of *PUMA*, which potentially initiates apoptosis.[68]

1.1.3.3.RASSF1A and cell cycle regulation

Several studies have demonstrated that RASSF1A is involved in several aspects of cell cycle regulation. RASSF1A modulates G1/S phase arrest and mitotic

regulation and negatively regulates the accumulation of cyclin D1 through a posttranscriptional mechanism resulting in inhibition of the cell cycle progression.[23, 58, 69] Cyclin D1 is a regulatory subunit for cyclin dependent kinase 4 and 6 that is expressed and involved in G1/S phase transition of the cell cycle.[70] Moreover, RASSF1A overexpression inhibits activator protein-1 (AP-1) activity by down-regulating c-Fos in a gastric carcinoma cell line leading to cell growth inhibition with G1 arrest.[71] Ectopic expression of RASSF1A in gastric or lung carcinoma cell lines down-regulates cyclin D1 expression, thereby resulting in G1/S phase arrest.[71, 72] Additionally, RASSF1A may induce G2/M arrest in a breast cancer cell line and a embryonic kidney cell line.[58, 73]

Aurora-A kinase is a centrosome kinase that plays a pivotal role in G2/M cell cycle transition.[74] RASSF1A can be phosphorylated by Aurora-A, leading to disruption of microtubule association and an inability to induce M-phase cycle arrest.[75] Additionally, RASSF1A can induce mitotic arrest by interacting with Cdc20 and inhibiting the formation of the APC/C-Cdc20 complex (anaphase-promoting complex/ cyclosome-cell division cycle 20 homolog) during early prometaphase.[58, 69] The APC/C complex is part of the ubiquitin-conjungation-system and targets proteins for proteasomal degradation. Through inhibition of this complex, cyclin A and B do not become degraded by proteasome resulting in mitotic arrest.[76] However, Liu *et al.* have been unable to detect RASSF1A association with Cdc-20 through yeast two-hybrid assay, GST-pull down or immunoprecipitation in 293T and HeLa cells.[77] It is further suggested that instead of promoting mitosis, a significant proportion of cultured fibroblasts from *Rassf1a*

null mouse embryos exhibit delayed mitosis which resulted in cytokinesis failure.[65] Therefore, RASSF1A is possibly not simply associated with Cdc20 and induces mitotic arrest. Further investigation is required for understanding the role of RASSF1A in regulating mitosis.

1.1.3.4.RASSF1A and other functions

In addition to inducing cell cycle arrest and apoptosis, Dallol *et al.* have demonstrated that RASSF1A is involved in controlling cell migration as RASSF1A-depleted cells increased cell migration, while ectopic expression of RASSF1A diminished the ability of cancer cells to migrate.[78] This finding suggests that RASSF1A may also inhibit metastasis through regulating cell migration. Additionally, RASSF1A is involved in negative regulation of β-catenin/Wnt signaling. Loss of *Rassf1a* in mice cooperates with loss of adenomatous polyposis coli (*Apc*) gene to accelerate intestinal tumorigenesis with increased nuclear accumulation of β-catenin, which can further contribute to the oncogenic effects of aberrant Wnt/β-catenin signaling.[79] Furthermore, RASSF1A is a potent inhibitor of Ras-Raf1-ERK1/2 in cardiomyocytes and prevents cardiac hypertrophy.[80] Armesilla *et al.* demonstrates that RASSF1A interacts with the plasma membrane calcium ATPase 4 (PMCA4) that can also modulate cardiac hypertrophy.[81]

1.1.4. Other RASSF Family Members

RASSF1-6 contain C-terminal RA domains, whereas the newly joined members RASSF 7-10 are characterized by N-terminal RA domains. Most of the family

members exhibit tumor suppressor properties (RASSF2, 4, 5, 6, and 8) and reduced expression in tumors (RASSF2, 4, 5, 6 and 10).[50, 82] RASSF2, 4, 5, 6 and 9 are thought to associate with K-Ras or H-Ras to induce apoptosis.[50, 83] RASSF5 is thought to also associate with MST 1 and 2 to exert apoptotic functions.[50] Overexpressed RASSF2, 3 and 4 can also associate with MST1, and RASSF2 can regulate MST1 and 2 proapoptotic kinase activities.[84-86] In addition, RASSF2 and 7 are thought to participate in cell cycle regulation, where overexpressed RASSF2 arrests cells in G0/G1 phase, and RASSF7 knockdown cells fail to form a spindle and arrest in mitosis.[2, 8] However, further investigations are required for the mechanisms of the tumor suppressor properties of these members.

1.1.5. RASSFs and Inflammation

Although most RASSF research is focused on the tumor suppressor properties, a few studies have suggested RASSFs are possibly also involved in inflammatory processes. RASSF5C (referred to as RAPL/NORE1B) also associate with lymphocyte polarity and adhesion, T cell migration and directional migration of vascular endothelial cells, which are essential in immune cell trafficking and immune-surveillance.[78, 87-90] Ishiguro *et al*. proposed that RASSF5C could potentially attenuate T cell receptor (TCR) induced nuclear factor-kappa B (NF-κB) activation but not NF-κB activation induced by TNFα or lipoteichoic acid.[91] NF-κB is an important transcription factor in pro-inflammatory response and also immune cell development and maturation. Therefore, RASSF family members can possibly be involved in inflammatory processes. Additionally, Allen *et al.* reported that RASSF6 suppresses the NF-κB pathway and proposed that RASSF6

may play a role in dictating the degree of inflammatory response to the respiratory syncytial virus.[6] Lock *et al.* suggest that RASSF8 knockdown cells exert increased NF-κB activity through promoter luciferase assay and confirmed with IκBα immunoblotting.[92] However, they demonstrated that p65 localized to adherens junctions at the membrane proximal when NF-κB is inactivated, which is contradictory to the majority of current researches that inactive NF-κB has cytoplasmic localization. Methylation studies have also revealed that the RASSF1A promoter region is not only hypermethylated in pancreatic cancer patients (64% in primary adenocarcinomas and 83% in primary adenocarcinomas) but also in pancreatitis patients (44%).[93] However, the detailed mechanism of RASSFs in the inflammatory process needs to be further investigated.

1.2. Inflammation

Inflammation involves a wide variety of physiological and pathological processes. A current definition of inflammation can be found in the textbook of *Pathology*, which is "a reaction of the microcirculation by movement of fluid and leukocytes from the blood into extravascular tissues".[94, 95] The blood vessels surrounding the sites of inflammation become more permeable, allowing fluid that is rich in plasma protein and leukocytes to infiltrate into tissues. Based on duration and cell type involved, inflammation can be divided into two major types: acute inflammation and chronic inflammation.

1.2.1. Acute Inflammation

Acute inflammation is an immediate response at sites of injured tissue and infection.[96] For example, microbial infections, particularly bacterial infections induced inflammation are triggered by receptors of the innate immune system, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization-domain protein (NOD)-like receptors (NLRs).[97] TLRs and NLRs are pattern recognition receptors (PRRs) that recognize various pathogenic components such as lipopolysaccharides (LPS) from bacterial cell walls, DNA and RNA fragments and protein components from pathogens. The initial recognition of infection is mediated by tissue-resident macrophages, dendritic cells and mast cells, leading to the production of a variety of inflammatory mediators, including chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades.[97] The major immediate effect of these mediators is to elicit an inflammatory exudate locally: plasma proteins and leukocytes (mainly neutrophils) that are normally restricted inside the blood vessels now gain access to the extravascular tissues at the site of infection or tissue injury. Neutrophils become activated when reaching the afflicted tissue site, either by direct contact with pathogens or through the actions of cytokines secreted by resident cells. Neutrophils attempt to kill or make the environment hostile to pathogens by releasing the toxic contents from their granules, which include reactive oxygen species (ROS) and proteases.[98] These highly potent effectors do not discriminate between microbial and host targets, so collateral damage to host tissues is unavoidable.[99]

1.2.2. Chronic Inflammation

If the acute inflammatory response fails to eliminate the pathogen, the inflammatory process persists and acquires new characteristics. Macrophages and lymphocytes gradually infiltrate into the infected or injured tissue and replace the local neutrophils. By utilizing the adaptive immune system, macrophages cooperate with lymphocytes to eliminate pathogenic components and dead cells in a more specific manner compared to the neutrophils. Macrophages engulf pathogens and kill them by proteolysis. Moreover, macrophages present antigen to major histocompatibility complex (MHC) class II-restricted T helper cells (i.e. CD4+ helper cells), thereby initiating an antigen specific immune response. Cytokines produced during the inflammation process are responsible for amplifying signals for the immune response as well as stimulating the growth of fibroblasts and endothelial cells by secretion of growth factors, such as FGF, PDGF, and TGF-β. Chronic inflammation is usually accompanied by tissue repair activity and permanent change in tissue architecture, such as angiogenesis and fibrosis. If the repair response is unsuccessful, the injured tissue might undergo remodelling which involves changing of cell types.[94, 96]

1.3. Intestinal Immunity

1.3.1. Mucosal Barriers and Intestinal Homeostasis

The mucosal surface of the intestinal tract is covered by epithelial cells that constitute an effective physical barrier between the intestinal lumen, which is full of dietary antigen and microflora, and the circulatory system, yet allows exchange

of nutrients and fluid (Figure 1.4).[100] The mucosal defense mechanism can be categorized into three key components: pre-epithelial, epithelial and post-epithelial.[101] The pre-epithelial mucus barrier is composed of mucins (high molecular weight glycosylated proteins) with other proteins and lipids that form a continuous hydrated gel with neutral pH and hydrophobic property.[102] The mucus barrier prevents microflora from coming in contact with and adhering to the plasma membrane as well as protects the epithelium against chemical and mechanical injuries.[103] The second barrier of the mucosa is the epithelial cell layer. The paracellular spaces between epithelial cells are sealed by an apical junctional complex, which is composed of tight junction and subjacent adherens junction. Tight junctions are multi-protein complexes composed of transmembrane protein claudins interacting with peripheral membrane proteins and cytoskeletal actins. Adherens junctions are comprised of cadherins, a family of transmembrane proteins that form strong interactions with intracellular catenins, which regulate local actin assembly. Adherens junctions provide the strong adhesive bonds that maintain cellular proximity and intercellular communication.[104] The third layer of the mucosal barrier is the post-epithelial layer, also called lamina propria, which is located beneath the basement membrane and contains immune cells, including macrophages, dendritic cells, plasma cells, lamina propria lymphocytes and occasionally neutrophils.[105]

Minor mucosal barrier defects allow pathogenic microbes and dietary antigens to cross the epithelium and contact the immune cells in the lamina propria area, thereby initiating inflammatory response and disequilibrium of the homeostasis of

the mucosa. The pathogenic components can be engulfed and presented on the plasma membrane of antigen-presenting cells, such as dendritic cells and macrophages, which direct the differentiation of T helper 1 (T_H1) or T_H2 cells. Cytokines such as TNFα, interferon γ (IFNγ), and interleukin-13 (IL-13) are released to amplify the inflammatory process. Conversely, epithelial cell-derived factors such as transforming growth factor-β (TGF-β) can promote antigen-presenting cells to initiate regulatory T (T_{Reg}) cell differentiation. The T_{Reg} cells present latency-associated peptide and may secrete IL-10 and TGF-β to prevent disease development.[105]

In addition to the mucosal barrier, pathogenic and commensal microorganisms can also determine the outcome of homeostasis or an infection. Bacteria can be sensed extra- and intracellularly through the recognition of pathogen-associated molecular patterns (PAMPs) by PRRs, including TLRs and NLRs. Detection by PRRs results in the activation of NF-κB, which promotes the transcription of target genes encoding pro-inflammatory cytokines and chemokines and can also promote tissue homeostasis and mucosal tolerance in the absence of barrier damage. Colonization of certain pathogenic bacteria in the intestine can induce mucosal barrier damage and intestinal pathology.[106]

1.3.2. Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is characterized by chronic, relapsing episodes of inflammation of the gastrointestinal tract of unknown cause. There are two major types of IBD: Crohn's disease (CD) and ulcerative colitis (UC).[107] IBD is

considered to result from inappropriate activation of the mucosal immune system driven by the normal luminal flora. However, multiple factors can contribute to the pathogenesis of IBD. This aberrant response is most likely facilitated by defects in both mucosal barrier function and the immune system.[108] IBD can be either sporadic or familial. To date, 99 potential IBD susceptible loci have been mapped including chromosome 16q12 (encoding for NOD2 [nucleotide-binding oligomerization domain 2]/CARD15 [caspase recruitment domain family, member 15], 1q32 (encoding for IL-10), 1q31 (encoding the IL-23 receptor) and an uncharacterized linkage on 3p21.[109-113]

Studies of murine genetic models of IBD have revealed that: (a) a compromised epithelial layer is possibly sufficient to result in intestinal inflammation; (b) autoreactive T cells can be inappropriately activated, contributing to pathogenesis; (c) a variety of other haematopoietic cells are able to mediate or regulate intestinal inflammation; (d) different cytokines and chemokines may play a role in pathogenesis of colitis in mice and IBD patients; (e) regulation of NF-κB signaling is essential in intestinal homeostasis; and (f) the resident enteric flora seems necessary for colitis induction although no specific pathogen isolated from the intestinal flora of spontaneous colitis models has been shown to cause disease.[110, 114]

1.4. NF-κB

The nuclear factor kappa-light-chain-enhancer of activated B cells (nuclear factor-κB, or NF-κB) transcription factor has been shown to have a pivotal role in many

biological processes, including inflammation, immunity, cell survival as well as chronic inflammation-associated tumorigenesis.[115, 116] Sen and Baltimore in 1986 first found NF-κB in B-cells that transcribe immunoglobulin κB light chain genes and interacts with a defined site in the kappa immunoglobulin enhancer.[115] In intensive studies, NF-κB has been demonstrated to target expression of more than 150 gene in response to numerous stimuli.[117] Most of the target genes have been confirmed to have distinct NF-κB binding sites. In response to pathogen recognition and inflammatory mediators, NF-κB is activated to target transcription of cytokines, chemokines and growth factors in order to promote inflammatory response. Besides, the NF-κB activity is also believed to participate in the resolution of inflammation. Additionally, it is thought that continuous nuclear NF-κB activity protects cancer cells from apoptosis and in some cases stimulates their growth. Therefore, many current anti-tumor therapies seek to block NF-κB activity in order to inhibit tumor growth or to sensitize the tumor cells to more conventional therapies, such as chemotherapy.[118]

1.4.1. NF-κB protein family

The larger NF-κB family consists of two subfamilies: the NF-κB family and the "Rel" family. All members share a REL-homology domain (RHD) that is responsible for DNA binding, dimerization, nuclear translocation and IκB (inhibitor of kappa light chain gene enhancer in B cells) binding.[119] The mammalian Rel subfamily includes c-Rel, RelB and RelA (also known as p65). The Rel proteins contain transactivation domains (TAD) that are required for activating transcription of a large variety of genes. Members of the mammalian

NF-κB subfamily (p105 and p100) are characterized by their C-terminal ankyrin repeats, which inhibit RHD domain from binding to DNA. The precursor p105 and p100 can become shorter (p105 to p50 and p100 to p52) and activate DNA-binding proteins, possibly by either limited proteolysis or arrested translation. As p105 and p100 do not possess TAD domains, they are generally not activators of transcription unless they dimerizes with members of the Rel subfamily.[118, 120] Collectively, active NF-κB dimers bind to 9-10 base pair DNA sites (also known as κB sites) with a large variability. As reviewed by Pahl, NF-κB dimers can be induced by over 150 different stimuli and participate in transcription regulation of over 150 target genes.[117] Most of the target genes have been confirmed to contain distinct NF-κB binding sites.

NF-κB dimers are retained in the cytoplasm and remained inactive by binding to ankyrin repeats containing proteins. The ankyrin repeats in p100 or p105 are also found in IκB proteins, including IκBα, IκBβ, and IκBγ.[120] The ankyrin repeats bind to the RHD of NF-κB, masking its nuclear localization sequence, thereby retaining NF-κB in the cytoplasm.[121]

1.4.2. Activation of NF-κB

Generally, NF-κB is kept inactive in most cells by sequestration to members of the IκB family of inhibitory proteins in the cytoplasm. In response to a large variety of agents, chemical, bacterial and viral components, IκB is rapidly degraded by the ubiquitin–proteasome pathway, allowing NF-κB to enter the nucleus to activate gene transcription of a wide spectrum of genes.[122]

As we know now, more than 150 stimuli are capable of activating NF-κB and in turn NF-κB activates more than 150 target gene expressions. The activation of NF-κB is broadly classified into two schemes, canonical and non-canonical pathways (also known as classical and alternative pathways respectively). In the canonical pathway, as indicated in Figure 1.5, stimulation of cells leads to the rapid phosphorylation of IκBα at serine 32 and serine 36 by a large kinase complex called IκB kinase (IKK). IKK consists of two catalytic subunits, IKKα and IKKβ, and an essential regulatory subunit termed NEMO, also known as IKKγ. Phosphorylated IκBα is then lysine 48-polyubiquitylated and targeted for degradation by the 26S proteasome, thereby liberating NF-κB.[123, 124] The term NF-κB commonly refers to the p50/RelA heterodimer, which is the major NF-κB complex in most cells.

The non-canonical pathway of NF-κB activation is mainly for p100/RelB processing into p52/RelB complexes during B- and T-cell development. This pathway is only responsible for certain receptor signals and proceeds through an IKK complex that is comprised of only two IKKα subunits without NEMO. Receptor stimulation leads to activation of the NF-κB-inducing kinase (NIK), which phosphorylates and activates the IKKα complex. Activated IKKα in turn phosphorylates p100, leading to its partial proteasome degradation and activation of the p52/RelB complex.[118, 124]

In either pathway, the unmasked NF-κB complex can then enter the nucleus to activate target gene expression. In the canonical pathway, IκBα is one of the target genes of activated NF-κB. Newly-synthesized IκBα can enter the nucleus,

associate with NF-κB and subsequently remove the NF-κB from the DNA. The IκBα-NF-κB complex can then be exported back to the cytoplasm to restored to the original latent state.[125]

A variety of recent evidence, however, indicates that the NF-κB activity is not simply regulated by the inhibitory protein IκB. In addition, RelA and p50 are regulated by ubiquitination, acetylation, methylation, phosphorylation, oxidation/reduction, and prolyl isomerization. Moreover, as a consequence of induction of NF-κB activity (at least by the tumor necrosis factor) IKKα is also induced to enter the nucleus where it becomes associated with κB site promoters/enhancers to phosphorylate histone H3 which enhances the transcription of κB site-dependent genes.[126]

TNFα induced canonical NF-κB activation pathway is a potent and well studied pathway that is very important in the pro-inflammatory response and anti-apoptotic effect.[127] Aggregation and activation of TNF-R1 by ligation of TNFα allow association of the TNF-R-associated death domain protein (TRADD). TRADD subsequently recruits adapter molecules including TNF receptor associated factor 2 (TRAF2) and TRAF5.[128-130] TRAFs subsequently may either recruit the IKK complex directly or indirectly through the serine/threonine kinase, receptor interacting protein 1 (RIP1).[131, 132] Upon ubiquitination, RIP1 can bind directly to the NF-κB essential modulator (NEMO or IKKγ) and recruit IKK independent of TRAF2.[133] Signaling downstream of RIP1 requires transforming growth factor-β activated kinase 1 (TAK1) for the activation of IKK.[134] The activated IKK complex subsequently phosphorylates IκBα and causes its

ubiquitination and proteasomal degradation. As a result, the released NF-κB dimer can be imported into the nucleus and promote gene transcription.

1.4.3. NF-κB and inflammatory response

NF-κB transcription factors regulate genes in many aspects of immunity and inflammatory response. It is well established that the canonical NF-κB pathway, based on IKK-dependent IκB degradation, is essential for innate immunity.[135] NF-κB knockout mice have defects in innate immunity response to bacterial infection.[136, 137] Recognition of the pathogenic components by the TLR receptors on the surface or inside of the epithelial, endothelial or immune cells can lead to activation of NF-κB. Subsequently, activated NF-κB promotes transcription of genes encoding chemokines, cytokines, adhesion molecules, enzymes that produce secondary inflammatory mediators, and inhibitors of apoptosis. The cytokines and chemokines such as IL-1β, IL-6, TNF-α, MCP-1 and IL-8 are important for activation and recruitment of neutrophils and macrophages to the infected or injured tissue where NF-κB is activated. The Adhesion molecules targeted by NF-κB such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), endothelial–leukocyte adhesion molecule 1 (ELAM) and matrix metalloproteinases (MMPs) are essential for the migration, capture and exiting the vasculature of the immune cells to the site of inflammation.[127, 135, 138, 139] Recruited neutrophils are the key mediators of acute inflammation and the NF-κB targeted anti-apoptotic genes are important for the survival of these cells.[140] NF-κB is important for the production of the enzymes that generate prostaglandins and reactive oxygen species such as cyclooxygenase-

2 (COX-2) and nitric oxidize synthase (NOS).[141] The immediate targets of NF-κB-dependent proinflammatory cytokines, such as TNFα, can in turn activate NF-κB. Therefore, NF-κB is crucial to the propagation and elaboration of cytokine responses. TNFα is particularly important for both local and systemic inflammation.[127]

NF-κB activity is also implicated to be essential for hematopoiesis, immune cell maturation, and lymphoid organ development. Mouse gene knockout studies of NF-κB subunits or signaling components suggest that NF-κB signaling is important for development and survival of neutrophils, dendritic cells, and lymphocytes, as well as differentiation of T_H lymphocytes into T_{H1} and T_{H2} cells.[127, 142-145]

As NF-κB promotes inflammatory response, it would be expected that NF-κB is primarily be turned off during the resolution phase of inflammation, which includes elimination of granulocytes (neutrophils) and mononuclear cells (macrophages and lymphocytes) to normal numbers in the tissue and circulation.[146] NF-κB activity is known to be essential for the survival of the immune cells. Recent work suggests that NF-κB also has a more active role for resolution.[147] During acute inflammation, there are multiple negative feedback pathways that help to restrict NF-κB activity. Besides IκBα, another target gene of NF-κB activity, B-cell lymphoma-3 (BCL-3) is suggested to mediate repression of proinflammatory genes, and also facilitates expression of the anti-inflammatory gene IL-10 during LPS stimulation.[148] Additionally, inhibition of NF-κB during the resolution phase can prolong the inflammatory process and prevent proper

tissue repair.[149] It was subsequently discovered that IKKα-deficient mice display increased inflammatory responses in models of local and systemic inflammation.[150]

Due to the importance of NF-κB in multiple biological processes, its activity is tightly regulated by inhibitory proteins in addition to negative feedback pathways, including the deubiquitinating enzyme A20 and CYLD by removing K63-linked activating ubiquitination from RIP1, NEMO or TRAFs. Therefore, IKK activation is attenuated resulting in restricted NF-κB activation.[151, 152] Another inhibitor protein named single Ig IL-1 receptor-related molecule (SIGIRR) is found to negative regulate IL-1, IL-18, IL-33 and specific TLR ligand-dependent NF-κB activation through interacting with TLR pathway components, but not on TNF-dependent NF-κB activation.[153] Loss of proper negative regulation of NF-κB can result in prolonged pro-inflammatory signaling, tissue damage, and chronic inflammation. For example, intestinal epithelium A20 expression has been implicated in preventing DSS-induced experimental colitis in mice.[154] SIGIRR deficient mice exert constitutive NF-κB activation by commensal bacteria and increased susceptibility to chemical induced intestinal inflammation and cancer, indicating its role in controlling gut homeostasis.[155] The mechanism of inhibiting NF-κB is relatively poorly understood compared to the activation pathway, thereby requires further investigation.

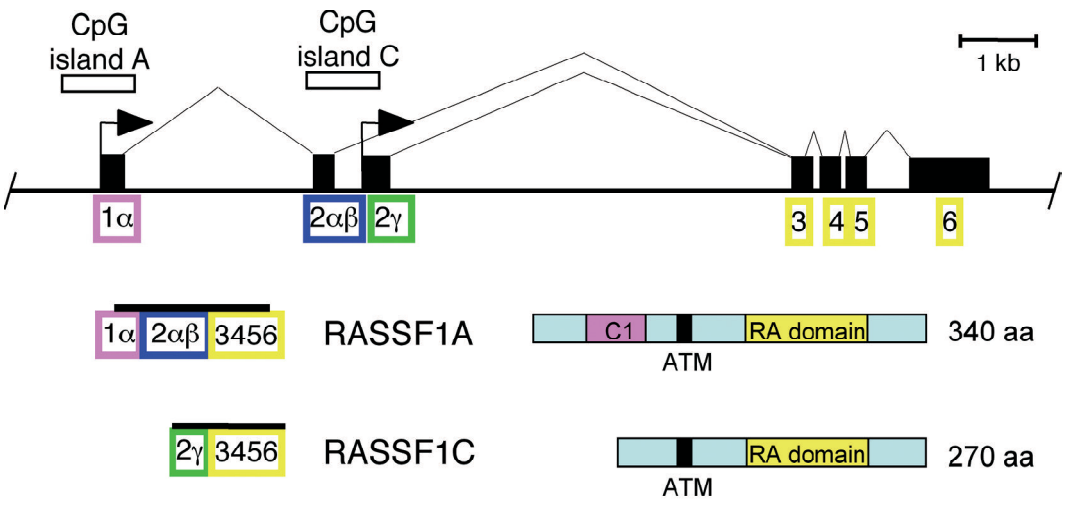

Figure 1.1: The schematic map of the RASSF1 gene and two major isoforms. The two promoters of RASSF1 (arrows) are located in CpG islands (open square). Two major isoforms (RASSF1A, RASSF1C) are made by alternative promoter usage and splicing of the exons (black boxes). The lower panel shows the comparison of the exons used by RASSF1A and RASSF1C as well as the protein sequences. RASS1A contains the C1 domain (purpule) that is encoded by Exon 1α, the putative ATM-kinase phosphorylation site (black) and the RA domain (yellow). Adapted from Dammann *et al.,* 2005.

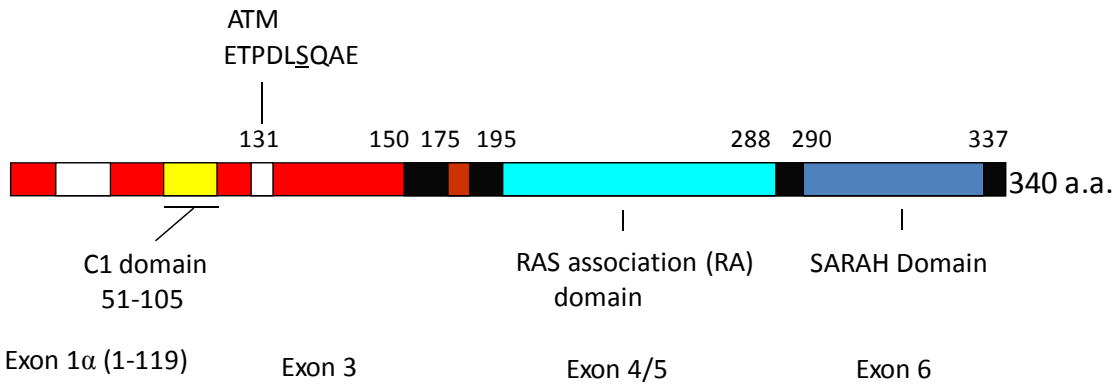

Figure1.2: Schematic RASSF1A protein sequence and domains. The C1 domain contains a zinc finger binding motif that is involved in associations with death receptor. The ATM site is phosphorylated by ataxia telangiectasia mutated (ATM) kinase during DNA damage control. The RAS association domain (RA) is involved in association with the Ras family of oncogenes. Lastly, the SARAH domain is a protein interaction domain in the Salvador/RASSF/Hpo pathway that promotes apoptosis. Approximate positions of exons are also indicated.

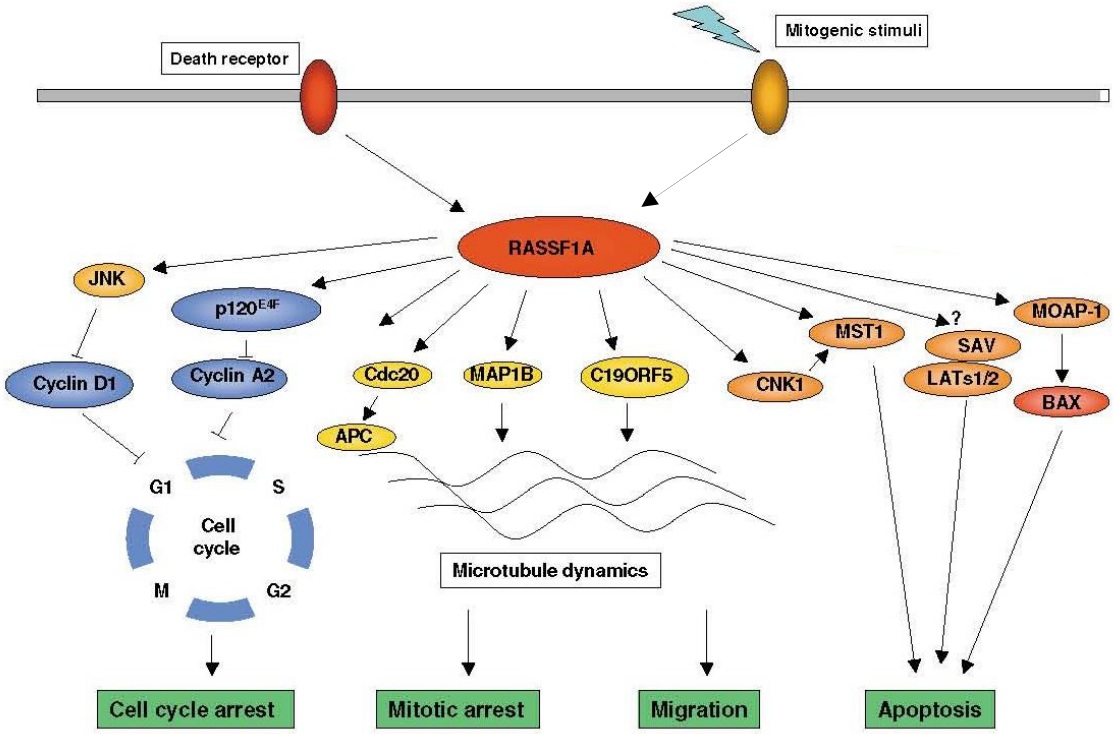

Figure 1.3: Summary of some of the known biological processes and pathways involving RASSF1A. RASSF1A responses to mitogenic or apoptotic stimuli and regulates various biological processes including cell cycle arrest, mitotic arrest, migration, and apoptosis. RASSF1A induces cell cycle arrest by suppressing the levels of cyclin D1 through activation of JNK pathway and enhancing the ability of $p120^{E4F}$ to suppress cyclin A2. RASSF1A stabilizes microtubule networks by associating with microtubule-associated proteins including the APC/C-Cdc20 complex, MAP1B and C19ORF5, thereby regulating mitosis. Interactions of RASSF1A with CNK1, MST1, Salvador and MOAP1 allow RASSF1A to modulate apoptosis. Adapted from Donninger *et al,* 2007.

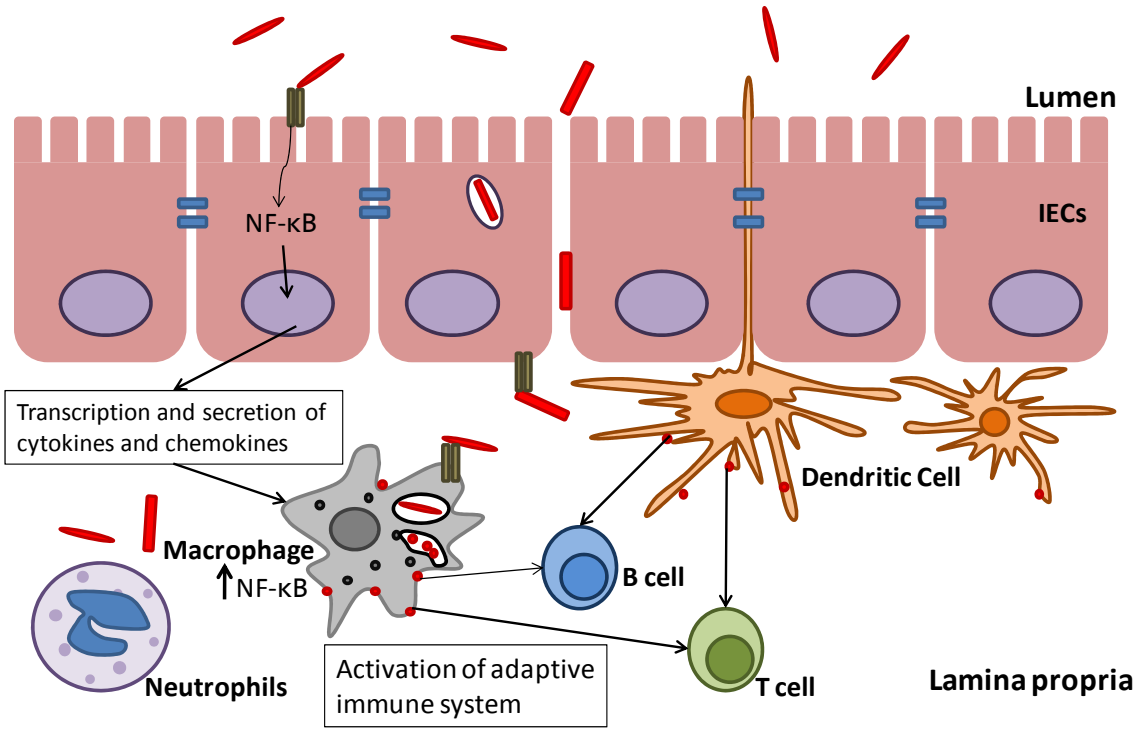

Figure 1.4: Homeostasis of the intestinal mucosa. The intestinal epithelial cells (IEC) form a barrier between the intestinal lumen and lamina propria area beneath the epithelium. Pathogenic bacteria can invade the epithelial cells directly, or enter into the lamina propria when the mucosal barrier becomes leaky. Subsequently, the pathogenic components are recognized and captured by the immune cells in the lamina propria including local macrophages and dentritic cells. The injured tissue, macrophages, and dendritic cells elicit proinflammatory cytokines, chemokines and other mediators to recruit other immune cells to the site of injury. Neutrophils are recruited first in the acute phase for pathogenic clearance. Macrophages and dendritic cells also communicate with B- and T-lymphocytes and activate adaptive immune response. Among these processes, NF-κB signaling is believed to play a role in regulating inflammatory response and immunity by promoting the transcription of inflammatory mediators.

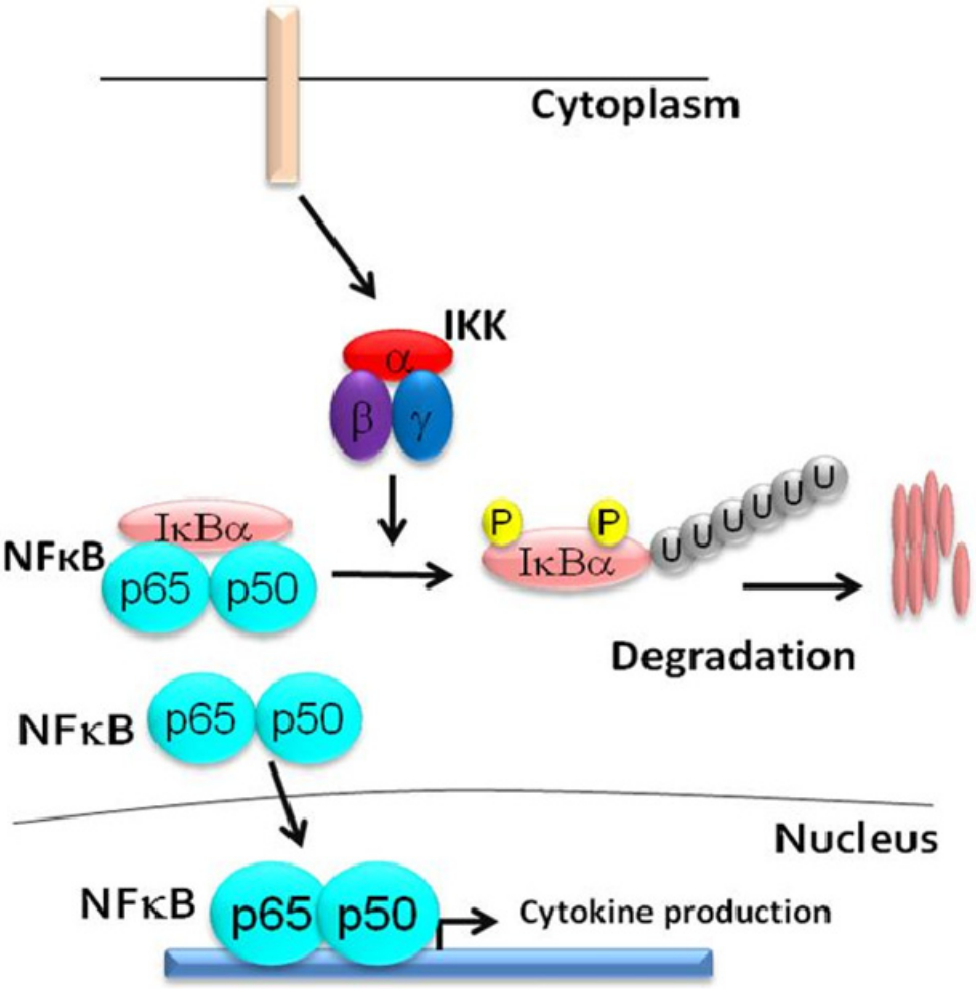

Figure 1.5: Simplified canonical NF-κB signaling. The NF-κB dimer of p65 and p50 is sequestered in the cytoplasm by binding to IκBα. Upon receptor activation, IKK can be activated and phosphorylate IκBα at Ser32/36, resulting in ubiquitinylation and proteasomal degradation of IκBα. The NF-κB dimer is then released and can imported into nucleus. Activated NF-κB dimer can bind to the enhancer region of the target genes and activate gene transcription including cytokine production.

Chapter 2

Materials and Methods

2.1. MATERIALS

2.1.1. Chemicals, Reagents and other Materials

Acrylamide	Invitrogen
Agrose	Invitrogen
Ammonium Persulfate (APS)	BioRad
β-mercaptoethanol	BioShop
Betain	Sigma
Bromophenol Blue	Sigma
Dextran Sulfate Sodium (DSS)	MP Biomedical
Diethyl Ether	Caledon
Dithiothreitol (DTT)	EMD
DirectPCR Lysis Reagent (Tail)	Viagen Biotch
dNTP mix	Qiagen
Enhanced Chemiluminescence (ECL)	GE Healthcare
Ethidium Bromide	BioRad
Ethylenediaminetetraacetic acid(EDTA)	EMD
Ethylene Glycol Tetraacetic Acid (EGTA)	EMD
Ethanol	Commercial Alcohols
Fluorescein Isothiocyanate (FITC)-Dextran(FD-4)	Sigma
Glycerol	Anachemia
Glycine	Fisher Scientific

Hydrochloric Acid	Caledon
Lipopolysaccaride (LPS)	Sigma
Methanol	Caledon
MyCoy's 5A media	Fisher Scientific
M-PER Mammalian Protein Extraction Reagent	Thermo Scientific
NP-40(IGEPAL)	Sigma
Polyethylenimine (PEI)	Polysciences, USA
Polyvinylidene Fluoride (PVDF) Membrane	Millipore
Skim Milk Powder	Nestlé
Sodium Azide	Fisher Scientific
Sodium Dodecyle Sulphate (SDS)	BioRad
Sodium Hyperchlorite (4%)	Sigma
Sodium Pyrophosphate	Sigma
Sodium Vanadate	Sigma
SuperSignal® West Dura Extended Duration Substrate	Thermo Scientific
Tris	Fisher Scientific
Trypsin	Fisher Scientific
Tween 20	Fisher BioReagents
Whatman Chromatography Paper	Fisher Scientific
Z-fix	Anatech LTD.

Protein A Sepharose	GE Healthcare
Protein G Sepharose	GE Healthcare

2.1.2. Antibodies

Primary Antibodies:

Mouse anti-HA	In house produced
Mouse anti-IRAK1	Santa Cruz sc-5288
Mouse anti-phospho-IκBα(Ser32/Ser36)	Cell Signaling 9246
Rabbit anti-IκBα	Cell Signaling 9242
Rabbit anti-IRAK4	Cell Signaling 4363
Rabbit anti-MyD88	Santa Cruz sc-11356
Rabbit anti-TLR4	eBioscience 14-9920
Rabbit anti-TRAF6	Santa Cruz sc-8409

Secondary Antibodies:

Anti-Mouse IgG Horseradish Peroxidase-Linked Whole antibody	GE Healthcare UK
Anti-Rabbit IgG Horseradish Peroxidase-Linked Whole antibody	GE Healthcare UK

2.1.3. Buffers and Other Solutions

4× Separating Buffer: 1.5 M Tris pH 8.7, 0.4% SDS

4× Stacking Buffer: 0.4 M Tris pH 6.8, 0.4% SDS

4× SDS-PAGE Loading Buffer: 0.174 M Tris pH 6.8, 25% Glycerol, 5% SDS, 0.02% Bromophenol Blue

10× PCR Buffer: 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 20 μg/mL gelatine

10× SDS-PAGE Running Buffer: 0.125 M Tris pH 8.3, 0.96 M Glycine, 0.5% SDS

10× Tris-Borate-EDTA (TBE) Buffer: 900 mM Tris-Borate, 20 mM EDTA

10× T4 Polynucleotide Kinase Reaction Buffer: 700 mM Tris pH7.6, 100 mM $MgCl_2$, 50 mM DTT

Cytoplasmic Extraction Buffer: 10 mM Hepes pH 7.6, 60 mM KCl, 1 mM EDTA, 1 mM DTT

Nuclear Extraction Buffer: 20 mM Tris pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% Glycerol

Nuclear Extraction Dilution Buffer: 20 mM Tris pH 7.6, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 10% Glycerol

Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4

SB Lysis Buffer: 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1.5 mM EDTA, 0.5% Triton X-100

Semi-Dry Transfer Buffer: 50 mM Tris, 380 mM Glycine, 0.1% SDS, 20% Methanol

Stripping Buffer: 52 mM Tris pH 6.8, 2% SDS

TAE Buffer: 400 mM Tris Acetate pH 8.5, 2 mM EDTA

TBS-Tween Buffer: 500 mM Tris pH 7.4, 100 mM NaCl, 0.05% Tween-20

***Taq* DNA Polymerase Dilution Buffer:** 20 mM Tris pH7.5, 0.1 mM EDTA, 1 mM DTT, 0.1 KCl, 0.5% Tween-20, 0.5% NP-40, 50% Glycerol

Tris-EDTA (TE) Buffer: 10 mM Tris (pH 7.5), 1 mM EDTA

Wet Transfer Buffer: 25 mM Tris, 192 mM Glycine, 20% Methanol

2.2. METHODS

2.2.1. Animal Experiments

The mouse containing conditional allele to Exon 1α of *Rassf1a* (background 129/Sv-C57BL/6) was obtained as described by Weyden et al. (2005) [54]and mated to a Villin-Cre expressing mouse (background C57BL/6) from JAX® Laboratory.[156] The mice were maintained on a mixed 129/Sv-C57BL/6 background. Genotyping was carried out on DNA obtained from ear notches.

2.2.1.1. Polymerase-chain reaction (PCR) genotyping

Genomic DNA of selected mouse was extracted by adding 200 µL of DirectPCR lysis reagent containing freshly prepared 0.4 mg/mL Proteinase K to one ear notch in an eppendorf tube. The ear biopsy was digested at 55°C overnight with shaking until no tissue clumps were present and followed by incubation at 85°C for 45 min in a water bath. The digested samples were spun down at 10k ×g for 1 min. Supernatant was aliquoted and stored at - 20°C for further PCR analysis. The genomic DNA was amplified in 25 µL reaction mixtures, including 1× PCR buffer, 0.25 µM dNTP, 6.5 U *Taq* DNA polymerase and 100 ng of each primer. Three sets of primers were used for genotyping individual mice (RSF-C/RSF-3 for the presence of conditional allele of *Rassf1a*, RSF-5/RSF-3 for deletion of

exon 1α of *Rassf1a*, oIMR1878, oIMR1879, oIMR0015 and oIMR0016 for detecting the presence of Cre Transgene). The primer sequences were as follows: RSF-3 (reverse primer), 5'-CCA GGC TTC CTT CTC ACT CCT CTG CCG C-3'; RSF-C (forward primer), 5'-CTC GCC CCT GTC AAA GAA AGC TGC TCT GGG GTT CT-3'; RSF-5 (forward primer), 5'-CTC GCC CCT GTC AGA CCT CAA TTT CCC-3', IMR1878 (Cre Transgene), 5'-GTG TGG GAC AGA GAA CAA ACC-3'; IMR1879 (Cre Transgene), 5'-ACA TCT TCA GGT TCT GCG GG-3'; IMR0015 (internal positive control forward), 5'-CAA ATG TTG CTT GTC TGG TG-3'; IMR0016 (internal positive control reverse), 5'-GTC AGT CGA GTG CAC AGT TT-3'. The PCR cycle profile for RSF-5/RSF-3 and RSF-C/RSF-3 was 1 cycle at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 65°C for 1 min, 72°C for 30 s, with a final cycle of 72°C for 10 min and keep at 4°C till the end. The PCR profile for Cre Transgene was 1 cycle at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 62°C for 1 min, 72°C for 1.5 min, with a final step of 72°C for 1 min and keep at 10°C till the end. All PCR products were separated in a 1.5% agarose gel containing ethidium bromide by electrophoresis in 1× TAE buffer. RSF-C/RSF-3 produces a 400 bp fragment for wild type allele of *Rassf1a* and a 480 bp fragment for conditioned allele. RSF-5/RSF-3 produces a 400 bp fragment only in the mouse tissue that has Exon 1α of *Rassf1a* deleted. The Cre Transgene genotyping produces a 200 bp fragment for the wild type allele and an 1100 bp fragment for the Cre Transgene. The mice expressing Villin-Cre and have homologous *Rassf1a* conditional allele are noted as $Rassf1a^{IEC\text{-}KO}$ mice and the corresponding wild type mice are noted as $Rassf1a^{IEC\text{-}WT}$.

2.2.1.2 Acute dextran sulphate sodium (DSS) treatment

The 8-10 weeks old $Rassf1a^{IEC\text{-}KO}$ and $Rassf1a^{IEC\text{-}WT}$ mice were separated in individual cages one day before starting the experiment. Normal drinking water was replaced by 3% DSS (36-50 kDa) water the next day and counted as day 0 for DSS treatment. The mice were exposed to DSS for 7 days and followed by normal drinking water for additional 8 days. During the 15-day period, the mice

were monitored for body weight changes, piloerection, bloatedness, rectal bleeding, and slowed movement according to Table 2.1. The mice were euthanized when they lost more than 20% of their body weight or had high level of inactivity (no movement upon touching).

2.2.1.3 Cardiac puncture

Blood samples of DSS treated and untreated mice were collected by cardiac puncture. The mice were anesthetized by inhaling ether. Blood was drained from the heart by 23G needles and 1 mL syringes and collected in 1.5 mL eppendorf tubes. Mice were then euthanized by cervical dislocation. The blood samples were coagulated at room temperature for 2 hours and centrifuged at 10,000 g for 10 min. The clear serum was collected and stored at -80°C for further analysis.

2.2.1.4 Enzyme-linked Immunosorbent Assay (ELISA)

Blood cytokine levels were analyzed by ELISA kit obtained from Thermo Scientific. Serum samples were diluted 1:3 for IL-6, TNF-α, IL-1β, IL-12, and IFN-γ using standard diluents from the kit. Colon tissue MPO concentration was measured by ELISA kit from Hycult Biotech. Colon sections was flushed with 1 × PBS and lysed by homogenization in M-PER lysis buffer (approximally 1 cm colon in 500 μL lysis buffer). Protein concentrations of the tissue extracts were determined by Bradford method. Colon lysates were diluted 1:4 by using standard diluents and measured for MPO concentration. The final concentration was represented as MPO μg/g of protein extracts.

2.2.1.5 Tissue histology assessment

Proximal colon or the whole colon together with kidney from individual mice were collected and fixed by Z-fix (Anatech). Both transverse and longitudinal section of the colon samples were subjected to Hematoxylin and Eosin (H&E) staining and performed by animal histology core facility of the Alberta Diabetes Institute. The colon histology sections were blindly scored by Dr. Aducio

Thiesen (Department of Anatomical Pathology, University of Alberta) for unbiased analysis according to Table 2.2. Kidney transverse sections were prepared by H&E staining in the histology core facility and blindly analyzed by Dr. Todd Alexander (Department of Physiology, University of Alberta) for renal cortex pathology.

2.2.1.6 Crypt cell isolation

Crypt cells of colon samples were collected based on the method modified from Xiao *et al*.[155] Briefly, one entire colon was removed and flushed with ice cold PBS using a 23G needle and a 3 mL syringe until the eluate is completely clear of stool. The clean colon was cut open longitudinally and cut into smaller pieces by using scissors. The colon pieces were transferred into 0.04% sodium hypochlorite solution and incubated for 30 min at room temperature with shaking. The colon pieces were then transferred to 1× PBS with 1 mM EDTA and 1 mM EGTA and incubated for 30 min at room temperature with continuous shaking. In order to release the crypt cells from the rest colon tissues, a 25 mL pipette was used to mix the colon pieces until significant amount of crypt cells were visualized under microscope. The cells were pelleted at 2,000 ×g for 10 min and washed with 1× PBS. The cell pellet was store at -80°C until further analysis.

2.2.1.7 Bone marrow cells isolation

Selected mice were sacrificed by CO_2 asphyxiation or cervical dislocation. The mouse body was spayed with 70% ethanol. Whole femur was removed and transferred to a sterile cell culture dish supplied with ice cold DMEM media (10% bovine growth serum). Both hip and knee joints were removed. Bone marrow was flushed by a 30G needle and 1 mL of DMEM media into a centrifuge tube. The bone marrow cells were centrifuged at 16,000 ×g for 5 min.

2.2.1.8 Intestinal permeability analysis

The intestinal permeability assay to assess barrier function was performed using a Fluorescien Isothiocyanate (FITC)-conjugated dextran method, as described.[157]

Food was withdrawn for overnight and the mice were orally gavaged with FITC-dextran (4.4 kDa, 60mg/100 g body weight). Four hours later, blood serum was collected by cardiac puncture. Fluorescence intensity of each sample was determined by fluorometry (excitation, 492 nm; emission, 525 nm; PerkinElmer plate reader). FITC-dextran concentrations were determined based on standard curves generated by serial dilution of FITC-dextran.

2.2.1.9. Serum creatinine analysis

Mouse serum was collected and blindly analyzed by Wanling Pan and Dr. Todd Alexander (Department of Physiology, University of Alberta) by using creatinine kit (R&D systems Inc., KGE005).

2.2.1.10. Systolic blood pressure measurement

The systolic blood pressure before and after DSS treatment was blindly measured by Jeff Odenbach and Dr. Carlos Fernandez-Patron (Department of Biochemistry, University of Alberta). Blood pressure measurements were carried out using a computerized tail cuff plethysmography (Kent Scientific Corporation).[158] Briefly, conscious mice were maintained at 32 – 35^{o}C using a heating pad and restrained during all blood pressure measurements. Averages of 10 inflation/deflation cycles were conducted to obtain mean systolic blood pressure. Animals were allowed to stabilize after transport and before blood pressure measurements were taken at the same time of day for the genotypes.

2.2.2 NF-κB Electrophoretic Mobility Shift Assay (EMSA)

2.2.2.1 Isolation of nuclear and cytoplasmic fraction

Cell pellet was incubated with Cytoplasmic Extraction (CE) buffer containing 0.2% NP-40 and protease inhibitors in a 1.5 mL eppendorf tube for 15 min on ice. The volume of CE buffer is 5 times of the pellet size, for example, 100 μL of buffer was added to 20 μL of pellet. The pellet was broken up by flicking the tube. The homogenate was centrifuged at 3,300 ×g for 5 min. The supernatant (cytoplasmic fraction) was removed and mixed with glycerol to a concentration of 10%. The

remaining pellet was washed with the same volume of CE buffer without NP-40 and centrifuged. The supernatant was discarded. Subsequently, the pellet was resuspended on ice for 20 min in Nuclear Extraction (NE) buffer half the volume of CE buffer, ie, 50 μL. The homogenate was centrifuged for 10 min at 16,000 ×g. The supernatant was transferred to a new tube and dilute 1:1 with Nuclear Fraction Dilution buffer. Protein concentration was determined by Bradford protein assay. The cytoplasmic fractions and nuclear fractions were all stored at -80°C for further analysis.

2.2.2.2 Preparation of radioactively labelled probe for NF-κB

In order to produce the NF-κB specific probe, the sense and antisense oligonucleotides were labelled at 5' ends separately with [γ-^{32}P]ATP by T4 polynucleotide kinase (T4 PNK). The oligonucleotides sequences were as follows: 5'-TCAGAGGGGACTTTCCGAGAGG-3' (wild type probe sense strand with NF-κB specific binding site underlined); 5'-CCTCTCGGAAAGTCCCCTCTGA-3' (wild type probe anti-sense strand); 5'-TCAGAGGcGACTTTCgGAGAGG-3' (mutant probe sense strand); 5'-CCTCTCcGAAAGTCgCCTCTGA-3' (mutant probe anti-sense strand). The reaction mixture with a total volume of 20 μL was composed of 0.4 μg oligonucleotide, 1× T4 PNK reaction buffer, T4 PNK, 0.67 μM [γ-^{32}P]ATP with 2 mCi/mL radioactivity. The labelling reaction was carried out at 37°C for 2 hrs. After labelling, 5 μL of 0.1 M EDTA and 70 μL of 1× TE buffer were aliquoted into the reaction mixture. Labelled oligonucleotides were purified using a G-50 sephadex column (GE Healthcare Life Sciences). The purified sense and anti-sense oligonucleotides were combined and allowed for annealing for 2-3 hrs at room temperature. The duplex oligonucleotide was counted for radioactivity by scintillation counter (Biochemistry Department, University of Alberta) and stored at -80°C.

2.2.2.3 Electrophoretic mobility shift assay (EMSA)

The binding of NF-κB probe to nuclear extract reaction was carried out at room temperature for 45 min. The reaction mixture in a total volume of 25 μL was

composed of 3-8 μg nuclear extract, 1× NF-κB binding buffer, 1μg poly dI:dC, 2.5 mM DTT, 150 mM EDTA, 25% Glycerol. All reagents were purchased from LI-COR Odyssey. Non-denaturing 5% Acrylamide gel was prepared and pre-run at 100 V for 30 min in 0.5× TBE buffer. Samples together with a lane of orange dye (LI-COR Odyssey) were loaded onto the gel and run at 200 V until the dye is close to the bottom of the gel. The gel was transferred to a blotting paper and dried on a vacuum gel dryer with heat (80°C) for 1 hr. The dried gel was exposed to X-ray films for 24-48 hrs at -80°C.

Alternative to the radioactive method, IRDye 700 end-labeled oligonucleotides and the Odyssey Infrared Imaging System were also used to detect NF-κB activity. The oligonucleotide sequences are as follows: 5' - AGT TGA GGG GAC TTT CCC AGG C - 3' (sense strand with NF-κB binding site underlined); 5' - G CCT GGG AAA GTC CCC TCA ACT - 3' (anti-sense strand with NF-κB binding site underlined). The experiment was performed according to the company instructions (829-07924, LI-COR).

2.2.3 Western Blotting

Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 1× running buffer. Proteins on the gel were transferred onto a PVDF membrane (MilliPore) in transfer buffer at 250 mA for 3 hrs. Subsequently, the membrane was blocked with 10% skim milk solution in TBS-Tween buffer for 1 hr at room temperature. The membrane was then incubated with primary antibody (diluted in 5% milk and supplied with 0.02% NaN3) overnight at 4°C on a shaker. Afterwards the membrane was washed 3 times with TBS-Tween buffer and incubated with secondary antibody for 1-2 hrs at room temperature on a shaker. Following the incubation, the membrane was washed 3 times with TBS-Tween buffer and incubated with the enhanced chemiluminescence (ECL) detection reagent (GE Healthcare) or SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific) for 3 min followed by detection using X-ray films or a gel imaging system from Alpha Innotech.

2.2.4 Statistical Analysis

The survival rate of each mouse group was calculated by using the Kaplan Meier method. The p-value was calculated from chi-square statistic. For comparing the difference between two sample groups, Student's *t*-test was used and the associated p-value was obtained. Both p-value and sample numbers were indicated in the figure legend. The standard error of means were calculated for each group and labelled in the graph.

Table 2.1. Disease activity (DAI) scoring chart

Weight loss	Rectal bleeding	Piloerection	Bloatedness	Movement	Score
0%	normal	no sign	no sign	very active	0
0-5%	stool redness	lost shine	mild	slightly slowed	1
6-10%	slightly visible		moderate		2
11-15%	obvious	obvious	severe	obviously slowed, hunched	3
>15%	severe				4
	gross with blood clots	severe		very little movement, severely hunched	5

Disease activity index (DAI) is the combined scores from each category. Modified from Murthy *et al.,* 1993.[159]

Crossed cells in the table mean that corresponding scores were not used for the observation.

Table 2.2. Histological scoring chart

Group	Description	Score
Enterocytes injury		
Normal	Rare intraepithelial lymphocytes	0
Mild	Intraepithelial neutrophils	1
Moderate	Mucosal necrosis and/or luminal pus	2
Severe	Necrosis muscularis mucosa	3
Lamina propria neutrophil infiltrate		
Normal	No extravascular presence	0
Slightly increased	Single neutrophils	1
Markedly increased	Cell aggregates	2
Lamina propria lymphocytes infiltrate		
Normal	One small lymphoid aggregate	0
Slightly increased	More than one small aggregate	1
Markedly increased	Large aggregates and/or greatly increased single cells	2
Epithelial hyperplasia		
Normal		0
Mild		1
Moderate		2
Pseudopolyps		3

Adapted from Saverymuttu *et al.,* 1986 and Madsen *et al.,* 2001.[160, 161]

Chapter 3

Intestinal Epithelial Expression of Rassf1a Protects Mice from Dextran Sulphate Sodium (DSS) Induced Experimental Colitis

3.1. BRIEF INTRODUCTION

Ras-association domain family protein 1A (RASSF1A) is a tumor suppressor protein. Loss of expression of RASSF1A due to aberrant promoter hypermethylation is one of the most frequent changes in numerous cancers.[53] It has been well documented that chronic inflammation can predispose individuals to developing cancer of the same tissue.[162] For example, patients with persistent inflammation of the bowel (such as in inflammatory bowel disease [IBD, Ulcerative Colitis and Crohn's disease]) have an increased risk of developing colorectal cancer and those with persistent pancreatitis may develop pancreatic cancer.[163-166] Interestingly, RASSF1A promoter hypermethylation is frequently found both in the primary pancreatic cancers (adenocarcinomas 64%, endocrine tumors 83%) and pancreatitis samples (44%).[93] This evidence suggests that RASSF1A may play an important role in regulating inflammation, and the loss of function during chronic inflammation may interfere with its role in preventing tumor formation.

Experimentally, dextran sulphate sodium (DSS) is widely used to induce colitis in mice as a model to study IBD. Preliminary results obtained by Mohamed El-Kalla (M. Sc. dissertation, University of Alberta, 2009) suggest that the *Rassf1a* whole body knockout (*Rassf1a*$^{-/-}$) mice are more susceptible to DSS-induced colitis like intestinal inflammation compared to the wild type mice (*Rassf1a*$^{+/+}$). Following DSS treatment, *Rassf1a*$^{-/-}$ mice display decreased survival rate, increased weight loss and rectal bleeding, shortened colon length, increased intestinal permeability, increased inflammatory score of histology sections and

increased serum cytokine/chemokine concentration (IL-6, IL-12, MCP-1) compared to wild type mice (Figure 3.6 left panel for IL-6, results not shown for IL-12 and MCP-1). These observations suggest that Rassf1a expression is crucial in protecting the mice from chemically induced intestinal inflammation.

However, the pathogenesis of intestinal inflammation was not clearly understood from the *Rassf1a* whole body knockout study as multiple factors could potentially mediate this effect, including homeostasis of luminal commensal bacteria, integrity of the intestinal epithelium lining following DSS-induced injury and the influence of resident and infiltrating immune cells on the intestinal epithelium. As the epithelium is the first line of defence against pathogenic components within the colon, I would like to determine if the loss of Rassf1a expression in the intestinal epithelium is important to the pathogenesis of DSS-induced intestinal inflammation that was initially observed in the *Rassf1a*$^{-/-}$ mice where *Rassf1a* is missing in all cells.

To investigate this, we obtained an intestinal epithelium cell (IEC) specific *Rassf1a* knockout mouse model (*Rassf1a*$^{IEC\text{-}KO}$). In this chapter, it is demonstrated that the *Rassf1a*$^{IEC\text{-}KO}$ mice displayed similar symptom to the whole body knockout mouse (*Rassf1a*$^{-/-}$) following DSS treatment, including decreased survival, increased weight loss and rectal bleeding, shortened colon length, increased intestinal permeability, increased inflammatory score of histology sections and increased serum cytokine concentration (IL-6). Results in this chapter are presented in comparison to the whole body knockout mice. Together, the data suggest that Rassf1a expression in the epithelium is essential in

protecting the mice from chemically induced intestinal inflammation. I speculate that loss of RASSF1A expression in the intestinal epithelium may predispose individuals to develop uncontrolled intestinal inflammation.

3.2. RESULTS

3.2.1. Rassf1a expression is lost in the colon epithelium cells of *Rassf1a*$^{IEC-KO}$ mice

In this study, the Cre-Lox system was utilized to generate intestinal epithelium cell specific *Rassf1a* knockout mice. The RASSF1 gene contains eight exons and generates seven isoforms (RASSF1A-G). As Exon 1α is only restricted to *RASSF1A*, we obtained a transgenic mouse that contains *Lox*P sites on either side of Exon 1α from Adams *et al*. (Cambridge, United Kingdom).[54] We also obtained another transgenic mouse that expresses the Cre recombinase under the control of villin promoter from Jackson laboratory. Cre recombinase recognizes *Lox*P sites and catalyzes the recombination reaction resulting in the excision of DNA sequences flanked by the *Lox*P sites (exon 1α in our case), thereby generating a knockout allele of the gene of interest. Villin is considered to be expressed specifically in the intestinal epithelium, predominantly in the villi and crypt cells of the gastrointestinal tract.[167] Therefore, by mating these two strains of mice, we expect to generate an intestinal epithelium cell specific *Rassf1a* knockout mouse (*Rassf1a*$^{IEC-KO}$).

In order to select the *Rassf1a*$^{IEC-KO}$ mice, I carried out polymerase chain reaction (PCR) to genotype these mice by using three sets of primers. As indicated in

Figure 3.1a and b, primer sets RSF-C and RSF-3 were used to detect *Lox*P site inserted at 3' end of Exon 1α. This reaction produced a fragment of 480 base pair (bp) for the floxed allele with insertion of *Lox*P sites and a fragment of 400 bp for the non-floxed allele. Primer set RSF-5 and RSF-3 was used to detect the knockout of Exon 1α catalyzed by Cre recombinase. This reaction produced a fragment of 400 bp only for the knockout allele. The presence of transgenic villin-Cre was also detected by PCR reactions and produced a fragment of 1100 bp for the Cre transgene. Therefore, the mice selected for *Rassf1a*$^{IEC\text{-}KO}$ are homologue for *Rassf1a* floxed allele with the presence of Cre transgene and absent for the RSF-5/RSF-3 PCR product from the ear notch, as the knockout of *Rassf1a* is only required in the colon (Figure 3.1b).

In order to confirm that *Rassf1a* is only knocked out in the colon but not the other tissues, I harvested spleen, liver, heart, lung, colon and kidney tissue from the *Rassf1a*$^{IEC\text{-}KO}$ mice and carried out PCR reactions on these tissue samples. None of the spleen, liver, heart, and lung tissue showed positive reaction for the RSF-5/RSF-3 reaction, suggesting Rassf1a is still present in these tissues. Both colon and kidney samples were RSF-5/RSF-3 reaction positive, suggesting that *Rassf1a* was knocked out in the colon and also in the kidney (Figure3.1c). Studies have indicated that villin can also be expressed in the epithelial cells of proximal tubules in kidneys.[168] In order to rule out the possibility that the absence of *Rassf1a* in the kidney may contribute to disease progression during DSS treatment, blood pressure measurement, serum creatinine level analysis, and kidney histological section staining were carried out through collaboration with Dr.

Carlos Fernandez-Patron (Department of Biochemistry, University of Alberta) and Dr. Todd Alexander (Department of Physiology, University of Alberta). The results suggested that kidney functions and tissue architectures were not altered during DSS treatment (results are shown in detail in 3.2.8-10).

Subsequently, I performed western blotting to verify that Rassf1a was absent in the intestinal epithelium cells, especially the colon crypt cells of *Rassf1a*$^{IEC\text{-}KO}$ mice. Crypt cells were collected from the colon of the mice and isolated for protein extraction. Erk 1 and 2 expression levels were used as loading control. Rassf1a western blotting indicated that Rassf1a was absent in the crypt cells of *Rassf1a*$^{IEC\text{-}KO}$ mice (Figure 3.1d).

Therefore, the results above indicated that the intestinal epithelial cell specific *Rassf1a* knockout mice were generated and could be used to study the role of RASSF1A in intestinal disease progression.

3.2.2. The *Rassf1a*$^{IEC\text{-}KO}$ mice demonstrated decreased survival following dextran sulphate sodium (DSS) treatment

Dextran sulphate sodium (DSS) administered in drinking water is established as a murine inflammatory injury model of colitis that mimics human inflammatory bowel disease (IBD). Studies suggest that DSS can induce intestinal inflammation by irritating the colonic mucosa, resulting in erosion and regeneration of intestinal crypt, and dysplasia of the colonic mucosa.[169, 170] Although evidence indicated that DSS exerts stimulatory effect on immune system, the mechanism of DSS-induced colitis is not clearly understood.[171-174]

To study the importance of the loss of Rassf1a expression in the intestinal epithelium, I administered 3% DSS in drinking water to the *Rassf1a*$^{IEC\text{-}KO}$ mice and the corresponding wild type mice (*Rassf1a*$^{IEC\text{-}WT}$). The *Rassf1a*$^{IEC\text{-}KO}$ mice were maintained on a 129/Sv-C57BL/6 mixed background, which is different from the *Rassf1a* whole body knockout mice that were on a pure C57BL/6 background. Therefore, the 129/Sv-C57BL/6 mice with *Rassf1a* floxed but without Cre transgene were used as an appropriate wild type control to the *Rassf1a*$^{IEC\text{-}KO}$ mice and designated as *Rassf1a*$^{IEC\text{-}WT}$. Eight to ten weeks old mice with mixed sex and weight >20 g were used in these experiments. I treated the mice with 3% DSS water for 7 days which was replaced with normal drinking water for another 8 days (Figure 3.2b). I recorded weight changes as well as clinical symptoms of colitis including piloerection, bloatedness, rectal bleeding and slowed movement (as indicated in Table 2.1). The mice were euthanized when they had severe rectal bleeding (score 5), very inactive movement (score 5) or lost more than 25% of original body weight. As shown in Figure 3.2a, the *Rassf1a*$^{IEC\text{-}KO}$ mice have a significantly decreased survival rate compared to the *Rassf1a*$^{IEC\text{-}WT}$ mice. Only 25% of the *Rassf1a*$^{IEC\text{-}KO}$ mice survived following DSS treatment while more than 90% of the *Rassf1a*$^{IEC\text{-}WT}$ mice survived at day 15. I have also recorded the sex for each mouse, but the survival rate is not significantly different between each sex group. This observation was very similar to the survival rate of the whole body *Rassf1a* knockout mice (*Rassf1a*$^{-/-}$) and its corresponding wild type mice (*Rassf1a*$^{+/+}$) following DSS treatment. The *Rassf1a*$^{-/-}$ mice only obtained 10% survival rate whereas the *Rassf1a*$^{+/+}$ mice

maintained 100% survival rate at day 15. The decreased survival rate of the *Rassf1a*$^{IEC\text{-}KO}$ mice following DSS treatment suggested that the expression of Rassf1a in the intestinal epithelium was essential to protect the mice against DSS-induced inflammation injury.

Food intake was not observed in this project. In the future, food intake is necessary to measure as the differences in the food intake may affect the outcome of DSS treatment. The initial weights of *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice were not significantly different, suggesting these mice have the same basal food consumption. We assume that as the mice become sick, their food intake will decrease due to increased discomforts and slowed movement. Despite this situation, the food intake issue does not affect the disease outcome of the DSS treatment as the *Rassf1a*$^{IEC\text{-}KO}$ mice can not recover after the DSS treatment while the *Rassf1a*$^{IEC\text{-}WT}$ mice can.

3.2.3. The *Rassf1a*$^{IEC\text{-}KO}$ mice had increased weight loss and disease severity following DSS treatment

DSS irritates the intestinal epithelium resulting in intestinal inflammation. As mentioned previously, clinical symptoms of the exposure to DSS including weight loss, piloerection, bloatedness, rectal bleeding and slowed movement. I recorded weight loss of individual mouse, and calculated as percentage of the original body weight. The *Rassf1a*$^{IEC\text{-}KO}$ mice demonstrated significantly decreased body weight compared to the *Rassf1a*$^{IEC\text{-}WT}$ mice (Figure 3.3b). Similar weight loss was also observed previously for the *Rassf1a*$^{-/-}$ and the *Rassf1a*$^{+/+}$ mice (Figure

3.3a). Furthermore, disease activity was scored based on the severity of the symptoms for piloerection (0 - 5), bloatedness (0 - 3), rectal bleeding (0 - 5), slowed movement (0 - 5) and weight loss (1 - 5%, score 1; 6 - 10%, score 2; 11 - 15%, score 3; 16 - 20%, score 4). The scores were totalled for each animal and averaged for each group. The *Rassf1a*$^{IEC\text{-}KO}$ mice showed significantly increased disease activity compared to the *Rassf1a*$^{IEC\text{-}WT}$ mice (Figure 3.4b). Again, this result was comparable to results in the *Rassf1a*$^{-/-}$ when compared to its corresponding wild type (the *Rassf1a*$^{+/+}$ mice) (Figure 3.4a). Increased weight loss and increased disease activity for the *Rassf1a*$^{IEC\text{-}KO}$ mice following DSS treatment suggest that Rassf1a expression in the intestinal epithelium is important to protect the mice from DSS-induced intestinal injury and colitis.

The symptoms we used for observing severities following DSS experiment is partially subjective such as bloatedness, piloerection and slowed movement, although I tried to reduce the scores and categories for distinguish these symptoms. However, the disease activity index result is supported by blinded histo-pathological scoring of experimental colitis as discussed later in section 3.2.7. In the future, more objective observations should be included for scoring disease severity, such as stool consistency.

3.2.4. The *Rassf1a*$^{IEC\text{-}KO}$ mice displayed increased intestinal permeability and shortened colon length following DSS treatment

The intestinal epithelium separates luminal contents from the lamina propria immune cells, blood vessels and lymph tissue. When injured by chemical or

pathogenic insults such as DSS, the intestinal epithelium becomes more permeable. In this case, luminal bacteria can spread into the lamina propria area, contact directly with immune cells and trigger pro-inflammatory response. Following tissue damage and inflammatory response, tissue repair and fibrogenesis usually proceed resulting in scarring of the tissue. Consequently, the inflamed colon becomes shorter in length due to fibrosis.

To investigate the possibility of increased intestinal permeability in the *Rassf1a*$^{IEC\text{-}KO}$ mice, we carried out an analysis of colonic permeability using fluorescein isothiocyanate (FITC)-dextran through the collaboration with Lei Liu and Dr. Eytan Wine (Department of Pediatrics, University of Alberta). FITC-dextran is composed of a polymer of anhydroglucose and a fluorescent tag. Oral administration allows FITC-dextran to be absorbed through the intestine into the blood stream. The intensity of the fluorescent signal detected in the blood serum is positively correlated with the degree of the intestinal permeability.[175] Food was withdrawn overnight prior to the experiment to equalize the absorption of FITC-dextran molecules through the intestine. Food particles can trap the FITC-dextran molecules from being absorbed by the intestine. Following overnight fasting, FITC-dextran (4 kDa) solution was orally gavaged to an individual mouse at a concentration of 0.6 mg/g of body weight by Dr. Eytan Wine. Four hours later, we collected blood serum by performing cardiac puncture on the mice, and determined blood levels of FITC-dextran by fluorometry. As shown in Figure 3.5a, the serum FITC signal was significantly increased in the DSS treated *Rassf1a*$^{IEC\text{-}KO}$ mice compared to the *Rassf1a*$^{IEC\text{-}WT}$ mice. The untreated mice as

well as the DSS treated *Rassf1a*$^{IEC\text{-}WT}$ mice retained low serum fluorescent signal. Therefore, intestinal permeability was significantly increased in the DSS-treated *Rassf1a*$^{IEC\text{-}KO}$ mice. Increased intestinal permeability was also observed in the conventional *Rassf1a* knockout mice (Figure 3.5a). These results suggest that the presence of Rassf1a expression in the intestinal epithelial cells is essential to protect from unwanted intestinal permeability following DSS-induced inflammation injury.

In addition, I dissected the large intestine and measured the distance from the proximal colon to the distal colon of the mice at day 8 following DSS treatment or the untreated mice (Figure 3.5b). The DSS treated *Rassf1a*$^{IEC\text{-}KO}$ mice revealed significantly decreased colon lengths compared to the DSS treated *Rassf1a*$^{IEC\text{-}WT}$ mice (Figure 3.5c) with the untreated *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice having relatively the same colon lengths at 8-10 weeks of age. These results further support the importance of Rassf1a expression in the intestinal epithelium and how this is essential to protect the mice from DSS-induced intestinal inflammation injury.

3.2.5. The *Rassf1a*$^{IEC\text{-}KO}$ mice showed increased serum IL-6 concentration

During the inflammatory response, increased cytokines and chemokines concentrations can usually be detected in the blood stream. Cytokines and chemokines are signaling molecules produced by immune or non-immune cells with a wide range of functions in immunity such as to recruit and activate neutrophils, macrophages and lymphocytes for pathogen elimination and tissue

repair. IL-6 is a pleiotropic pro-inflammatory cytokine secreted by monocytes, macrophages and certain non-lymphoid cell types in response to tissue damage or infection. High concentrations of serum IL-6 in inflammatory bowel disease (IBD) patients are associated with disease severity, and increased serum IL-6 levels during remission are predictive of disease relapse.[176] Other studies also suggest high serum IL-6 levels are detected in patients with colorectal cancer and are possibly involved in colitis-associated tumorigenesis.[177]

In order to investigate the serum IL-6 concentration of the *Rassf1a*$^{IEC\text{-}KO}$ mice following DSS treatment, enzyme-linked immunosorbent assay (ELISA) was used to detect IL-6 levels from the mice serum. The *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice were treated with 3% DSS in drinking water for 7 days and subjected to cardiac puncture for blood collection at day 8. As shown in Figure 3.6 (a), the *Rassf1a*$^{IEC\text{-}KO}$ mice demonstrated significantly increased serum IL-6 concentration compared to the *Rassf1a*$^{IEC\text{-}WT}$ mice following DSS treatment. The untreated *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice both showed similar levels of IL-6 concentration. These results indicate that the *Rassf1a*$^{IEC\text{-}KO}$ mice are more susceptible to DSS induced intestinal inflammation. Similarly, the serum IL-6 concentration results of the tissue specific knockout mice are comparable to the conventional knockout mice, suggesting that Rassf1a expression in the intestinal epithelial cells is essential to protect the mice from DSS-induced intestinal inflammation.

3.2.6. The *Rassf1a*$^{IEC\text{-}KO}$ mice showed increased colon tissue MPO concentration following DSS treatment

Multiple immune and non-immune cell types are involved in the inflammatory response by secreting factors or directly destroying pathogens. At the site of bacterial infection or tissue injury, appearance of neutrophils is usually an indication of inflammation. Neutrophils are phagocytic cells that constitute the first line of defense of the innate immune system. One of the characteristic features of neutrophils is the presence of numerous cytoplasmic granules that contain enzymes such as proteases, oxidases and lipases to degrade pathogenic components after engulfing by phagocytosis. Myeloperoxidase (MPO) is a marker enzyme of neutrophils that presents in the granules at a high concentration (about 1 mM in the granules) and carries out H_2O_2-dependent oxidation of halides that can react with and kill microbes.[178] Therefore, measurement of the amount of MPO can be used as an indication of the neutrophil activity.

In order to analyze MPO concentration in the colonic area, MPO-specific ELISA was performed on colon lysates collected from the mice. At day 8 following 7 days of DSS treatment, whole colon homogenates were prepared in M-PER lysis buffer (Thermo Scientific). M-PER lysis buffer contains nondenaturing detergent that allows the protein extracts to be analyzed by ELISA method. Colon lysates were also collected from untreated mice and used as controls. As shown in Figure 3.7, the *Rassf1a*$^{IEC\text{-}KO}$ mice exhibited significantly increased MPO concentration compared to the *Rassf1a*$^{IEC\text{-}WT}$ mice following DSS treatment. Both untreated groups displayed very low MPO activity. These results suggest that the

Rassf1a$^{IEC\text{-}KO}$ mice are more susceptible to DSS-induced insults and further support that Rassf1a expression in the intestinal epithelial cells is important to protect the mice from DSS-induced intestinal inflammation.

3.2.7. The *Rassf1a*$^{IEC\text{-}KO}$ mice showed increased colon histopathological scores following DSS treatment

Histologically, the DSS experimental colitis is mostly characterized by shortening and disruption of crypt structure, injury of epithelium and mucosa, thickening of lamina propria area with infiltrating neutrophils, macrophages and lymphocytes.[179] In order to investigate the histopathology of the DSS-induced experimental colitis, colon histology sections were stained using hematoxylin and eosin (H&E) method and a blinded analysis for histological disease scores was carried out. Hematoxylin stains blue for arginine rich proteins mostly histones in the nuclei. Eosin stains pink for the eosinophilic proteins in the cytoplasm.

Therefore, the H&E stained histology sections could be analyzed for tissue architecture integrity under a light microscope. Colonic sections were prepared from the *Rassf1a*$^{IEC\text{-}KO}$ and the *Rassf1a*$^{IEC\text{-}WT}$ mice at day 8, one day after the 7 day DSS treatment. Then the colons were immersed in Z-fix fixation solution and subjected to H&E staining. In Figure 3.8a, the DSS treated *Rassf1a*$^{IEC\text{-}KO}$ mice demonstrated more severely damaged crypt structure and mucosa as well as increased infiltrating cells into the lamina propria compared to the DSS treated *Rassf1a*$^{IEC\text{-}WT}$ mice. Both the untreated *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice displayed healthy colonic tissue architecture. Moreover, the colon histology

sections were blindly scored by Dr. Aducio Thiesen (Department of Anatomical Pathology, University of Alberta) for unbiased analysis. The histopathology was scored for enterocyte injury (0-3), infiltrating lamina propria neutrophils (0-2), infiltrating lamina propria lymphocytes (0-2) and epithelial hyperplasia (0-3). As shown in Figure 3.8b, the DSS treated *Rassf1a*$^{IEC\text{-}KO}$ mice demonstrated significantly increased histopathological scores (average score 6.4) compared to the DSS treated *Rassf1a*$^{IEC\text{-}WT}$ mice (average score 3.0), whereas the untreated mice retained relatively the same and low scores (between 0-1). The increased scores were mostly observed in the categories of the enterocyte injury and infiltrating neutrophils, which also support the previous results of decreased survival, increased DAI, increased intestinal permeability, decreased colon length, increased serum IL-6 concentration, and colon MPO concentration in the DSS treated *Rassf1a*$^{IEC\text{-}KO}$ mice. These results further indicated that the *Rassf1a*$^{IEC\text{-}KO}$ mice developed more severe colitis-like intestinal inflammation compared to their respective wild type mice. Therefore, Rassf1a expression in the intestinal epithelial cells is essential to protect the mice from the DSS-induced experimental colitis.

3.2.8. Kidney tissue architecture was not altered following DSS treatment

We have observed that the Rassf1a expression in the intestinal epithelium is important for the intestinal physiology. However, as mentioned in result 3.2.1, knockout of the *Rassf1a* gene was also detected in the kidney tissue of the *Rassf1a*$^{IEC\text{-}KO}$ mice by using PCR genotyping and most likely in the epithelium cells in the proximal tubules. It is important to distinguish whether loss of

Rassf1a in the kidney is also physiologically important to our study. The kidney plays a vital role in the body to filter toxins and waste products out of the blood and balances the levels of electrolytes in the body so as to control blood volume and blood pressure. Damaged kidneys can result in the slow build up of harmful wastes and excess contaminants in the blood, which can consequently cause damage to other organs and result in high blood pressure. Eventually, a dysfunctional renal system can lead to renal failure and end-stage renal disease that is fatal to the body. However, no evidence to date suggests that DSS can induce renal diseases in mice. Therefore, although the *Rassf1a* is knocked out in the kidney of the $Rassf1a^{IEC\text{-}KO}$ mice, we hypothesize that the renal function is not altered in the $Rassf1a^{IEC\text{-}KO}$ mice following DSS treatment.

Renal diseases such as glomerulonephritis, interstitial nephritis and acute tubular necrosis usually associate with necrosis and fibrogenesis of the glomeruli or the renal tubules, and increased infiltrating immune cells into the inflamed area. These pathological changes can be detected from the kidney biopsy by using H&E staining. In order to rule out the possibility that the loss of *Rassf1a* in the kidney influenced the disease outcome following DSS treatment, several methodologies were carried out to determine kidney function including histological analysis of kidneys sections from the DSS treated or untreated $Rassf1a^{IEC\text{-}KO}$ and $Rassf1a^{IEC\text{-}WT}$ mice.

First, I harvested kidneys from the mice at day 8 following 7 days of DSS treatment as well as from the untreated mice. The kidneys were immediately immersed in the Z-fix fixing solution and subjected to sectioning and H&E

staining. The kidney histology slides were analyzed by Wanling Pan and Dr. Todd Alexander (Department of Physiology, University of Alberta). As shown in Figure 3.9, no significant histopathological changes were detected from the renal cortex of the DSS treated or untreated *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice. The glomeruli and tubule structures remained distinct and healthy without infiltrating immune cells. Therefore, kidney tissue architecture remained intact in the DSS treated or untreated *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice. The conclusion from this analysis is that the disease outcome of DSS treatment of the *Rassf1a*$^{IEC\text{-}KO}$ mice probably results from the knockout of *Rassf1a* in the intestinal epithelial cells and not kidney cells.

3.2.9. Serum creatinine levels remained the same following DSS treatment

Creatinine is a natural metabolite from muscle cells by hydrolysis of creatine and phosphocreatine. Creatinine is produced at a relatively constant rate by the body and filtered through the kidneys by glomerular filtration and proximal tubule secretion.[180] Serum creatinine levels measurement is widely used as a biomarker for renal function.

In order to further support our hypothesis that renal functions are not altered in the *Rassf1a*$^{IEC\text{-}KO}$ mice, the mice serum was subjected to serum creatinine measurement. I collected blood serum from the *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice at day 8 following 7 days of DSS treatment as well as from the untreated mice. Serum creatinine measurement was carried out by Dr. Todd Alexander (Department of Physiology, University of Alberta) using alkaline picrate

colorimetric assay. As demonstrated in Figure 3.10, the serum creatinine levels remained relatively unchanged for the untreated *Rassf1a*$^{IEC\text{-}KO}$ and the *Rassf1a*$^{IEC\text{-}WT}$ mice as well as the DSS treated mice. These data suggest that renal function is not altered for the *Rassf1a*$^{IEC\text{-}KO}$ mice, further supporting our hypothesis that the disease outcome of DSS treatment of the *Rassf1a*$^{IEC\text{-}KO}$ mice results from the knockout of *Rassf1a* in the intestinal epithelial cells.

3.2.10. Systolic blood pressure remained the same following DSS treatment

As mentioned earlier, the kidney not only functions to filter toxins and wastage out of the blood but also balance blood volume in the body. Dysfunctional renal system can result in increased blood volume and thereby increased blood pressure. Blood pressure measurement is another commonly used method to determine renal function. In order to further support our hypothesis that the renal function is not altered in the *Rassf1a*$^{IEC\text{-}KO}$ mice, the systolic blood pressure was investigated for the untreated or DSS treated *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice. The systolic blood pressure was blind analyzed by Jeff Odenbach and Dr. Carlos Fernandez-Patron (Department of Biochemistry, University of Alberta). The blood pressure was measured from the tail artery of the mice ten times for each mouse in a day including eight manual measurements and two automatic measurements. Blood pressure was also measured for untreated mice on day 5 and day 7 following DSS treatment of the *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice. As demonstrated in Figure 3.11, systolic blood pressure remained the relatively the same and in the normal range through the DSS treatment for the *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice. These data further support our hypothesis that the renal

function is not altered in the *Rassf1a*$^{IEC-KO}$ mice with or without DSS treatment although *Rassf1a* is likely knocked out in the kidney tissue. The disease outcome of DSS treatment of the *Rassf1a*$^{IEC-KO}$ mice results from the knockout of *Rassf1a* in the intestinal epithelial cells.

3.3. CONCLUSION

In this chapter, I have characterized a novel function of intestinal epithelial Rassf1a in protection against chemical induced intestinal inflammation. Together with the previous investigation of the conventional Rassf1a knockout by Mohamed El-Kalla, we have discovered a new potential biological function of Rassf1a, which is protection against inflammatory injury. The *Rassf1a*$^{IEC-KO}$ mice were obtained and characterized for their biological and pathological features following DSS administration in drinking water. The *Rassf1a*$^{IEC-KO}$ mice demonstrated increased susceptibility for the DSS-induced experimental colitis compared to the respective wild type mice.

Following DSS treatment, the *Rassf1a*$^{IEC-KO}$ mice displayed significantly decreased survival, decreased percent body weight, increased disease activity index, increased intestinal permeability, decreased colon lengths, increased serum IL-6 concentration, increased colon tissue MPO concentration and increased colon histopathological scores. These results are also comparable to the conventional *Rassf1a* knockout mice, suggesting *Rassf1a* expression in the intestinal epithelial cells is essential to protect the mice from DSS-induced intestinal inflammation. Through PCR analysis of various tissues from the

Rassf1a$^{IEC\text{-}KO}$ mice, *Rassf1a* is likely to be also absent in the kidney in addition to the colon. However, results from renal cortex histopathological analysis, serum creatinine measurements and systolic blood pressure measurements suggested the renal function was not altered for the untreated *Rassf1a*$^{IEC\text{-}KO}$ mice or for the DSS treated mice. In conclusion, the disease outcome of DSS treatment of the *Rassf1a*$^{IEC\text{-}KO}$ mice results from the absent of Rassf1a in the intestinal epithelial cells. Rassf1a expression in the intestinal epithelium is essential for intestinal homeostasis and protects against chemical induced colitis in mice. Interestingly, IBD has a genetic linkage of 3p21 where harbors RASSF1A.[110] Our studies may also contribute to the understanding of IBD pathogenesis.

3.4. DISCUSSION AND FUTURE DIRECTION

3.4.1. Cytokine, chemokine and other inflammatory mediators

Besides serum IL-6 analysis, I also analyzed serum levels of IL-12, IL-1β, monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor α (TNFα), and interferon γ (IFNγ). However, I did not observe increased levels of serum IL-12, IL-1β, TNF-α, MCP-1 or IFNγ of the DSS treated *Rassf1a*$^{IEC\text{-}KO}$ mice compared to the *Rassf1a*$^{IEC\text{-}WT}$ mice (data not shown). Preliminary results from Mohamed El-Kalla showed the *Rassf1a*$^{-/-}$ mice had increased serum levels of IL-12 and MCP-1 compared to the *Rassf1a*$^{+/+}$ mice following DSS treatment. The absent of Rassf1a in the IEC may result in increased cytokine and chemokine production predominantly restricted to the intestinal area. Therefore, in the future, the colon lysates should be used to carry out the analysis of inflammatory

mediators, where the differences in cytokine and chemokine concentrations may be easier to detect.

In addition, further analysis of genes that is regulated by Rassf1a in the DSS experiment is required, such as inflammatory mediators IL-18, IL-23, TGF-β, macrophage inflammatory protein 2 (MIP-2) or keratinocyte chemoattractant (KC), interferon gamma-induced protein (IP)-10, MMP-2, MMP-9, cyclooxygenase 2 (COX-2), IL-10 and transforming growth factor β (TGF-β). TNFα and IL-1β are pro-inflammatory cytokines released by immune or non-immune cells in response to pathogenic insults to activate and recruit neutrophils and macrophages and promote inflammatory response.[181] IL-12 and IL-23 are cytokines released primarily by activated macrophages and dendritic cells to initiate and regulate adaptive immune response.[182] IL-18 is a pro-inflammatory cytokine that is secreted by injured tissue and responsible for host defense against infections by enhancing T cell and natural killer cell maturation.[183] IFN-γ is released by microbial infected cells and is important for various biological functions including up-regulation of pathogen recognition, antigen processing and presentation, the antiviral state, inhibition of cellular proliferation and effects on apoptosis.[184] MCP-1, MIP-2 and KC are chemokines for chemotaxis of macrophages and neutrophils to the injured tissue.[185] IP-10 is a chemokine is secreted by endothelial cells, fibroblasts, and macrophages in response to IFN-γ, leading to chemoattraction of macrophages, dendritic cells, T cells, and natural killer cells, as well as exhibiting antitumor activities and inhibits angiogenesis.[186, 187] COX-2 is an inducible enzyme during inflammatory response and responsible

for the production of prostanoids, which induce vascular changes to facilitate inflammatory process.[188] IL-10 is an anti-inflammatory cytokine secreted by macrophages and injured tissue that is important for ameliorating inflammation.[189] TGF-β released by macrophages, platelets and injured tissue is important to ameliorate TLR signaling and induce fibrogenesis and tissue repair.[147]

We are currently analyzing some of these cytokines, chemokines and inflammatory mediators by ELISA method. Loss of Rassf1a in the intestinal epithelium may also affect the expression of these factors in the blood serum or the colon tissue. We also collected colon tissue for microarray analysis of the *Rassf1a*$^{IEC\text{-}KO}$, *Rassf1a*$^{IEC\text{-}WT}$, *Rassf1a*$^{-/-}$, and *Rassf1a*$^{+/+}$ mice to investigate the influences of the loss of RASSF1A to global gene expression profiles following DSS experiment. Due to the time limitation, I could not obtain the results from the microarray analysis. These analyses will be very helpful in understanding the function of RASSF1A in DSS induced experimental colitis.

3.4.2. Possible mechanism of Rassf1a in the DSS experimental colitis

Although we have observed an essential role of Rassf1a in intestinal physiology, the mechanism of Rassf1a in regulating the intestinal homeostasis is unclear. Possible mechanisms of Rassf1a in regulating intestinal homeostasis include negatively regulating transcription of pro-inflammatory signals, positively regulating epithelial cell integrity and tissue repair.

3.4.2.1. Transcription factors. The up-regulated transcription factors during inflammatory process include the nuclear factor kappa B (NF-κB), activator

protein 1 (AP-1) and IFN regulatory factors (IRFs). Among these transcription factors, NF-κB is thought to be the key regulator in cytokine and chemokine concentration to promote inflammatory response and regulating immune system. AP-1 and IRFs are also implicated in inflammatory responses by up-regulating pro-survival signals for immune cells.[190-192] We have observed the role of Rassf1a in negatively regulating pro-inflammatory cytokines and neutrophils infiltration suggesting NF-κB activity is increased in the *Rassf1a*$^{IEC-KO}$ mice following DSS treatment. These results lead to the next aim of my thesis study described in Chapter 2, which is the role of Rassf1a in regulating NF-κB activity in the DSS-induced intestinal inflammation. Other transcription factors are also discussed in Chapter 2.

3.4.2.2. Epithelial cell integrity. Since RASSF1A is essential for the integrity of microtubule networks which is important for cellular structure, loss of RASSF1A in the intestinal epithelium may also result in decreased epithelial integrity and intestinal disease. E-cadherin complexes with β-catenin at the cell membrane that are important for adhesion junctions between intestinal epithelium cells and resealing of the epithelial barrier.[193] Additionally, Adenomatous poliposis coli (Apc) associate with glycogen synthase kinase, GSK-3β, downregulates cytosolic β-catenin and prevents it from transcriptional activation of genes involved in proliferation.[194] Loss of Rassf1a and Apc cooperate to induce accumulated nuclear levels of β-catenin and result in colon tumorigenesis in mice.[79] Therefore, we speculate the *Rassf1a*$^{IEC-KO}$ mice also have decreased membrane associated β-catenin accompanied with increased nuclear accumulation of β-catenin compared

to the respective wild type mice following DSS treatment. The β-catenin expression is currently investigated in our group by immunohistostaining of the colon histology sections. Loss of Rassf1a in the intestinal epithelium may result in decreased epithelial integrity.

3.4.2.3. Tissue repair. Damaged and impaired intestinal surface barrier is observed in various intestinal diseases and may result in an increased penetration of pathogenic components from the intestinal lumen to the lamina propria, leading to inflammation, uncontrolled immune response, and disequilibrium of the intestinal homeostasis.[195] Therefore, rapid resealing and reestablishing the continuity of the surface epithelium following damage is essential to preserve the normal homeostasis.[196-199] The mechanisms mainly involve epithelial restitution, proliferation, and differentiation. Epithelial restitution is the migration of adjacent epithelial cells to reseal the injured surface without proliferation of these cells.[200] Epithelial proliferation is necessary to replenish the decreased cell pool. Also, differentiation of the pre-mature epithelial cells is needed to maintain the normal mucosal functions. Deeper lesions require additional repair mechanisms that involve inflammatory responses and non-epithelial cell populations.

Various types of factors are involved in intestinal epithelial restitution and tissue repair, including epidermal growth factor (EGF), vascular endothelial cell growth factor (VEGF), platelet-derived growth factor (PDGF), keratinocyte growth factor (KGF), fibroblast growth factor (FGF), IL-1 and IFN-γ. These factors mainly conduct the function of epithelial restitution and tissue repair by increasing TGF-β signaling to promote cell proliferation.[102] In addition, trefoil factor (TFF)

peptides released by goblet cells in the intestinal epithelium also appear to stimulate epithelial restitution in conjunction with mucin glycoproteins through a TGF-β-independent mechanism.[201] Therefore, loss of Rassf1a can also result in dysfunctional epithelial restitution and tissue repair upon DSS treatment. Expression of TGF-β and TFF peptides in the serum and colon tissue is being investigated in our group by ELISA method. Moreover, the epithelial cell proliferation is also being investigated by proliferating cell nuclear antigen (PCNA) staining of the colon histology sections. We speculate loss of Rassf1a in the intestinal epithelium may result in decreased TGF-β, TFF peptides and crypt cell proliferation rate resulting in dysfunctional tissue repair following DSS treatment.

Furthermore, lysophosphatidic acid (LPA) is a key factor inflammation and is rapidly released from thrombin-activated platelets, growth factor stimulated fibroblasts and injured epithelial cells to promote tissue repair by activating G-protein coupled receptors (GPCRs) that is expressed in many cells.[202, 203] LPA can promote cell proliferation, platelet aggregation, angiogenesis, and also wound healing.[102, 204] It is also thought that LPA can stimulate GPCR mediated NF-κB activation by involving various protein kinase C (PKC) isoforms.[205] RASSF1A has recently been found to be a direct phosphorylation target of conventional and novel PKC isoforms by involving the C1 domain.[60] Therefore, RASSF1A can also be involved in LPA mediated anti-inflammatory and tissue repair process. As a future aim, we would like to investigate the LPA expression level in the serum and the colon tissue following DSS treatment. We speculate that loss of

Rassf1a expression in the intestinal epithelium can result in dysregulation of LPA signaling and dysfunctional tissue repair.

3.4.2.4. TLR pathway. DSS is thought to cause destruction of the mucosal barrier, whereas the direct target of DSS is currently unknown. Recently, DSS stimulation was found to relate to the TLR pathway activation and hyaluronic acid (HA) concentration.[174, 206] HA usually polymerizes and composes the extracellular matrix (ECM). The free HA released by depolymerization or *de novo* synthesis can become signaling molecules and associate with receptors such as TLR4. Activated TLR4 pathway directs NF-κB activation and thereby induces pro-inflammatory mediator production.[127] Traditionally, TLR4 recognizes lipopolysaccharides (LPS), which is a component of the bacterial cell wall. Preliminary experiments carried out in colon cancer cell lines, such as HCT116 and HT29, suggested that RASSF1A was able to associate with TLR pathway components such as myeloid differentiation primary response gene 88 (MyD88), Interleukin-1 receptor-associated kinase 1 (IRAK1), IRAK4 and TNF receptor associated factor 6 (TRAF6) (unpublished observation). However, the association mechanism between RASSF1A and these components requires comprehensive investigation. The DSS-induced RASSF1A association with TLR pathway is also currently being investigated in our group. We speculate that over-expression of RASSF1A in the cells can negatively regulate TLR signaling pathway and NF-κB activity induced by DSS, resulting in decreased cytokine production.

3.4.3. Kidney analysis

Kidney functions remained the same between the *Rassf1a*$^{IEC\text{-}KO}$ and the *Rassf1a*$^{IEC\text{-}WT}$ mice treated or untreated with DSS through the histological analysis, the systolic blood pressure measurement and the serum creatinine analysis. However, limitations also exist among these experiments. The creatinine-picric acid assay we used is not as sensitive for mice as compared to humans because mice serum has interference such as chromagen. High pressure liquid chromatography (HPLC) is more sensitive to detect the critical amount of creatinine, but relatively difficult to do. In the future, other methods are required for critically analyzing the kidney function of the *Rassf1a*$^{IEC\text{-}KO}$ mice following DSS treatment, such as investigating proteinurine. Additionally, more sensitive assays for detecting early kidney injury urinary are interleukin-18 (IL-18), neutrophil gelatinase-associated lipocalin (NGAL) or kidney injury molecule-1 (KIM-1) measurements, which are associated with proximal tubule destruction.

3.5. SUMMARY

We have successfully characterized the novel function of intestinal epithelial Rassf1a which is protection against DSS-induced intestinal inflammation. Loss of Rassf1a in the intestinal epithelium can result in increased colitis like symptoms following DSS treatment. We further hypothesize that RASSF1A is a negative regulator of inflammation. However, further investigation is required to understand the mechanisms of RASSF1A to regulate inflammation through

studying the inflammatory mediators, transcription factors, tissue repair processes, as well as the signaling pathways that is regulated by RASSF1A.

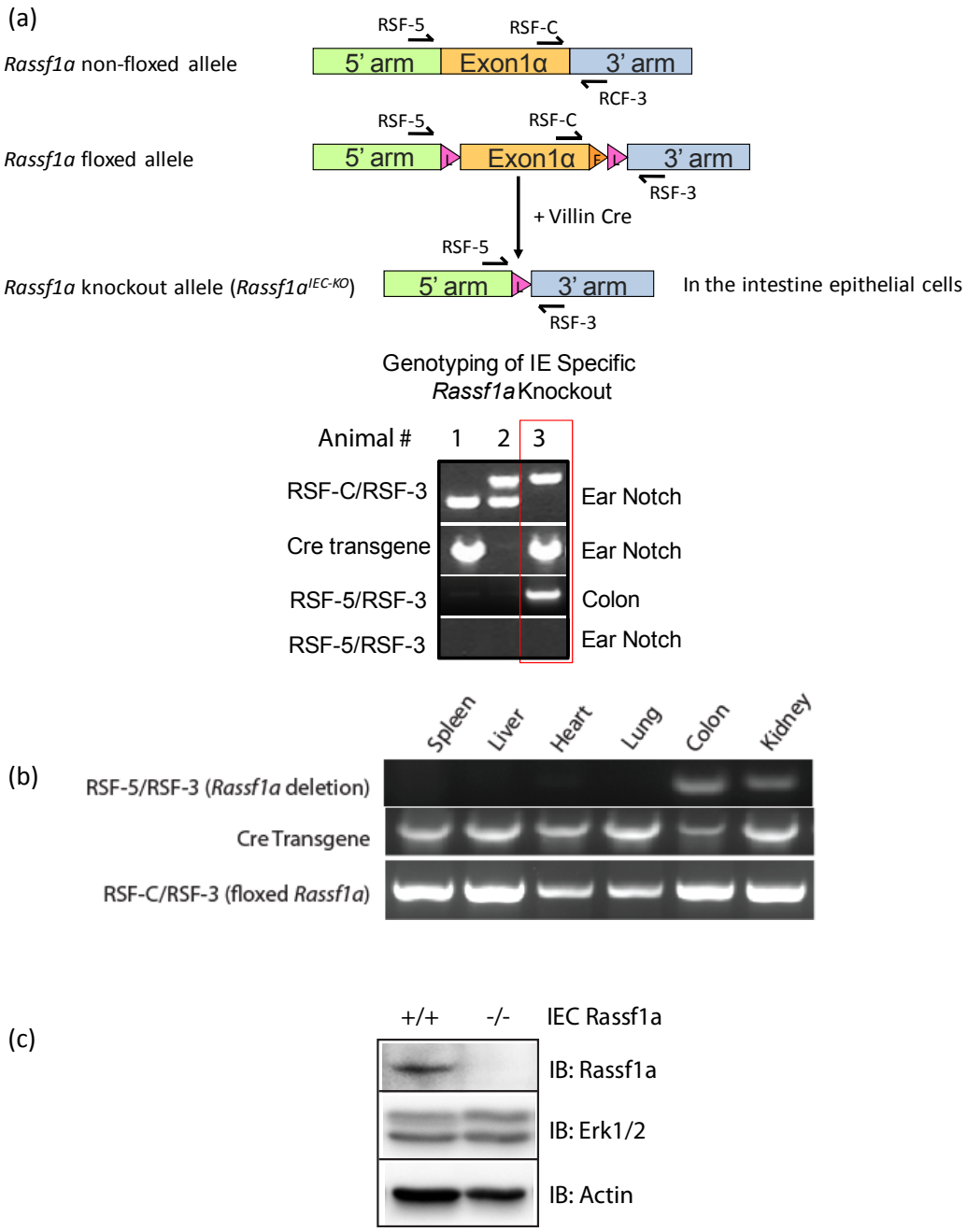

Figure 3.1 *Rassf1a* is deleted in the intestinal epithelial cells. (a) Analysis of a conditional knockout to RASSF1A. Multiplex PCR using the primer set *RSF-C/RSF-3* generated a 480 bp fragment for the floxed allele and a 400 bp fragment for the non-floxed allele. PCR using *RSF-5/RSF-3* generated a 400 bp fragment for the *Rassf1a* knockout allele specifically in the intestinal epithelial cells. PCR reaction of the Cre recombinase transgene produced an 1100 bp fragment. (b) PCR genotyping of various mouse tissues showing the knockout of *Rassf1a*, the presence of the Cre transgene and *Lox*P sites. (c) Western blot of RASSF1A in the intestinal epithelial cells (IEC) of mice. Erk1/2 and actin immunoblotting were used as loading controls.

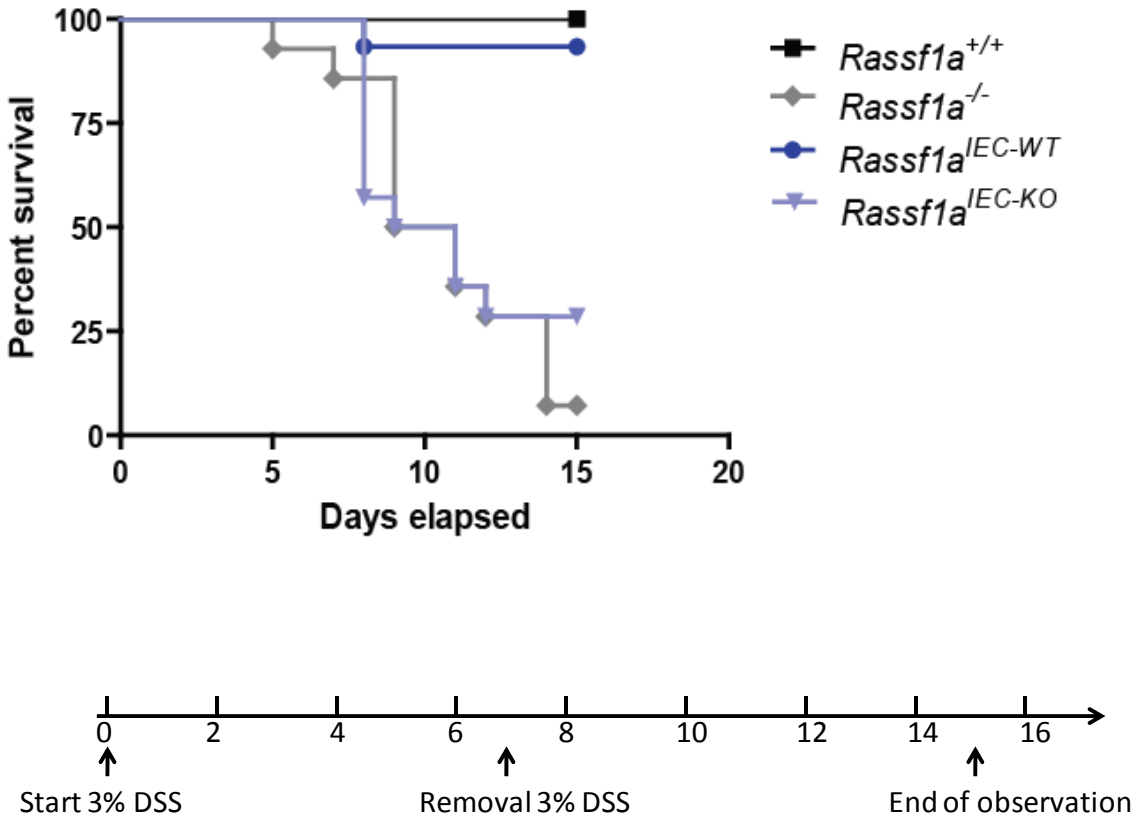

Figure 3.2 *Rassf1a*$^{IEC\text{-}KO}$ mice were more susceptible to DSS treatment. *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice were treated with 3% DSS in drinking water with the timeline indicated at the bottom (n = 15 for each group, $p < 0.0001$). Percent survival rates were compared to *Rassf1a*$^{+/+}$ and *Rassf1a*$^{-/-}$ mice (n = 13 for *Rassf1a*$^{+/+}$ and n = 14 for *Rassf1a*$^{-/-}$, $p < 0.0001$). The *Rassf1a*$^{+/+}$ and *Rassf1a*$^{-/-}$ mice data was obtained previously by Mohamed El-Kalla (M. Sc. dissertation, University of Alberta, 2009).

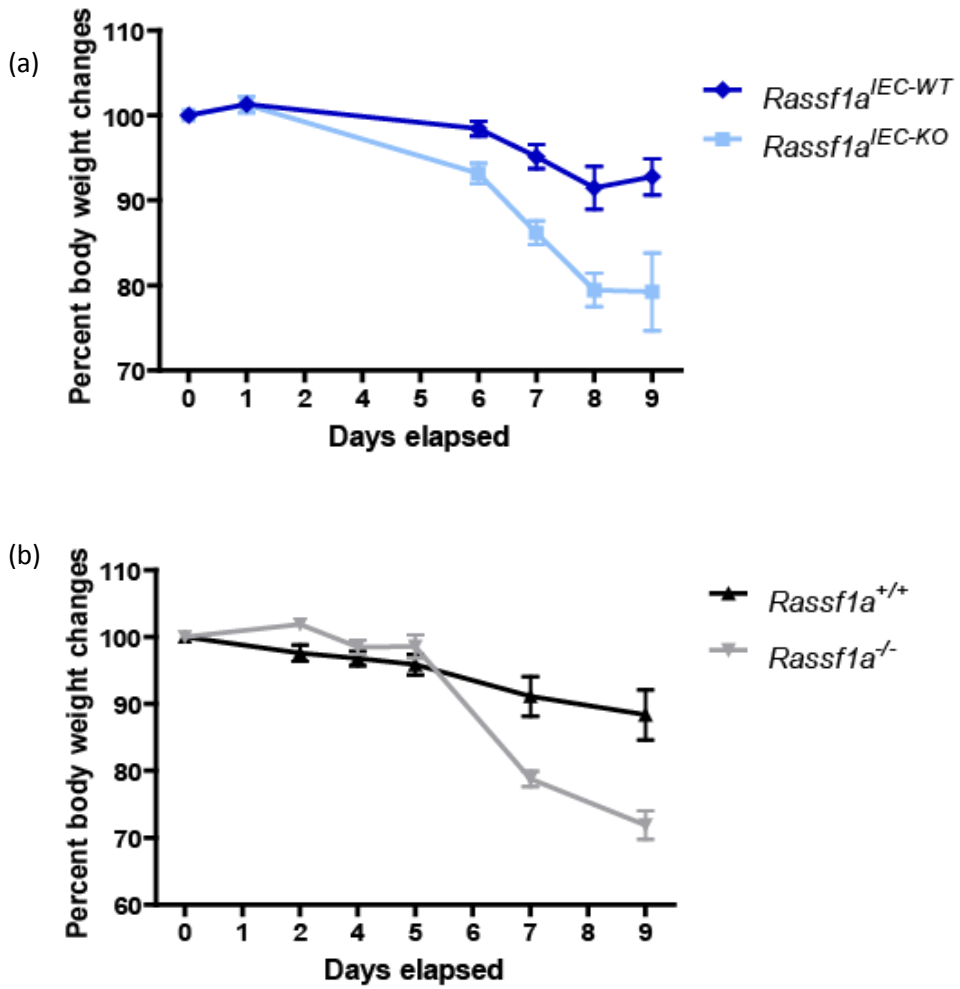

Figure 3.3 *Rassf1a*$^{IEC\text{-}KO}$ mice showed increased weight loss following DSS treatment. Percent body weight changes were obtained from (a) *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice following 3% DSS treatment (n = 11 for each group, p = 0.0009 at day 7, p = 0.0028 at day 8, p = 0.0163 at day 9) and compared to (b) *Rassf1a*$^{+/+}$ and *Rassf1a*$^{-/-}$ mice (*Rassf1a*$^{+/+}$ n = 8, *Rassf1a*$^{-/-}$ n = 10, p = 0.0004 at day 7, p = 0.0013 at day 9), data obtained by Mohamed El-Kalla (M. Sc. dissertation, University of Alberta, 2009). Student's *t* test was used for statistical analysis.

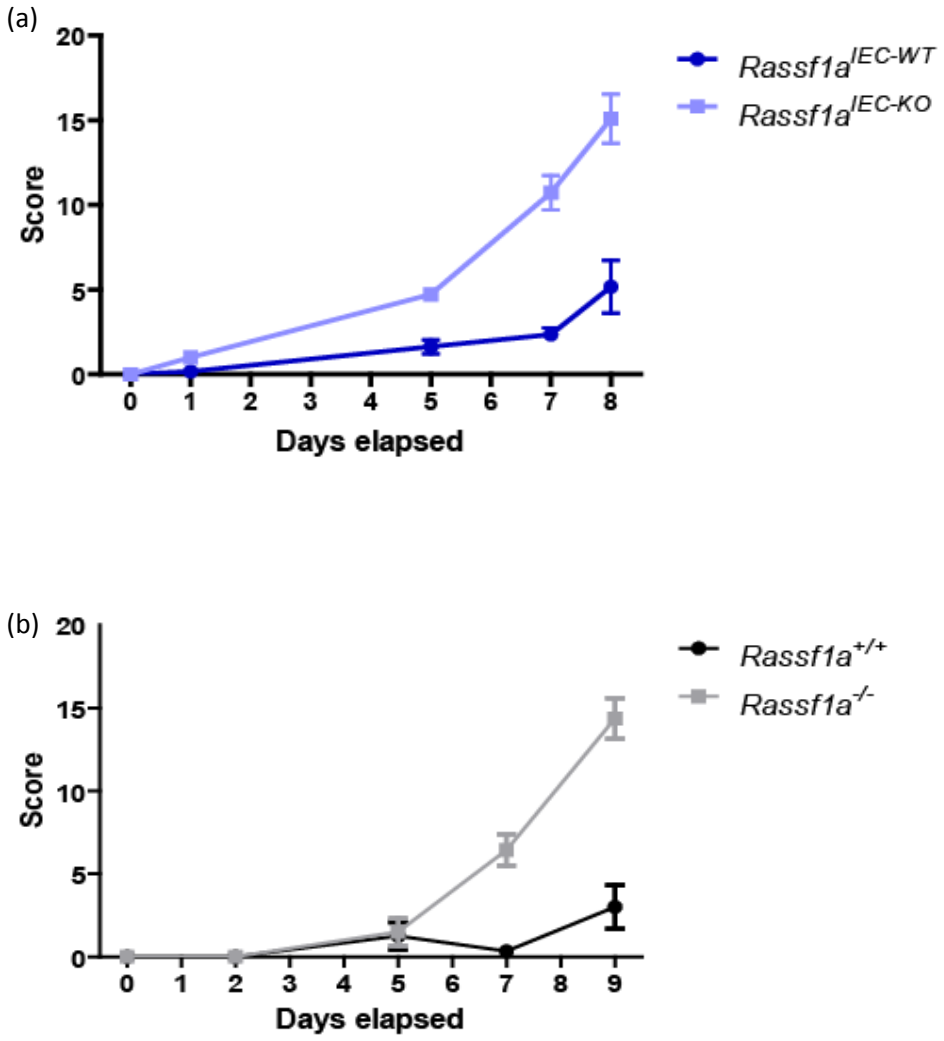

Figure 3.4 *Rassf1a*$^{IEC\text{-}KO}$ mice showed increased disease activity index (DAI) score following DSS Treatment. Disease activity was scored based on the severity of the symptoms for piloerection (0 - 5), bloatedness (0 - 3), rectal bleeding (0 - 5), slowed movement (0 - 5) and weight loss (1 - 5%, score 1; 6 - 10%, score 2; 11 - 15%, score 3; 16 - 20%, score 4; 21 - 25 %, score 5). The scores were totaled for each animal and averaged for each group. (a) *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice ($n = 11$, $p < 0.0001$ at day 5, $p < 0.0001$ at day 7, $p = 0.0002$ at day 8). (b) *Rassf1a*$^{+/+}$ and *Rassf1a*$^{-/-}$ mice ($n = 13$, $p = 0.0002$ at day 7, $p < 0.0001$ at day 9), data obtained by Mohamed El-Kalla (M. Sc. dissertation, University of Alberta, 2009). Student's *t* test was performed for statistical analysis.

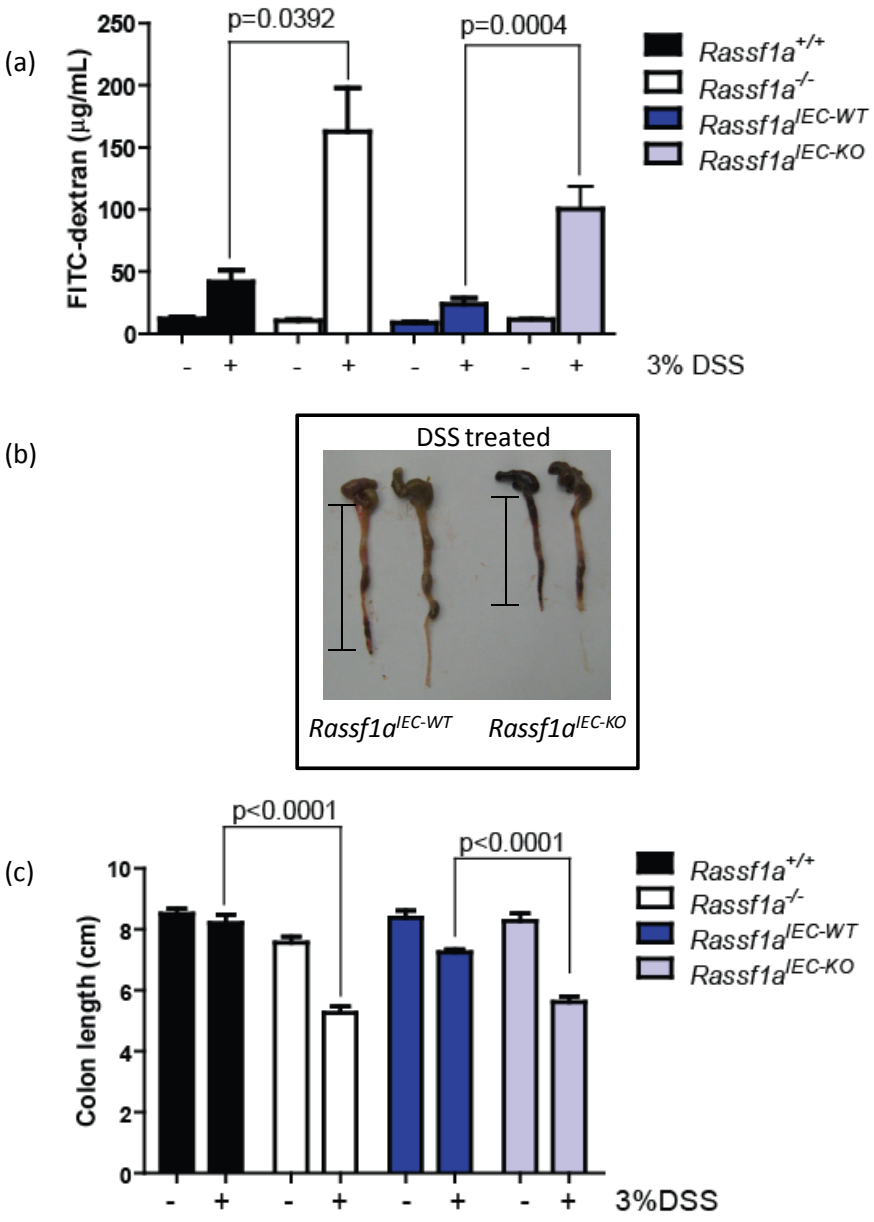

Figure 3.5 *Rassf1a*$^{IEC\text{-}KO}$ mice showed increased intestinal permeability and shortened colon length following DSS treatment. (a) Intestinal permeability was analyzed by oral administrating FITC-dextran (4 kDa) solution to the *Rassf1a*$^{IEC\text{-}KO}$, *Rassf1a*$^{IEC\text{-}WT}$, *Rassf1a*$^{+/+}$ and *Rassf1a*$^{-/-}$ mice treated with 3% DSS or the untreated mice. Blood serum was collected and measured for fluorescent signals (n = 6 for untreated mice, and n = 10 for DSS treated mice). (b) Colon lengths were measured from proximal colon to distal colon for the treated or untreated *Rassf1a*$^{IEC\text{-}KO}$, *Rassf1a*$^{IEC\text{-}WT}$. (c) Statistical plot of colon length for the *Rassf1a*$^{IEC\text{-}KO}$, *Rassf1a*$^{IEC\text{-}WT}$, *Rassf1a*$^{+/+}$ and *Rassf1a*$^{-/-}$ mice treated or untreated with 3% DSS. Student's *t* test was used for statistical analysis (n = 8 for untreated mice and n = 20 for DSS treated mice). The *Rassf1a*$^{+/+}$ and *Rassf1a*$^{-/-}$ mice data was obtained Mohamed El-Kalla (M. Sc. dissertation, University of Alberta, 2009) and me.

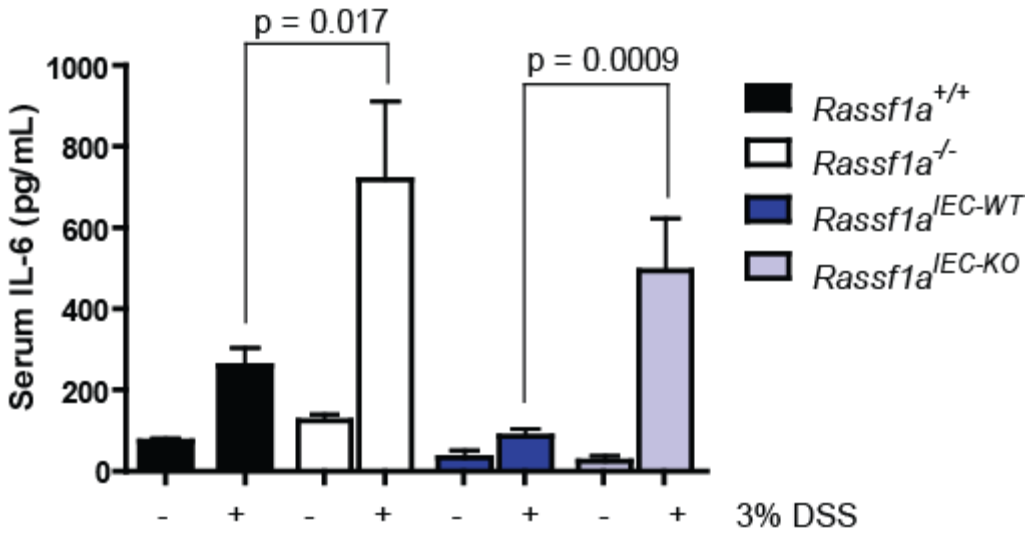

Figure 3.6 *Rassf1a*$^{IEC-KO}$ mice showed increased serum IL-6 concentration following DSS treatment. Serum IL-6 concentration was analyzed by ELISA from the DSS treated or untreated *Rassf1a*$^{IEC-KO}$, *Rassf1a*$^{IEC-WT}$, *Rassf1a*$^{+/+}$ and *Rassf1a*$^{-/-}$ mice (n = 10 for each group). The *Rassf1a*$^{+/+}$ and *Rassf1a*$^{-/-}$ mice data was obtained Mohamed El-Kalla (M. Sc. dissertation, University of Alberta, 2009) and me.

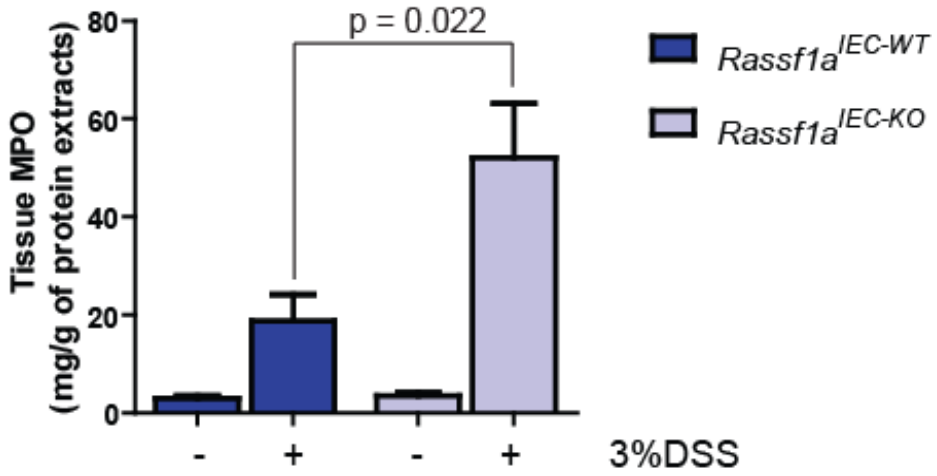

Figure 3.7 *Rassf1a*$^{IEC\text{-}KO}$ mice showed increased colon tissue MPO concentration following DSS treatment. MPO from the colon tissue was analyzed by ELISA for the treated (n = 5) or untreated (n = 10) *Rassf1a*$^{IEC\text{-}KO}$, *Rassf1a*$^{IEC\text{-}WT}$. Student's *t* test was used for statistical analysis.

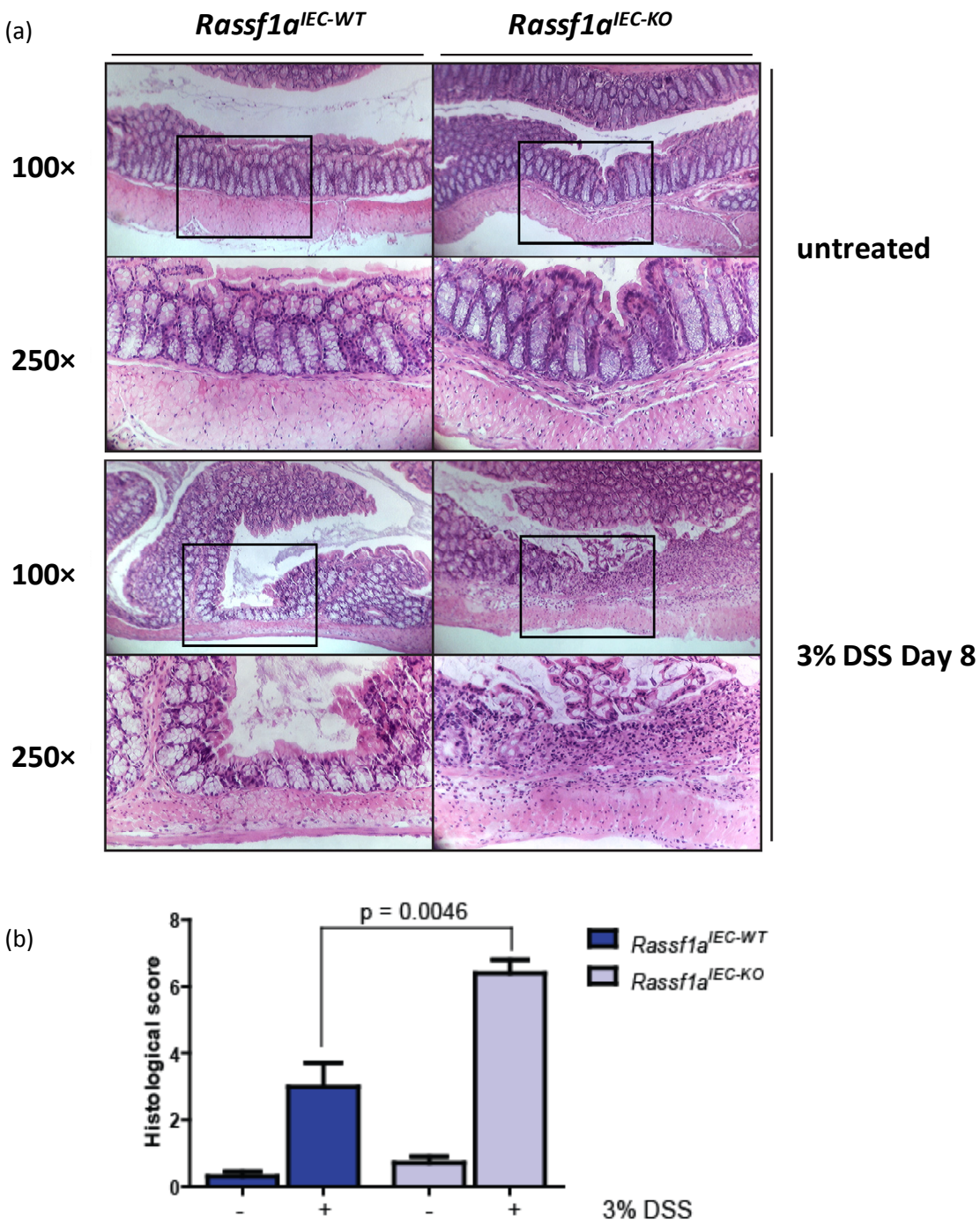

Figure 3.8 *Rassf1a*$^{IEC\text{-}KO}$ mice showed increased colon histopathological score following DSS treatment. (a) Reperesentative images of colon histology of DSS treated and untreated *Rassf1a*$^{IEC\text{-}KO}$, *Rassf1a*$^{IEC\text{-}WT}$ mice. (b) Histological scores were analyzed for enterocytes injury (0 - 3), epithelial hyperplasia (0 - 3), lamina propria lymphocytes (0 - 2), lamina propria neutrophils (0 - 2). The scores were analyzed blindly by Dr. Aducio Thiesen (Department of Anatomical Pathology, University of Alberta). Student's *t* test was used for statistical analysis (n = 10 for each group).

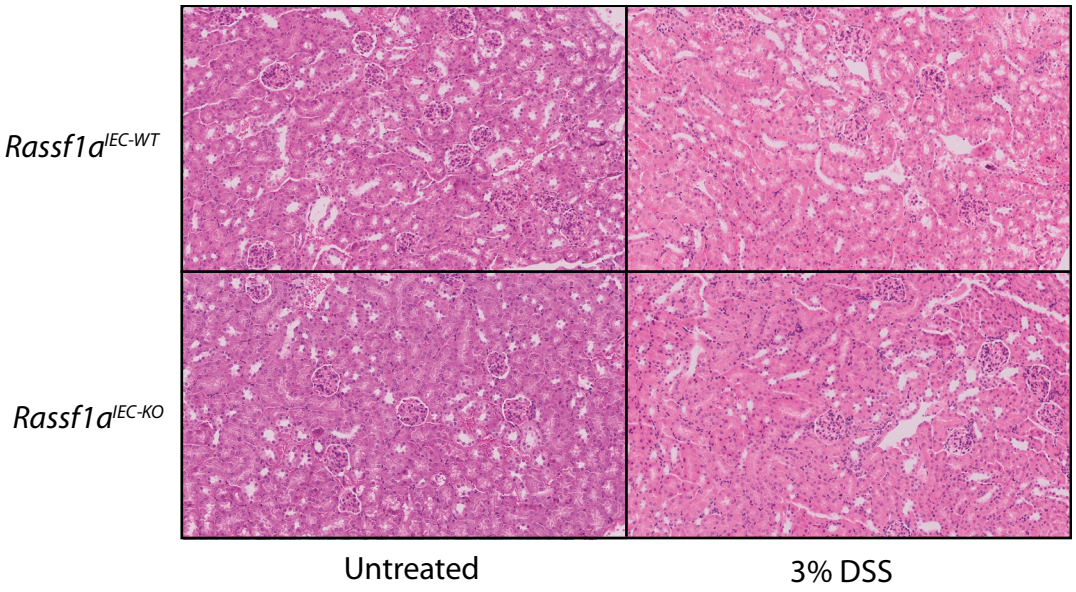

Figure 3.9: Kidney tissue architecture was not altered following DSS treatment. Representative images of renal cortex sections stained with Hematoxylin and Eosin (H&E) from *Rassf1a*$^{IEC\text{-}WT}$ and *Rassf1a*$^{IEC\text{-}KO}$ mice following DSS treatment for 8 days and compared to untreated mice (3 mice from each group were used for kidney histological analysis). The slides were blindly analyzed by Dr. Todd Alexander (Department of Physiology, University of Alberta).

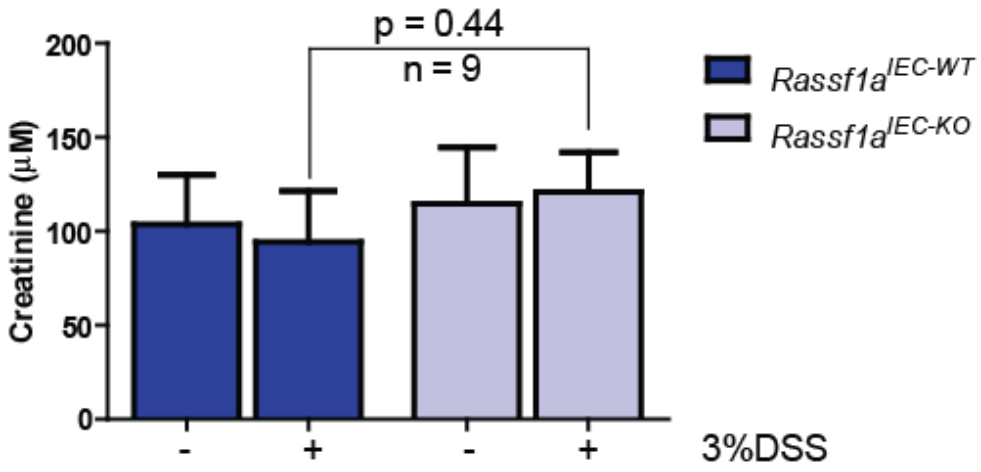

Figure 3.10 Serum creatinine levels remained the same following DSS treatment. Serum creatinine levels were analyzed for *Rassf1a*$^{IEC\text{-}WT}$ and *Rassf1a*$^{IEC\text{-}KO}$ mice following DSS treatment for 8 days and compared to untreated mice by Wanling Pan from Dr. Todd Alexander (Department of Physiology, University of Alberta). Student's *t* test was performed for statistical analysis (n = 9 for each group, *Rassf1a*$^{IEC\text{-}WT}$ and *Rassf1a*$^{IEC\text{-}KO}$, DSS treated and untreated).

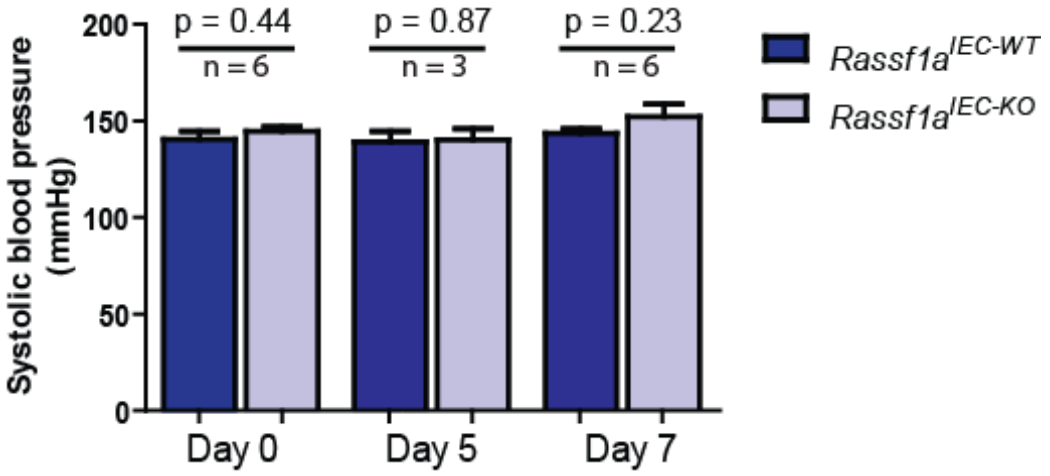

Figure 3.11 Systolic blood pressure remained the same following DSS treatment. Systolic blood pressure was blindly measured at day 0, 5 and 7 following DSS treatment for both *Rassf1a*$^{IEC\text{-}WT}$ and *Rassf1a*$^{IEC\text{-}KO}$ mice by Jeff Odenbach and Dr. Dr. Carlos Fernandez-Patron (Department of Biochemistry, University of Alberta). Student's *t* test was performed for statistical analysis. The numbers of mice for each group were indicated in the graph.

Chapter 4

The Role of Rassf1a in Regulating NF-κB Activity

4.1. BRIEF INTRODUCTION

A characteristic feature of the inflammatory response is elevated nuclear factor-kappa B (NF-κB) activity. NF-κB plays an essential role in the inflammatory processes by up-regulating the expression of pro-inflammatory cytokines and chemokines in both innate and adaptive immunity responses.[127] Damaged epithelial cells or microbial invaded resident immune cells can have increased NF-κB activity and consequently increased cytokine and chemokine production. Such factors are then able to target endothelial cells which then attract circulating immune cells (i.e. neutrophils) to the site of inflammation. In addition, cytokines and chemokines resulting from the NF-κB activity and those that are circulating within the body can also promote the maturation, survival and recruitment of macrophages and lymphocytes.

In the last chapter, we observed that the loss of Rassf1a expression resulted in increased susceptibility to DSS-induced experimental colitis. Therefore, RASSF1A can be suggested to protect against DSS-induced inflammation and possibly negatively regulate intestinal inflammation. However, the mechanism of RASSF1A negative regulation remains to be elucidated. As NF-κB plays a central role in regulating inflammatory response, we hypothesize that *Rassf1a* expression especially in the intestinal epithelium is important in negatively regulating NF-κB signaling thereby preventing an overt immune response. To support our hypothesis, we have observed increased serum IL-6 concentration and increased colon tissue neutrophil activity in both conventional and IEC specific *Rassf1a* knockout mice following DSS administration. IL-6 is one of the target

genes of NF-κB. Furthermore, NF-κB activity is important for the chemotaxis of neutrophils.

In order to examine the NF-κB activity following DSS administration, colon crypt cells and bone marrow cells were isolated from the IEC specific knockout mice (*Rassf1a*$^{IEC\text{-}KO}$) and their respective wild type mice (*Rassf1a*$^{IEC\text{-}WT}$) following DSS administration. Subsequently, these cells were analyzed for the NF-κB DNA binding activity by using electrophoretic mobility shift assay (EMSA). IκBα expression and phosphorylation were determined by Western Blot analysis. The results demonstrated that the NF-κB DNA binding activity was increased in both the colon crypt cells and bone marrow cells of the *Rassf1a*$^{IEC\text{-}KO}$ mice following DSS administration. However, the phospho-IκBα /total IκBα expression ratio was not significantly increased in the colon crypt cells of the *Rassf1a*$^{IEC\text{-}KO}$ mice upon DSS treatment, but may approach statistical significance by increasing sample numbers. These data suggested that absence of Rassf1a in the intestinal epithelium associated with elevated NF-κB activity both within the intestinal epithelium and the bone marrow. We further suggest that the absence of Rassf1a in the intestinal epithelium can lead to uncontrolled innate and adaptive immune responses.

In general, our studies suggest that RASSF1A negatively regulate NF-κB activity following the DSS-induced experimental colitis. However, the detailed mechanism this regulation has yet to be further investigated.

4.2. RESULTS

4.2.1. RASSF1A negatively regulates NF-κB DNA-binding ability

Activation of the NF-κB pathway promotes the dimerization of NF-κB subunits and the subsequent transport of the dimer to the nucleus. Subsequently, active dimers bind to the promoter regions of the target genes to initiate gene transcription. Therefore, NF-κB activity can be detected by measuring nuclear NF-κB and its binding capacity to the target sequence. Rassf1a is absent in the intestinal epithelial cells of the *Rassf1a*$^{IEC\text{-}KO}$ mice. Damage to the epithelial barrier may induce NF-κB activation in the epithelial cells and lead to cytokines and chemokines production, further potentiate and recruit immune cells to the site of injury. DSS administration in drinking water induces experimental colitis in mice. Therefore, the crypt cells were collected from the mice and analyzed for NF-κB DNA binding activities. Bone marrow is known to be rich in immune cell progenitors where NF-κB activity is very important for survival and maturation of these cells. Active inflammatory response in the intestine may result in the orchestration of immune system that involves NF-κB activity. Therefore, bone marrow cells were also collected from these mice for the study of NF-κB activity although Rassf1a is intact in the bone marrow of both genotypes. We hypothesize that absence of Rassf1a in the intestinal epithelium may result in increased NF-κB DNA binding activity in the intestinal epithelial cells as well as in the bone marrow cells following DSS treatment.

I isolated the nuclear extracts from the bone marrow cells from the *Rassf1a*$^{IEC\text{-}WT}$ and *Rassf1a*$^{IEC\text{-}KO}$ mice and used to perform eletrophoretic mobility shift assay (EMSA). Protein concentrations were determined using Bradford protein assay to ensure equal quantity of nuclear extracts per sample. Samples were then incubated with the LI-COR NF-κB binding oligonucleotides (sense strand 5'-AGT TGA GGG GAC TTT CCC AGG C-3'; binding sequence underlined) end labelled with IRDye® 700 infrared dye. This oligonucleotide sequence was initially discovered by EMSA analysis of the proteins that bind to the immunoglobulin kappa light chain enhancers in mouse B cells.[115] Currently, the aforementioned sequence is widely used in EMSA protocols for detecting potential NF-κB activity. The mixtures of nuclear extracts and NF-κB binding oligonucleotides were subjected to non-denaturing polyacrylamide gel electrophoresis and binding capacity was detected using LI-COR Odyssey imaging system. Densitometric analysis was performed to quantify any alterations in NF-κB binding capacity.

Figure 4.1a demonstrated a band shift of the NF-κB associated oligonucleotides which migrated slower than the unbound oligonucleotides. The *Rassf1a*$^{IEC\text{-}KO}$ mice had significantly increased NF-κB DNA binding activity in the bone marrow cells compared to the *Rassf1a*$^{IEC\text{-}WT}$ mice following DSS administration. The intensities of the bands were quantified and demonstrated in Figure 4.1b. These results suggest that the absence of Rassf1a in the intestinal epithelium can result in hyper-activated NF-κB activity systematically and lead to uncontrolled hyper-activation of immune response. The graph of Figure 4.1b is a collection of both Dr. Shairaz Baksh and my results.

I collected the colon crypt cells from the DSS treated or untreated *Rassf1a*$^{IEC\text{-}WT}$ and *Rassf1a*$^{IEC\text{-}KO}$ mice, which can be visualized under the light microscope as shown in Figure 4.2a. I also isolated the nuclear extracts from the colon crypt cells and incubated with the LI-COR NF-κB binding oligonucleotides. However, the preliminary results showed very weak NF-κB DNA binding activity with the crypt cell nuclear extracts. Fortunately, Dr. Shairaz Baksh was able to detect the DNA binding activity by using γ-^{32}P labelled oligonucleotides (γ-^{32}P-5'-AGT TGA GGG GAC TTT CCC AGG C-3'; binding sequence underlined) with 3-5 days of radiographic exposure. The sequence of the radioactively labelled oligonucleotides is the same with the LI-COR sequence used previously. As shown in Figure 4.2b, the *Rassf1a*$^{IEC\text{-}KO}$ mice had significantly increased NF-κB DNA binding activity in the colon crypt cells compared to the *Rassf1a*$^{IEC\text{-}WT}$ mice following DSS administration. The intensities of the bands were quantified and demonstrated in Figure 4.2c. The data of Figure 4.2c was obtained from experiments conducted by Dr. Shairaz Baksh and myself. These results suggest that the absence of Rassf1a in the intestinal epithelium can result in hyper-activated NF-κB activity in the intestinal epithelial cells, which may result in increased cytokine and chemokine production and recruitment of immune cells to the site of injury.

In order to analyze NF-κB DNA binding activity critically, in the future it may need to include untreated *Rassf1a*$^{IEC\text{-}KO}$ mice to make sure the elevated NF-κB activity is due to DSS treatment. Additionally, the eluted bone marrow cells various stem cells including hematopoietic progenitor cells that give rise to

leukocytes, erythrocytes, thrombocytes, mesenchymal stem cells that give rise to osteoblasts, chodrocytes, and myocytes, as well as endothelial stem cells. Also the bone marrow contains stroma cells including fibroblasts, macrophages, adipocytes, osteoblasts, osteoclasts, and endothelial cells. As bone marrow contains these various cell types, in the future, flow cytometry may be necessary to perform in order to determine the portion of leukocyte progenitors, because leukocytes are the cells that are activated and recruited in respond to inflammatory signals.

The NF-κB DNA binding activities detected was relatively weak in the crypt cell nuclear extracts compared to the bone marrow extracts. The reason may due to the degeneralized consensus sequence of the NF-κB binding site. In 1986, the NF-κB binding site was initially thought to be specifically 5'-GGGACTTTCC-3' within the intronic enhancer of the immunoglobulin kappa light chain gene in mature B- and plasma cells.[115] This sequence was generally widely used to detect the potential NF-κB binding activity in the cells. However, as we know now, more NF-κB binding sites have been identified in the promoters/enhancers of an increasing number of inducible genes in most cell types containing a consensus sequence (5'-GGGRNNYYCC-3', where R is purine, N is any nucleotide and Y is pyrimidine).[207] Therefore, the degeneralized NF-κB binding sequence for various target genes in different cell types might explain the low NF-κB activity in crypt cells. To circumvent this issue, another NF-κB binding site for IL-6 transcription (5'-GGGATTTTCCC-3') can be used to alternatively detect NF-κB activity.[208] We have observed significantly increased serum IL-6 and colon tissue MPO

concentration in the *Rassf1a*$^{IEC-KO}$ mice following DSS administration compared to the respective wild type mice. This indicates that the NF-κB activity is likely increased for cytokine and chemokine production in intestinal epithelial cells.

Furthermore, to ensure the specificity of the NF-κB binding to DNA, mutant NF-κB probes, cold competitor probes, as well as antibodies against p65 as in supershift assay may be needed to critically analyzing NF-κB activity.

4.2.2. RASSF1A and the activation of NF-κB

To further support our hypothesis, we investigated the regulation of NF-κB pathway in *Rassf1a*$^{IEC-KO}$ and the *Rassf1a*$^{IEC-WT}$ mice with or without DSS treatment. Inactive NF-κB dimers are sequestered in the cytoplasm by binding to the inhibitory protein IκBα. Upon activation of the pathway, IκBα is phosphorylated by the IκBα kinase (IKK) complex at serine 32 and serine 36. Subsequently, phosphorylated IκBα is ubiquitinated and targeted for proteasomal degradation, whereby NF-κB becomes activated and transported into the nucleus to promote target gene expression. Therefore, increased phosphorylated IκBα over the total level of IκBα is an indication of NF-κB activation.

In order to investigate the expression level of phosphorylated IκBα and the total level of IκBα, cytoplasmic fractions of the bone marrow cells and the crypt cells were isolated from the *Rassf1a*$^{IEC-KO}$ and the *Rassf1a*$^{IEC-WT}$ mice and subjected to immunoblot analysis. I first immunoblotted the membranes against phospho-IκBα (ser32/ser36), then stripped and re-probed against total IκBα. As indicated in Figure 4.3a, the phospho-IκBα level was increased in the crypt cells of

Rassf1a$^{IEC\text{-}KO}$ mice relative to the *Rassf1a*$^{IEC\text{-}WT}$ mice following DSS treatment. In order to properly compare the differences of the phospho-IκBα/total IκBα between each group, the band intensities were quantified using Alpha Innotech imaging system. As shown in Figure 4.3b, the increase of the phospho-IκBα/total IκBα ratio from the crypt cells of the *Rassf1a*$^{IEC\text{-}KO}$ mice was not statistically significant compared to the *Rassf1a*$^{IEC\text{-}WT}$ following DSS treatment. However, this difference may be approaching significant increase with increased sample numbers.

In contrast, we were not able to consistently detect significant levels of the phosphorylated IκBα or the total level of IκBα in the cytoplasmic fractions of bone marrow cells. Previous gene knockout studies of various components of the NF-κB signaling suggest that the NF-κB activity is essential for the development and survival of hematopoietic cells in the bone marrow.[209] The NF-κB pathway in the bone marrow hematopoietic cells can be relatively more activated compared to the intestinal epithelial cells. The low levels of IκBα may then be due to low expression levels or high turnover rate in these cells. Therefore, alternative methods to examine NF-κB activity may be through immunoblot analysis of nuclear p65 expression levels *in vivo* and *in vitro*, or *in vitro* promoter luciferase assay.

4.3. CONCLUSION

Our results have successfully detected NF-κB DNA binding activity in the bone marrow cells and colon crypt cells by EMSA, and IκBα protein expression in

colon crypt cells by Western Blot analysis. The NF-κB DNA binding activity was increased in both the bone marrow cells and crypt cells from the *Rassf1a*$^{IEC-KO}$ mice compared to the *Rassf1a*$^{IEC-KO}$ mice. The phospho-IκBα/total IκBα ratios in the colon crypt cells were not significantly increased in the *Rassf1a*$^{IEC-KO}$ mice compared to the *Rassf1a*$^{IEC-WT}$ mice upon DSS administration, but may approach statistical significance with increased sample numbers. These results suggest that Rassf1a is a potential NF-κB negative regulator in intestinal inflammation. It is likely, that the absence of Rassf1a in the intestinal epithelial cells can result in increased NF-κB activity both locally and systematically following DSS administration, and subsequently causing hyper-elevated cytokine production and uncontrolled inflammation. Additionally, nuclear p65 detection by Western Blot analysis and immunofluorescence staining, and *in vitro* promoter luciferase assay can be used as alternative methods to further investigate the NF-κB activity in the crypt cells, bone marrow cells and cell cultures.

4.4. DISCUSSION AND FUTURE DIRECTION

During inflammatory processes, multiple transcription factors are involved in addition to NF-κB, such as activator protein (AP)-1, interferon regulatory factor (IRF) and hypoxia-inducible factor (HIF).[190, 210] The AP-1 transcription factor is composed of members of the Jun and Fos family of DNA binding proteins that can be activated by growth factors, cytokines, chemokines, hormones and multiple environmental stresses to promote cell proliferation, differentiation and apoptosis.[191, 211, 212] AP-1 activity is largely regulated by mitogen-activated protein kinase (MAPK) pathways. The MAPK signaling pathways regulates

crucial inflammatory mediators and thus may serve as potential molecular targets for anti-inflammatory therapy. Multiple MAPK pathways have been identified, among which involve the extra-cellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK) and p38 cascades. Such pathways are significantly activated and directly involved in inflammatory diseases such as IBD.[213-216] Additionally, RASSF1A can inhibit tumorigenesis by decreasing AP-1 expression in a gastric cancer cell line.[71] Furthermore, many genes involved in fibrogenesis and wound healing including the transforming growth factor (TGF)-β gene contain AP-1 binding sites therefore are consequently regulated by AP-1 activity.[217, 218] We can then hypothesize that in the DSS-induced acute intestinal inflammation model, Rassf1a expression may regulate AP-1 activity and TGF-β signaling.

Another transcription factor family that plays a critical role in the immune response is the IRF transcription factor. IRFs were first characterized as regulators of type I IFNs and IFN-inducible genes. However, this family is now recognized as playing a pivotal part in the regulation of many aspects of innate and adaptive immune responses. Activation of pattern recognition receptors (PRRs) by bacterial or viral components can induce IRF activation to promote the production of type I IFNs, pro-inflammatory cytokines and chemokines to elicit the antimicrobial innate response. Gene knockout studies of IRF family members also suggested the importance of IRFs in the development of dendritic cells, macrophages, natural killer cells, and T lymphocytes. In addition to its contributions to immunity, IRFs also regulate oncogenesis.[192, 219] Previous studies

have suggested the expression of various IRFs were decreased in cancers, such as chronic myeloid leukemia, breast cancer, and hepatocellular carcinoma.[219] Owing to the importance of IRFs in immunity and oncogenesis, we would like to further investigate the role of RASSFA in regulating IRFs in the DSS induced colitis mouse model. The DNA binding activities of IRFs in the DSS treated conventional and IEC specific knockout mice are currently investigated in our group. As IRFs are essential for anti-microbial defense, we speculate Rassf1a deficient mice may have decreased IRF activity in the DSS-induced colitis mice.

HIFs are transcription factors that are induced by both systematic and cellular hypoxia conditions. Furthermore, a broad variety of inflammatory mediators and pathogenic components can also induce HIF. HIF signaling cooperates with other signaling pathways such as NF-κB signaling to ensure activation and recruitment of immune cells such as neutrophils and macrophages.[220, 221] Additionally, intestinal epithelial expression of HIFs is believed to have a protective role against mouse experimental colitis likely by increasing survival and repair of epithelium cells during the inflammatory process.[222, 223] HIF can induce epithelial heat shock protein-90α expression and subsequently recruit fibroblast for epithelial repair.[224] HIF is also responsible for inducing growth factors such as vascular endothelial growth factor (VEGF) to increase angiogenesis which is important for pathogenic clearance and tissue repair.[221] However, prolonged activation of HIF-1 under conditions of inflammation, may contribute to the survival of damaged tissue and cells, thus promoting the development of tumors.[221] Since HIFs are protective against experimental colitis, we speculate

loss of Rassf1a may result in dysregulation of HIF transcription factor activities in the DSS-induced experimental colitis.

4.5. SUMMARY

Loss of Rassf1a in the intestinal epithelium resulted in increased NF-κB DNA binding activity in bone marrow cells and colon crypt cells following DSS treatment. This result further supports the previous observation that the *Rassf1a*$^{IEC\text{-}KO}$ mice are more susceptible to DSS-induced colitis with decreased survival, increased intestinal permeability, increased pathological scoring of colitis, increased cytokine concentration, and increased neutrophil infiltration. Increased NF-κB activity resulting from the absence of Rassf1a in the intestinal epithelium may cause uncontrolled pro-inflammatory signaling induced by DSS and thereby intestinal inflammation.

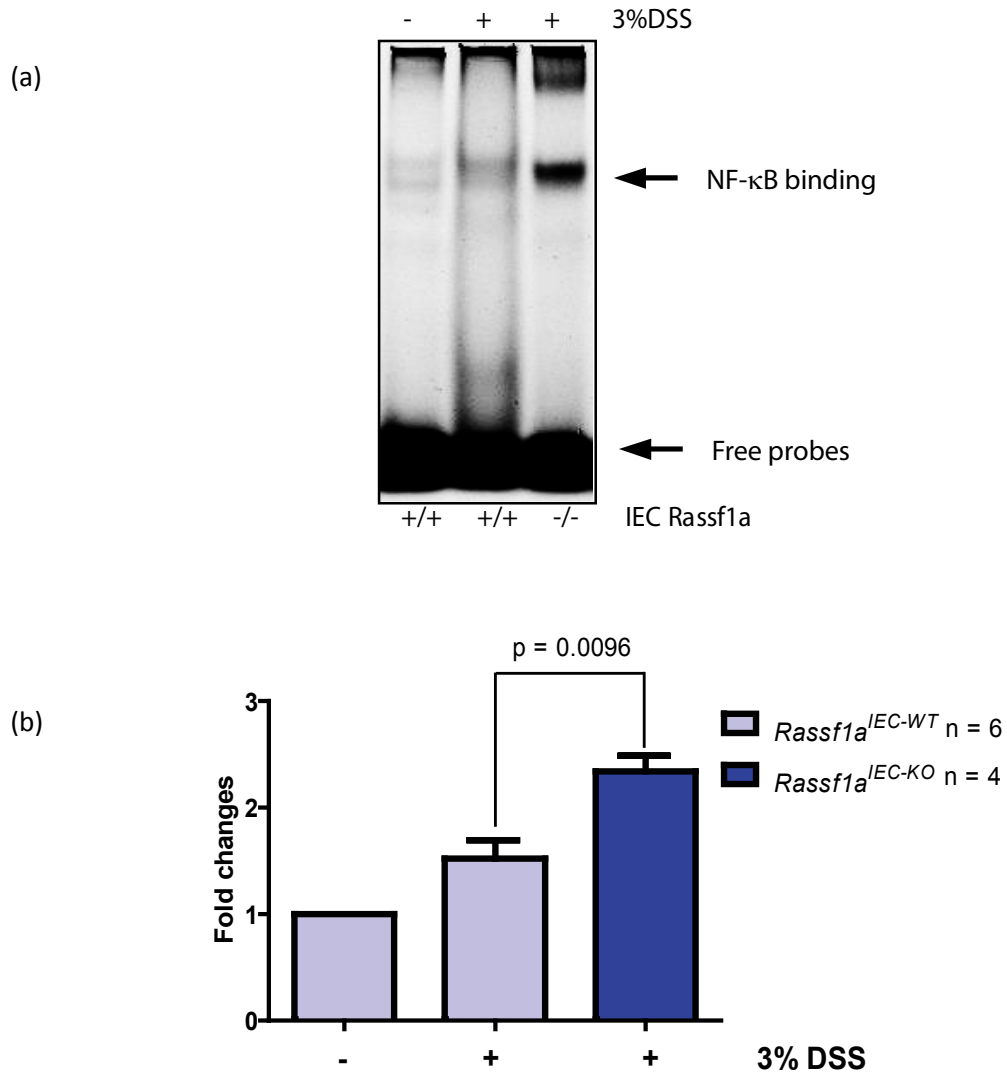

Figure 4.1 Rassf1a negatively regulates NF-κB DNA binding activity in the bone marrow cells. (a) NF-κB DNA binding assay for the nuclear extracts of bone marrow cells from both the *Rassf1a*$^{IEC\text{-}WT}$ and *Rassf1a*$^{IEC\text{-}KO}$ mice. Nuclear extracts were isolated and incubated with IRDye 700 labeled NF-κB binding oliges (5'-AGTTGAGGGGACTTTCCAGGC-3', with binding site underlined) and separated by using non-denaturing electrophoresis. The shifted complex with NF-κB oligos and the unbound oligos are indicated with arrows. (b) Analysis of band intensities for the NF-κB DNA binding activity of bone marrow cells by using LI-COR Odyssey imaging system. Quantifications of DSS treated *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice were normalized to the untreated *Rassf1a*$^{IEC\text{-}WT}$ mice. Student *t* test was used for statistical analysis with *p* values and n numbers indicated in the graph. The result was obtained by Dr. Shairaz Baksh and me.

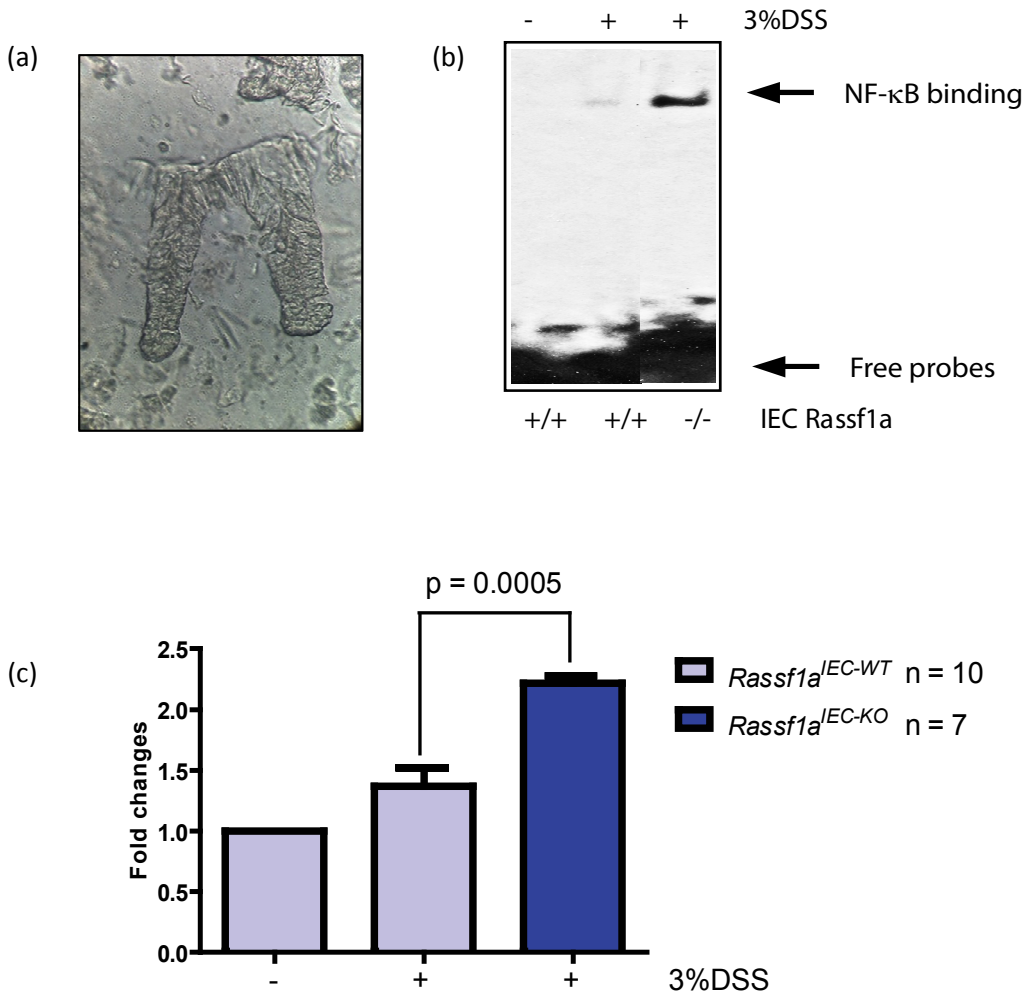

Figure 4.2 Rassf1a negatively regulates NF-κB DNA binding activity in the colon crypt cells. (a) NF-κB DNA binding assay for the nuclear extracts of colon crypt cells from both the *Rassf1a*$^{IEC\text{-}WT}$ and *Rassf1a*$^{IEC\text{-}KO}$ mice. Nuclear extracts were isolated and incubated with γ-P32-ATP labeled NF-κB binding oliges (5'-AGTTGAGGGGACTTTCCAGGC-3', with binding site underlined) and separated by using non-denaturing electrophoresis. The shifted complex with NF-κB oligos and the unbound oligos are indicated with arrows. (b) Analysis of band intensities for the NF-κB DNA binding activity of bone marrow cells by using Image J software. Quantifications of DSS treated *Rassf1a*$^{IEC\text{-}KO}$ and *Rassf1a*$^{IEC\text{-}WT}$ mice were normalized to the untreated *Rassf1a*$^{IEC\text{-}WT}$ mice. Student *t* test was used for statistical analysis with *p* values and n numbers indicated in the graph. The result was obtained by Dr. Shairaz Baksh and me.

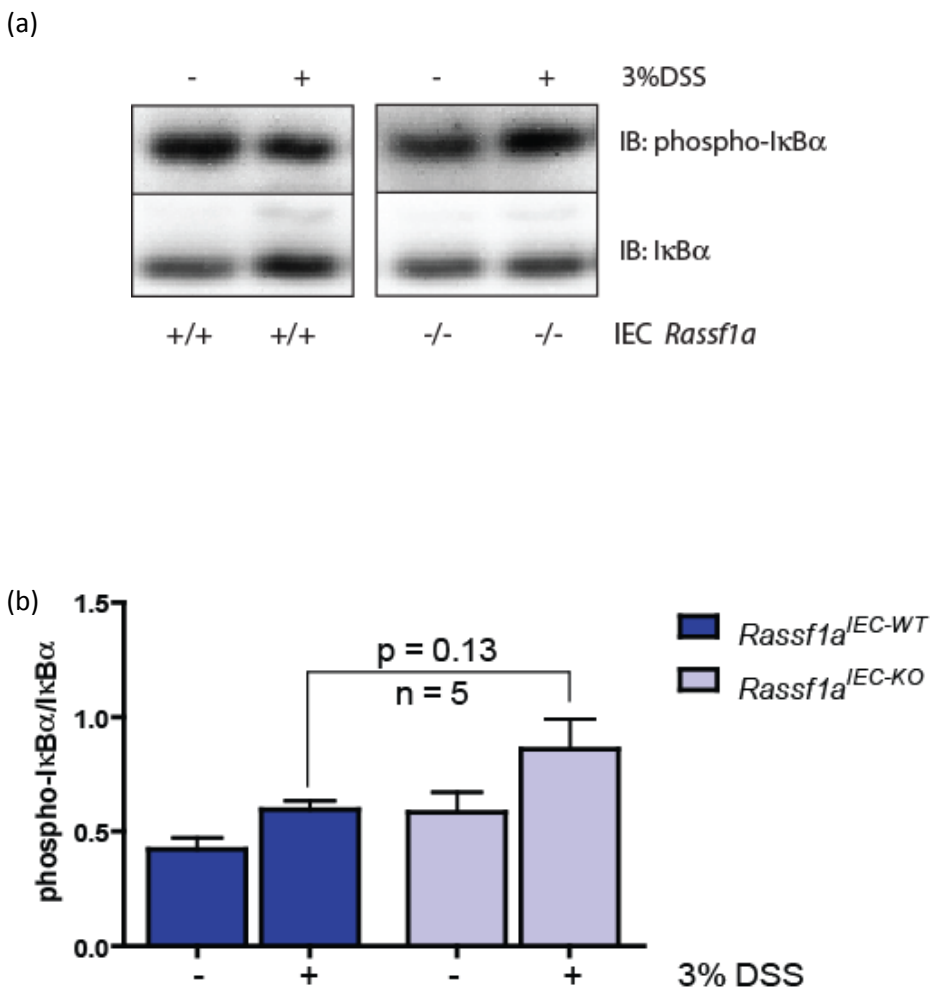

Figure 4.3 Rassf1a and NF-κB activation in colon crypt cells. (a) Western blotting against phospho-IκBα and IκBα antibodies for the cytoplasmic fractions isolated from the crypt cells of the *Rassf1a*$^{IEC\text{-}WT}$ and *Rassf1a*$^{IEC\text{-}KO}$ mice following DSS treatment. (b) Band intensity analysis of the phospho-IκBα and IκBα western blots by using Alpha Innotech imaging system. Ratios of phospho-IκBα/IκBα were averaged and analyzed by Student's *t* test with *p* value and n numbers indicated in the graph (n = 5 for each group).

Chapter 5

Concluding Remarks

In my thesis study, I have successfully generated and characterized the intestinal epithelial cell specific *Rassf1a* knockout mice (*Rassf1a*$^{IEC\text{-}KO}$) in the dextran DSS-induced experimental colitis model. The *Rassf1a*$^{IEC\text{-}KO}$ mice demonstrate increased susceptibility to DSS-induced colitis compared to the respective wild type mice, including decreased survival, increased disease activity, increased weight loss, increased intestinal permeability, decreased colon length, increased serum cytokine IL-6 concentration, increased colon neutrophil MPO activity, and increased colon histopathological score. These results suggest that Rassf1a expression in the intestinal epithelium is essential for protecting mice from colitis. Together with the conventional *Rassf1a* knockout mice study we have characterized a novel role for RASSF1A, which is to protect against induced intestinal inflammation, possibly through negative regulation of inflammation (Figure 5.1).

Furthermore, I have started to investigate the mechanism of Rassf1a in negative regulation of DSS induced colitis. Loss of Rassf1a in the intestinal epithelium demonstrated increased NF-κB DNA binding activity in bone marrow cells and colon crypt cells following DSS treatment. This suggests the hyper-activated NF-κB activity resulting from loss of Rassf1a may contribute to developing induced intestinal inflammation, as shown in the summary model in Figure 5.1.

Although *Rassf1a* is likely partially knocked out in the kidney tissue of the *Rassf1a*$^{IEC\text{-}KO}$ mice, various kidney analyses suggested kidney functions were unaffected in the DSS experiments. However, further investigation may be required for critical analysis of kidney functions.

As we have discovered a novel function of RASSF1A, many future investigations are required. Detailed future directions are discussed in each result chapters. Briefly, further analysis of genes that is regulated by Rassf1a in the DSS experiment is required, such as inflammatory mediators IL-18, TGF-β, KC/MIP-2, IP-10, MMP-2, MMP-9, LPA, and transcription factors AP-1, IRFs, HIFs. Multiple mechanisms in intestinal homeostasis may be regulated by Rassf1a such as tissue repair and epithelial restitution. In the future, we would like to investigate the role of Rassf1a in regulating epithelial cell integrity by analyzing β-catenin expression, TGF-β-dependent tissue repair, trefoil factors production, and crypt cell proliferation. In addition, we would like to investigate the molecular mechanisms that involve RASSF1A to regulate NF-κB activity. Preliminary *in vitro* investigation in colon cancer cell lines such as HCT116 and HT29 suggests that RASSF1A may inhibit NF-κB activity through association with the components of Toll-like receptor pathway (unpublished observation), but the stimulant dose and time dependent association mechanisms require to be further specified. We are also conducting microarray analysis for the colon tissues of the $Rassf1a^{IEC\text{-}KO}$, $Rassf1a^{IEC\text{-}WT}$, $Rassf1a^{-/-}$, and $Rassf1a^{+/+}$ mice to investigate the influences of the loss of RASSF1A to global gene expression profiles following DSS experiment. The results from these experiments will be very useful in understanding the role of RASSF1A in DSS induced experimental colitis. Furthermore, as RASSF1A is required to prevent the mice from chemical induced intestinal inflammation, we would like to know whether RASSF1A can

be involved in other inflammatory injury models, such as asthma and arthritis models.

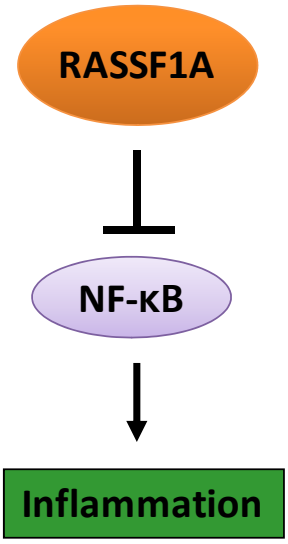

Figure 5.1 The summary model of RASSF1A in regulating inflammation. RASSF1A expression in the intestinal epithelium protects the mice from DSS-induced inflammation injury, possibly through negative regulation of NF-κB activity.

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